Emerging Infectious Diseases of the 21st Century

I. W. Fong · David Shlaes · Karl Drlica
Editors

Antimicrobial Resistance in the 21st Century

Second Edition

Springer

Emerging Infectious Diseases of the 21st Century

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Emerging Infectious Diseases of the 21st Century
ISBN 978-3-319-78537-0 ISBN 978-3-319-78538-7 (eBook)
https://doi.org/10.1007/978-3-319-78538-7

Library of Congress Control Number: 2018944271

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Printed on acid-free paper

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The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

The era of antimicrobial resistance is now upon us. Resistance problems are especially visible with hospitalized patients, as resistant infections readily spread among weakened hosts in close contact. But treatment choices are also diminished for some community infections, such as tuberculosis, pneumonia, and sexually transmitted diseases. Managing this new era in medicine is challenging, in part because we rely so heavily on antimicrobials. The problems are exacerbated by global travel, since resistance is readily disseminated. Calls to reduce consumption have raised general awareness and led to some reduction in the prevalence of resistance, but on a global level antimicrobial consumption remains high, both in agriculture and in human populations. Thus, antibiotic resistance will not disappear soon. Since new antimicrobials are becoming increasingly difficult to find, it is likely that we will need new strategies for suppressing resistance and for incentivizing the discovery and development of new antimicrobial therapies.

One approach is through education. For us, that has meant putting together a second edition of *Antimicrobial Resistance in the 21st Century* that covers many more topics than the first edition. Since we do not know from where new insights will emerge, we have chosen to provide students and clinicians with a technical introduction to the scientific literature concerning resistance. We have also included commentary on the processes leading to drug approval, since bringing new antimicrobials to market will be an important part of managing resistance. In terms of writing style and level of detail, the chapters should be considered scientific review articles. Thus, readers can expect to be well versed on the topics covered.

Much of the first edition of *Antimicrobial Resistance* concerned the resistance situation with a variety of pathogens. We have updated those chapters and added 16 new topics of a more general nature. The second edition begins with drug-resistance chapters on pneumococci (Chap. 2), MRSA (Chap. 3), Gram negative bacilli (Chap. 4), mycobacteria (Chap. 5), anaerobic bacteria (Chap. 6), HIV (Chap. 7), and Herpes virus (Chap. 8). As a part of the anaerobe discussion (Chap. 6), the concept of breakpoints is discussed and the clinical definition of resistance is introduced. A concept emerging from these surveys is that resistant bacterial subpopulations are being seen

at high frequency, pointing to an even more serious resistance problem in the future. This phenomenon, termed heteroresistance, is discussed in Chap. 9.

Understanding the biology of resistance now involves a variety of studies. One is the epidemiology of resistance (Chap. 10). An important concept is that the within-host emergence of resistance during treatment differs from the between-host transmission of resistance in both concept and suppression strategies. Another important aspect of resistance biology is the role of plasmid-mediated resistance (Chap. 11). Plasmids are mobile DNA elements that can carry multiple resistance genes; consequently, selection for one type of resistance can confer resistance to many antimicrobials. We also consider the degree to which we have contaminated the environment with antimicrobials (Chap. 12), and we develop the idea of tolerance and persistence (Chap. 13; these two terms generally refer to the pathogen not being killed by the antimicrobial, even though pathogen growth is blocked). These phenomena allow disease to relapse after treatment is stopped, thereby giving the pathogen another chance to evolve to the resistant state. Indeed, antimicrobial tolerance appears to be an important adaptation of some strains to the hospital environment – they become more problematic during infection even though they are less fit for transmission (Chap. 14).

Many different genetic mechanisms underlie resistance, two of which are discussed as examples that likely cross pathogen species lines. One example concerns pathogen genes that participate in two-component signaling systems (Chap. 15); a second focuses on fluoroquinolone resistance (Chap. 16). In the latter situation, the drug and target protein make specific contacts that are important for drug binding. Amino acid substitutions that interfere with the binding confer high-level resistance. Knowing this information leads to new ideas for bypassing resistance using fluoroquinolone-like agents that do not use the same binding pattern.

Solving the resistance problem has often been left to the development of new antimicrobial classes. Natural products have been the source of most antimicrobials to date, and they continue to be investigated (Chap. 17). One of the emerging debates is whether success is more likely with new derivatives directed at old targets or whether new targets should be the focus (Chap. 18). An example of an old target is represented by the non-quinolone topoisomerase inhibitors, which target a novel binding site on DNA gyrase (Chap. 19). Another approach is to understand the mechanism by which antibacterials kill pathogens, since that might lead to small-molecule enhancers of lethal activity. A promising lead concerns stress-mediated accumulation of toxic reactive oxygen species (Chap. 20). The novel inhibitors and adjuvants emerging from basic studies can be tested for efficacy and for their ability to restrict the emergence of resistance using an in vitro system in which changing drug concentration is modeled and the effects on pathogen populations are measured (Chap. 21). A key idea is that treating to cure disease is not enough – we must also treat to restrict resistance.

Once a new compound has shown good activity with a variety of clinical isolates, it is moved toward regulatory approval. Among the types of data that are important for approval are pharmacodynamic-pharmacokinetic measurements (Chap. 22). Clinical trials and comparisons are also important, especially because regulatory

philosophy is subject to change (Chap. 23). Since bringing a new antimicrobial to market is expensive (on the order of one billion dollars), considerable thought has gone into finding innovative economic strategies for commercialization of new antibiotics (Chap. 24). Taken as a whole, *Antibiotic Resistance in the 21st Century* can serve as a text for a college-level biology course.

We wish to thank the chapter authors for the time and effort they put into presenting their areas of expertise in an interesting and authoritative manner. We also thank Rita Beck and Deepak Devakumar at Springer for proposing the project and for facilitating the manuscript process.

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About the Editors

I. W. Fong is the Editor of the Emerging Infectious Diseases of the 21st Century series [Springer]. He was the Chief Editor for six books and the sole author for another six books published in the series. He completed his residency training in Internal Medicine at the University of Toronto and as a Fellow in Infectious Diseases at the University of Washington, Seattle. Dr. Fong has published studies concerning a variety of infectious diseases that include therapeutics and pharmacology of antibiotics, AIDS and treatment of opportunistic infections, mechanistic and treatment studies of mucosal candidiasis, and pathogenic studies on infection and induction of atherosclerosis in animal models. He was Chief of Infectious Diseases at St. Michael's Hospital (Toronto) for 34 years; he is still on staff in Infectious Diseases and is a Professor of Medicine, Department of Medicine at the University of Toronto, Canada.

David Shlaes author of *Antibiotics, The Perfect Storm* (Springer) and *The Drug Makers* (Lulu), has had a 30-year career in anti-infectives spanning academia and industry with a long-standing scientific interest in antimicrobial resistance. He trained in Infectious Diseases at Case Western Reserve University in Cleveland. He then joined the faculty and ultimately became a Professor of Medicine there. Dr. Shlaes left academia to become Vice President for Infectious Diseases at Wyeth Pharmaceuticals in 1996 where he was an important leader in the development of tigecycline. In 1998, he was the cover feature in the April issue of *Business Week* that was dedicated to antibiotics research. He also served as a member of the Forum for Emerging Infections of the National Academy of Sciences for 7 years. In 2002, Dr. Shlaes became Executive Vice President, Research and Development for Idenix Pharmaceuticals, a company located in Cambridge, MA, that focused on the discovery and development of antivirals. In 2005, he established a consulting company. During his consulting years he contributed significantly to the development of avibactam, eravacycline, and lefamulin. During his working career, he lived in Paris, France for several years. Although Dr. Shlaes has retired from Anti-infectives Consulting, he remains an Editor for the journal *Antimicrobial Agents and Chemotherapy*, writes a blog – *Antibiotics the Perfect Storm* – and continues to be active in antibiotic policy making.

Karl Drlica is a molecular biologist (Ph.D. University of California, Berkeley) whose early work focused on DNA gyrase and the control of DNA supercoiling. His studies contributed to the discovery that bacterial supercoiling is homeostatically regulated by topoisomerases having opposing activities and that environmental conditions (oxygen tension, salt concentrations) can alter global supercoiling levels. Thus, bacterial chromosome structure is sensitive to conditions outside the cell. The finding by his laboratory that transcription can alter supercoiling opened studies on local control of supercoiling. In the 1990s, when immunosuppressed patients in New York City suffered an outbreak of multidrug-resistant tuberculosis, Drlica shifted his focus to the fluoroquinolone inhibitors of bacterial DNA topoisomerases. Studies of fluoroquinolone mechanism and resistance were aimed at combatting the expanding problem of antimicrobial-resistant bacterial infections, in particular tuberculosis. In collaboration with Xilin Zhao, Drlica developed the idea that resistant mutant subpopulations are selectively enriched within a specific range of antimicrobial concentration. This concept revealed a fundamental flaw in our antimicrobial dosing strategies, since with most drug-pathogen combinations the concentrations within patients fall in the mutant-enriching range and thus encourage the emergence of resistance. Drlica's work is currently focused on improving lethal activity of antimicrobials to suppress the enrichment of induced and preexisting mutant subpopulations. Drlica has also served on NIH Study Sections, on the editorial board of several scientific journals, and as a consultant for patent disputes involving gene cloning and fluoroquinolones. His publications include three books (*Understanding DNA and Gene Cloning, Double-Edged Sword, Antibiotic Resistance*), and with Dr. Fong he has edited two others. Drlica has carried out his work as a member of the faculty of the University of Rochester and the Public Health Research Institute (now a part of Rutgers University) with visiting scientist positions at the Pasteur Institute (Paris), University of California (Berkeley), and the Indian Institute for Science (Bangalore).

Chapter 1
Introduction: Coordinated Global Action Is Needed to Combat Antimicrobial Resistance

I. W. Fong

Antimicrobial resistance is a global dilemma that threatens the health and safety of populations in all countries of the world. Urgent actions are needed to be taken before it reaches a critical stage, when large numbers of people in communities cannot be treated for life-threatening infections due to lack of effective drugs. Although the threat is most imminent from antibacterial resistance to commonly used antibiotics for infections seen regularly in intensive care units and hospitals, it is more prevalent and widespread and involves a wide spectrum of microbes. This second edition of "Antimicrobial Resistance and Implications for the 21st Century" provides not only updates and advances since the original edition but provides a wider spectrum of topics on the issue. Although most chapters of this new edition address issues of common bacterial resistance, others provide up-to-date reviews on resistance trends with viruses, including human immunodeficiency virus [HIV] and human herpes group of viruses.

To understand the evolution of microbial resistance, it is appropriate to review historical aspects. Development of antimicrobial chemotherapy is usually attributed to Paul Ehrlich ["father of chemotherapy"] based on his quest to find a cure for parasitic infections, toward the latter part of the nineteenth century, with natural dyes and heavy metals [mercury and arsenicals]. Penicillin was subsequently discovered in 1928 and administered clinically in the 1940s; sulfonamides were introduced clinically in 1937 [1]. Thus, the "antibiotic era" was under way by the early 1940s. Penicillin and other antibiotics were initially derived from environmental fungi and bacteria, often with improvements made by chemical synthesis. Hence, the origin of antibiotics is through naturally derived substances produced to antagonize or inhibit the growth of other microorganisms, probably due to an evolutionary process that protects environmental niches of the producing organisms.

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© Springer International Publishing AG, part of Springer Nature 2018

I. W. Fong et al. (eds.), *Antimicrobial Resistance in the 21st Century*, Emerging Infectious Diseases of the 21st Century, https://doi.org/10.1007/978-3-319-78538-7_1

Streptomycin and the precursor of cephalosporins were also obtained from soil microbes in the 1940s. Penicillin was first used in 1941, and by 1944 penicillinase-producing *Staphylococcus aureus* was described. Streptomycin was introduced clinically in 1944 for treatment of tuberculosis, but resistance soon developed during treatment [2]. By the mid-1950s, most of the major antibiotic families, including aminoglycosides, chloramphenicol, tetracycline, and macrolides, had been developed [1]. Synthetic chemical agents with antibacterial activity were introduced in the early 1950s with para-aminosalicylic acid and isoniazid as antituberculosis agents; nitrofurantoin was found around the same time, followed by trimethoprim in 1956. Nalidixic acid, discovered in the early 1960s, was the precursor of the fluoroquinolones, with norfloxacin and ciprofloxacin developed in the 1980s. Rifampin was introduced for tuberculosis in 1968.

The spectacular success of antimicrobial therapy led to widespread use and emergence of resistance. Pharmaceutical companies saw profit in new, more potent derivatives, which gave rise to broad-spectrum antipseudomonal penicillins and second-generation cephalosporins in the 1970s, with subsequent introduction in the 1980s of third-generation cephalosporins. Later, beta-lactamase inhibitors combined with broad-spectrum penicillins and carbapenems, new glycopeptides, newer macrolides, later-generation quinolones, linezolid [a new class of oxazolidinone], and glycylcyclines [tigecycline] were introduced. Currently, there are more than 100 antimicrobial compounds available.

1.1 Evolution of Resistance

It is important to understand the evolution of antimicrobial resistance in order to tackle the problem. It was initially thought that antimicrobial resistance was a modern, man-made phenomenon. Although penicillinase-producing bacteria were recognized soon after the discovery of penicillin, it was not recognized as a problem until clinical use of penicillin became widespread. By the end of the 1950s, 80% of *Staphylococcus aureus* isolated from hospital patients were penicillin resistant due to β-lactamase production [1]. This observation led to the development of penicillinase-stable penicillins [methicillin, cloxacillin, and oxacillin] which were introduced in the early 1960s to treat *S. aureus*. But resistance to methicillin was seen within a year or so of its clinical introduction. Thus, this was a sign that microbial resistance would be a problem, but in most cases this was overcome with development of new, more potent compounds.

Many agents were developed that initially failed to compete well in the marketplace. The lack of use kept resistance from emerging, and now they are being used more frequently to fill a niche. Vancomycin is one of these agents. It was approved for use in 1958, but it was used sparingly due to concerns of toxicity and efficacy. When methicillin-resistant *S. aureus* [MRSA] appeared in hospitals during the 1970s and then spread to other health-care facilities, vancomycin became more frequently used, beginning in the 1980s [3]. Similarly, polymyxins were developed in

the 1950s, but toxicity kept them from widespread use for systemic infections. Now they are gaining clinical use with infections caused by *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and carbapenemase-producing *Enterobacteriaceae* that are resistant to all other antibiotics [4]. Full vancomycin resistance among MRSA strains is rare, probably because mutational changes cause impaired fitness for MRSA as susceptibility decreases. As a result, vancomycin-intermediate resistance [VISA] is slowly becoming a problem. Resistance to polymyxins appears to be emerging readily with increased use.

Over the years, development of new antibiotics to counter resistant bacteria led to appearance of novel resistant strains to these new drugs, which become widespread with increasing use of the antibiotics. This pattern has been seen with every new class of antibiotic developed over the years. There is strong correlation between the frequency and quantity of antibiotics used in humans and animals and the rate of development of antibacterial drug resistance. The logarithmic growth of resistant bacteria since the 1970s is reflected by the number of β-lactamase enzymes identified during the antibiotic era. Before 1970, there were only several β-lactamase enzymes described, and now about 900 β-lactamase enzymes have been identified [2].

Mobile genetic elements, extrachromosomal self-replicating structures [plasmids] and transposons, found in bacterial cells provide a resistance threat that maybe unconquerable. Our understanding of horizontal transfer of bacterial resistance was heralded by the discovery of antibiotic resistance plasmids that could be disseminated by bacterial conjugation in the mid-1950s [2]. Since 1989 we have gained much greater knowledge of the genetics of bacterial resistance following the discovery of integrons. Integrons are versatile genetic elements, commonly found in bacterial genomes, that allow efficient capture and expression of exogenous genes. Plasmids and transposons are considered mobile integrons. Integrons play a major role in the spread of antibiotic resistance, particularly in Gram-negative pathogens; the majority of these pathogens carry integrons with resistant genes [5]. The process of microbial resistance is complex, and besides plasmids and DNA mutations [acquired or heritable and transferable], other mechanisms include biofilms, which harbor hypermutator bacteria that select for resistance more frequently, and phenotypic tolerance, a situation in which bacteria are not killed by antimicrobials [6]. Antibiotic pressure predisposes to resistance and tolerance. Despite the call for intensive research on mechanisms of microbial resistance and development of novel compounds to counteract the spread in 2012 [6], no innovative agent is on the horizon.

The origins, evolution, genetics, and biochemistry of antibiotic resistance have been studied over the last 60 years. Figure 1.1 outlines the history of antibiotic discovery and subsequent development of antibiotic resistance. The emergence of antibiotic resistance of pathogenic bacteria after antibiotic development and clinical use, plus the absence of resistance in bacteria of the pre-antibiotic era [7], suggested that resistance is a modern phenomenon. However, metagenomic analyses of authenticated ancient DNA from 30,000-year-old Beringian permafrost sediments identified genes encoding resistance to β-lactam, tetracycline, and glycopeptide

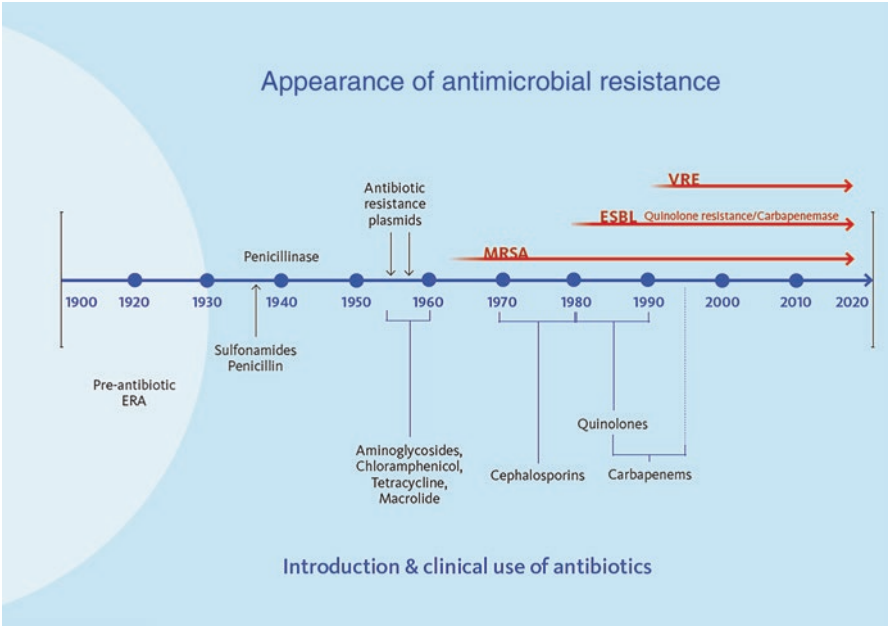

Fig. 1.1 Discovery of antibiotics and evolution of antimicrobial resistance. Abbreviations: MRSA methicillin-resistant *S. aureus,* VRE vancomycin-resistant enterococci, ESBL extended β-lactamase

antibiotics [8]. Thus, antibiotic resistance is a natural phenomenon that predates the modern selective pressure associated with clinical and animal use. This explains the rapid emergence of resistance to new antibiotics; resistance will continue to emerge with drugs now in development. Hence, it is predictable that new antibiotics will select for preexistent resistance determinants that have been present and circulating in universal, environmental microbial pangenome for hundreds of years [8]. Thus, the era of antibiotics has shifted naturally to the era of antibiotic resistance. Our challenge is to minimize the problem.

1.2 Defining the Problem

It is now evident that antimicrobial resistance is inevitable. Viable solutions are needed to postpone the inevitable; development of novel antibiotics is only a temporary remedy, especially since most "new" agents are chemical refinements of old ones. Of the 8 new antibiotics approved by the US Food and Drug Administration [FDA] since 2010, only one [bedaquiline for tuberculosis] is from a new drug class [9]. Thus, resistance to most of these agents will likely develop rapidly. A comprehensive, multipronged, coordinated approach is needed to combat

antimicrobial resistance. The World Health Organization [WHO] is best suited to lead the fight and is already involved in the process. What are the necessary steps to combat microbial resistance?

Curbing the global overuse of antimicrobials is probably the greatest challenge. Unnecessary use of antibiotics for humans and animals is a major concern, even after two decades of effort to reduce the flagrant abuse and overuse. A ban on nontherapeutic use of antibiotics in animals and agriculture has been recommended since 1969, but it has been very difficult to gain worldwide acceptance [4]. For example, the European Union banned use of antibiotics for growth promotion in animals in 2006, but this practice is still widespread in many other countries. Recent analyses estimate that from 2010 to 2030 global utilization of antibiotics in the livestock industry will increase by two thirds and that it will double in the growing economies of Brazil, China, India, Russia, and South Africa [10]. There appears to be no political will to ban antibiotics as growth stimulants for animal husbandry. Companies that use deception to provide antibiotics to animals under cover of different names should be charged hefty fines. As of 2010 only the Netherlands and Scandinavia had successfully reduced antibiotic resistance levels by enforcing antibiotic restrictions [4]. The Dutch government instituted a policy of requiring a 70% reduction of antibiotic use in animals between 2009 and 2015 and prohibited use of new antimicrobials. These initiatives resulted in a 56% reduction in animal antimicrobial use between 2007 and 2012 [11]. Thus, rollbacks can be achieved, but how will multidrug-resistant bacteria that developed in other countries be kept out of countries that restrict use?

The availability of inexpensive antibiotics is still largely uncontrolled in many developing countries, and these drugs can be obtained from pharmacies without a prescription. Even in Europe, persons can purchase antibiotics over the counter or through the Internet in 19 countries. In 12 countries antibiotics can be obtained on the black market or veterinary clinics [WHO Regional Office for Europe, antimicrobial resistance [http://www.euro.who.int/en/health-topics/disease-prevention/antimicrobial-resistance]. These practices continue to play a role in the abuse and overuse of antimicrobials. One approach may involve education. For example, enabling pharmacists to deliver accurate information and counseling on proper antibiotic should be implemented.

Antibiotic-resistant bacteria respect no borders; international travelers can acquire and spread these microbes. In a prospective longitudinal study of Dutch international travelers, 34% of 1847 travelers acquired extended β-lactamase-producing *Enterobacteriaceae* [ESBL] that persisted for 12 months in 11% of the respondents [12]. Among travelers to southern Asia, 75% acquired ESBL; the frequency was 89% with travelers to India. Antibiotic use is a strong predictor of carrying resistant genes, and travelers should be discouraged from using antibiotics for self-limited infection such as traveler's diarrhea.

1.3 Moving Forward

It has been argued that the lack of access to life-saving antibiotics is as important an issue as antibiotic resistance. It has been estimated that universal access to antibiotics could prevent 445,000 deaths out of 590,000 deaths from pneumonia [75% reduction] in 101 countries [13]. However, increased use of pneumococcal and *Haemophilus influenza* type B vaccines could prevent up to 11.4 million days on antibiotics – a 47% reduction in 75 countries. Carriage of multiresistant bacteria is not restricted to travelers or developing countries. In a study from Germany, of 4376 patients admitted to general wards, third-generation cephalosporin-resistant *Enterobacteriaceae* were detected from rectal swabs in almost 10% of patients immediately after admission [14]. Risk factors for presence of these multiresistant bacteria included prior antimicrobial treatment, travel outside Europe, stay in a long-term care facility, and use of proton pump inhibitors for gastroesophageal reflux.

Many high-income countries, including the US and parts of Europe, have created national plans as well as regulation to address antibiotic resistance issues. However, the brunt of the problem will be borne by low-income and middle-income countries that cannot afford the newer, expensive drugs.

Health care-associated infections are a major source of the problem, and intensive care units are "generators" of resistant bacteria. The empiric institution of broad-spectrum antimicrobials for infections in very ill patients is the force behind this problem. There is a great need for inexpensive, rapid and reliable microbiological/molecular diagnostic tests to alleviate some of the empiric overuse of antibiotics in health-care settings. Conventional culture methods usually take >2 days for identification and susceptibility determination, but a rapid multiplex polymerase chain reaction [rmPCR] can provide identification in 1.3 h [15]. Moreover, rapid point-of-care tests are needed to distinguish viral and noninfectious inflammatory conditions from bacterial infections. Antibiotic stewardship in hospitals in North America and other countries reduces antibiotic use, improves patient outcome, decreases adverse events such as superinfection with *Clostridium difficile* and antibiotic resistance [to a modest degree], and is cost-effective [16]. Wider adoption of stringent stewardship programs is needed for all community hospitals globally, but it will be difficult to implement in resource-poor countries.

Inappropriate antibiotic use is still widespread for acute upper respiratory tract infections despite attempts to curb the abuse in outpatient primary-care practice by education. Efforts had been made to improve prescription behavior and provide guidelines for antibiotic use across the USA, but success has been limited. Overall antibiotic use for acute respiratory infections has significantly declined in children [17], but use in adults remains high, especially for broad-spectrum antibiotics and macrolides [18], as confirmed by recent data from the Veterans Affairs health system [19]. A review of outpatient antibiotic use in the USA reported that about 13% of all visits [about 154 million per year] resulted in antibiotic prescriptions of which at least 30% were considered unnecessary [20]. Thus, unnecessary use of antibiotic

for acute respiratory or minor infections remains high. One method worth exploring is for public health officials and medical associations to send frequent e-mail messages to primary-care physicians concerning the dangers of antibiotic overprescribing [no proven benefit]. Another option is to provide financial incentives for not prescribing antibiotics by medical insurance companies. Delayed prescribing [delay between receiving the prescription and collecting the drugs] has shown some success in reducing antibiotic use [21]. In Thailand, the Antibiotic Smart Use program has shown that alternative treatment options were important in restricting antibiotic outpatient use, such as oral rehydration and zinc for diarrheal diseases, and herbal drugs packaged in antibiotic-like capsules for viral upper respiratory infections [22].

What is being implemented to combat antimicrobial resistance? Several countries are now taking steps to improve antibiotic prescribing, and the World Health Day in 2011 was dedicated to antimicrobial resistance. The Infectious Disease Society of America in 2011 outlined a road map to counter antimicrobial resistance: regular surveillance and data collection on resistance patterns and prevalence, universal antibiotic stewardship for hospitals, and provision of research and development [R&D] incentives for drug companies to facilitate licensing of novel antimicrobials [23]. But as pointed out, new antimicrobials will simply delay the problem. More recently, the Presidential Advisory Council on Combating Antibiotic Resistant Bacteria and Innovative Medicines Initiative suggested public-private partnership to provide financial resources to assist R&D [24]. However, proposed funding cuts by the Trump administration to the CDC's antimicrobial resistance [AMR] fund by 14% and the NIAID by 23% threatens the progress in the fight against antimicrobial resistance [Boucher et al. Proposed US funding cuts threaten progress on antinicrobial resistance. Ann Int Med 2017; 167:738–9]

The WHO or United Nations could provide leadership to facilitate multinational global collaboration in this effort. The WHO has just released priority pathogens list for R&D of new antibiotics: priority 1 [critical] includes carbapenem-resistant *A. baumannii, P. aeruginosa*, and multiresistant *Enterobacteriaceae;* priority 2 [high] includes vancomycin-resistant *Enterococcus faecium* [VRE], vancomycin-intermediate MRSA, clarithromycin-resistant *Helicobacter pylori*, fluoroquinolone-resistant *Campylobacter* and *Salmonella* spp., and third-generation-resistant *Neisseria gonorrhoeae*; and priority 3 [medium] includes penicillin-non-susceptible *Streptococcus pneumonia,* ampicillin-resistant *H. influenzae*, and fluoroquinolone-resistant *Shigella* spp. [25]. R&D for new drugs for multiresistant tuberculosis and artemisinin-resistant malaria were previously noted as high priority by the WHO. Development of new antibiotics to combat resistant bacteria is a short-term solution to meet current needs. Resistance will eventually develop to these agents as well. Innovative biological substances for therapeutics where resistance is unlikely to develop are needed; research in this area should be encouraged. This could include use of probiotics to counter and prevent enteric colonization of resistant bacteria or bacteriophages to lyse colonized resistant organisms such as MRSA and VRE.

On a global scale, there is much that can be done to reduce the risk of infection and decrease the need for antibiotics, mainly in low-income countries. These activities include wider and more universal use of vaccines, such as the pneumococcal conjugate vaccine, *H. influenzae* type B vaccine, pertussis vaccine in pregnancy, rotavirus vaccine, and measles vaccine, which could save lives and dramatically reduce the use of pediatric antibiotics worldwide. Yearly universal influenza vaccination of children and adults could also reduce the outpatient use of antibiotics for respiratory infections in all countries. A major problem in developing and low-income countries is poor sanitation and lack of clean water, which predisposes persons to a variety of infectious diarrheas that leads to antibiotic overuse and increased antimicrobial resistance. In general, better infection control practices in health-care institutions could reduce the need for antibiotics and lead to reduced prevalence of resistance. In the USA alone, it is predicted that within 5 years multiresistant bacteria could cause 340,000 deaths per year, but immediate implementation of a national intervention strategy involving all elements of the healthcare network [hospitals, nursing homes, etc.] through infection control and universal antibiotic stewardship could save 37,000 lives and avert 619,000 infections over the next 5 years [26]. Current estimates are that antibiotic-resistant bacteria cause 2 million illness and 23,000 deaths each year in the USA with a annual cost to the health care system of over $20 billion [CDC. Antibiotic resistance threats in the United States, 2013. www.cdc.gov/drugresistance/pdf/ar-trhreats-2013-508.pdf]

1.4 Concluding Thoughts

Should antibiotic regulation and stewardship be instituted at the national societal level? Public education on antibiotic use and national guidelines have had limited impact. Policies that involve withdrawal of subsidies for expensive antibiotics can have an effect as shown in Australia with quinolone prescriptions [27]. Overuse and abuse of antibiotics in humans and animals is causing pollution of the coastal aquatic ecosystems with antibiotic-resistant pathogens, and concern for effects on human and animal health should be of similar concern as global climate change. A recent study from China has documented widespread pollution of the estuaries along the coastal environment of China with over 200 different antibiotic-resistant genes that affect almost all major classes of antimicrobials [28]. The United Nations has now recognized the universal importance of antimicrobial resistance on human and animal health. On September 21, 2016, a high-level meeting was convened with Heads of State for commitment to taking a broad, coordinated approach in addressing the issue of antimicrobial resistance across multiple sectors of human and animal health and agriculture [http://via-jwat.ch/2nb4Dec2]. In summary, antimicrobial resistance is a global threat to humanity with no immediate end in sight and it is considered an intetrnational crisis. The cost in lives and to health care systems worldwide are huge and will continue to rise in the foreseeable future. Evolution science indicates that

microbes will continue to develop resistance to future antimicrobials and cannot be prevented, but we can limit the speed and magnitude of antimicrobial resistance by a coordinated, multiprong approach.

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Part I
Examples of Resistance

Chapter 2
Antimicrobial Resistance Among *Streptococcus pneumoniae*

Catia Cillóniz, Carolina Garcia-Vidal, Adrian Ceccato, and Antoni Torres

2.1 Introduction

Antibiotic resistance is a direct result of antibiotic consumption [1, 2]. In the United States, it is estimated that antibiotic resistance is responsible for more than 2 million infections and 23,000 deaths each year, with a direct cost of $20 billion and additional productivity losses of $35 billion [3, 4]. Data from Europe showed that approximately 25,000 deaths are attributable to antibiotic-resistant infections, with a related cost of $1.5 billion annually [5]. The use of antibiotics in primary care is high; the most frequent indications for their use are respiratory tract infections [6].

Streptococcus pneumoniae (pneumococcus) is the leading cause of community-acquired pneumonia and is considered to be a major cause of death of children under 5 years old worldwide. In a recent report on global antibiotic resistance, published by the World Health Organization (WHO) in 2014, pneumococcus was considered to be one of the nine bacteria of international concern [7]. Other infections caused by pneumococcus include bacteremia, otitis media, and meningitis. In bacterial meningitis, pneumococcus is associated with mortality rates ranging from 16% to 37%. About 30–50% of adult survivors present permanent residual symptoms [8, 9]. The study by Van Boeckel et al. [10], regarding global antibiotic consumption from 2000 to 2010, reported that it grew by more than 30%, from approximately 50

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I. W. Fong et al. (eds.), *Antimicrobial Resistance in the 21st Century*, Emerging Infectious Diseases of the 21st Century, https://doi.org/10.1007/978-3-319-78538-7_2

billion to 70 billion standard units. Penicillins, cephalosporins, and macrolides were the three most consumed antibiotics in 2010. The three countries that consumed the most antibiotics in 2010 were India with 13 billion standard units, China with 10 billion, and the United States with 7 billion standard units (a standard unit is the number of doses sold; the IMS Health database identifies a dose as a pill, capsule, or ampoule).

Resistance of pneumococcus against ß-lactams and macrolides is a major concern worldwide. For example, in Southern European countries, the prevalence of this resistance may be above 20% [11, 12]. The increased utilization of antibiotics, the dissemination of several resistant clones, the ability of pneumococcus to undergo serotype replacement and capsular switching, and the horizontal transmission of antibiotic resistance genes make this pathogen very difficult to control. This chapter summarizes currently available information regarding pneumococcal antibiotic resistance.

2.2 Basis of Antimicrobial Resistance in *Streptococcus pneumoniae*

The nasopharyngeal carriage rate of pneumococcus is higher in children, mainly during the first years of life (nasopharyngeal carriage rates range from 20% to 50% in healthy children). In contrast, with the healthy adult population, nasopharyngeal carriage rates range from 5% to 30%. Transmission of pneumococcus from children to household contacts or adults is the principal cause of nasopharyngeal carriage and the spread of antibiotic-resistant clones. Pneumococcus undergoes genetic transformation and can acquire DNA from other *streptococci*; during asymptomatic nasopharyngeal carriage, selection of resistant pneumococcus occurs especially in children, because they carry pneumococcus more often and for longer periods. Moreover, children are more frequently exposed to antibiotics. Interestingly, the use of fluoroquinolones in children is limited, because in animal models using young animals, development of articular cartilage damage in weight-bearing joints has been described [13, 14]. This adverse effect may explain why the rate of pneumococcus resistance to fluoroquinolones remains low. A direct correlation has been reported between the use of the fluoroquinolone antibiotics and prevalence of fluoroquinolone resistance in pneumococcus [15–18]. Table 2.1 describes the principal mechanisms of resistance to this antibiotic class by pneumococcus; Fig. 2.1 shows the timeline of antimicrobial resistance of pneumococcus.

Table 2.1 Basis of antimicrobial resistance in *Streptococcus pneumoniae*

Antibiotic	Effect	Mechanism resistance	Risk factors
ß-lactam	Inhibit the final steps of peptidoglycan synthesis (cell wall) by binding to high-molecular-weight penicillin-binding proteins (PBPs)	Alteration of the cell wall PBP, resulting in decreased affinity for penicillin	Previous antibiotic use of ß-lactam antibiotics in the last 3–6 months
			Prior hospitalization in the last 3 months
			Attendance in a day-care center
			Residence in long-term care facilities
			Chronic pulmonary disease mainly chronic obstructive pulmonary disease (COPD)
			Human immunodeficiency virus (HIV) infection
Macrolides	Inhibit protein synthesis by binding 23S ribosomal target sites in bacteria	Target site (ribosomal) alteration by an enzyme that methylates 23S rRNA subunits and is encoded by the *erm*B (erythromycin-resistance methylase) gene: high level of macrolide resistance and complete cross-resistance to macrolide lincosamide streptogramin B type Active efflux pumps encoded by the *mef*E or *mef*A (macrolid efflux) gene: low-level of resistance only to macrolides	Previous hospital admission
			Resistance to penicillin
			Previous use of macrolides
			Recurrent otitis media
			Cases related to serotypes such serotype 6A, 6B, 14, 23F, 19F
			Attendance in day-care centers
Fluoroquinolones	Inhibit DNA synthesis by interacting with intracellular drug targets, DNA gyrase, and topoisomerase IV	Spontaneous point mutations in the quinolone resistance-determining region (QRDR)	Prior use of fluoroquinolones
			Chronic obstructive pulmonary disease (COPD)
			Residence in a long-term center
			Elderly persons
			Cerebrovascular disease

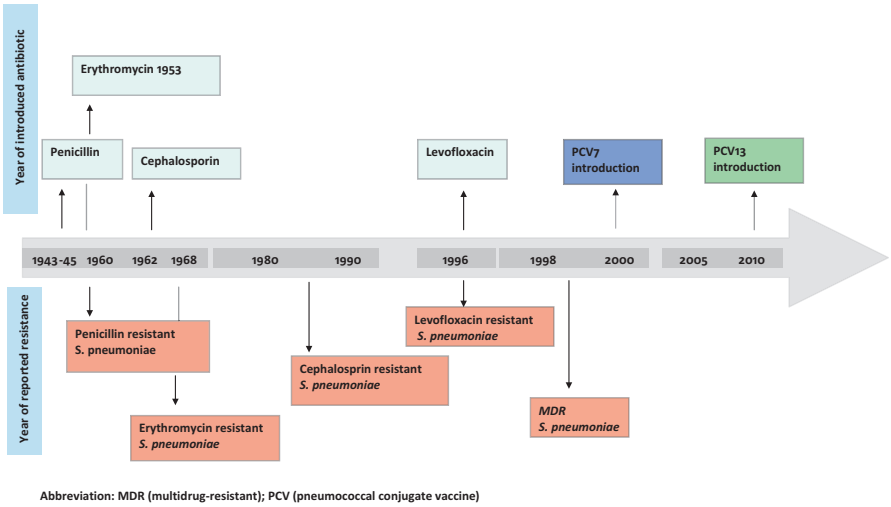

Fig. 2.1 Timeline of antibiotic resistance of *Streptococcus pneumoniae. Abbreviation:* MDR multidrug-resistant, PCV pneumococcal conjugate vaccine

2.2.1 *Penicillin and β-Lactam-Resistant* Streptococcus pneumoniae

β-lactam antibiotics include penicillins, cephalosporins, and carbapenems. These compounds inhibit the final steps of peptidoglycan (cell wall) synthesis by binding to high-molecular-weight penicillin-binding proteins (PBPs). These antibiotics have a broad spectrum of activity against Gram-positive and Gram-negative bacteria. β-lactam antibiotics are considered to be time-dependent killers, meaning that increasing concentration significantly above the minimal inhibitory concentration (MIC) does not increase killing. The compounds have efficacy when concentrations are approximately four times the MIC of the microorganism. To determine the efficacy of β-lactam antibiotics, the preferred pharmacodynamic parameter is time (T) > MIC. For the majority of β-lactams, effectiveness is achieved at T > MIC for more than 40–50% of the dosing interval [19].

Amino acid alterations of the cell wall PBP result in decreased affinity for penicillin, which is the main mechanism of penicillin resistance. Several PBPs have been identified, including 1a, 1b, 2x, 2a, 2b, and 3. Alterations to the properties of PBPs are brought about by transfer of portions of the genes encoding the PBPs from other streptococcal species, resulting in mosaic genes [20].

The Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) define penicillin resistance of pneumococcus via empirical breakpoint determination [21]. Breakpoints established by the CLSI in 2012 for pneumococci defined penicillin resistance as:

- *Infections other than meningitis*: susceptible < 2 μg/ml, intermediate < 4 μg/ml, and resistant ≥ 8 μg/ml
- *Meningitis*: susceptible ≤ 0.06 μg/ml, intermediate ≥ 0.12 μg/ml, and resistant ≥ 2 μg/ml

The breakpoints for penicillin susceptibility are based on three criteria: microbiological data, pharmacokinetic/pharmacodynamics of β-lactam antibiotics, and clinical outcome of pneumococcal infections. In a patient treated with a dose of intravenous penicillin, the levels achieved in the lung will be 100 times greater than those reached in the brain. Thus, use of low concentrations of β-lactam for pneumococcal infections, such as otitis media or meningitis, could lead to treatment failure. In contrast, with pulmonary infections the levels of β-lactam reached are generally sufficient to clear infection. Therefore, treating the same pathogen will require different doses of a given β-lactam depending on the site of infection. Likewise, we must considerer pneumococcal resistance in different sites of infection differently, and breakpoints for resistance will be different.

2.2.2 *Macrolide Resistance in* Streptococcus pneumoniae

Macrolides inhibit bacterial protein synthesis by binding to the 23S rRNA component of the 50S ribosomal subunit in bacteria. There are two main mechanisms of macrolide resistance in pneumococcus. One involves target-site (ribosomal) alteration by an enzyme that methylates 23S rRNA, an enzyme that is encoded by the *erm*B (erythromycin-resistance methylase) gene. The resistance phenotype is called MLS_B (macrolide, lincosamide, streptogramin B type) and is responsible for a high level of macrolide resistance. In a low proportion of cases, *erm*B gene variation that modifies the binding site for macrolides and lincosamides confers complete cross-resistance to clindamycin [22].

The second mechanism of resistance involves active efflux pumps encoded by the *mef*E or *mef*A (macrolide efflux) genes. These mutations result in low-level resistance to macrolides but not to the other two agents. The *mef*A gene is predominant in Europe, whereas *mef*B gene predominates in North America.

The relative frequency of the two macrolide resistance mechanisms varies by geographic region [23–36] (Table 2.2): in European countries, approximately 90% of the isolates of pneumococcus presented the MLS_B phenotype, which is associated with high levels of macrolide resistance, whereas in North America between 50% and 65% of the resistant pneumococcus isolates contained efflux mutations that were associated with lower levels of macrolide resistance [12]. In Asian countries, strains that showed both mechanism of resistance are a major concern, with between 12% and 40% of the resistant isolates displaying both mechanisms [31, 37–40]. In South American countries, isolates reporting both mechanisms vary between 4% and 20% [34, 41]. Worldwide resistance to macrolides in pneumococ-

Table 2.2 Worldwide genotype distribution of macrolide resistance in *Streptococcus pneumoniae*

Country/year of study	No of isolates tested	% genotype distribution	Reference
Europe			
Turkey – 2008–2009	80	44% *ermB* 11% *mefA* 44% *ermB* + *mefA*	Sirekbasan et al. [23]
Spain – 1999–2007	187	90% *ermB* *9% mefE* *1% mefA*	Calatayud et al. [24]
Greece – 2005–2009	1105 (carriers)	29% *ermB* 24% *ermB* + *mefE* *42% mefE* *5% mefA*	Grivea et al. [25]
Belgium – 2007–2009	249	90% *ermB* 2% mefE 3% *ermB* + *mefE*	Lismond et al. [26]
North America			
Canada– 1998–2004	865	47% *mefA* 43% *ermB* 6% *ermB* + *mefA*	Wierzbowsk et al. [27]
USA – 2007	4535	18% ermB 62% *mef* (A/E) gene 15% *mef (A/E)* + *ermB*	Hawkins et al. [28]
Asia			
Lebanon – 2010–2015	132	38% *ermB* 29% *mef (A/E)* 31% *mef (A/E)* + *ermB*	El Ashkar et al. [29]
Japan – 2013–2014	960	76% *ermB* *32% mef (A/E)* *11% mef (A/E)* + *ermB*	Kawaguchiya et al. [30]
Iran	186	44% *ermB* *16% mef (A/E)* *40% mef (A/E)* + *ermB*	Azadegan et al. [31]
South Korea – 2008–2009	2184	49% *ermB* *20% mefA* *30% mefA* + *ermB*	Kim et al. [32]
South America			
Colombia – 1994–2011	225	98% *ermB* *2% ermB* + *mef E*	Ramos et al. [33]
Argentina – 2009–2010	126	77% *mefA* 19% *ermB* *4% mefA* + *ermB*	Reijtman et al. [34]
Africa			
Morocco – 2007–2014	655	90% ermB 6% *mef E* 35 *ermB* + *mefE*	Diawara et al. [35]
Tunisia – 1998–2004	100	88% *ermB* *12% mefA*	Rachdi et al. [36]

cus has increased recently and is associated with the extensive global use of macrolides, principally for community-acquired respiratory tract infections.

2.2.3 *Fluoroquinolone Resistance in* Streptococcus pneumoniae

Fluoroquinolones inhibit DNA synthesis by forming drug-enzyme-DNA complexes with DNA gyrase and topoisomerase IV. The main mechanism of resistance to fluoroquinolones is mediated by amino acid substitutions in these two essential enzymes. As with other bacteria, resistant pneumococcus exhibits spontaneous point mutations in a region of GyrA (gyrase) and ParC (topoisomerase IV) called the quinolone resistance-determining region (QRDR). Some pneumococci may also exhibit an efflux-mediated mechanism, although the clinical significance is unclear. The presence of dual mechanisms of resistance has been reported in strains having high levels of resistance, often from cases of treatment failure [12, 42, 43]. In some cases multiple mutations in the target proteins accumulate [44], which supports the idea that repeated antimicrobial challenge gradually erodes the effectiveness of fluoroquinolones.

2.2.4 *Resistance to Other Antibiotics*

Currently, the European Respiratory Society (ERS)/European Society of Clinical Microbiology and Infectious Diseases (ESCMID) guidelines recommend the use of tetracyclines (broad-spectrum bacteriostatic antibiotics that act by binding to the 30S ribosomal subunit and thereby inhibit bacterial protein synthesis) as a first choice for treatment of lower respiratory infections [45]. On the other hand, the American Thoracic Society (ATS)/Infectious Disease Society of America (IDSA) [46] recommends doxycycline for healthy patients with pneumococcal community-acquired pneumonia with low risk of drug-resistant pneumococcus and for patients with penicillin allergy [46].

The ribosomal protection protein (RRP), which binds to the ribosome and forces the drug from its binding site, is the main resistance mechanism of pneumococcus to tetracycline and doxycycline. This form of resistance is mediated by an alteration in the *tetM* gene. In 2012 a study by Dönhöfer et al. showed that TetM can directly remove and release tetracycline from the bacterial ribosome by an interaction between domain IV of the 16S rRNA and the tetracycline binding site [47].

Due to the increase in resistance of pneumococcus to several antibiotics over the last decade and several reported cases of treatment failure, vancomycin, a glycopeptide antibiotic that acts by inhibiting proper cell wall synthesis, was added to the standard antibiotic treatment for pneumococcal meningitis. There are several reports

about treatment failure with vancomycin due to the emergence of vancomycin-tolerant pneumococcus. However, there is no report of vancomycin-resistant pneumococcus.

Tolerant pneumococcus survives but does not replicate during therapy with antibiotics. When antibiotic therapy is finished, pneumococci are able to resume growth. This phenomenon is associated with a reduction of autolysin activity, which is part of an endogenous bacterial cell-death pathway [48].

2.2.5 *Multidrug-Resistant (MDR)* Streptococcus pneumoniae

It is estimated that the worldwide prevalence of multidrug-resistant (MDR) *S. pneumoniae* is high, ranging from 36% in Asia to 15% in Europe [12, 49], although the prevalence is geographically variable. Multidrug resistance in pneumococcus is defined as resistance to three or more antibiotic classes. Pneumococcus MDR generally involves reduced susceptibility to β-lactams, macrolides, tetracyclines, and sulfonamides; resistance to quinolones in MDR pneumococcus is less frequent.

The majority of MDR strains of pneumococcus are derived from resistant genetic clones, with a few clones dominating the pneumococcus isolates on a worldwide basis [49]. Data from European studies show that the MDR phenotype is most frequent among serotypes 1, 14, 15A, 19A, 19F, and 23F [50]. In the United States and Canada, however, the most frequent serotypes associated with MDR pneumococcus are 15A, 15B, 15C, 22F, 23A, 33F, and 35B [51–54]. Studies from Asian countries report that 11A, 15A, 19A, and 19F are the serotypes most frequently associated with MDR pneumococcus [53, 55, 56]. In African countries, 19A and 19F are the most frequently associated with MDR pneumococcus [57]. Collectively these data indicate that the spread of MDR pneumococcus globally has high variability among countries. The introduction of conjugate pneumococcal vaccines contributed to the large reduction of the burden of pneumococcal disease and the reduction of antimicrobial resistance in *S. pneumoniae*. Nevertheless, the emergence of non-vaccine serotypes that show multidrug resistance is a major concern.

2.3 Risk Factors for Infection by Drug-Resistant Pneumococcus

Several studies identify factors associated with an increased risk of infection by pneumococcus resistant to the most frequently used antibiotics. The three main factors are host factors (age, comorbidities), environmental factors (geographic regions with high population density and proximity to high-resistance regions, day-care centers with children, long-term nursing facilities with elderly persons), and factors related to the use of antibiotics (previous antibiotic therapy, duration of antibiotic therapy).

2.3.1 Risk Factors Related to Penicillin Resistance

The use of a β-lactam antibiotic in the previous 3–6 months is the main risk factor associated with penicillin-resistant pneumococcal infection [12, 21, 58–60]. A study by Ruhe et al. [61] regarding the duration of previous antibiotic treatment and its association with penicillin-resistant bacteremic infection revealed that the risk depends on the class of prior antibiotic exposure and the duration of therapy. The study analyzed 303 patients with pneumococcal bacteremia. In 98 (32%) cases of bacteremia caused by penicillin-non-susceptible *S. pneumoniae*, statistical analysis showed that the use of β-lactams, sulfonamides, and macrolides within the last 1–6 months before presentation was associated with penicillin-non-susceptible *S. pneumoniae* bacteremia ($p < 0.05$). In a second study with the same bacteremic population, Ruhe et al. [62] identified 33 (11%) cases of bacteremia caused by high-level resistant *S. pneumoniae*. In these cases, three risk factors for high-level penicillin-resistant pneumococcal infection were identified: β-lactam antibiotic use in the previous 6 months, previous residence in a risk area (defined as stays in day-care facilities, prisons, homeless shelters, nursing homes, or other long-term care facilities), and respiratory tract infection in the previous year.

Age extremes (<5 years or > 65 years) are a recognized risk factor for penicillin-resistant pneumococcal infections [12, 17, 63]. As pointed out above, nasopharyngeal carriage of pneumococcus in healthy children ranges from 20% to 50%, and in the healthy adult population, nasopharyngeal carriage rates range from 5% to 30% [64, 65]. Consequently, it is not difficult to understand why several studies have shown that day-care centers are a risk factor for colonization and infection of children due to penicillin-resistant pneumococcus [66–68]. Similarly, institutionalized adults, especially those older than 65 years of age, have increased risk for penicillin-resistant pneumococcal infections [69]. Moreover, the presence of specific comorbidities, such as human immunodeficiency virus (HIV) and chronic pulmonary disease, especially chronic obstructive pulmonary disease (COPD), is a recognized risk factor for penicillin-resistant pneumococcal infection [58].

Several studies have addressed the association between antibiotic consumption and resistance selection. A study by van Eldere et al. [70], concerning the impact of antibiotic usage in ambulatory patients in Belgium, involved 14,448 *Streptococcus pneumoniae* isolates collected between 1994 and 2004. This work showed a modest relationship between consumption and resistance; additional factors were high population density and proximity to high-resistance regions, particularly for the development of multiple resistances in pneumococcus. In this Belgian population, the highest levels of resistance were to erythromycin, followed by resistance to tetracycline and penicillin; the highest prevalence of co-resistance to two antibiotics was for erythromycin-tetracycline.

In 2001 the prevalence of non-susceptibility to erythromycin in the Belgium study peaked at 36.7% and stayed mostly stable until 2004. Prevalence of non-susceptibility to tetracycline reached its highest level (31.7%) in 2000; penicillin non-susceptibility hit 17.7% in 2000 and declined to 11.6% in 2004. The prevalence

of co-resistance to erythromycin-tetracycline was 26.7% in the period 2002–2003 and decreased slightly to 25.9% in 2004.

The overall antibiotic consumption in Belgium was 26.4 DID (daily doses per 1000 inhabitants per day) in 1995 and decreased slightly to 23.3 DID in 2004. The most frequently consumed antibiotics were broad-spectrum penicillins (9 DID in 2000 to 6.4 DID in 2004). Macrolides showed a similar pattern (6 DID in 2000 to 4.5 DID in 2004) as did cephalosporins (4.7 DID in 2000 to 3.7 DID in 2004). Tetracycline was the second most prescribed class in 1995, but usage declined in 2004 to 1.9 DID. Overall, consumption and resistance were roughly parallel.

Another study concerned antimicrobial drug use in ambulatory care and resistance trends in Europe [71] for 21 countries during the period 2000–2005. The work showed that variation in consumption coincided with the prevalence of resistance at the country level [71]. Antimicrobial drug use decreased (>15%) in Bulgaria, Czech Republic, France, and Germany, but it increased (>15%) in Croatia, Denmark, Greece, and Ireland. The most widely used antibiotics were penicillins (including broad-spectrum penicillins). Macrolides were the second most widely used category; the third consisted of cephalosporins, monobactams, and carbapenems. Fluoroquinolones occupied the fourth position. Four (France, Luxemburg, Belgium, and Portugal) of the six countries reporting the highest antimicrobial usage (Greece, France, Luxembourg, Portugal, Croatia, and Belgium) also reported the highest resistance proportions.

An interesting, small, case-controlled study about penicillin dust exposure with pharmaceutical workers in Tehran (Iran) reported that the workers in the penicillin production line carried a greater percentage of resistant pneumococcus [72]. The study included 60 cases (workers on a penicillin production line) and 60 controls (workers in food production), and data were obtained via survey, air sampling, and throat swab. In the penicillin production line arm of the study, the mean overall concentrations of penicillin dust were 6.6 mg/m^3, while it was 4.3 mg/m^3 in the food production line ($p = 0.001$). *S. pneumoniae* was detected in 45% (27) individuals in the dust-exposed group, 92.6% of which showed penicillin resistance. In the control group, *S. pneumoniae* was detected in 35% of the subjects, while 71.4% of the *S. pneumoniae*-positive cases were drug resistant ($p = 0.014$).

2.3.2 Risk Factors Related to Macrolide Resistance

Recent therapy by macrolides is the main risk factor for macrolide-resistant nasal colonization and pneumococcal infection [1, 12, 73, 74]. The study by Dias et al. [75], which evaluated the role of antimicrobial and vaccine use in the trends of resistance to penicillin and erythromycin in Portugal from 1994 to 2004, found that the use of macrolides was the main factor associated with an increase of

penicillin and erythromycin non-susceptible isolates among adults ($p < 0.01$) and erythromycin non-susceptible isolates among children ($p = 0.006$). The study also suggested that the heptavalent vaccine is failing to reduce antimicrobial resistance, possibly due to the increased consumption of azithromycin ($p = 0.04$). Other works showed that there is an increased risk of macrolide-resistant infection in cases related to certain pneumococcus serotypes, in particular 6A, 6B, 11A, 14, 23F, and 19F [76, 77].

Other important risk factors are age below 5 years [78–81], attendance in a day-care center [82–84], middle ear infection [85–87], and nosocomial acquisition [26]. As with β-lactams, there is strong evidence correlating the prevalence of macrolide resistance of pneumococcus and overall macrolide consumption within specific geographic areas [70, 71, 88].

2.3.3 Risk Factors Related to Fluoroquinolone Resistance

Previous exposure to fluoroquinolones is considered the main risk factor for fluoroquinolone resistance [89–92]. Other risk factors, reported worldwide, are COPD, nosocomial acquisition, and residence in a nursing home [43, 93, 94]. A retrospective review of cases of invasive pneumococcal infections in adults in Spain reported that residence in public shelters (OR 26.13, $p = 0.002$), previous hospitalization (OR 61.77, $p < 0.001$), human immunodeficiency virus (HIV) infection (OR 28.14, $p = 0.009$), and heavy smoking (OR 14.41, $p = 0.016$) are risk factors associated with acquiring an infection by levofloxacin-resistant pneumococci [95–97].

2.3.4 Risk Factors Related to Multidrug-Resistance

The reported risk factors for multidrug-resistant pneumococcal infection are extremes in age (< 2 years and > 65 years), presence of co-morbidities, such as chronic heart disease, chronic lung disease, chronic liver disease, chronic renal disease, prior exposure, especially repeated exposure, to antibiotic therapy in the previous 3 months, and being an immunosuppressed host [21, 46, 49, 66, 98–100]. Also, infections with pneumococcal serotypes such as 6A/B, 19A, 19F, 15A, 35B, 23A, 22F, and 33F were risk factors. Of these, the strongest risk factor is repeated exposure to antibiotic therapy. Figure 2.2 provides a schematic explanation for how antibiotic resistance arises and spreads in bacterial populations. We conclude that the increasing prevalence of multidrug-resistant strains complicates treatment options for *S. pneumoniae* infections and in some cases resistance leads to treatment failure.

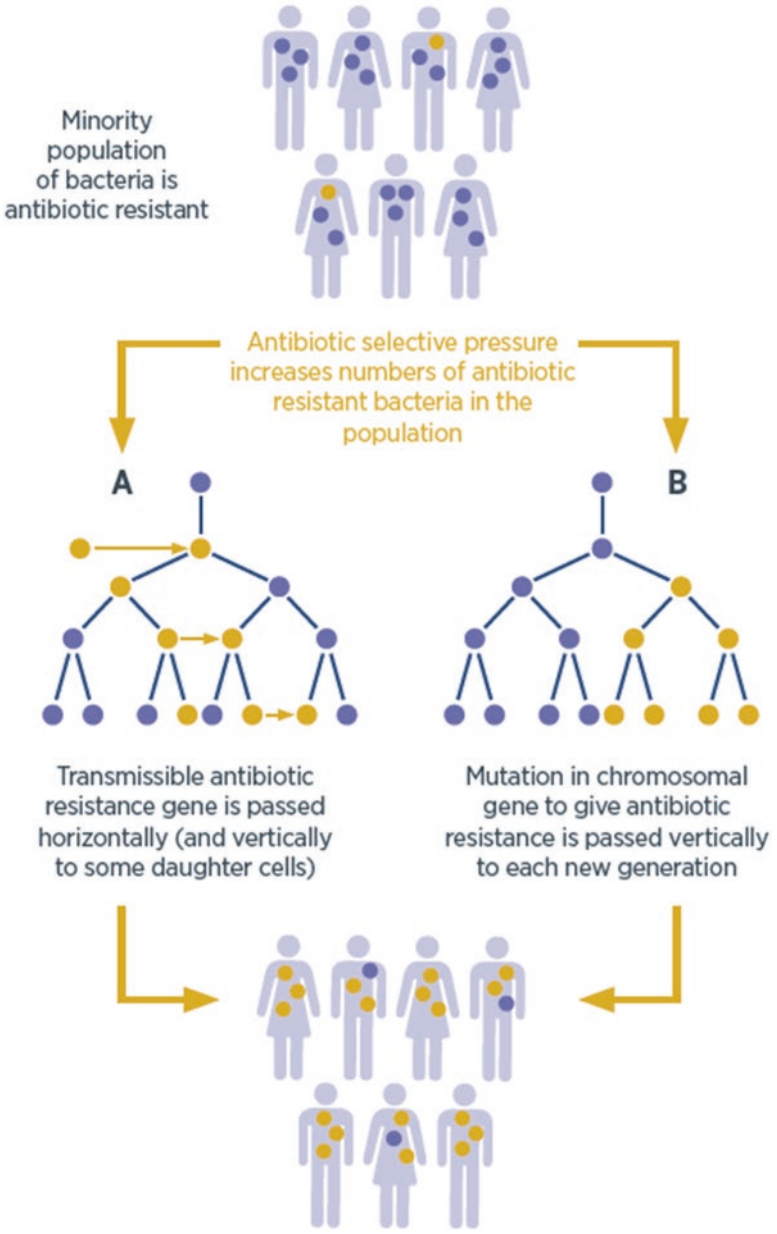

Fig. 2.2 How antibiotic resistance arises and spreads in bacterial population. (Figure reproduced by the permission of the author: Laura Piddock and Victoria Wells. Longevity Bulletin: Antimicrobial Resistance, Chapter 3: How antimicrobial resistance emerges. Issue 8, May 2016)

2.4 Pneumococcal Serotypes and Antibiotic Resistance

There are 98 reported pneumococcal serotypes (capsule type); 92 were identified using the Quellung method, and the additional serotypes were identified using molecular techniques [101–103]. These serotypes are grouped into 48 serogroups based on their antigenic similarities [104]. Several epidemiological studies suggest that relationships exist between specific serotypes/serogroups and the age of the host, site of infection, comorbidities, geographic region, pneumococcal invasiveness, and disease severity [105–108]. As pointed out above, serotype differences also relate to antimicrobial resistance. The differing behavior among serotypes may reflect differences in nasopharyngeal carriage, with the highest rates in children, especially in the first year of life. As pointed out above, risk factors for nasopharyngeal carriage in children include winter season, age below 6 years, presence of younger siblings, and attendance in day-care centers. In adults, risk factors for nasopharyngeal carriage include cigarette smoking, asthma, and acute upper respiratory infection [64, 109, 110].

Colonization in children may persist for a mean of 4 months, but it is much shorter in adults, usually 2–4 weeks [16]. This long period of carriage and the frequent exposure to antibiotics by children explain why they are considered the main source of resistant strains of pneumococcus [99].

2.5 Global Resistance Trends

In recent decades there has been a global acceleration in pneumococcal antibiotic resistance that coincides with the increased use of antibiotics [2]. The report, *Antibiotic Resistance Threats in the United States, 2013* [111], highlights the importance of drug-resistant pneumococcus. This report covers bacteria causing severe human infections and the antibiotics used to treat those infections. The main objective of this report was to provide an overview of the complex problem of antibiotic resistance and to encourage immediate action to keep the situation from getting worse. In this report the CDC prioritized bacteria into one of three categories: urgent threats, serious threats, and concerning threats. Drug-resistant *S. pneumoniae* was considered to be a serious threat. Pathogens in this category require prompt and sustained action.

Navarro et al. [112], in a 2010 surveillance report on invasive pneumococcal disease in 26 EU/EEA countries, considered isolates with MIC $\geq$ 0.12 mg/L as non-susceptible to penicillin (this cutoff value is for meningeal isolates and is the most widely used for surveillance studies). The highest rates of non-susceptibility to penicillin were found in Romania (42.2%), Cyprus (36.4%), and France (27.5%). The highest rates of non-susceptibility to cefotaxime were found in Romania (23.8%) and Ireland (9.3%).

The European Antimicrobial Surveillance, published in 2014, showed that of the 10,456 invasive pneumococcal disease cases reported by 28 EU/EEA countries, Romania, Spain, and Croatia showed the highest rates of non-susceptibility to penicillin (47%, 28%, and 26%, respectively, for these countries). The lowest rates were reported for Cyprus, Belgium, and the Netherlands, at 0%, 1.3%, and 2.1%, respectively [95]. We note that these surveillance data might not be strictly comparable among all countries, as the clinical breakpoints used to determine penicillin susceptibility differed, depending on guidelines used and the site of infection [113]. Nevertheless, the striking differences likely reveal key differences in antimicrobial use.

Rates of macrolide resistance range widely, from 20% to 90%. This variability is likely related to geographical differences [114–116]. A US surveillance study by Jones et al. [114] reported that 56% of isolates (from 19,000 samples analyzed) showed macrolide resistance. The 2014 European Report of antimicrobial resistance showed that Romania, Slovakia, and Malta (48%, 41%, and 38%, respectively) reported the highest rates of non-susceptibility to macrolides; the lowest rates were reported for Cyprus (0%), Latvia (4.1%), and the Netherlands (4.3%) [113]. A recent Spanish study of 643 patients with community-acquired pneumonia found that 22% had macrolide-resistant pneumococcus and 98% of those showed high-level resistance [117].

The rate of fluoroquinolone resistance of pneumococcus in the United States and Europe remains low (<1% and <3%, respectively) [12, 113, 118, 119]. One study, the Antimicrobial Resistance Surveillance in Europe [113], reported resistance data for 30 European countries from the period 2009 to 2012 for 8 bacterial pathogens as invasive isolates (blood and cerebrospinal fluid). Twenty-four European countries reported susceptibility data for fluoroquinolones in 6263 isolates (57% of all reported pneumococcus isolates). Among these, 5.2% were resistant to fluoroquinolones, and 4.4% of the fluoroquinolone-resistant isolates were also penicillin non-susceptible. Similarly, an American study by Jones et al. [114], which was a 14-year longitudinal (1998–2011) survey of *S. pneumoniae* that analyzed 18,911 isolates (collected from community-acquired respiratory tract infections, bacteremias, and pneumonia), reported only 1.2% non-susceptibility to fluoroquinolones (levofloxacin). In contrast, Asian countries reported higher levels, from 10% to 12%. For example, a study from Hong Kong that analyzed antimicrobial resistance data for *S. pneumoniae* from the period 2001–2007, using samples from respiratory tissue, wounds, blood, and other fluids, reported that 11% had reduced susceptibility to levofloxacin [32, 120]. Similarly, a prospective surveillance study of 2184 *S. pneumoniae* isolates collected from patients with pneumococcal infections from 60 hospitals in 11 Asian countries from 2008 to 2009 reported resistance to fluoroquinolones at 1.7%, 0.4%, 1.5%, and 13.4% for levofloxacin, moxifloxacin, gatifloxacin, and ciprofloxacin, respectively (Kim et al. [91]). Isolates from Taiwan (6.5%) and South Korea (4.6%) showed the highest rates of levofloxacin resistance.

2.6 Impact of Vaccines on Resistance

Two types of pneumococcal vaccines are currently available: the polyvalent pneumococcal polysaccharide vaccine (PPV) and the pneumococcal conjugate vaccine (PCV).

The PPV23 vaccine includes 23 purified capsular polysaccharide antigens of *Streptococcus pneumoniae* (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F); it was licensed in the United States in 1983. PPV23 induces antibodies primarily through a T-cell-independent immune response that enhances phagocytosis, thereby killing the bacterium [121]. The immune system of young children does not produce an adequate response to the polysaccharide capsule; consequently, the vaccine is not used in this age group.

The pneumococcal conjugate vaccine 7-valent (PCV7), which included seven pneumococcal sertotypes (4, 6B, 9V, 14, 18C, 19F, and 23F), was introduced in the United States in 2000. It is recommended for infants and young children. This vaccine is highly effective in preventing invasive disease, with percentages of efficacy of about 90%. The routine use of PCV7 has resulted not only in a tremendous reduction in invasive pneumococcal infections in children but also decreased rates of pneumococcal disease in adults.

Also, after 4 years of the introduction of PCV7 in the United States, the incidence of invasive pneumococcal disease caused by penicillin-non-susceptible *S. pneumoniae* and multidrug-resistant *S. pneumoniae* decreased. In 1999, the rate of invasive disease caused by penicillin-non-susceptible strains was 6.3 cases per 100,000 – it decreased to 2.7 cases per 100,000 in 2004. Similarly, in 1999 the rate of cases caused by strains not susceptible to multiple antibiotics was 4.1 cases per 100,000 and decreased to 1.7 cases per 100,000 in 2004 [122]. The study by Whitney et al. [123] demonstrated that the PCV7 vaccine prevents invasive disease in both healthy and chronically ill children. Despite the success of PCV7, studies have noted an increase in the incidence of invasive pneumococcal disease (IPD) caused by non-vaccine serotypes, such as 1, 3, 5, 6A, 6C, 7F, 12F, 19A, and 22F [124]. These serotypes are related to penicillin-non-susceptible clones. The emergence of serotype 19A, which correlates with high-level penicillin and multidrug resistance, is a main concern globally [125]. This serotype presents a dual macrolide-resistance phenotype (*erm* B and *mef*A).

A new 13-valent pneumococcal polysaccharide-protein conjugate vaccine (PCV13) was approved by the Food and Drug Administration in February 2010 for the prevention of IPD in infants and young children. PCV13 contains capsular polysaccharides from serotypes 1, 3, 4,6A, 7F, 9 V, 14, 18C, 19A, 19F, and 23F. In March 2010, the Advisory Committee for Immunization Practices (ACIP) recommended that PCV13 replace PCV7 for the vaccination of children. New studies show a similar reduction in IPD following the introduction of the PCV13 vaccine, as seen previously with the PCV7 vaccine. The study by Moore et al. [126] analyzed IPD cases (33,688 cases, of which 89% contained serotyping results) during July 2004–June 2013 and classified as being caused by the PCV13 serotypes against

which PCV7 has no effect (PCV13/nonPCV7). The work found a reduction in IPD in adults associated with PCV13 introduction in children. In all adult age groups, PCV13/nonPCV7-type IPD (especially serotypes 19A and 7F) declined by 58–72%, which was comparable to that observed early after PCV7 introduction. The PCV13 led to overall reductions of IPD of 12–32% [126]. However, the phenomenon of serotype replacement, which is thought to be caused by non-vaccine serotypes (NVT) that occupy nasopharyngeal natural niches vacated after pneumococcal vaccination, is again observed with pneumococcal serotypes 11A, 15A, 23B, and 35B, the most frequent serotypes. Serotypes 15A and 23B show a high proportion of penicillin non-susceptibility [127].

2.7 Impact of Antibiotic Resistance on Outcome

The relationship between antibiotic resistance of pneumococci and clinical outcome is an important consideration for clinicians, because treatment failure related to antimicrobial resistance is not clear-cut. There are several factors that influence clinical outcome in pneumococcal infections, such as comorbidities (host factors) and invasiveness of the pneumococcus serotype (virulence of the microorganism) that contribute to poor outcome [12].

2.7.1 β-Lactam Resistance and Clinical Implications

The relevance of penicillin-resistant *S. pneumoniae* to clinical outcome in cases of pneumococcal community-acquired pneumonia (CAP) is controversial. Several studies showed that treatment failure in CAP cases does not occur when appropriate therapy and doses are used, even in those patients infected with non-susceptible strains and treated with β-lactams. For example, in 2010 a Spanish study analyzed 1041 patients with pneumococcal pneumonia in which 114 (11%) presented septic shock. The main risk factors were current smoking, chronic corticosteroid therapy, and serotype 3 pneumococcus. No difference was found regarding genotypes or patterns of antibiotic resistance between patients with or without septic shock [128]. Similarly, a study by Morgandon et al. [129], concerning severe pneumococcal pneumonia in patients admitted to intensive care units (ICU), reported that risk factors for mortality were age, male sex, and renal replacement therapy. Comorbidities, macrolide administration, concomitant bacteremia, or penicillin susceptibility did not influence outcome in these cases. These studies suggest that the outcome with community-acquired pneumococcal pneumonia is probably associated with the clinical presentation of pneumonia rather than the antibiotic resistance of the pneumococcus strain. A plausible explanation is that antibiotic concentrations achieved in the lung are usually higher than the pneumococcal MIC for more than 40–50% of the dosing interval, even with resistant strains. It will now be interesting to

determine whether infection by strains having a very high level of resistance to β-lactams (MIC ≥ 16 μg/ml) correlates with clinical failure with pneumonia patients.

A different situation is seen with pneumococcal otitis media or meningitis when treated with a β-lactam – treatment failure is associated with resistant strains. The speculation is that treatment failure is due to the difficulty in obtaining sufficiently high antibiotic levels at these sites of infection. For this reason, most guidelines recommend the use of concomitant vancomycin for patients with pneumococcal meningitis until the pneumococcal MIC for a β-lactam is known [130].

2.7.2 Macrolide Resistance and Clinical Implications

The high rate of macrolide resistance in pneumococcus is a major concern worldwide. Reports of treatment failure in cases of otitis media, meningitis, pneumonia, and bacteremic pneumonia are in the literature [12, 131] for patients who had infections caused by macrolide-resistant strains. For this reason, monotherapy with macrolides is not recommended as an empirical treatment in any infection caused by pneumococcus.

Much less information is available for the relationship between macrolide-resistant *Streptococcus pneumoniae* and clinical outcome than with patients treated with β-lactams. A recent work by Cillóniz et al. [117] concerning the effect of macrolide resistance on the presentation and outcome of 643 patients with CAP reported that 22% were macrolide resistant. They found no evidence suggesting that patients hospitalized for macrolide-resistant *S. pneumoniae* pneumonia were more severely ill upon presentation or had worse clinical outcomes if they were treated with guideline-compliant regimens, including β-lactams, versus noncompliant regimens. A randomized prospective trial is needed to determine whether there is a relationship between macrolide resistance and poor outcome in patients with severe community-acquired pneumonia with whom β-lactam-macrolide combination therapy might improve outcome.

2.7.3 Fluoroquinolone Resistance and Clinical Implications

Treatment failure has been observed with patients treated with fluoroquinolones who had infections caused by fluoroquinolone-resistant strains [43, 132]. However, the global rates of fluoroquinolone resistance remain low [32, 99, 114, 118, 133], making correlation between resistance and outcome statistically marginal. In a 2013 study, Kang et al. [134] evaluated the impact of levofloxacin resistance on 136 adult patients with invasive pneumococcal disease (IPD). In this work, pneumonia was the most frequent disease (68%), followed by primary bacteremia (11%) and meningitis (11%). The rate of levofloxacin resistance in invasive pneumococcal isolates

was 3.7% (5/136) of the isolates. The overall 30-day mortality rate was 26.5% (36/136). In univariate analysis, the factors associated with 30-day mortality in patients with IPD were corticosteroid use, presentation with septic shock, and development of acute respiratory distress syndrome (ARDS). The authors found an association between levofloxacin resistance and increased mortality, although statistical significance was not reached ($p = 0.083$). However, multivariate analysis revealed that presentation with septic shock, corticosteroid use, development of ARDS, and levofloxacin resistance were independent factors associated with 30-day mortality.

Several worldwide reports about antimicrobial resistance in pneumococcus noted that in countries where the rates of β-lactam resistance and macrolide resistance are high, the prevalence of fluoroquinolone resistance is also high [70, 114]. It may be that in those situations the consumption of fluoroquinolones is also high.

2.8 Future Considerations

Pneumococcal infections and antimicrobial resistance remain a global health problem.

Since global antibiotic consumption contributes to the emergence of antibiotic-resistant bacteria such as *S. pneumoniae*, one approach for reducing the problem is to reduce the need for antibiotics through better public health. Changing social norms about how and when to use antibiotics is central to preserving antibiotic effectiveness in all countries. For example we should avoid the use of antibiotics in agriculture and the food industry. The study by Boeckel el al., concerning global trends in antimicrobial use in food animals, reported that the demand for meat globally has led to antibiotic consumption in animals to rise by 70% over the past decade.

The pneumococcus is unusual because vaccines are available. The pediatric pneumococcal conjugate vaccine has had a striking effect on vaccinated children and even non-vaccinated children and adults for the pneumococcal serotypes included in the vaccine. However, non-vaccine serotypes have emerged and are now associated with high-level antimicrobial resistance. Therefore, continuous surveillance programs are needed to determine optimal empiric treatment for a given locality. Surveillance programs are also needed to control the impact of pneumococcal campaigns on serotype distribution, emergence of non-vaccine serotypes, and antimicrobial resistance.

Not all members of an antibiotic class are equally effective against the pneumococcus. Some have a lower MIC than others, and some kill more rapidly. At approved doses, some reach infected tissues more effectively than others. These properties need to be carefully defined to guide clinical use. For example, with compounds that induce mutagenic responses, rapid killing is likely to be important. Additional insight may emerge from geographical locations that use particular derivatives and have very high rates of resistance. A clear example of this is the resistance of pneumococcus to macrolides. In Europe the main resistance mechanism is the ribosomal

mutation that confers high resistance to macrolides, whereas in the United States, the dominant mechanism of resistance is active efflux, which confers low levels of resistance to macrolides. These data suggest the importance of clinical studies in different geographical areas before recommending particular antibiotics. A completely different question is how to slow transmission among young children and elderly persons in long-term care facilities. Solutions may involve reducing antimicrobial consumption, the main driver of newly acquired resistance.

Continued surveillance to quantify pneumococcal resistance is also needed to detect the emergence of new strains exhibiting high-level resistance to penicillin. Moreover, we need to better understand the clinical relevance and impact of antibiotic resistance on pneumococcal infections, since there is not always a clear relationship between resistance and treatment failure.

Major Points

- *Streptococcus pneumoniae* remains an important pathogen worldwide. Pneumococcal infections are related to high rates of morbidity and mortality especially in young children, older adults, and immunocompromised persons.
- Worldwide pneumococcal infections remain a big challenge for physicians because of its resistance to penicillin and increasing resistance to macrolides.
- Efforts to reduce antibiotic consumption should be encouraged by educational programs and guidelines for healthcare professionals.
- The best way to prevent pneumococcal infection is by the implementation of conjugate pneumococcal vaccinations.
- It is important to monitor the evolution of pneumococcal disease, focusing on serotype replacement.
- Studies focusing on the development of new vaccine designs should be addressed in order to avoid serotype replacement.

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Chapter 3
Emergence of MRSA in the Community

Lacey P. Gleason, David C. Ham, Valerie Albrecht, and Isaac See

3.1 Introduction: *Staphylococcus aureus* and Antimicrobial Resistance

Staphylococcus aureus has been recognized as a cause of human infection for over 100 years, and its role in causing clinical syndromes, such as sepsis and abscesses, was first described by Ogston in the late nineteenth century [119]. *S. aureus* can colonize human hosts without causing disease, but infections with *S. aureus*, especially antimicrobial-resistant varieties such as methicillin-resistant *S. aureus* (MRSA), contribute significantly to the burden of infectious diseases in humans.

Penicillin was first introduced to treat patients with bacterial infections in 1941, and resistance to penicillin was first reported in *S. aureus* within 1–2 years [87]. These resistant strains were first found in hospitals after the Second World War, where patients were exposed to this new antimicrobial agent [7]. *S. aureus* had quickly acquired the ability to produce penicillinase, an enzyme that inactivates penicillin. An "epidemic strain" of penicillin-resistant *S. aureus*, which was characteristically lysed by bacteriophage 80 and 81, was noted to cause hospital outbreaks in Australia, Canada, and the United States in the 1950s, particularly in hospitalized children and otherwise healthy young adults [50].

In Denmark in the late 1960s, the first large-scale study of penicillin-resistant *S. aureus* discovered that not only was the majority of *S. aureus* found in hospitals resistant to penicillin but also the resistance gene had spread to a majority of *S. aureus* strains collected from patients in community settings [79]. Within a decade, the majority of community *S. aureus* strains in the United States were penicillin-resistant [132]. New drug development provided a solution to penicillin-

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I. W. Fong et al. (eds.), *Antimicrobial Resistance in the 21st Century*, Emerging Infectious Diseases of the 21st Century, https://doi.org/10.1007/978-3-319-78538-7_3

resistant strains with the release of the semisynthetic penicillins (e.g., methicillin, oxacillin, nafcillin) that resisted penicillinases produced by the majority of *S. aureus* strains.

The first *S. aureus* isolates resistant to methicillin were isolated from patients in England within months of the introduction of methicillin in 1959 [80]. Reports of MRSA in the United States soon followed [8]. As with penicillin-resistant *S. aureus*, MRSA strains were first seen in hospitals, prompting concerns that MRSA would spread outside the hospital. Over 50 years later, MRSA has established itself as a common cause of infections in community settings.

In this chapter, we discuss the epidemiology and mechanisms of resistance of community-associated MRSA (CA-MRSA), an approach to management of CA-MRSA infections, recommendations for prevention of MRSA in the community, and future directions for research, focusing mostly on CA-MRSA in the United States.

3.2 Epidemiology and Mechanisms of Resistance in MRSA in the Community

3.2.1 Recognition of Emergence of CA-MRSA in the United States

In 1999, the Centers for Disease Control and Prevention (CDC) published a report concerning four children from 12 months to 13 years of age who had died from MRSA infections [17]. None of the children had risk factors for healthcare-associated MRSA (HA-MRSA), which at that time included recent hospitalization or surgery, residence in a long-term care facility, or a history of injection drug use. Although CA-MRSA had been recently reported in children [1, 71], most previously described MRSA infections in the United States were associated with healthcare settings or injection drug use in adults [93, 135]. These four cases demonstrated that not only could patients develop MRSA disease outside of the hospital but also that CA-MRSA disease could be severe or fatal. In addition to the four pediatric deaths, CA-MRSA infections were reported in other populations, such as prisoners [18] and military personnel [81]. In response to these reports, CDC initiated active surveillance to describe the epidemiology and drug resistance patterns of MRSA isolates in the community in the United States [56].

This and other early studies demonstrated that there were CA-MRSA strains with unique characteristics compared to methicillin-susceptible *S. aureus* (MSSA) and traditional healthcare-associated MRSA strains. Infections from these CA-MRSA strains often had different epidemiologic risk factors, clinical manifestations, and microbiological characteristics than HA-MRSA strains.

In the literature, the designations CA-MRSA and HA-MRSA have been used to describe both distinct MRSA strains and infections with different epidemiologic

risk factors. CA-MRSA infection is defined epidemiologically as an MRSA infection with onset in the community in an individual lacking established HA-MRSA risk factors. Specifically, for surveillance conducted through the CDC-sponsored Emerging Infections Program, an MRSA infection is classified as community associated if *S. aureus* isolates are cultured from an outpatient or less than 2 days after hospitalization and the patient has had no hospitalization, surgery, dialysis, or residence in a long-term care facility within the previous year and no indwelling central vascular catheter in the 2 calendar days prior to infection [26]. The remainder of the chapter will primarily focus on CA-MRSA infections as defined epidemiologically by the CDC Emerging Infections Program. In this chapter, we will specifically use the term "CA-MRSA strains" to refer to the strains most closely associated with MRSA infections in the community.

3.2.2 Microbiology and Mechanisms of Resistance

S. aureus is a non-motile, non-spore-forming Gram-positive coccus that appears in grape-like clusters. The bacteria are catalase-positive, facultative aerobes that are usually unencapsulated. They can survive on fomites in the environment for months.

Colonization with *S. aureus* (i.e., growth of the organism in or on the body without disease) is common in humans. Although colonization may occur in many parts of the body (including the axillae, perineum, groin, rectum, skin, and umbilical stump in neonates), the anterior nares are the most consistent site of colonization [4].

Most individuals are either transiently or persistently colonized by *S. aureus* at some point during their lives. Staphylococcal carriage studies have found that 16–36% of individuals are persistently colonized, 15–70% are intermittently colonized, and 6–47% are never colonized [158, 168]. Colonization with *S. aureus* is usually thought of as a precursor of *S. aureus* infection; individuals colonized with MRSA are more likely than non-colonized individuals to develop infection [47, 161].

Microbiologic differences between MRSA isolated from patients with CA- and HA-MRSA infections have been identified based on molecular typing, antimicrobial susceptibility testing, and identification of methicillin resistance and toxin genes (Table 3.1). However, these differences are becoming less distinct as MRSA strains that emerged in the community develop resistance to additional classes of antimicrobial agents and enter healthcare settings [151, 156].

3.2.2.1 Mechanisms of Resistance

Penicillin resistance in *S. aureus* is conferred by a plasmid-associated gene (*blaZ*) that codes for beta-lactamase. Methicillin resistance is usually conferred by an altered chromosomally-encoded penicillin-binding protein (PBP2a) that causes

Table 3.1 Molecular characteristics of methicillin-resistant *Staphylococcus aureus* strains typically considered community-associated and healthcare-associated, 2017

Characteristic	Community-associated	Healthcare-associated
Pulsed-field gel electrophoresis (PFGE) type	USA300 commonly, USA400, USA1000, USA1100 less commonly	USA100 commonly, USA200 less commonly
SCCmec type	IV	II
Presence of Panton-Valentine Leukocidin (PVL) toxin	Common	Rare
Antimicrobial susceptibility[a]	Generally susceptible to antimicrobials other than β-lactams and erythromycin	Generally resistant to multiple agents
Clindamycin	Often susceptible	Usually resistant
Erythromycin	Usually resistant	Usually resistant
Fluoroquinolone	Variable	Usually resistant
Trimethoprim-sulfamethoxazole	Usually susceptible	Usually susceptible

[a]Antimicrobial susceptibility patterns may change over time

resistance to all beta-lactam antimicrobial agents (including penicillin) and cephalosporins. PBP2a is encoded by the *mecA* gene that is carried on a distinct mobile genetic element, the *staphylococcal* chromosomal cassette (SCC*mec*). SCC*mec* can be mobilized for transfer between organisms in vitro [84], although this has historically been thought to be a rare occurrence [27]. SCC*mec* contains two genes (cassette chromosome recombinase A and B [*ccrA* and *ccrB*]) that encode recombinases that integrate the cassette into its chromosomal locus.

Eleven types of SCC*mec* have been described. Types II and IV are the primary types seen in the United States. SCC*mec* Type IV has been identified in MRSA strains from CA-MRSA infections in the United States and worldwide. MRSA strains classically associated with healthcare transmission in the United States most commonly contain SCC*mec* Type II and less commonly Types I and III. SCC*mec* Type IV is also typical in some healthcare-associated strains, such as USA800 (see Sect. 3.2.2.4 on molecular typing below). Types II and III often carry genes conferring resistance to other antimicrobial agents (e.g., aminoglycosides, tetracyclines, erythromycin, and clindamycin), whereas Type IV typically does not. This difference in community- and healthcare-associated SCC*mec* types often leads to different antimicrobial agent susceptibility patterns between healthcare- and community-associated MRSA infections.

3.2.2.2 Mechanisms of Virulence

Virulence factors enhance the ability of bacteria to cause infection by evading the host's defenses, increasing adherence to tissues, or spreading through tissues. Examples of virulence factors in *S. aureus* include production of coagulase, toxins,

and proteins intrinsic to the cell wall. *S. aureus* produces coagulase, which interacts with fibrinogen causing plasma to clot. This clumping creates a loose polysaccharide capsule that can interfere with phagocytosis. The combination of these virulence factors may cause localization of an infection, such as in an abscess, a common clinical manifestation of CA-MRSA infection.

Panton-Valentine leukocidin (PVL) is a cytotoxin (coded by the *lukS-PV* and *lukF-PV* genes) first identified in methicillin-susceptible *S. aureus* [121]. PVL kills leukocytes by creating pores in the cell membrane of affected cells or by activating apoptosis pathways. Pore formation leads to increased cell wall permeability and leakage of protein from the cell causing cell death and tissue necrosis. PVL genes have been associated with severe abscesses, necrotizing pneumonia, and increased complications in osteomyelitis [96, 102]. PVL genes are found in most CA-MRSA strains, such as with pulsed-field gel electrophoresis type USA300. PVL, however, is not limited to CA-MRSA, as the toxin is also found in the majority of MSSA strains isolated from patients with community-acquired skin and soft tissue infections (SSTIs) [64]. While PVL is rare in other *S. aureus* strain collections such as colonization or clinical isolates from bloodstream infections, it is highly associated with SSTIs [144].

In addition to PVL, other toxins may be produced by *S. aureus*: α-toxin, which causes tissue necrosis and acts on cell membranes; exfoliative toxins ETA, ETB, and ETD, which are encoded on different genetic elements and cause skin separation in diseases such as bullous impetigo and staphylococcal scalded skin syndrome; enterotoxins A–E, G–J, and R–T (SEA-SEE, SEG-SEJ, SER-SET) which can cause vomiting and diarrhea associated with food poisoning; and toxic shock syndrome toxin I (TSST-1) which induces production of interleukin-1 and tumor necrosis factor leading to shock [64]. Peptidoglycans, which comprise 50% by weight of the cell wall of staphylococci, can have endotoxin properties as well. Other cell wall polymers (e.g., teichoic acid) and cell surface proteins (e.g., protein A and fibronectin- and collagen-binding proteins) may also be virulence factors for *S. aureus* [64]. Recent DNA sequencing of the most common molecular type of CA-MRSA (USA300) suggests that encoded gene products might enhance the ability of the strain to live on the host's skin [41].

3.2.2.3 Antimicrobial Susceptibility Testing

Antimicrobial agent susceptibility testing is commonly used in clinical laboratories to guide the clinical treatment of *S. aureus* infection. Disk diffusion and broth microdilution are the most standardized and accurate testing methods. In disk diffusion tests, a disk impregnated with an antimicrobial agent is placed on an agar plate containing a lawn of bacteria to test whether the antimicrobial agent inhibits the growth of bacteria. (However, the vancomycin disk diffusion test does not detect vancomycin-intermediate *S. aureus* isolates.) One variation of the disk diffusion test is the E-test, a plastic strip with a gradient of antimicrobial agent concentrations used to determine the minimal inhibitory concentration (MIC) of specific

antimicrobial agents. Broth microdilution tests determine the lowest concentration of antimicrobial agents that inhibit bacterial growth in a broth medium using a standard inoculum size. In an agar screen test, a standardized suspension of the microorganism is inoculated directly onto an agar plate impregnated with an antimicrobial agent. Rapid automated instrumentation, such as with devices offered by Vitek™, Microscan™, and others, are most commonly used in laboratories to determine the susceptibility pattern of *S. aureus*.

Clindamycin resistance may be constitutive or inducible, and testing for this resistance can impact clinical treatment decisions. Resistance to clindamycin shares some common mechanisms with resistance to erythromycin, the latter of which is encoded by two different genes: *msrA* and *erm* [145]. The *msrA* gene encodes an adenosine triphosphate (ATP)-dependent efflux pump that confers resistance to erythromycin but not clindamycin. The *erm* (or erythromycin ribosomal methylase) gene confers constitutive resistance to erythromycin and either constitutive or inducible clindamycin resistance. MRSA isolates with inducible clindamycin resistance are resistant to erythromycin and sensitive to clindamycin on routine testing but can be induced to express resistance to clindamycin in vitro.

Rates of inducible clindamycin resistance among strains of MRSA vary widely across the United States, from less than 10% [136] to greater than 90% [54]. A population-based analysis of MRSA in the United States found the rate of inducible clindamycin resistance to be almost 18%. Inducible clindamycin resistance was much higher among USA100 isolates compared to USA300 isolates (25.9% vs 2.9%) [94]. Inducible clindamycin resistance can be identified with the D-zone test, a double-disk diffusion test in which the zone of inhibition is measured around both erythromycin and clindamycin disks [52]. The "D" is formed when the zone of inhibition around the clindamycin disk is blunted on the side adjacent to the erythromycin disk. A positive D-zone test indicates inducible clindamycin resistance. The Clinical and Laboratory Standards Institute (CLSI), formerly the National Committee for Clinical Laboratory Standards (NCCLS), recommends performing a D-zone test on all erythromycin-resistant, clindamycin-susceptible *S. aureus* isolates before reporting clindamycin susceptibility results [28].

3.2.2.4 Molecular Typing of MRSA

Molecular typing of MRSA strains is used to link cases in a cluster, locate sources of specific outbreaks, and conduct macroepidemiology and evolutionary studies. Using the antimicrobial agent susceptibility profile to determine genetic relatedness of strains of *S. aureus* is unreliable. Historically, pulsed-field gel electrophoresis (PFGE) was one of the most commonly used methods for MRSA strain typing in outbreak investigations. Pulsed-field types are still commonly recognized in the United States and around the world. Sequence-based typing methods such as multi-locus sequence typing (MLST) and *spa* (Staphylococcal protein A) typing have been used in more recent years for the analysis of long-term epidemiology

and evolution of MRSA. MLST and *spa* typing have been found to be highly concordant, and both typing methods are easier and less costly than performing PFGE, providing unambiguous typing results that can be compared between laboratories and over time [120]. An early application of whole genome sequencing was to characterize MRSA outbreaks in healthcare settings [89].The requirement for bioinformatics expertise is one factor that has limited adoption by healthcare institutions. Nonetheless, advances in WGS and the accessibility of a large number of assembled bacterial genomes have made possible new methods for strain-level epidemiologic tracking of isolates. This includes the ability to apply MLST schemes on a genome-wide scale [37]. Phylogenetic analysis, derived from sequencing results, has been used to study the population structures of outbreaks and to build transmission networks and help identify factors associated with USA300 strains [127].

In the United States, a limited number of MRSA strains have been implicated in most community outbreaks. A recent description of clinical MRSA isolates from 43 centers across the United States indicated that the USA300 pulsed-field type was the most common type in all regions and from all specimen sources [40]. Other community MRSA genotypes (USA400, USA1000, and USA1100) also cause disease [103]. Molecular typing has further classified the USA300 strain as ST8 and most commonly *spa* type t008. The highly conserved USA300 strain (USA300–0114) has been implicated in multiple outbreaks across the United States in diverse populations that are not epidemiologically related, such as athletes, prisoners, and children [85].

3.2.2.5 Molecular Origins of MRSA

Phenotypic and molecular characterization of CA-MRSA isolates demonstrate that they are different from major circulating HA-MRSA clones. The microbiological differences between strains of MRSA isolated from CA-MRSA and HA-MRSA infections give us clues as to the origins of MRSA in communities, and a number of studies have addressed the genomic evolution of USA300. Recent findings provide support for the idea that there has been clonal emergence of USA300 and consequent spread throughout the United States, rather than evolutionary convergence [156]. However, many questions remain regarding the evolution of USA300 and how it has adapted to the community. Some investigators have suggested that an arginine catabolic mobile element in the USA300 genome was transferred recently (from an evolutionary sense) from coagulase-negative staphylococcus and may be important for the success of this MRSA clone. The *speG* ACME gene product confers increased survival to innate immune defenses on the skin [123, 124]. However, ACME is absent from some USA300 isolates from household clusters, leaving some uncertainty about the necessity of this element for evolutionary success [3].

3.2.3 Epidemiology of CA-MRSA

3.2.3.1 Geographic Characteristics

Cases of CA-MRSA infection have been reported worldwide. The predominant MRSA strains (i.e., MLST sequence types) responsible for community-associated disease vary around the world as does presence or absence of PVL in those strains, though SCC*mec* type IV predominates among strains causing CA-MRSA worldwide [38]. Reports of USA300 MRSA causing community-associated and healthcare-associated disease outside the Americas have been published [61, 131]. However, despite evidence of multiple introductions of USA300 in some countries, it has not achieved the same type of epidemic success outside of the Americas [118, 153]; the reasons for this are not known. The incidence of CA-MRSA infections varies severalfold across the United States [88].

Within the United States, CA-MRSA is found in both urban and rural settings. However, the USA300 MRSA strain was previously reported to be rare as a cause of CA-MRSA infections in rural Alaska [5, 33]. Rural areas have been described as having lower rates of invasive CA-MRSA compared to urban areas [141]. However, as injection drug use is a significant risk factor for invasive *S. aureus* infection and there are concerns that injection drug use is increasing in non-urban areas in the United States recently [167], these trends may change in the future.

3.2.3.2 Age and Sex

As discussed earlier, some of the first reports of MRSA infection without traditional healthcare-associated risk factors were in children [17, 71]. The age distribution for noninvasive CA-MRSA syndromes, such as SSTI, is not well described. However, from population-based surveillance data for MRSA in the United States, invasive (i.e., isolated from a normally sterile body site) CA-MRSA infections are most common in persons aged 50 or older and in children less than 1 year of age [26]. Age differences exist in nasal colonization rates as well. A national study of *S. aureus* nasal colonization among noninstitutionalized persons at least 1 year of age showed that persons age 60 years or older had the highest odds of MRSA nasal carriage; however, this does not specifically refer to carriage of USA300 MRSA or other community MRSA strains. For example, the same study reported that among persons colonized with MRSA, younger persons most often had USA300 or USA400 community MRSA strains [62]. In addition, older age was associated with decreased odds of USA300 MRSA nasal colonization in one study [55].

Trends for MRSA colonization by sex have varied. *S. aureus* nasal carriage has been reported to be more common in men than women in most studies [62, 76]. Some studies have shown higher prevalence of MRSA nasal carriage in men [76], but a large population study of nasal carriage in the United States in 2001–2004 did not find significantly higher prevalence of MRSA carriage in men [62]. Most studies

have reported higher rates of MRSA bloodstream infections in men vs women; reasons for this are unclear. It has been postulated that these reasons might relate to differences in behavior (e.g., studies showing that hygiene differs by sex) or to physiological factors (e.g., hormonal differences) (reviewed in [76]).

3.2.3.3 Race, Ethnicity, and Socioeconomic Status

Rates of CA-MRSA infection vary between racial and ethnic groups. Compared with Australians of European descent, high rates of CA-MRSA infection have been noted in Maori and Pacific Islanders in Australia [72]. Similarly, Pacific Islanders in Hawaii have much higher rates of CA-MRSA infection than Hawaiians of Asian descent. In one investigation of MRSA in Hawaii, 76% of patients with CA-MRSA were Pacific Islanders, but only 35% of patients who received care in the facility were Pacific Islanders [48]. Rates of CA-MRSA SSTIs are high among Alaskan natives [128].

Population-based surveillance data from the continental United States have consistently shown higher incidence rates of invasive CA-MRSA infection among black versus white persons, in both adult and pediatric populations [65, 67, 78, 88, 129]. African-American race has also been described as an independent risk factor for colonization with the USA300 strain [55]. Analysis of public health surveillance has demonstrated that when socioeconomic factors such as income and crowding are accounted for, no significant differences in invasive community-associated MRSA rates by race remain [141]. However, the specific mechanisms by which differences in socioeconomic status lead to racial disparities in invasive community-associated MRSA have not yet been elucidated. It may occur through differences in concurrent diseases such as diabetes that result from socioeconomic disparities or from factors such as limited access to healthcare and crowded housing conditions, which are more common in some racial groups [48]. For example, a study in urban Chicago has described incarceration and public housing as risk factors for CA-MRSA SSTIs [74]. The importance of socioeconomic factors as a contributor to CA-MRSA rates is underscored by a study showing transient residence and substance abuse as factors associated with CA-MRSA compared to patients with MSSA [159].

3.2.3.4 Other Epidemiologic Risk Factors for Colonization

HIV infection, illicit drug use, temporary housing, and incarceration are associated with colonization with USA300 MRSA [126]. In addition, antimicrobial use is also a risk factor for MRSA colonization in the community [47, 105, 106].

3.3 Considerations in Management of MRSA Infection in the Community

Clinical treatment guidelines for MRSA were published by the Infectious Disease Society of America (IDSA) in 2011 [97]. This section will review major recommendations from those guidelines as well as new developments that have occurred since then.

3.3.1 Clinical Presentation

CA-MRSA infections can occur as any one of a myriad of clinical syndromes. Among the most common are SSTIs, pneumonia, and invasive infections such as osteomyelitis, endocarditis, and other bloodstream infections [56]. Wound, skin, and soft tissue MRSA infections account for approximately 90% of CA-MRSA infections [56]. The majority of SSTIs are abscesses or cellulitis, but up to one quarter are superficial infections such as impetigo [112]. MRSA and other *S. aureus* SSTIs have often been misdiagnosed as "spider bites," particularly by patients [30, 42] even when the relevant spiders (e.g., brown recluse) are not present in those regions [160]. Whether invasive CA-MRSA infections from the USA300 strain or other PVL-positive strains are more severe than other MRSA infections remains unclear [92, 113, 144]. MRSA pneumonia has been reported as a bacterial superinfection after influenza infection in both adults and children [11, 25, 66, 125]. MRSA can be a cause of otitis media in children [134] and accounted for as much as 12% of cases of otorrhea in one series [77]. MRSA has also been a reported cause of pyomyositis [82] and Waterhouse-Friderichsen syndrome in children [2]. In addition, cases of CA-MRSA necrotizing fasciitis have been reported [104].

CA-MRSA may also cause recurrent infections, as there is no known natural immunity to *S. aureus* after infection. Severity of disease can vary by site of infection. In one study, approximately one quarter of patients with CA-MRSA infection were hospitalized for their infection [56], with a greater percentage among those with severe or invasive disease [112].

3.3.2 Management of MRSA Skin or Soft Tissue Infections

MRSA has increased in prevalence as a cause of purulent SSTIs since the early 2000s and is the most common cause in many communities [151]. Some of the major considerations for treatment of these infections outlined in the IDSA guidelines, along with a brief discussion of the literature, are listed below:

1. Incision and drainage should be routine for skin lesions that can be drained. Some clinicians suggest the use of ultrasonography to distinguish if there is a drainable collection [148]. Incision and drainage has been the primary mode of treatment for skin and soft tissue abscesses for many centuries and is essential. Although the 2011 IDSA guidelines suggest that additional data are needed to describe whether antimicrobials are needed for simple abscesses/boils, a randomized trial at five US emergency departments showed that trimethoprim-sulfamethoxazole (TMP-SMX) treatment led to higher cure rates among patients with a drained cutaneous abscess compared with patients who received placebo [152]. Antimicrobial treatment may also reduce recurrent infections [45, 139, 152] and the need for subsequent procedures [152]. Available literature suggests that clinicians should weigh the costs and benefits of prescribing antimicrobial agents: for example, considering an individual patient's risk for recurrent infections.
2. Collect diagnostic specimens for culture. Cultures should be obtained from patients with both draining and non-draining purulent lesions. Obtaining isolates helps guide treatment of individual patients and monitor the antimicrobial agent susceptibility patterns in the community. Cultures are also recommended by some experts when there is a severe local infection, signs of systemic illness, inadequate response to initial treatment, or concerns about a cluster or outbreak of cases [148].
3. Use of antimicrobial agents. For adult patients, the IDSA guidelines suggest using clindamycin, TMP-SMX, a tetracycline, or linezolid for empiric coverage of CA-MRSA in outpatients with SSTI when empiric coverage is needed, with the additional suggestion that if coverage for β-hemolytic streptococci is also needed, a beta-lactam be added if TMP-SMX or a tetracycline is prescribed. For hospitalized patients with complicated SSTI, empiric therapy is suggested to include vancomycin, linezolid, daptomycin, telavancin, or clindamycin. Local antimicrobial susceptibility data for outpatient *S. aureus* SSTIs should guide empiric treatment decisions. The 2011 IDSA guidelines specifically suggest empiric therapy for CA-MRSA be prescribed for outpatients with purulent cellulitis, hospitalized patients with complicated SSTI, and abscess associated with certain conditions—severe or extensive disease or rapid progression with cellulitis, signs/symptoms of systemic illness, immunosuppression, extremes of age, lack of response to initial treatment, septic phlebitis, or anatomically difficult-to-drain location. The guidelines state that the role of CA-MRSA in outpatient nonpurulent cellulitis is unknown, and despite use of state-of-the-art techniques for molecular identification, the microbiologic etiology of nonpurulent cellulitis in the community and role of MRSA in particular remain unknown [32]. Notably a recent study has shown no difference in outcomes with the use of cephalexin alone compared with the use of cephalexin plus TMP-SMX in patients with uncomplicated cellulitis [109].
4. Perform careful and thorough personal and environmental hygiene. MRSA can be transmitted from person to person or to the environment through contact with draining skin and soft tissue lesions. After incision and drainage, wounds should

be adequately covered, bandages should be appropriately disposed of, and hand hygiene should be continued to prevent further spread of MRSA. In addition, items that have contacted infected skin such as towels should not be reused or shared.

3.3.3 Management of Severe or Invasive MRSA Infections

Severe or invasive MRSA infections include sepsis, pneumonia, endocarditis, osteomyelitis, and the progression of localized infections such as of the skin or soft tissue. Empiric therapy for MRSA is recommended in IDSA guidelines for severe cases of hospitalized community-acquired pneumonia, defined as (1) requiring ICU admission, (2) having necrotizing or cavitary infiltrates, or (3) being associated with empyema [97]. Recent studies of community-acquired pneumonia have found MRSA to be an uncommon pathogen, and though such cases are severe, it is unclear whether clinical presentation of MRSA significantly differs from that of other community-acquired pneumonia pathogens to distinguish it on clinical ground alone [108, 143]. In addition to antimicrobial agent therapy, incision and drainage is mandatory for drainable SSTIs and should be considered for severe or deep infections, such as septic joints or osteomyelitis. In addition, for infections related to an implanted device, the device should be removed if feasible [97].

Vancomycin and daptomycin are recommended as first-line agents for adults with MRSA bacteremia. Duration of therapy depends on whether bacteremia is determined to be complicated or uncomplicated and whether or not associated with endocarditis [97].

3.3.4 Newer Developments in Antimicrobial Treatment of MRSA Infections

Several antimicrobials have been developed more recently with in vitro activity against MRSA, many of which also have indications for skin infections or other syndromes that may be CA-MRSA infections (Table 3.2). These include dalbavancin, oritavancin, ceftaroline, and tedizolid [14]. Some clinicians have suggested combination therapy (e.g., either vancomycin or daptomycin, in conjunction with a β-lactam) for complicated MRSA bacteremia [39]. Clinical trials are underway to further define the role of combination therapy [154]. In addition, a new clinical treatment guideline for *S. aureus* bacteremia is planned by IDSA and may clarify the role of newer agents or combination therapy for bacteremia for treatment of CA-MRSA syndromes.

Table 3.2 Description of major antimicrobials or antimicrobial classes that have been used to treat methicillin-resistant *Staphylococcus aureus* infections

Antimicrobial	Mechanism of action	Other comments
Ceftaroline	Binding to penicillin-binding proteins (fifth-generation cephalosporin)	High affinity for penicillin-binding protein 2A (PBP2A), leading to greater in vitro activity against MRSA than other cephalosporin antibiotics
Clindamycin	Inhibition of bacterial protein synthesis	Inducible resistance can occur
Dalbavancin	Same as vancomycin	Dosing for skin infections approved as either single dose or two doses 1 week apart
Daptomycin	Cyclic lipopeptide; binds to bacterial cell membranes	Inactivated by surfactant and not recommended for treatment of MRSA pneumonia
Linezolid	Inhibition of ribosomal protein synthesis	Myelosuppression reported; could cause serotonin syndrome in conjunction with some medications (e.g., antidepressants)
Oritavancin	Inhibits cell wall synthesis (similar to vancomycin) and disrupts cell membrane barrier function	Once/week dosing
Quinupristin-dalfopristin	Inhibits peptide bond formation in ribosome	Arthralgias/myalgias are common adverse events
Tedizolid	Same as linezolid	Approved for skin infections; advantage over linezolid is once/day dosing
Telavancin	Inhibits cell wall synthesis (similar to vancomycin) and disrupts cell membrane barrier function	Synthetic derivative of vancomycin but once/day dosing
Tetracyclines (class)	Inhibition of bacterial protein synthesis	Not recommended in children because of effects on bones and teeth
Tigecycline	Inhibits protein translation	FDA boxed warning about increased risk of death
Trimethoprim-sulfamethoxazole	Blocks production of folic acid	
Vancomycin	Inhibits cell wall synthesis	Monitoring of serum levels recommended by IDSA guidelines; dosing in obese patients controversial; often drug of choice for patients with bacteremia

3.3.5 Strategies to Eliminate S. aureus *Colonization as Part of Treatment for Infected Patients*

Decolonization has been suggested as a component of treatment for recurrent or persistent MRSA infections, but its value is unclear. Decolonization regimens have been effective in reducing colonization in the short term, but recolonization is common [90, 122]. Carriage at sites other than the nares and reports of resistance to

mupirocin may limit the effectiveness of nasal decolonization [98]. Proposed regimens for decolonization include intranasal mupirocin twice a day for 5–10 days and antiseptic body wash, such as with chlorhexidine, for 5–14 days [97, 150]. Dilute bleach baths have been suggested for patients with recurrent MRSA infections, but children who underwent routine hygienic measures plus twice weekly bleach baths for 3 months did not experience a significant reduction in recurrent SSTI requiring medical attention within a year of treatment compared with those using routine hygienic measures [83]. A randomized controlled trial testing the effect of skin cleaning with chlorhexidine gluconate (CHG) three times per week for 6 months in a jail showed no difference in MRSA carriage between groups cleaning with CHG cloths compared with water-soaked cloths [35]. When household transmission is suspected, implementation of personal and environmental hygiene measures and evaluation of symptomatic contacts are recommended in IDSA guidelines, which state that decolonization may be considered [97]. Decolonization with a 5-day regimen of hygiene, nasal mupirocin, and chlorohexidine body washes of all household members was associated with a significant decrease in self-reported recurrent SSTI at 12-month follow-up compared to a group in which only the index patient was decolonized [57]. However, SSTI still recurred in the majority of cases [57]. The use of oral antimicrobial therapy is generally not recommended for decolonization in current guidelines [97].

3.4 Transmission and Outbreaks of MRSA in the Community

3.4.1 Transmission of CA-MRSA

The following information describes some of what is known about transmission of MRSA in the community from studies in non-outbreak settings. In general, direct person-to-person transmission of *S. aureus* is believed to occur via contact, as opposed to respiratory droplets or aerosols [147]. Whole genome single-nucleotide polymorphism (SNP) analysis of community MRSA isolates from a hospital system in New York demonstrated that isolates from the same household are much more closely related than community isolates from different households, suggesting transmission and possibly persistence within households [155]. Analysis of USA300 isolates from Los Angeles and Chicago also showed through whole genome sequencing that isolates often clustered into closely related groups by household [3]. In addition, the study suggested that introduction of MRSA colonization within the household often preceded the first symptomatic infection of the household member. Household studies have suggested that factors associated with household transmission of MRSA include persons in the household requiring assistance for daily activities or sharing topical products or bath towels [114, 116]. Colonized and infected individuals can also contaminate the household environment [58]. Therefore in summary, environmental surfaces, shared items, and hands/skin may serve as vehicles for transmission.

3.4.2 *Outbreaks of MRSA in the Community*

In addition to the above studies, a significant amount of information has been learned about CA-MRSA transmission from outbreaks in the community. Although some experts have questioned whether risk factors for transmission might differ in outbreak versus non-outbreak settings, the lessons learned from outbreaks can still be useful.

Since the early 1980s when MRSA was recognized as a pathogen that can cause outbreaks in the community in groups such as intravenous drug users, outbreaks of CA-MRSA have been reported in a number of diverse groups: Native American, Alaskan Native, and Pacific Islander communities [5, 6, 48, 63, 72]; prisoners [18, 22]; amateur and professional sports participants, such as football players, wrestlers, rugby players, fencers, and divers [9, 21, 85, 149, 163]; child care center attendees [1]; military personnel [13, 170]; men who have sex with men [91]; methamphetamine and injection drug users [29, 53]; survivors of natural disasters [23]; recipients of tattoos [24]; and isolated religious communities [31]. MRSA can cause infections in animals and pets and has been reported to cause infections in humans who have had contact with infected animals [165].

Although these groups are diverse, they have common factors that may underlie the transmission of MRSA in the community. Based on investigations of community outbreaks, five factors that contribute to transmission of MRSA in the community can be characterized as the "Five Cs" as described below. However, it is important to note that in a study at an urban emergency department in the United States, most patients with MRSA skin infections had none of these characteristic risk factors associated with CA-MRSA outbreaks [107]:

1. Crowding. Outbreaks have occurred in populations living in crowded quarters such as prisons and military barracks. Living in a house with more than one person per bedroom has been independently associated with developing a CA-MRSA skin or soft tissue infection [29].
2. Contact, skin-to-skin. Participants in contact sports have frequent skin-to-skin contact, which may act as a method of transmitting MRSA SSTI. Outbreaks among professional and college football teams have been attributed to frequent skin-to-skin contact [9, 85]. Similarly, wrestlers who have significant skin-to-skin contact have experienced outbreaks of MRSA infection [21]. High-risk sexual behavior [91] and sexual contact with someone with a skin infection [29, 91] have both been associated with CA-MRSA SSTIs. These factors have been described both in rural and urban communities.
3. Cut or compromised skin. Breaks in the skin are a portal for MRSA bacteria to enter the body. For example, in an outbreak of MRSA infections among a college football team, MRSA infections were associated with abrasions from artificial grass ("turf burns") and cosmetic body shaving [9]. In an outbreak among military recruits, most of the MRSA SSTIs were on exposed skin of the arms, legs, and knees, where abrasions are common during field training [170]. Skin-picking behavior has also been associated with MRSA SSTIs [29]. Injection drug use,

where the skin is compromised by insertion of contaminated needles, has been associated with MRSA infections [104, 169], but injection may not be the only method by which MRSA is transmitted among drug users [29].

4. Contaminated surfaces and shared items. Although environmental transmission of MRSA may not be the most common mode of transmission, the environment may have played a role in some outbreaks of MRSA in the community. Outbreaks have been associated with whirlpools [9] and MRSA contaminated sauna benches [6]. An outbreak among fencers was unusual because there is typically little skin-to-skin contact in that sport; however, investigators surmised that the cluster of cases was due to shared fencing equipment [21]. In a correctional facility in Mississippi, sharing personal items such as linens was associated with infection [18], while sharing bars of soap was implicated in an outbreak among members of a college football team [117].
5. Cleanliness. Cleanliness includes both personal bathing and laundering of clothing, linens, and towels, all of which have been noted as potential contributing factors to CA-MRSA infection among prison inmates [18]. Investigations of MRSA transmission in prisons suggest that lack of access to basic hygiene is a contributing factor [22]. Homelessness has also been associated with MRSA SSTIs [169].

In addition to the "Five Cs," previous use of antimicrobial agents has also been shown to be a factor in the development of CA-MRSA [5, 85]. An outbreak of CA-MRSA skin infections in southwestern Alaska found that patients with skin infections received significantly more antimicrobial agents in the year before the outbreak compared to community members without skin infections [6]. In an outbreak of MRSA in a closed religious community in the United States, investigators found the use of antimicrobial agents was associated with infection [31].

3.5 Prevention of MRSA in the Community

Prevention strategies for CA-MRSA need to include public health officials, medical providers and infection control practitioners, and patients and community members. The following are considerations for these different groups for prevention of CA-MRSA.

3.5.1 Public Health Officials

1. Consider initiating public health investigations when MRSA is detected in a group of individuals in the community who are linked epidemiologically. When considering whether to investigate, public health officials should weigh the number and clustering of time and space of cases, the setting of the cluster, the sever-

ity of illness, the presence of ongoing transmission, and the likelihood that an intervention could be successfully implemented.
2. Enhance surveillance. Both prospective and retrospective surveillance are important to identify cases of MRSA in the community and intervene in outbreak settings. Consider notifying contacts of patients with MRSA infection to identify new cases in outbreak settings and to ensure that they are receiving proper treatment.

3.5.2 Medical Providers and Infection Control Practitioners

1. Use appropriate treatments for infections. Treatment considerations for MRSA infections were discussed in an earlier section.

In addition, since CA-MRSA infections have been associated with previous antimicrobial use, antimicrobials should be used appropriately, both when treating patients with CA-MRSA infections and when prescribing antimicrobials for other conditions in the community.

2. Educate providers to assess for additional symptomatic contacts. Clinicians should ask patients with MRSA infections if other contacts and household members also have suspicious lesions or infections, so that contacts can be appropriately treated and further transmission limited.
3. Prevention of MRSA in healthcare settings. Recommendations for preventing MRSA in healthcare settings are reviewed below, although it is not clear if these have significant impact on preventing CA-MRSA infections. Recommendations from CDC's Healthcare Infection Control Practices Advisory Committee for preventing MRSA infections in the healthcare setting where MRSA is considered an epidemiologically important multidrug-resistant organism generally include (1) promotion of appropriate hand hygiene, (2) use of contact precautions for MRSA-colonized and MRSA-infected patients, (3) appropriate cleaning and disinfection of patient equipment and environmental surfaces, and (4) educating healthcare personnel about MRSA prevention and transmission [146]. However, the use of contact precautions has recently been questioned, and additional research would be helpful to define the added benefits of contact precautions to other measures for controlling transmission of MRSA in acute care hospitals [110]. Additional measures are suggested for consideration if MRSA is not adequately controlled using standard measures. Active surveillance, isolation, and cohorting of MRSA-positive patients can also be implemented [12, 146]. In addition, based on results from a positive clinical trial, the Society for Healthcare Epidemiology of America also recommends universal decolonization of ICU patients with chlorhexidine and nasal mupirocin in hospital locations that continue to have high MRSA rates after the implementation of basic MRSA control strategies [12, 75].

3.5.3 Patients and Members of the Community

1. Educate patients on treatment and prevention. Patients should be encouraged to keep wounds covered, to maintain good personal and hand hygiene, to avoid sharing potentially contaminated items, and to seek care early if they believe they might have an infection (CDC MRSA website https://www.cdc.gov/mrsa/community/index.html). Prevention recommendations may need to be tailored for high-risk groups (e.g., athletes).
2. Care for and contain wounds. Wounds should be covered with clean, dry dressings until healed. Patients with open skin wounds, such as draining SSTIs that cannot be covered, may need to be excluded from activities that could lead to transmission. For example, if sport-specific rules do not exist, in general, athletes should be excluded if wounds cannot be properly covered during participation, and athletes with open wounds or infections should not use common-use water facilities like swimming pools or therapy pools. Patients should be encouraged to seek care from a medical provider and not treat wounds themselves by picking or popping sores (CDC MRSA website https://www.cdc.gov/mrsa/community/index.html).
3. Encourage personal hygiene, especially hand hygiene. Patients should wash hands regularly especially after dressing changes. Use soap and water or alcohol-based hand gels to clean hands and encourage regular bathing or showering, especially after exercise. Do not share personal items that may transmit infection such as towels, washcloths, razors, and clothing. Launder contaminated clothes and linens with detergent, soap, or bleach, and dry thoroughly. Athletic uniforms should be washed and dried after each use (CDC MRSA website https://www.cdc.gov/mrsa/community/index.html [142]).
4. Maintain a clean environment. For example, when MRSA skin infections occur, cleaning and disinfection should be performed on surfaces likely to contact uncovered or poorly covered infections, such as in facilities where patrons and staff have close contact (e.g., homeless shelters) or shared equipment or surfaces (e.g., gyms). Surfaces should be cleaned with detergent-based cleaners or Environmental Protection Agency (EPA)-registered disinfectants (https://www.epa.gov/pesticide-registration/selected-epa-registered-disinfectants).

3.6 Future Directions

3.6.1 Vancomycin Resistance

Vancomycin is a primary treatment for severe, invasive MRSA infections. Clinical isolates of *S. aureus* with intermediate resistance to vancomycin (MICs of 8–16 mug/mL) were first reported in Japan in the late 1990s [73]. Intermediate resistance

to vancomycin in strains of *S. aureus* may be due to the development of thicker cell walls in the bacteria.

Resistance to vancomycin is conferred by the presence of a vanA operon, which is thought to be transferred from vancomycin-resistant enterococci [166]. In 2002, reports of *S. aureus* resistant to vancomycin (MICs ≥ 32 ug/mL) came from two states (Michigan and Pennsylvania) in the United States [19, 20]. To date, 14 vancomycin-resistant *S. aureus* (VRSA) isolates have been identified in the United States [162]. All have occurred in patients with significant prior healthcare encounters, such as for chronic wounds and dialysis. In addition, all except for the 13th isolate have belonged to lineages traditionally associated with healthcare; the 13th isolate was a USA1100 MRSA [95]. In addition, VRSA from a community MRSA lineage has been reported in Brazil, where vancomycin resistance appears to have been acquired while a patient was on vancomycin treatment [133].

3.6.2 Vaccine and Other Novel Prevention/Treatment Approaches

New mechanisms of preventing and treating *S. aureus* infection are being studied. Vaccines against *S. aureus* are being developed. Although significant difficulties have been encountered with previous studies of potential *S. aureus* vaccines, there are clinical trials of vaccine candidates ongoing [10, 59, 100, 138]. In addition, some studies of antibodies to treat or prevent *S. aureus* infections are also underway [59]. Another research area that has attracted some interest recently is the use of bacteriophages for prevention or treatment of *S. aureus* infections, though the application of such an approach is likely distant [86].

3.6.3 Novel Potential Sources for MRSA in the Community

3.6.3.1 Pets and MRSA

MRSA is also an important pathogen in veterinary medicine [60], and some researchers have explored the relationship between animal and human MRSA infections. There have been case reports of suspected transmission of MRSA between owners and pets [16, 43, 44, 101], and models of MRSA acquisition in dogs identify contact with humans as an influential source [69, 70]. Little is known about the potential for companion animals to serve as reservoirs of human infections, but a small percentage of healthy cats and dogs have been shown to carry multidrug-resistant staphylococci [36]. Approximately 6–9% of pets living in the same household as a patient infected with MRSA carried genetically concordant strains of MRSA [49, 51, 111], but the odds of obtaining a positive culture from pets rapidly decrease from the time of the patient's MRSA diagnosis [111]. Additional studies

might clarify the dynamics of, risk factors for, and importance of this potential horizontal transmission between pets and humans.

3.6.3.2 Livestock and MRSA

The use of antimicrobials in industrial agriculture is associated with the presence of antimicrobial-resistant bacteria [68]. In Europe, livestock-associated MRSA has been described as a unique MRSA clone (MLST sequence type 398) associated with livestock exposure, that can cause a range of infections including bacteremia, pneumonia, osteomyelitis, endocarditis, and SSTIs [46, 99, 157]. Some have raised concerns in the United States for occupational and environmental exposures, as MRSA has been found in dust and surface samples within industrial hog operations, air and soil samples in the surrounding environment, and surface waters near industrial hog operation spray fields [68]. Among patients admitted to a rural, tertiary care hospital without occupational exposure to industrial hog operations, individuals living in areas with higher swine density have higher odds of MRSA carriage [137]. Proximity to swine crop fields is also associated with CA-MRSA and SSTIs [15]. However, in one study, individuals who have occupational exposure to industrial hog operations were not at increased risk of MRSA colonization compared to community referents [115]. In addition, a study of individuals in the United States found that livestock exposure was a risk factor for colonization with *S. aureus* but not for SSTIs overall, and only one participant with livestock exposure was found to have the livestock-associated MRSA strain [164]. The ST398 MRSA strain has not been detected to date in multisite public health surveillance in the United States for invasive MRSA (Centers for Disease Control and Prevention, unpublished data). At this point, it is unclear whether observed differences in MRSA colonization in areas with concentrated livestock operations are reproducible and, most importantly, if they meaningfully contribute to the burden of CA-MRSA infections in the United States.

3.6.4 Changes in Molecular Epidemiology of MRSA Strains

CA-MRSA strains have been increasingly described as the cause of disease and outbreaks in healthcare settings. USA300 MRSA was the most common MRSA strain causing bloodstream infections in a Chicago hospital and has been reported to make up an increasing proportion of hospital-onset MRSA infections [34, 130]. Surveillance from CDC's EIP suggests that the increase in the proportion of hospital-onset MRSA infections caused by USA300 has occurred principally because the incidence of MRSA infections caused by other strains in hospitals has declined [140]. Data from the same surveillance system show that the incidence of invasive USA300 infections has not decreased over the past decade, and additional strategies for preventing USA300 MRSA infections in both healthcare and the community are needed.

The epidemiology of CA-MRSA infections continues to evolve. We will need to be vigilant in our identification and treatment of MRSA infections in the future to prevent further spread and development of new resistant strains. Innovative methods of preventing CA-MRSA disease would provide a large benefit to public health.

Acknowledgments/Disclaimer The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention. Use of trade names and commercial sources is for identification only and does not imply endorsement by the Centers for Disease Control and Prevention, the Public Health Service, or the US Department of Health and Human Services. The authors would like to thank the authors of the previous version of this chapter (Adam Cohen, Daniel Jernigan, and Rachel Gorwitz) for permission to include some materials from the previous edition.

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Chapter 4
Resistance of Gram-negative Bacilli to Antimicrobials

Charles R. Dean, Gianfranco De Pascale, and Bret Benton

4.1 The Expanding Problem of Multidrug-Resistant (MDR) Gram-negative Bacilli

Much has transpired in the realm of antibiotic resistance in the 10 years since the first edition of this text. This chapter began in 2007 with the line, "At the beginning of the twenty first century, we now find ourselves experiencing a taste of what life was like prior to the advent of the antibiotic age…," and this reality is continuing to sink in. So much so, in fact, that antibiotic resistance is now routinely broached in the popular media and has the attention of government agencies and philanthropic groups and to some extent may be prompting a return to antibiotic discovery within the pharmaceutical industry. In 2009, the Infectious Diseases Society of America (IDSA) released the updated call to action for a coordinated effort to bring antibiotic development to the forefront, specifically regarding the "ESKAPE" pathogens, *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. [1]. These pathogens cause the majority of US hospital infections, and resistance is a major issue. The Gram-negative bacilli are well represented in this group and pose a very significant emerging problem, particularly in the case of pan-antibiotic-resistant *A. baumannii*, multidrug-resistant (MDR) *P. aeruginosa*, and carbapenem-resistant *Enterobacteriaceae* (CRE). More recently the IDSA has begun "the 10x20 Initiative" (http://www.idsociety.org/10x20/). In February of 2017, the World Health Organization established its priority list for drug-resistant pathogens, and in the "critical" category are carbapenem-resistant *A. baumannii* and *P. aeruginosa* and carbapenem-resistant, extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* (http://www.who.int/mediacentre/news/releases/2017/bacteria-antibiotics-needed/en/). The notion of tackling antimicrobial resistance was

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I. W. Fong et al. (eds.), *Antimicrobial Resistance in the 21st Century*, Emerging Infectious Diseases of the 21st Century, https://doi.org/10.1007/978-3-319-78538-7_4

also addressed by the economist Jim O'Neill, who articulated the human costs and economic and security threats associated with a failure to act (https://amr-review.org/sites/default/files/160518_Final%20paper_with%20cover.pdf). Discussions about how to incentivize antibiotic discovery have followed and the establishment of research funding through agencies such as the Biomedical Advanced Research and Development Authority (BARDA, https://www.phe.gov/about/BARDA/Pages/default.aspx) and Combating Antibiotic-Resistant Bacteria Biopharmaceutical Accelerator (CARB-X, http://www.carb-x.org); Wellcome Trust and Pew Charitable Trust have also come online to promote antibiotic discovery. These activities, although encouraging, still underscore the challenge upon us. Therefore, an understanding of resistance in Gram-negative pathogens is informative and will be discussed in the following sections.

This chapter addresses intrinsic resistance and mutationally or horizontally acquired resistance mechanisms. Intrinsic (or innate) resistance varies widely among different pathogens and is determined by the general makeup of a cell, where the overall complement of genes and their expression levels establish a baseline susceptibility to an antibacterial. Here we focus on two broad elements important for intrinsic resistance, the impermeability of the Gram-negative cell envelope, which impedes a compound's entry into the cell to exert its effect, and energy-dependent active efflux which extrudes a compound back out of the cell before it can engage its target. We begin there, since (i) these can be important hurdles to overcome in efforts to discover new antibiotics for Gram-negative pathogens and (ii) they can facilitate/exacerbate the emergence of mutationally or horizontally acquired resistance. The organism-specific genetic blueprint for intrinsic resistance provides the background within which mutations can be selected that further decrease susceptibility to antibacterial compounds. As well, the horizontal acquisition of new genetic material is an important route of acquired resistance. The progression to resistance is often multifactorial, and several acquired mechanisms can accumulate over time to cause clinically significant resistance and multidrug resistance (MDR). In that regard, the meaning of "resistance" is context-specific. In clinical antimicrobial susceptibility testing, resistance is based on a specific minimal inhibitory concentration (MIC) of an antibiotic tested under standardized conditions, and clinical resistance occurs if the MIC of the antibiotic is above an established clinical resistance "breakpoint" [2]. Here, we use the term more generally to convey the idea that the mechanisms discussed will alter (increase) the level of resistance (or decrease susceptibility), but not all resistance mechanisms will cause the specifically defined clinical resistance (shift over the breakpoint). The first edition of this chapter pertained mainly to antibiotics that had been in clinical use for some time (e.g., fluoroquinolones). These sections are updated here, but two additional aspects are now included. In 2007, tigecycline was just entering the clinic, and we update on what has happened in the approximately 12 years it has been in widespread clinical use (Sect. 4.2.4). Second, polymyxins were reintroduced into the clinic as a last line of defense against MDR Gram-negative pathogens. In a relatively short time, resistance has emerged and has begun to erode the clinical utility of these compounds, and this is discussed in Sect. 4.2.6.

4.2 Resistance in Gram-negative Bacilli

4.2.1 The Gram-negative Cell Envelope: Efflux and Outer Membrane Impermeability

4.2.1.1 Active Efflux

Bacteria have a broad range of efflux pumps that can actively extrude molecules from the cell. Efflux pumps can serve natural physiological roles such as extrusion of metabolites but also function in efflux of toxic molecules that enter the cells. Efflux of toxic molecules serves to lower their intracellular accumulation to reduce access to the intracellular target(s), thereby protecting the bacteria. The five broad efflux pump superfamilies most important in bacteria are the ATP-binding cassette (ABC) family, the major facilitator superfamily (MFS), the small multidrug resistance (SMR) family, the multidrug and toxic compound extrusion (MATE) family, and the resistance-nodulation-cell division (RND) family (reviewed in [3] (Fig. 4.1)). The ABC family differs from the other families in that they derive energy to drive active efflux from hydrolysis of ATP, whereas the other families derive energy from the proton gradient maintained at the bacterial cytoplasmic membrane. The pump proteins that mediate compound recognition and energy-dependent extrusion for all families are situated in the bacterial cytoplasmic membrane. Members of all pump families except RND pumps are found in both Gram-positive and Gram-negative bacteria. The RND family pumps are unique to Gram-negative bacteria and have additional components and an overall architecture necessary for efflux across the Gram-negative outer membrane (OM) (Fig. 4.1). Depending on the context, all of the families can contribute to resistance in Gram-negative pathogens, but non-RND family pumps can only efflux compounds into the periplasmic space between the cytoplasmic and OM but not to the outside of the cell. Furthermore, RND pumps are

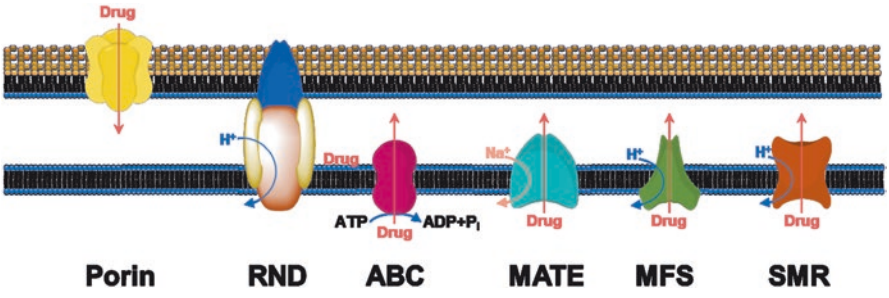

Fig. 4.1 General architecture of efflux pump families and placement in the Gram-negative cell envelope. The Gram-negative envelope has two membranes (the inner membrane, shown here as a symmetrical bilayer in blue, and an outer membrane, which is asymmetrical) and has phospholipid (blue) at the inner leaflet and lipopolysaccharide (gold) at the outer leaflet. RND family pumps have an architecture that spans both membranes. Some compounds can enter the cells via water-filled porins (yellow)

notable for their large amorphous compound-binding pockets [4, 5] which confer the ability to recognize and extrude a very broad range of structurally unrelated molecules. For these reasons, RND family pumps are regarded as the most significant efflux pumps overall in Gram-negative bacteria in terms of antibacterial resistance. However, it is also clear that there is cooperation between networks of pumps of different families when their substrates overlap [6]. In those cases, the single-component pumps may efflux a substrate into the periplasm, and the RND pump may then expel the compound from the periplasm to the outside of the cell. RND pumps are tripartite structures, comprised of the inner membrane-located RND pump component, an OM channel component (outer membrane factor (OMF)), and a periplasmic membrane fusion protein (MFP) that links these components (Fig. 4.1). This architecture spans the double membrane of the Gram-negative cell to allow compound extrusion across the OM through the OMF, driven by the proton-motive force (PMF) at the inner membrane. RND pumps are typically named in the order MFP-pump-OMF, and the best studied RND pumps are AcrA-AcrB-TolC (shortened to AcrAB-TolC) of *E. coli* and MexAB-OprM of *P. aeruginosa.* RND family pumps have been found in all Gram-negative bacteria so far studied, and most RND pumps have a broad substrate range, allowing them overall to accommodate most classes of antibiotics, biocides, dyes, organic solvents, detergents, bile salts, β-lactamase inhibitors, and other molecules [3]. Moreover, some bacteria possess several different RND pumps with partially overlapping substrate specificities, increasing their ability to deal with toxic compounds (Table 4.1). The complement of efflux pumps in a particular Gram-negative species likely reflects the variability of its environment. For example, the ubiquitous environmental organism *P. aeruginosa* has a large and highly regulated genome that encodes 12 different putative RND family efflux pumps [7, 8], presumably enhancing survival in the presence of toxic molecules, including natural product antibacterials encountered in the environment. In contrast, *Haemophilus influenzae*, which is adapted mainly to the human respiratory tract, has only one RND pump.

4.2.1.2 Mechanism of Efflux by RND Family Pumps

Significant advancements have been made in the understanding of RND pump assembly and function in recent years. The pump proteins AcrB in *E. coli* and MexB in *P. aeruginosa* organize as a trimeric structure in the cytoplasmic membrane with each protein having an extension into the periplasm made up of a porter and funnel domain. MFP components are anchored in the inner membrane by a palmitate acyl chain and have four domains: membrane proximal, β-barrel, lipoyl, and α-helical. The MFS protein AcrA was shown to organize as a hexamer. Finally the OMF is organized as a trimer in the OM with large domains extending into the periplasm. Interaction between AcrA and AcrB and AcrA and TolC has been demonstrated in vitro, consistent with AcrA acting as a linker between the AcrB pump and the TolC OMF. TolC assumes a closed shape when not partnered with AcrA, and the interaction of TolC with the α-helical hairpins of AcrA is thought to mediate the

Table 4.1 Example RND efflux pumps in Gram-negative pathogens and range of antibiotics accommodated by each pump

Organism	Pump component			Antibiotics pumped
	MFP	RND	OMF	
A. baumannii	AdeA	AdeB	AdeC	AG, CM, FQ, TC (MC), TG
	AdeI[a]	AdeJ[a]	AdeK[a]	BL, CM, EM, FQ, TC (MC), TG
	AdeF	AdeG	AdeH	FQ, TG
B. cepacia	CeoA	CeoB	OpcM	CM, FQ, TM
E. coli	AcrA[a]	AcrB[a]	TolC[a]	BL, CM, FQ, ML, NO, RF
	AcrA	AcrD	Tolc	AG, FU, NO
	acrE	Acrf	TolC	FQ
H. influenzae	AcrA[a]	AcrB[a]	TolC[a]	EM, NO
K. pneumoniae	AcrA	AcrB	TolC	BL, CM, EM, FQ, TG
	OqxA	OqxB		TG
	KpgA	KpgB	KpgC	TG
P. aeruginosa	MexA[a]	MexB[a]	OprM[a]	AG, BL, CM, ML, NO, TC, TG, TM,CM, CP, FQ, TC
	MexC	MexD	OprJ	CM, FQ
	MexE	MexF	OprN	EM, TC
	MexJ	MexK	OprM/ OprH	CM, EM, FQ, TC
	MexV	MexW	OprM	AG, ML, TC, TG
	MexM	MexN	OprM	BL
	MexX	MexY	OprM	AG, ML, TC, TG
S. enterica serovar Typhimurium	AcrA[a]	AcrB[a]	TolC[a]	BL, CM, EM, FQ, NO, RF, TC
S. maltophilia	SmeA	SmeB	SmeC	AG, BL, FQ
	SmeD[a]	SmeE[a]	SmeF[a]	EM, FQ, TC (MC), TG

Table 4.1 summarizes data extracted from Li et al. [3]. For additional pumps and details regarding substrate ranges and pump regulation, consult this very comprehensive review

MFP membrane fusion protein, *RND* resistance-nodulation-division pump component, *OMF* outer membrane factor

Antibiotics: *AG* aminoglycosides, *BL* β-lactams, *CM* chloramphenicol, *EM* erythromycin, *FQ* fluoroquinolones, *FU* fusidic acid, *ML* macrolides, *NO* novobiocin, *RF* rifampicin, *TC* tetracyclines, *MC* minocycline, *TG* tigecycline, *TM* trimethoprim

[a]Denotes a pump that is expressed constitutively (housekeeping pump), but regulatory mutations can further upregulate expression

switch to an open state of TolC [9]. A direct interaction between AcrB and TolC has also been shown in vitro and also in cells using chemical cross-linking [10], but other models suggest an alternative mechanism of assembly where AcrB and TolC do not interact [11]. A very recent study showing in vitro reconstitution of AcrAB-TolC and MexAB-OprM using nanodisc technology and characterization by single-particle electron microscopy revealed a structure whereby the pump and OMF were linked by the MFP but did not directly interact [12]. Whether that structure represents the final functional pump assembly in a cellular context or if direct interaction

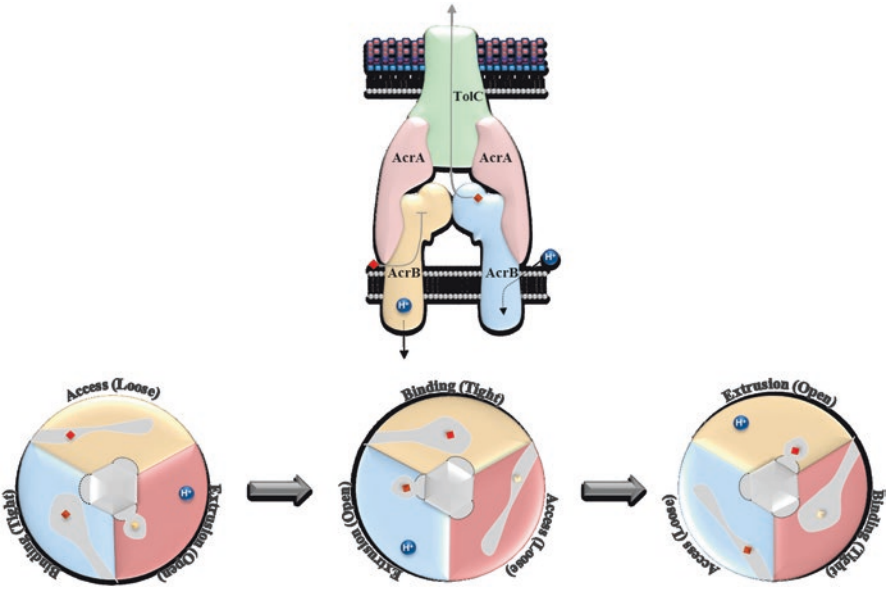

Fig. 4.2 Rotating functional mechanism of efflux by RND family pumps (represented by AcrAB-TolC); compounds enter at AcrB access conformation; AcrB undergoes a conformational change to the binding mode and then to the extrusion mode where the compound is released into the outer membrane channel. Side view of assembled pump with access and extrusion depicted (top); top view cross section of functional AcrB rotamers (bottom)

between the pump and OMF is required for function is not currently resolved, but these observations support the notion that the MFP component itself likely forms part of the exit duct between the RND pump component and the OMF. Structural studies with AcrB done by independent groups [13–15] and later simulation studies [16, 17] revealed that drug efflux occurs by a functional rotation mechanism (Fig. 4.2). Each of the three protomers of the assembled pump component (e.g., AcrB) can exist in one of three states referred to as "access" (or "loose"), "binding" (or "tight"), and "extrusion" (or "open") (Fig. 4.2). A complete functional cycle occurs as follows: a compound enters the access conformation of AcrB from the periplasm (likely from the outer leaflet of the cytoplasmic membrane) via a transmembrane domain called the vestibule. AcrB then changes conformation to the binding conformation, which opens the large compound-binding pocket to accommodate the entry of the compound, and finally AcrB rotates to the open (extrusion) conformation which releases the compound from the binding pocket into the funnel region toward the OMF (TolC). As mentioned above, the interaction of the MFP component with TolC keeps TolC in an open formation allowing compounds to be expelled outside the cell. Energy for this process is derived from transport of protons from the periplasm to the cytoplasm, and it is suggested that a proton is released to the cytoplasm when AcrB transitions from the tight to the open conformation [14]. Consistent with RND pumps requiring proton-motive force to function, energy

decouplers like CCCP inhibit efflux. The location of the vestibule in pump proteins like AcrAB is such that compounds enter from the periplasmic leaflet of the cytoplasmic membrane, thereby suggesting generally that RND pumps recognize compounds as they are entering the cell rather than after they ultimately reach the cytosol. This is consistent with early observations that certain RND family pumps reduced susceptibility to β-lactam antibiotics or β-lactamase inhibitors that target penicillin-binding proteins or β-lactamase enzymes, respectively, which are located in the periplasm [18–20], and with the reported importance of amino acid residues in periplasmic loops of the inner membrane pump components in determining substrate recognition [21–23]. As mentioned above, single-component pumps from other families may also play a possibly underappreciated role in acting cooperatively with RND pumps when they have overlapping substrate specificities and the cellular antibacterial target of a compound is cytosolic [6]. This has been fairly well established in the case of tetracycline-specific MFS (TetA/C) pumps which specifically efflux tetracycline into the periplasm where broader specificity RND pumps that recognize tetracycline, such as MexAB-OprM, can extrude the compound from the periplasm [24]. It remains to be determined in detail where these multi-pump interactions are important in terms of clinical resistance. Without the contribution of an RND pump in this sequential efflux, the compound may accumulate in the periplasm where it may readily diffuse back in across the cytoplasmic membrane. When effluxed out of the periplasm by the RND pump, it can diffuse away or alternatively must reenter the cell by again traversing the OM. This raises the concept of compound influx and the role of the Gram-negative OM permeability barrier as it relates to RND-mediated efflux, which is discussed in the next section.

4.2.1.3 The Gram-negative Outer Membrane (OM) Permeability Barrier and Its Interrelationship with Efflux

The OM of Gram-negative bacteria differs from the cytoplasmic membrane phospholipid bilayer in that it is asymmetrical, having an inner leaflet of phospholipid and an outer leaflet of lipopolysaccharide (LPS) (Fig. 4.3). The basic structure of LPS is comprised of lipid A, which forms the outer leaflet of the membrane bilayer, to which is attached the core oligosaccharide that extends out from the cell surface [25]. Lipid A core is often decorated with a highly variable polysaccharide repeating unit (O-antigen). Each lipid A molecule contains several acyl chains, and lipid A is packed together by Mg^{2+} cross-links between phosphates on the lipid A. Additional cross-linking between phosphates on the core oligosaccharide can also be important in some bacteria [26]. Because of this, the Gram-negative OM bilayer can provide a formidable permeability barrier to a wide variety of molecules, since it has a net negative charge combined with the hydrophobic layer provided by the lipid portion of the bilayer. Differences in lipid A structures and variation in lipid A cross-linking among Gram-negative bacteria can cause differences in the permeability barrier of the OM bilayer.

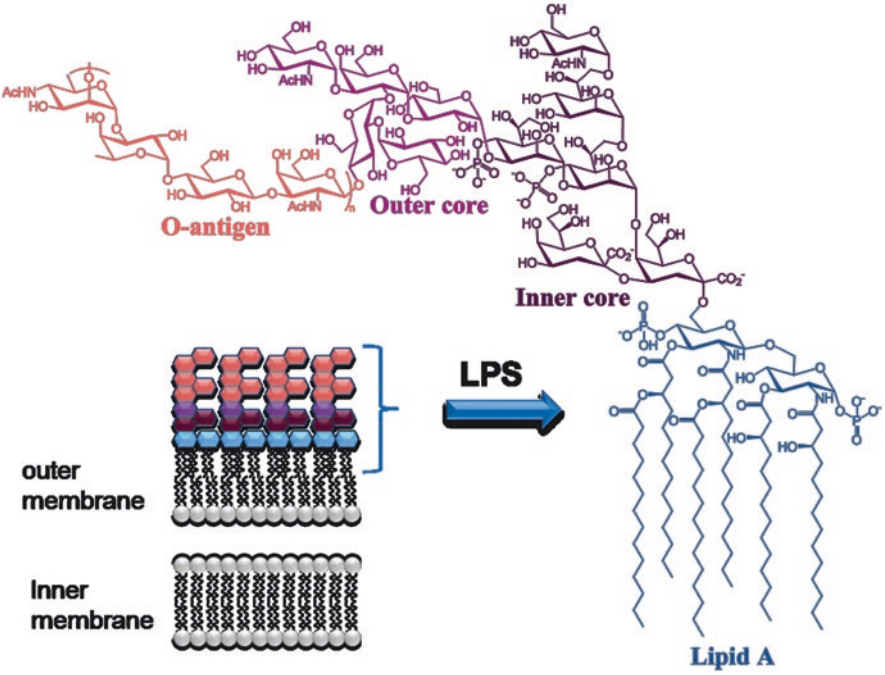

Fig. 4.3 Chemical structure of lipopolysaccharide from *E. coli* 0157:H7. The lipid A forms the outer leaflet of the asymmetrical outer membrane. Acyl chain number and lengths and level of lipid A and core phosphorylation vary among different Gram-negative bacteria

The requirement for nutrient uptake across the OM bilayer is generally met by water-filled protein β-barrel channels that span the bilayer, known as porins (see Fig. 4.1). Porins allow for the passage of small hydrophilic molecules across the OM, essentially establishing the overall OM as a molecular sieve. These channels are also thought to allow influx of certain hydrophilic antibiotic molecules that are small enough to traverse the porin channels [27]. Gram-negatives such as *E. coli* have several relatively nonspecific large porins such as OmpC and OmpF with molecular weight cutoffs of approximately 600 Da [28]. In contrast *P. aeruginosa* harbors a number of more specialized or restrictive (smaller) porins to allow influx of nutrients. This organism also has the general porin OprF, but this exists only occasionally in the conformation that allows the channel to be open [29]. This highlights that, along with variability in the lipid bilayer characteristics among different Gram-negative bacteria, the number and characteristics of the OM porin channels can also vary, causing large differences in the effectiveness of the OM permeability barrier. Reflecting this, the OM of *P. aeruginosa* was estimated to be more than tenfold less permeable than that of *E. coli* [30]. The best studied examples of antibiotic permeation via porins center on various β-lactams. For example, carbapenems can enter *E. coli* via OmpC porins, and some carbapenems such as imipenem enter *P. aeruginosa* via OprD. In the latter case, the natural function of

OprD is transport of basic amino acids, which bear some structural resemblance to certain carbapenems. Imipenem was also recently shown to enter *P. aeruginosa* via OpdP [31], suggesting that some antibiotics may access cells via multiple porins. Hydrophobic and/or larger compounds not able to enter by porins can enter the bacterium by diffusion across the membrane bilayer, although these processes are likely slower and are not well understood. As well, there are a limited number of specialized energy-dependent active transporters in the OM that import scarce nutrients such as iron (as siderophore or protein-bound complexes) [32] or cobalamin [33].

Overall, the OM of Gram-negative bacteria is highly evolved to provide a strong protective permeability barrier, but it still allows influx of important nutrients, either by passive diffusion in the case of porins or active transport in other cases. For most antibiotics though, the OM slows their influx considerably, and the combination of reduced antibiotic influx due to the OM permeability barrier and active efflux together typically determines the levels of susceptibility [34, 35]. RND family efflux pumps do not always exhibit a high velocity of antibiotic efflux, so beyond whether the pump recognizes a specific antibiotic, its effectiveness depends to a large extent on how slowly a given substrate antibiotic is entering the cell. If the influx rate is too fast, the pump may not "keep up," and even if the compound is a substrate, the pump may not confer meaningful resistance. In contrast, if the membrane barrier is slowing influx, efflux pumps can then become a very significant resistance factor. This was elegantly shown for oxacillin compared to ampicillin in *E. coli*. These antibiotics were shown to be very similar as substrates of the AcrB efflux pump, but oxacillin traversed porins more slowly. Deletion of the *acrB* gene had a much larger impact on susceptibility to oxacillin than to ampicillin [36, 37]. Furthermore, disruption of either efflux or the OM permeability barrier in *P. aeruginosa* strongly increased antibiotic susceptibility, with an even greater increase in susceptibility when both were disrupted simultaneously, showing the interplay between these two factors [38]. More recently it was reported that expression of an OM iron-siderophore transporter that had been engineered to create very large porin-like channels in the bacterial OM strongly increased susceptibility to a range of antibacterial compounds, further showing the important role of the OM permeability barrier and its interrelationship with active efflux in several Gram-negative pathogens [39, 585]. Correspondingly, mutations that impact either the OM permeability barrier or efflux in Gram-negative bacteria can decrease susceptibility to antibiotics, and this is discussed in the next section.

4.2.1.4 The Role of Efflux and the OM Permeability Barrier (Cell Permeability) in Decreasing Antibiotic Susceptibility

The combination of the OM permeability barrier and efflux is important for dictating the spectrum of Gram-negative pathogens that a clinically used or novel antibiotic under development will be sufficiently active. In those cases where useful antibacterial activity does occur (e.g., with currently used antibiotics), mutations

that increase the expression of efflux pumps, alter substrate recognition, or impact the OM permeability barrier (decreased compound influx) erode this activity over time and limit a compound's therapeutic longevity. Since many RND efflux pumps have broad substrate ranges, selection of pump upregulation with one compound will usually affect susceptibility to multiple antibiotics, contributing to multidrug resistance. These factors are particularly problematic since they contribute to the therapeutic demise of current antibiotics while also being the major impediment to the discovery of novel replacement antibiotics. As an example illustrating both of these points, tigecycline, a glycyl derivative of minocycline that evades the classic tetracycline-specific resistance mechanisms of ribosomal protection and efflux by tetracycline-specific single-component efflux pumps (e.g., TetA) (discussed in detail in Sect. 4.2.4), was still subject to intrinsic RND-mediated efflux in *P. aeruginosa* [40], and therefore its spectrum does not include this pathogen. Although tigecycline achieves useful antibacterial activity against other Gram-negatives and has been successfully implemented clinically, mutations leading to RND pump upregulation can erode this activity in organisms such as *Proteus mirabilis* [41], *K. pneumoniae* [42], *E. coli* [43], and *A. baumannii* [44].

The impact of cell impermeability on the discovery of new anti-Gram-negative antibiotics is difficult to overstate, as the vast majority of compounds are subject to some level of efflux and/or limited influx. In one study, the majority of antimicrobial compounds identified from direct antibacterial screening in *E. coli* were AcrAB-TolC pump substrates [45]. In a broader discussion of overall screening efforts conducted at AstraZeneca, the inability of compounds to accumulate in Gram-negative bacteria was cited as a significant impediment to novel antibiotic discovery using corporate compound collections [46]. Additional examples of novel compounds that inhibit specific bacterial targets but are subject to efflux include the peptide deformylase inhibitor LBM415, which is subject to AcrAB-TolC-mediated efflux in *H. influenza* [47], and CHIR-090 an LpxC inhibitor that has potent intrinsic activity against *P. aeruginosa* but selects in vitro for mutations that upregulate expression of several RND efflux pumps [48]. Similarly, standard antibiotics with good intrinsic activity against Gram-negative pathogens, such as fluoroquinolones, many β-lactams, and aminoglycosides, also select for mutations causing increased pump expression which can decrease susceptibility substantially [3].

The selection of mutations leading to pump upregulation underscores the idea that although RND pump expression is generally subject to intricate regulation, pump expression is not typically induced by antibiotics of clinical importance. An exception is the strong induction of the MexXY efflux pump of *P. aeruginosa* by compounds that perturb protein synthesis. Even in that case, however, induction occurs in response to a range of structurally and mechanistically unrelated protein synthesis inhibitors including aminoglycosides and tetracyclines [40], novel ribosome inhibitors such as argyrin B [49] or to mutations that impair ribosome function [50, 51]. Therefore, MexXY expression is responsive to ribosome impairment [52, 53] rather than to the specific antibacterial compounds, and in some cases such as argyrin B, the inducing compound may not be a pump substrate. Novobiocin was also shown to directly bind to the NalD repressor of the MexAB-OprM efflux pump and induce pump expression [54].

In general, pump upregulation leading to decreased susceptibility to antibiotics generally occurs by selection of stable mutations in the pump gene promoter region or more often in regulatory genes. The circuits controlling efflux pump expression can be highly complex, and overall this topic is beyond the scope of this section, but in general, efflux pump regulation often involves repressor proteins that bind operators upstream of pump genes to reduce pump expression unless relieved by either an intracellular signal or interaction with other modulatory elements (e.g., MexR and NalD, which control expression of MexAB-OprM in *P. aeruginosa* [55–57]), positive activators that bind and induce pump gene expression in response to intracellular signals (e.g., MexT which activates expression of MexEF-OprN in *P. aeruginosa* [58, 59]), or in some cases two-component histidine kinase sensor response regulator pairs (e.g., BaeRS and CpxRA [60–63]). In most Gram-negative bacteria, there is usually a housekeeping pump (e.g., MexAB-OprM of *P. aeruginosa* or AcrAB-TolC of *E. coli*) that is expressed constitutively, with additional pumps that are not appreciably expressed, at least under laboratory conditions (e.g., MexCD-OprJ of *P. aeruginosa* and AcrEF-TolC of *E. coli*). Expression of pumps such as MexAB-OprM and AcrAB-TolC can be increased by mutations in genes encoding their cognate repressors (e.g., *mexR* [64] and *nalD* [65] or other regulators (*nalC* [66]) for MexAB-OprM or *acrR* [67] for AcrAB-TolC). Mutations in genes controlling typically silent pumps, such as MexCD-OprJ and MexEF-OprN, can turn on expression to generally high levels with corresponding increases in resistance to their substrate antibiotics [59, 68]. Upregulated pumps are routinely found among clinical isolates [3]. Since pump upregulation can result from simple loss-of-function mutations in repressor genes, these mutants can be selected at high frequencies under antibiotic exposure levels within which the efflux pump can accommodate. Furthermore, since RND or other pumps can extrude common biocides, such as chlorhexidine, pump upregulation is likely selected in the environment by biocide-containing cleaning solutions [69].

The complex regulatory circuits controlling expression of some efflux pumps can also control the expression of OM porins through which some antibiotics cross the OM. For example, the highly complex MAR (multiple antibiotic resistance (reviewed in [3, 7, 70]) regulatory circuit controls AcrAB-TolC expression and porin expression in *E. coli*. Therefore mutants having both reduced antibiotic influx and increased efflux via AcrAB-TolC can emerge. Similarly, *P. aeruginosa* mutants that overexpress MexEF-OprN are also downregulated for expression of the porin OprD, the main entry route of carbapenems into the cell [59]. Reduced susceptibility to carbapenems in *nfxC* mutants is thought to be mediated mainly by reduced influx rather than efflux. Mutations affecting porin expression or function, including mutations within porin genes, have been described in several bacteria and in particular are associated with carbapenem resistance in *Enterobacteriaceae* and *P. aeruginosa* clinical isolates [71–74]. Mutations in genes encoding the two-component regulator ParRS in *P. aeruginosa* were shown to cause inducible or constitutive resistance to four classes of antibiotic (polymyxins, aminoglycosides, fluoroquinolones, and β-lactams) via a combination of increased efflux (MexXY/OprM), porin downregulation, and aminoarabinose modification of lipopolysaccharide (LPS)

[75]. The latter affects the ability of polymyxin (cationic peptides) to interact with LPS which is required for their entry into cells (discussed in Sect. 4.2.6). These examples serve to illustrate the extensive ability of many Gram-negative bacteria to survive exposure to toxic molecules by preventing their accumulation in the cell. The ability of mechanisms such as efflux and porin loss to enhance survival under exposure to antibiotics also supports the emergence of other resistance mechanisms such as specific target mutations, ultimately facilitating the emergence of very high levels of resistance.

4.2.1.5 Efforts to Address Compound Accumulation in Gram-negative Bacteria

Lack of sufficient compound accumulation in cells is arguably the single most important specific factor hindering the discovery and development of novel antibiotics for Gram-negative pathogens. The search for novel antibacterials with new mechanisms of action is predicated on avoidance of cross-resistance with existing mechanisms selected by currently used antibiotics. The presence of RND efflux pumps (and selection of pump over-expressors clinically), as a general broad resistance mechanism, often serves to defeat this strategy in cases where the novel compound is a pump substrate and has an intracellular target. It is reasonable to speculate that the efflux-/OM-mediated permeability barrier has coevolved with many intracellular targets that are essential for growth or viability in order to exclude most molecules with the characteristics required to optimally bind and inhibit intracellular essential targets. Therefore, strong interest has developed within the antibiotic discovery field in understanding the Gram-negative OM permeability barrier and efflux with a view toward two general goals: interfering with cell impermeability as a way of potentiating antibiotic activity by increasing the cellular accumulation of a partner antibiotic (combination therapy) and the understanding of the design of inhibitors that are less impacted by efflux and can penetrate cells effectively to reach their target. See the Innovative Medicines Initiative (IMI) translocation effort (https://www.imi.europa.eu/content/translocation) for more details.

Potentiation of the Cellular Activity of Antibiotics

One strategy to potentiate partner antibiotics that has garnered extensive interest over the years is the design of efflux pump inhibitors (EPIs). In theory, a potent EPI could improve the spectrum and potency of a range of currently used antibiotics whose usefulness is compromised by efflux. EPIs could also serve to enhance and extend the clinical usefulness of novel agents that are or may become affected by efflux. Several EPIs have been described over the last two decades.

The first EPI described in detail as an inhibitor of multiple RND family pumps, MC207,110 (*phe-arg*-β-napthylamide, PAβN) was originally identified by screening for compounds that potentiated the activity of pump substrate fluoroquinolone

antibiotics in *P. aeruginosa* [76]. Inhibitors such as MC 207,110 are pump substrates and are thought to act through competitive binding and interference with substrate antibiotic recognition [77]. MC207,110 is a lipophilic amine and as such falls into a chemical property space known for target promiscuity and associated challenges in achieving an acceptable safety profile [78]. Perhaps reflecting this, MC207, 110 was also shown to have some bacterial membrane-disrupting activity [76, 79]. In contrast to MC207,110, the EPI D13-9001 is more specific to the MexAB-OprM efflux pump within *P. aeruginosa* [80]. Compounds like D13-9001 would therefore need to be partnered with an antibiotic effluxed primarily by MexAB-OprM. The mechanism of pump inhibition involves binding of D13-9001 into a hydrophobic "trap" extending off the substrate translocation channel within MexB, ultimately compromising the pump's functional rotation [81, 82]. This mechanism is more likely than that of a substrate competition mechanisms, such as that of MC207,110, to block efflux of multiple antibiotics and to enable better inhibitor potency. Furthermore, D13-9001 is zwitterionic which places it in a potentially less promiscuous chemical property space. Newer pyranopyridine EPI molecules (e.g., MBX2319 and analogs [83–85] that take advantage of the hydrophobic trap mechanism have achieved substantial increases in potency. Recently described EPIs (NSC 60339 and analogs) were shown to have a very novel mechanism of inhibition of AcrAB-TolC, by binding the membrane fusion protein (AcrA), inducing structural changes, and possibly interfering with assembly of the functional pump [86]. Advances in the structural understanding of pump assembly and function, as well as the binding of several EPI molecules and the diversity of potential pump inhibitory mechanisms, may increase the ability to design new EPI molecules in the future.

Selection of a suitable partner antibiotic is potentially a complex issue, especially with clinically used antibiotics. This is because non-efflux-based resistance mechanisms (e.g., target or modifying enzyme-based mechanisms) affecting many standard antibiotics may have become widespread, and those mutants may be resistant even if efflux is fully inhibited. Secondly, many antibiotics are effluxed by several pumps within a given Gram-negative pathogen or across different bacteria, thereby requiring a broad spectrum of EPI activity to cover multiple pumps. To date no Gram-negative EPI has reached clinical use. MC207,110-based analogs were lipophilic cations, and ultimately unfavorable toxicity profiles could not be overcome [77]. It remains to be seen if this approach will be successful with other novel inhibitors. Finally, the intriguing finding that a significant percentage of *P. aeruginosa* clinical isolates recovered from cystic fibrosis patients have mutationally lost MexAB-OprM function and become susceptible to ticarcillin has prompted suggestions that this antibiotic may find use in treating this subpopulation [87].

An alternative approach to efflux inhibition may be disruption of the bacterial membrane, thereby improving the ability of a partner antibiotic to gain access to the cell. It is well established that mutations affecting the synthesis or assembly of the Gram-negative OM cause hypersusceptibility to a range of antibiotics, especially those that are more hydrophobic in nature or are large molecular weight (i.e., too large to pass through porins and whose exclusion from cells is mediated mainly by the permeability barrier of the OM bilayer). Targets impor-

tant for OM biosynthesis/assembly that may affect the OM permeability barrier if inhibited include the lipid A biosynthetic genes *lpxA*, *lpxB*, and *lpxC* [88, 89] and the LPS transport/assembly genes *lptD* and *lptE* in *E. coli* [90–92] and *P. aeruginosa* [93, 94]. Chemical inhibitors of targets such as these could induce disruption of the OM permeability barrier and strongly potentiate the activity of many antibiotics used in combination, although precisely where this may occur at a clinically useful level remains to be determined. Alternatively upon target inhibition, a corresponding progressive disruption of the permeability barrier may generate a cycle of increased uptake of the inhibitor itself, improving cellular potency, although again this remains to be shown for specific examples. Since LPS synthesis and assembly per se are essential in many Gram-negative bacteria, enzymes such as LpxC (first committed step in lipid A biosynthesis), LpxA/LpxD, and LptD have generated interest as targets for the design of novel inhibitors. For example, extensive medicinal chemistry efforts have resulted in potent small molecule inhibitors of LpxC with antibacterial activity [95–108], at least one of which reached early phase clinical evaluation [109]. *P. aeruginosa*-specific peptidomimetics targeting LptD (POL7001, POL7080) have very potent antipseudomonal activity [108, 110], suggesting the potential of this approach. Intriguingly, there are a small number of Gram-negative bacteria that can survive in the absence of lipid A biosynthesis, including the important pathogens *Neisseria meningitides* [111, 586] and some *A. baumannii* strains [112–114]. Loss of lipid A in the latter has been shown to result from mutations in lipid A biosynthetic genes *lpxA*, *lpxC*, or *lpxD* [113], and *lpxC* and *lptD* can be genetically deleted in some *A. baumannii* [115]. Therefore, inhibitors of these targets would not be expected to have good, or any, antibacterial activity against such strains. However, reflecting the potential of inhibitors of these targets to potentiate antibiotics, strains harboring these genetic mutations or wild-type strains exposed to LpxC inhibitors became susceptible to several antibiotics [113, 115, 116]. Importantly, an intact OM permeability barrier is also important for survival of pathogens (virulence) during infection, by providing protection from serum complement and other host immune factors.

Molecules such as LPS are also strong activators of toll-like receptors (e.g., TLR4). Consistent with this, an LpxC inhibitor with no appreciable in vitro antibacterial activity against *A. baumannii* was highly protective in a mouse infection model [117]. This was attributed to increased opsonophagocytic killing and lower levels of released LPS, leading to reduced inflammation. This example illustrates the additional potential of inhibitors of targets such as LpxC, used alone or in combination with other antibiotics, resulting from effects on the OM. *A. baumannii* represents an extreme example to show this, since some *A. baumannii* can tolerate a large or total loss of lipid A synthesis, providing the widest possible window to see this effect. It remains to be understood how broadly this might translate across different targets/inhibitors and different Gram-negative pathogens in clinically relevant scenarios. As with any novel antibacterial, in particular ones that must still reach an intracellular target such as LpxC, a variety of resistance mechanism are likely to be able to impact their cellular activity. In vitro, mechanisms such as target

mutations, target overexpression, partial bypass by mutations in *fabG*, and upregulated efflux can all reduce the activity of the LpxC inhibitor CHIR-090 in *P. aeruginosa* [48], and mutations in *fabZ* reduce susceptibility of *E. coli* to LpxC inhibitors [118]. In contrast to targets like LpxC which are intracellular, the OM itself is also a target, and compounds that could interact with and disrupt the OM might potentiate antibiotics without having to reach an intracellular target. Classic examples of this are cationic molecules, such as polymyxins, which interact with LPS via phosphates attached to lipid A and disrupt the OM permeability barrier causing sensitization to antibiotics [119–121]. Such an approach to potentiate antibiotics against Gram-negative pathogens (compound SPR741, an analog of polymyxin B nonapeptide) is currently being pursued [122].

Understanding Compound Penetration to Improve Access to Targets

While factors limiting a compound's OM and inner membrane (IM) permeability are fairly well understood, structure activity relationships for efflux remain incomplete. Strategies to improve compound access to intracellular targets must consider the specific compartment in the cell where the target resides. A target might reside anywhere from the cell surface to the periplasmic space between the inner and OM through to the cytosol of the cell. Different issues come into play for each of these. One way to reduce the complexity of cell penetration is to pursue targets located near or on the cell surface, circumventing the need for significant cell penetration. As mentioned above, polymyxin-based antibiotic potentiators such as SPR741 directly target the LPS on the cell surface. Similarly, the cationic peptidomimetic POL7001 targets LptD, an essential protein localized in the OM [110] at or near the cell surface. The number of OM protein targets that are essential for growth is low (i.e., LptD and BamA), and these targets exist as components of complex machinery which could be more difficult to disrupt or inhibit by small molecules, but this remains to be fully understood. To date, only larger peptidomimetic inhibitors of these targets have been described [110, 123], providing some possible insights into the types of molecules expected to be active in this context. On a related note, eliminating the need for cell penetration is a salient feature of the monoclonal antibody approach, which functions specifically by exploiting surface-exposed antigens. An example is a recently described bispecific antibody (MEDI13902) targeting PcrV (type III secretion) and the exopolysaccharide Psl [124, 125] which is currently undergoing clinical trials for prevention of nosocomial pneumonia. Progressing further into the cell, some targets reside in the periplasmic space between the IM and OM. The number of essential targets here is also limited but includes the clinically validated penicillin-binding proteins (PBPs) that are inhibited by the β-lactam class of antibiotics. Inhibitors of periplasmic targets need only to cross the OM, which lends itself to utilization of water-filled porins for compound access, with corresponding optimization for this route of entry. Entry through porins relies to a large extent on a size small enough to traverse the porin (porin cutoff is approximately 600 Da), compound polarity (hydrophilicity), and appropriate charge distribution.

The mechanisms by which translocation of compounds occurs via porins have been the subject of extensive investigation [126–128], and assays to evaluate porin translocation for use in drug design are being pursued [129, 130]. A recent study suggested that porin traversal may be optimal for small polar compounds with charged groups and a dipole moment having a component aligned perpendicular to its main axis [128]. Since β-lactams have periplasmic targets, they may represent the class of antibiotic that can best be optimized specifically for permeation through porins. It is likely that this contributes in some cases to reducing the impact of efflux since rapid influx can overwhelm the capacity of RND family efflux pumps even if the compound is a pump substrate [36, 37]. The majority of novel antibacterial targets however are located in the cytosol, and understanding compound penetration (and evasion of efflux) becomes more complex in that case than for compounds such as β-lactams. This stems from the necessity to traverse the two distinctly different membranes. The OM severely limits influx of larger or more hydrophobic compounds, which must diffuse across the asymmetrical OM bilayer which has low fluidity and presents a high barrier for lipophilic compounds, since they are excluded from entry through porins [3]. Smaller more hydrophilic molecules can traverse the OM through porins, but extensive optimization for polarity to maximize this can hinder entry across the symmetrical phospholipid inner membrane bilayer, which favors diffusion of hydrophobic molecules. Antibiotics directed at cytosolic targets may therefore need some element of amphiphilicity to cross both membranes, which also may increase recognition by RND efflux pumps [131]. A much better understanding of the chemical property space required for the design of cell active inhibitors of intracellular targets, and the representation of this chemical property space typically found in corporate screening libraries, is likely required to overcome the ongoing inability to deliver novel antibacterials in this area. Initial efforts toward this understanding were described by O'Shea and Moser [132], who examined the properties of a wide range of antibacterial compounds and, consistent with our understanding of the cell envelope, correlated properties such as molecular weight and polarity with Gram-negative antibacterial activity. A more recent look at data from a wide range of screening efforts at AstraZeneca also suggested that polarity and small size correlated with reduced efflux and cellular activity and increasing compound hydrophobicity could drive biochemical target inhibition but possibly at the expense of cellular activity [46]. However, increased polarity itself was not sufficient to ensure antibacterial activity [46], again suggesting a fine balance of properties is likely necessary. This is consistent with the notion that the cell envelope and efflux likely coevolved with intracellular targets to exclude molecules with the properties to strongly bind and inhibit essential targets. These properties may also be compound scaffold specific. Modulation of physicochemical properties (pKa and logD) improved the antibacterial activity of novel bacterial type II topoisomerase inhibitors [133]. A very recent study directly measured accumulation of compounds in *E. coli* using mass spectrometry, and computational analysis indicated that rigid, amphiphilic compounds with low globularity and containing an amine moiety accumulated better. These rules were applied to convert a compound that was active only against Gram-positive bacteria into one with *E. coli* activity [134]. This suggests it

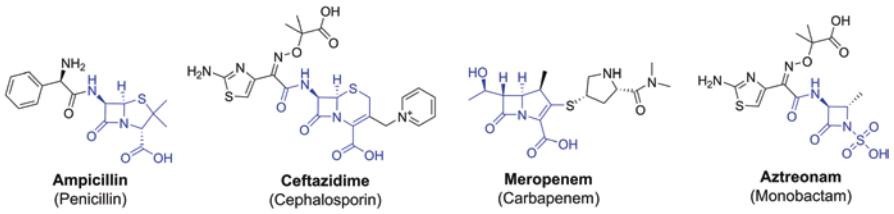

Fig. 4.4 Representative structures for each class of β-lactam. The core structure is depicted in blue; the specific side chains are depicted in black

may be possible to derive some general rules to engineer compounds with Gram-negative accumulation and cellular activity, but more research will be necessary to validate this concept. Furthermore, compound accumulation must be achieved together with low toxicity in order for resulting compounds to be therapeutically useful. Efforts are also underway to explore new methods complementary to mass spectrometry to measure cellular accumulation of compounds, which could further assist in defining rules for cell penetration [135, 136]. Finally, the Trojan-horse concept has also been applied to antibacterial design. In this scenario, an antibiotic is linked to a compound that is actively transported into cells, thereby exploiting the active uptake mechanism to drive intracellular accumulation of the chimeric antibiotic molecule. Among others, a recent example of this is compound cefiderocol (S-649266), a catechol cephalosporin that is proposed to utilize energy-dependent siderophore-iron uptake systems in Gram-negative bacteria for improved cellular access [137].

4.2.2 β-Lactams and β-Lactamase Inhibitors

The identification of the β-lactam benzylpenicillin in the 1920s essentially started the antibiotic era [138]. Initially used to treat soldiers in World War II, the lifesaving potential of these compounds was quickly realized, leading to the design of novel β-lactams that continues to this day. β-lactams fall into four classes: penicillins, cephalosporins, carbapenems, and monobactams (monocyclic β-lactams) (Fig. 4.4). Together these comprise by far the most widely used class of antibiotics worldwide. The history and details of the development of the multitude of β-lactam antibiotics are beyond the scope of this chapter and have been recently reviewed in [139]. Examples of β-lactams with a broader spectrum that can include serious Gram-negative pathogens such as *E. coli*, *K. pneumoniae*, and *P. aeruginosa* are the penicillins, such as ampicillin, amoxicillin, carbenicillin, and piperacillin; the cephalosporins such as ceftazidime and cefepime; the carbapenems such as imipenem, meropenem, and doripenem; and the monobactams, such as aztreonam.

β-lactams are bactericidal and target penicillin-binding proteins (PBPs) [140]. Gram-negative bacteria possess multiple PBPs which are important for cross-linking of the peptidoglycan that makes up the rigid bacterial cell wall [141].

β-lactams resemble segments of the growing peptidoglycan (e.g., D-Ala-D-Ala) and, after the formation of a low-affinity complex, covalently bind (acylate) the PBP at its active-site serine residue. Bacteria possess multiple PBPs, broadly classified into high-molecular-weight (HMW) and low-molecular-weight (LMW) categories [141]. In general, the LMW PBPs are monofunctional D-Ala-D-Ala carboxypeptidases, whereas the HMW PBPs are either bifunctional (class A, transpeptidase and transglycosylase) or monofunctional (class B, transpeptidase) [141]. Not all PBPs are essential for growth, but certain ones such as PBP3 (main target of aztreonam) are essential [141, 142]. Furthermore, many β-lactams can acylate the active-site serine of several PBPs which can contribute meaningfully to increased antibacterial activity. Inhibition of PBPs like PBP3 or multiple PBPs like PBP3 with PBP1a/PBP1b can ultimately lead to cell lysis [140, 142]. Recent studies have now elucidated that inhibition of PBPs also triggers a lethal malfunctioning of the cell wall synthetic machinery [143].

The high potency of many β-lactams against Gram-negative pathogens relies to a large extent on two factors, compound access and the nature of the targets themselves. PBPs are located in the Gram-negative periplasmic space. This means that β-lactams only need to cross the OM to access their targets. Therefore β-lactams can be polar molecules able to traverse water-filled porins, which have the fortuitous benefit of improving their safety. The second factor is that these relatively "exposed" PBPs are particularly good targets in conjunction with this class of inhibitor, since the compound-target interaction is covalent (essentially irreversible), and PBP inhibition causes severe impairment of a fundamental cellular process, leading ultimately to lethality. However, the PBP targets also form the basis of the overarching issue with resistance to β-lactams, which is the expression of β-lactamase enzymes. These enzymes are expressed in the periplasm and appear to have evolved from PBPs to attack and efficiently hydrolyze the β-lactam, mediating resistance [144]. Non-β-lactamase mechanisms that affect susceptibility to β-lactams in Gram-negative pathogens include efflux, loss of uptake porins, and amino acid substitutions in the target PBPs. These topics are addressed in the following sections.

4.2.2.1 β-Lactamases

The discovery of β-lactamases predated the clinical use of benzylpenicillin, but the widespread use of these agents in the clinic has, over time, led to the emergence of an astonishing number of β-lactamase variants [144, 145], which as a group can degrade most or all β-lactam antibiotics. Indeed the development of new β-lactam antibiotics is to some extent a continuing story of addressing the emergence of new β-lactamases [146, 147], as is the ongoing development of β-lactamase inhibitors (BLIs) for use in combination with β-lactams to restore their activity against β-lactamase-expressing strains (see Sect. 4.2.2.3). β-lactamases hydrolyze the β-lactam ring of all classes of β-lactam antibiotics by one of the two major mechanisms. The first is mediated by an active-site serine (Ser), via a covalent enzyme intermediate that is rapidly hydrolyzed causing inactivation of the antibiotic. β-lactamases that operate by this mechanism are therefore referred to as serine

Table 4.2 Classification of clinically relevant β-lactamases

Molecular class	Functional group	Description	Substrates	Representative families	Representative enzyme in clinical isolates
A	2be	Extended-spectrum β-lactamases (ESBLs)	Penicillins, cephalosporins, monobactams	TEM, SHV, CTX-M, PER, VEB	TEM-3, SHV-2, PER-1, VEB-1, CTX-M-15, CTX-M-9, CTX-M-14, CTX-M-3
A	2br	Inhibitor-resistant β-lactamases	Penicillins, narrow-spectrum cephalosporins	TEM, SHV	TEM-30, SHV-10
A	2f	Serine carbapenemases	Carbapenems, cephalosporin, cephamycins	KPC, IBC, IMI, NMC, SME, GES, SFC,	KPC-1, KPC-2, KPC-3, SME-1
B	3a	Metallo-carbapenemases	Carbapenems, penicillins, cephalosporins, cephamycins	IMP, VIM, NDM, SPM, GIM, SIM, AIM, DIM, FIM, POM	VIM-1 VIM-2 IMP-1 NDM-1
C	1	AmpC β-lactamases	Cephamycins, cephalosporins, narrow-spectrum monobactams, and penicillins	CMY, FOX, ACC, LAT, ACT, MOX, DHA, MIR,	CMY-1, CMY-2 ACT-1, DHA-1, DHA-2, CMY-13, CMY-4
D	2de	Extended-spectrum β-lactamases (ESBLs)	Cephalosporins, oxacillins	OXA	OXA-10, OXA-13, OXA15, OXA-18, OXA-45
D	2df	Carbapenemases	Carbapenems, oxacillins	OXA	OXA-48, OXA-23 OXA-40, OXA-51, OXA-58

β-lactamases. This mechanism is reminiscent of that for PBP inactivation by β-lactam antibiotics, as β-lactamases share an active-site Ser-XX-Lys motif with PBPs. A main difference in these processes is a comparatively very low rate of hydrolysis of the covalent adduct in the case of PBPs. The second mechanism is metal-mediated, whereby one or two bivalent metal ions activate a water molecule that attacks the β-lactam ring [148]. These β-lactamases are correspondingly referred to as metallo-β-lactamases. The large number of serine and metallo-β-lactamases is categorized via two different classification systems (Table 4.2). The Ambler classification is based on protein sequence homology that divides β-lactamases into four classes (A, B, C, and D). Classes A, C, and D are all serine β-lactamases, whereas class B is the metallo-β-lactamases. The second classification scheme in use for β-lactamases, defined by Bush-Jacoby, is based on enzymatic

functionality and divides β-lactamases into three major groups: group 1 cephalosporinases (class C), group 2 serine β-lactamases (classes A and D), and group 3 metallo-β-lactamases. Each major group is then divided into several subgroups based on specific attributes [145]. The first β-lactamase, TEM-1, identified in a clinical isolate was reported in the early 1960s in an *E. coli* isolate from a patient in Greece [149]. Since then, the number of β-lactamases identified has constantly grown. A recent report estimated that over 2000 unique β-lactamases sequences have been identified [150]. The major players in the clinic for infections caused by Gram-negative pathogens are the extended-spectrum β-lactamases (ESBLs), the AmpC cephalosporinases, and the serine and metallo-carbapenemases.

Extended-Spectrum β-Lactamases (ESBLs)

Extended-spectrum β-lactamases (ESBLs) confer resistance to nearly all β-lactam antibiotics except carbapenems and cephamycins. ESBLs were first identified in the mid-1980s in *K. pneumoniae* and *Serratia marcescens* [151]. The occurrence of ESBLs in clinical isolates has been constantly increasing in the past two decades. A recent Centers for Disease Control and Prevention (CDC) report estimated nearly 26,000 healthcare-associated *Enterobacteriaceae* infections are caused by ESBL-producing *Enterobacteriaceae* (19% of isolates) causing 1700 deaths each year (https://www.cdc.gov/drugresistance/threat-report-2013/index.html). The fast spread of ESBL-producing strains is due to the presence of these genes on mobile genetic elements, such as plasmids, usually carrying other antibiotic resistance genes [145, 152, 153]. Early ESBLs evolved from the TEM and SHV enzymes to be able to hydrolyze oxyimino-cephalosporins, and these are molecular class A, functional group 2be. Subsequently, the ESBL category expanded to include enzymes such as the CTX-M family, mainly present in *E. coli* and *K. pneumoniae*; the PER family identified in *Pseudomonas*, *Acinetobacter*, and *Salmonella* species; and the VEB family reported in *A. baumannii*. These β-lactamases are not genetically related to TEM or SHV β-lactamases but have similar hydrolytic profiles and are part of the functional group 2be [153–155]. The most recent ESBLs are the OXA family, originally reported *in P. aeruginosa*, isolated in Turkey and France. The OXA family, in contrast to the other ESBLs, belongs to molecular class D and functional group 2de [156].

ESBLs are prevalent in the clinic and present serious problems in hospital-acquired infections, leading to increased mortality worldwide. ESBL prevalence varies across different geographic regions. In a recent report on *Enterobacteriaceae* isolates collected in 63 US hospitals from 2012 to 2014, 13.7% of these isolates had an ESBL profile. Different trends were observed among different species of *Enterobacteriaceae*; in *E. coli* the ESBLs occurrence increased from 12.7% (2012) to 15.1% (2014), whereas in *K. pneumoniae*, rates decreased from 18.9% (2012) to 15.5% (2014). Also, a statistically significant variation was observed across different regions in the United States. In the South Atlantic Region, the ESBL rates decreased from 20.8% (2012) to 9.2% (2014). Conversely in the Pacific region, the

ESBL rates increased from 11.4% (2012) to 16.9% (2014). The predominant ESBLs identified in this study were CTX-M-15 (59% of ESBLs) followed by SHV (19% of ESBLs), both mainly in *K. pneumoniae* isolates, and CTX-M-14 (18% of ESBLs) [157]. In Europe the ESBL rates vary considerably by country. The prevalence of ESBLs in *E. coli* in the 2014 European surveillance varies from 3.3% in Iceland to 40.4% in Bulgaria. Even more alarming is the prevalence of ESBLs in *K. pneumoniae* with rates over 70% in Greece, Bulgaria, and Romania. On a positive note, the rates of ESBLs did not increase from 2009 to 2014, attributed to the increased use of carbapenems [158]. Also across Europe, the most prevalent ESBL types identified in clinical isolates were the CTX-M family β-lactamases, but the specific type varies considerably among countries, with CTX-M-9 and CTX-M-14 enzymes dominant in Spain and CTX-M-3 and CTX-M-15 dominant elsewhere [159].

Another growing family of ESBLs is the OXA-type enzymes that confer resistance to ampicillin and cephalothin, are characterized by their high hydrolytic activity against oxacillin and cloxacillin, and are very poorly inhibited by clavulanic acid. The OXA-type enzyme genes differ genetically from all other ESBLs. To date, over 500 different OXA-type variants have been reported, but not all are ESBLs. The OXA-type enzymes with activity against oxyimino-cephalosporins are OXA-10 and its variants (OXA-11, OXA-14, OXA-16, and OXA-17), OXA-13 and its variants (OXA-19 and OXA-32), and some other OXA enzymes (OXA-15, OXA-18, and OXA-45). These enzymes have been identified mainly in *P. aeruginosa* isolates [155, 160].

Even though ESBL incidence rates have not been increasing in the past few years, they are still very high in some parts of the world and are a major health concern. Further, ESBLs are often present on mobile genetic elements with other antibiotic-resistant determinants, including those for aminoglycosides and fluoroquinolones. The use of carbapenems to treat infections caused by ESBL-producing pathogens is increasing the emergence of carbapenem-resistant strains, starting the debate on how to better treat those pathogens. Using a β-lactamase inhibitor (see Sect. 4.2.2.3) with a β-lactam is in principle a targeted and effective approach. A detailed analysis on the benefit of β-lactam/β-lactamase inhibitor combinations for the treatment of ESBL-producing pathogens can be found in a recent review by Viale et al. [161].

Class C β-Lactamase (AmpC)

AmpC β-lactamases belong to class C and functional group 1. They confer resistance to cephamycins, such as cefoxitin and cefotetan, and cephalosporins, including oxyimino-cephalosporins such as ceftazidime, cefotaxime, and ceftriaxone. They are also able to hydrolyze to a lesser extent penicillins and aztreonam [162]. The majority of AmpC β-lactamases are not or are only weakly inhibited by inhibitors of class A enzymes such as clavulanic acid, sulbactam, and tazobactam. Some AmpC variants have been reported to be inhibited by tazobactam or sulbactam [162, 163]. Several AmpC β-lactamases are chromosomally encoded enzymes, found in

Acinetobacter spp., *C. freundii*, *Enterobacter* spp., *E. coli*, *Hafnia alvei*, *Morganella morganii*, *P. aeruginosa*, and *Yersinia enterocolitica*. AmpC basal expression is generally low but can be induced to high levels in some bacteria (e.g., *M. morganii* and *P. aeruginosa*) upon exposure to some β-lactams. The regulation of AmpC expression varies among different organisms. In bacteria where AmpC is inducible, the *ampC* gene is accompanied by *ampR*, encoding a member of the LysR transcriptional regulator family. Disruption of peptidoglycan synthesis by β-lactams causes accumulation of peptidoglycan fragments that dislodge oligopeptides of UDP-*N*-acetylmuramic acid normally bound to AmpR, causing a conformational change where AmpR then positively activates transcription of *ampC*. The activity of AmpR is controlled indirectly by the activities of AmpD, a *N*-acetyl-muramyl-L-alanine amidase, and an inner membrane permease, AmpG, which are both involved in recycling of peptidoglycan intermediates. Therefore, although *ampC* expression is inducible in these cases, constitutive upregulation can occur via mutations in the genes encoding these regulatory factors. In clinical isolates, the most common cause of AmpC hyperexpression is mutation in *ampD*. Mutations in *ampR* causing AmpC hyperexpression have been reported but are not as common. Mutations in *ampG* only result in constitutive low-level expression and are the least common [162]. In *P. aeruginosa* PAO1, AmpC expression is very tightly regulated by the presence of three AmpD genes with different affinities for their substrates. These AmpD genes are also reported to be involved in *P. aeruginosa* PAO1 virulence [164]. Other organisms like *E. coli* and *Shigella* lack AmpR, and regulation occurs via a weak promoter and a strong attenuator. In these cases, AmpC expression is not inducible by β-lactams, and hyperexpression of AmpC leading to resistance results from mutation in the *ampC* promoter or attenuator [165–167]. AmpC β-lactamases can also be expressed from plasmids. The first plasmid-encoded AmpC variant, CMY-1, was identified in 1989 from *K. pneumoniae* isolated from a wound infection in South Korea. The high degree of resistance to cefoxitin was due to the high-level constitutive expression of CMY-1 [162, 168]. Since the identification of CMY-1, several families of plasmid-encoded AmpC variants have been reported in clinical isolates, especially in *K. pneumoniae* and *E. coli*.

Based on the source of the *ampC* gene, several plasmid-encoded AmpC families have been reported: the two CMY families (CMY-1 and CMY-2), the FOX family, the ACC family, the LAT family, the MIR family, the ACT family, the MOX family, and the DHA family [162, 169, 170]. Plasmids encoding ACT-1, DHA-1, DHA-2, and CMY-13 typically contain an *ampR* gene, and as such expression of these β-lactamases is inducible, whereas the other plasmid-encoded AmpC variants lack *ampR* and are not inducible. The high level of expression for the non-inducible plasmid-encoded AmpC variants is mainly due to strong promoters and high-gene copy number. As with several other plasmid-borne antibiotic resistance genes, plasmids harboring AmpC β-lactamase genes often carry resistance determinants for fluoroquinolones, sulfonamides, tetracyclines, aminoglycosides, chloramphenicol, trimethoprim, and other β-lactamases (ESBLs and metallo-β-lactamases) [162, 168, 171, 172]. Clinical isolates of *K. pneumoniae* and *Salmonella enterica* carrying plasmid-encoded AmpC have been reported to be resistant to cephalosporins and

cephamycins as well as carbapenems. Detailed analysis of these strains showed that the resistance to cephalosporins and cephamycins was due to the plasmid-mediated CMY-4 β-lactamase for *Salmonella enterica* and DHA-1 for *K. pneumoniae*, whereas resistance to carbapenems also involved the lack of outer membrane porin proteins [173–177]. Combination of plasmid-encoded AmpC with deletions of porin genes (and possibly increased efflux) results in high-level resistance to most if not all β-lactams and leaves clinicians with few treatment options.

Carbapenemases

Carbapenems are considered the most effective β-lactams for the treatment of serious infections caused by Gram-negative bacteria and present a broad spectrum of antibacterial activity. Furthermore, carbapenems are relatively stable to most ESBLs and class C enzymes and are deemed to be safer to use than any other last-resort antibiotic. Therefore, the increasing number of reports of β-lactamases able to hydrolyze carbapenems over the last few years is of major concern. Carbapenemases are a heterogeneous group of β-lactamases, including members from classes A, B, and D [178, 179]. Most carbapenemases are able to hydrolyze a very broad spectrum of β-lactams. Several class A enzymes, functional group 2f, with carbapenem-inactivating activity, have been reported over the years. The first, SME-1, was reported in 1990 in a *Serratia marcescens* isolate from the United Kingdom [180]. Subsequently, GES-1 in *K. pneumoniae*, SFC-1 in *Serratia fonticola*, and IBC-1/IMI-1/NMC-A in *Enterobacter cloacae* have been reported [181]. One of the most recent and widespread class A carbapenemases is the *K. pneumoniae* carbapenemases (KPCs). KPC-1 was the first carbapenemase identified for this family of enzymes, reported in 1996 in North Carolina [182, 183]. KPCs, in contrast to the other class A carbapenemases that are chromosomally encoded, are usually on mobile genetic elements and since their discovery have spread to many other organisms including most species of *Enterobacteriaceae* (including *Enterobacter* spp., *Serratia marcescens*, and *Salmonella* spp.), *P. aeruginosa*, and several other genera. A recent study that analyzed 147 cases of infections due to carbapenem-resistant *K. pneumoniae* from 2013 to 2014, in one hospital in Northern Italy, showed that the major resistance determinant was KPC-3 (83.8%). The death rate was an alarming 24.0% in 2013 and 37.5% in 2014 [184]. This is of great concern especially in Southern European countries where CREs expressing KPC are spreading rapidly.

Several enzymes of the class D family (OXA type) are able to degrade carbapenems [185]. The first identified enzyme in this class able to hydrolyze imipenem was OXA-23. It was isolated in the United Kingdom in 1985 from an *A. baumannii* isolate and was originally characterized as ESBL [186]. Since this initial report, several other OXA enzymes have been described in *Enterobacteriaceae*, such as OXA-23-like, OXA-40-like, OXA-51-like, OXA-58-like, and OXA-48-like enzymes [185, 187]. The most widespread enzymes in this class are the OXA-48-like enzymes. OXA-48 was first isolated in a patient with UTI in Turkey in 2001 from a strain of *K. pneumoniae* and is now widely

spread across *Enterobacteriaceae* and other Gram-negative species [188]. In the recent past, OXA-48-like carbapenemases have been responsible for several outbreaks among carbapenem-resistant *K. pneumoniae* in Spain [189] and Greece [190], and currently no broadly active inhibitors of class D enzymes are on the market, in part due to high structural diversity within this class of enzymes [185].

Metallo-β-lactamases (MBLs) belong to Ambler class B, functional group 3, and all can inactivate carbapenems [191]. MBL hydrolysis of β-lactams is mediated by zinc and inhibited by metal chelators such as ethylenediaminetetraacetic acid (EDTA) but not clavulanic acid or other clinically used β-lactamase inhibitors. MBLs can hydrolyze to varying degrees members of all β-lactam classes except monobactams. Since the early 1990s, when the first MBL, IMP-1, was detected, the number of transmissible genes encoding MBLs has been constantly growing. The major MBLs currently present in the clinic are IMP-like, VIM-like, and NDM-like enzymes. Other MBLs such us the SPM, GIM, SIM, AIM, DIM, FIM, and POM have been reported but are not widely distributed [181]. More than 30 derivatives of IMP-like enzymes have been reported and are commonly found in Japan, China, and Australia causing sporadic outbreaks [187]. In contrast, the VIM-like carbapenemases have been reported from hospitals worldwide. The first VIM-like carbapenemase, VIM-1, was identified in Italy from a *P. aeruginosa* isolate in 1997 [192]. Currently, over 30 VIM-like carbapenemase have been reported around the world with VIM-2 being the most widespread MBL in *P. aeruginosa* [191]. VIM-like enzymes are often harbored in gene cassettes and are also associated with integrons [193]. The prevalence of VIM-like enzymes among MBL-producing *Enterobacteriaceae* isolates in Europe is very high, especially in countries like Italy and Greece. A multicenter European survey showed the presence of VIM-1-like enzymes in 98.9% of *Enterobacteriaceae* isolates producing MBLs [187].

The New Delhi metallo-β-lactamase (NDM), first reported in 2009, is the latest carbapenemase described that is threatening the usefulness of β-lactams globally. So far, 13 different variants of NDM have been reported; in several cases, the mutation in the bla_{ndm} gene seems to predict rates of β-lactam hydrolysis [194]. NDM producers have been isolated across the globe but predominately in the Asian continent and mainly in India. In the United Kingdom and the Middle East, outbreaks of NDM-producing strains have been reported in the recent past. Several reports have shown that NDM-1-producing pathogens are resistant to many other antibiotics, thus limiting options for treating these infections to a small number of agents such as polymyxins, fosfomycin, and aminoglycosides which are rarely used due to efficacy and/or safety concerns [194]. As well, many NDM-1-producing strains also possess ribomethylase, leading to aminoglycoside resistance (see Sect. 4.2.5.2). In 2010 in Canada, a NDM-1-producing *K. pneumoniae* outbreak was reported, which included a patient with no prior history of traveling to Asian countries that are high risk for these infections. In vitro susceptibility tests showed that the strain was resistant to the majority of available antibiotics except colistin and tigecycline [195]. Several studies summarized in a recent review by Zmarlicka et al. have suggested however that in vitro resistance to several β-lactams by NDM-1-producing organisms does not always translate to clinical outcomes, suggesting that some carbapenem/β-lactamase inhibitor combinations may still work in the clinic [194]. More data and in-depth analysis would be needed to fully understand this.

4.2.2.2 Non-β-Lactamase-Mediated Resistance

Since β-lactam antibiotics cross the Gram-negative OM via porins, mutations causing loss of porins or affecting their structure or expression level can reduce susceptibility by reducing influx. Well-characterized examples of this are loss of OmpK35 and/or OpmK36 in *K. pneumoniae* [196, 197] and loss of OprD in *P. aeruginosa* [198, 199]. Loss or downregulation of these may have a significant impact on susceptibility to carbapenems and other classes. Mutations leading to upregulation of RND family efflux pumps can also reduce susceptibility to β-lactams, and in some cases, efflux upregulation occurs concomitant with porin downregulation (see Sect. 4.2.2). These mechanisms cause modest shifts in susceptibility generally but become significant in isolates where β-lactamases are also expressed [174, 200]. Alterations in PBPs (target mutations) had not garnered a lot of attention in Gram-negative pathogens, although it stands to reason that the widespread use of β-lactams/β-lactamase inhibitors is applying selective pressure for the emergence of altered patterns of PBP expression and/or mutations in PBPs of these organisms. Consistent with this, altered expression patterns of PBPs have been reported in pan-β-lactam-resistant clinical isolates of *P. aeruginosa*, although no changes in the amino acid sequences were found [199]. Amino acid substitutions in PBP2 have been found in *E. coli* clinical isolates that affect susceptibility to carbapenems [201]. Recently amino acid insertions in PBP3 were identified in clinical *E. coli* isolates that affect susceptibility to a range of β-lactams including the monobactam aztreonam [202, 203]. The mechanisms of porin loss, efflux, and PBP changes in isolation only shift β-lactam susceptibility modestly, but cumulatively they can have a large impact, especially when combined with the expression of β-lactamases. That PBP3 insertions modestly shift susceptibility to aztreonam is concerning since monobactams are the only class of β-lactams that are intrinsically stable to NDM metallo-β-lactamases, for which no inhibitors are currently available, and PBP3 insertions have been reported in NDM-1 expressing *E. coli* isolates in certain geographic areas [202]. These strains often express various serine β-lactamases as well. β-lactamase inhibitors such as avibactam can address these β-lactamases, but underlying mutations altering PBPs combined with porin loss and efflux are likely to erode the effectiveness not only of β-lactams but also currently available β-lactam/β-lactamase inhibitor combinations.

4.2.2.3 β-Lactamase Inhibitors

Dissemination of β-lactamases prompted efforts to identify β-lactamase inhibitors (BLIs) to restore effectiveness of partner β-lactams used in combination. Currently, there are four BLIs in clinical use: clavulanic acid, sulbactam, tazobactam, and the newly approved avibactam (Table 4.3).

Table 4.3 β-Lactamase inhibitors on the market or in clinical development

Name	Chemical class	Combination with	Current status
Clavulanic acid	β-Lactam	Amoxicillin	Marketed (also generic)
Sulbactam[a]	β-Lactam	Ampicillin	Marketed (also generic)
Tazobactam	β-Lactam	Piperacillin or ceftolozane	Marketed
Avibactam	Diazabicyclooctane	Ceftazidime	Marketed
Avibactam	Diazabicyclooctane	Ceftaroline, aztreonam	Phase III, Pfizer Phase II, Pfizer
Relebactam	Diazabicyclooctane	Imipenem, cilastatin	Phase III, Merck
Nacubactam: RG6080, OP0505	Diazabicyclooctane	Meropenem	Phase I, Roche
Vaborbactam (RPX7009)	Boronate	Meropenem	New drug application (NDA) (Carbavance)
ETX2514	Diazabicyclooctane	Sulbactam[a]	Phase I, Entasis [204]
AAI101	β-Lactam (tazobactam analog)	Cefepime or piperacillin	Phase I, Allecra
Zidebactam	Diazabicyclooctane	Cefepime	Phase I, Wockhardt

[a]Sulbactam has antibacterial activity against *A. baumannii*

The first of these to be identified and brought to the clinic was clavulanic acid, a natural product isolated from *Streptomyces clavuligerus* [205], followed by the semisynthetic penicillanic acid sulfone class of inhibitors (sulbactam and tazobactam) [206, 207]. These BLIs all possess the basic core structure of a β-lactam which allows for recognition and binding to β-lactamase. However, key structural differences from β-lactams eliminate most or all intrinsic antibacterial activity against many bacteria and render them mechanism-based "suicide inhibitors" of sensitive β-lactamases [208]. The mechanism of β-lactamase inactivation by these inhibitors is complex, but in general, the active-site serine of the β-lactamase attacks the carbonyl group in the β-lactam ring of clavulanic acid leading to acylation of the β-lactamase. This is then followed by a series of secondary reactions in the enzyme active site that irreversibly inactivate the enzyme [209–211]. A main difference between the BLI molecules and β-lactams that facilitates this mechanism is that BLIs possess good leaving groups at the C-1 position of their five-membered rings. This allows for secondary ring opening and subsequent β-lactamase enzyme modification. Important factors for BLI efficacy include high acylation and low deacylation rates, which localize them for a longer time period in the enzyme active site and a low number of hydrolytic events (inhibitor molecules hydrolyzed per unit time) necessary for complete enzyme inactivation (termed turnover number or t_n). Differences exist in these factors between clavulanic acid, sulbactam, and tazobactam, and these are affected by differences in the active sites among β-lactamases. Clavulanic acid and tazobactam cover most class A β-lactamases including ESBLs. Sulbactam also covers these but is less potent against some enzymes. Tazobactam and sulbactam are better inhibitors of class C carbapenemases than clavulanic acid

and notably differ from clavulanic acid in that they do not induce expression of AmpC in bacteria where this enzyme is inducible [212]. However, none of these can cover strains producing metallo-β-lactamases such as NDM-1. Clavulanic acid is partnered in the clinic with amoxicillin or ticarcillin, sulbactam with ampicillin, and tazobactam with either piperacillin or ceftolozane.

Avibactam, the first non-β-lactam BLI approved for clinical use, is a broad inhibitor of class A (including KPCs), class C, and some class D β-lactamases. Avibactam is a member of the diazabicyclooctane (DBO) chemical class [213], and as such it has a different mechanism of inhibition from previous BLIs. This mechanism is not yet fully understood, but it appears that avibactam functions as a slowly reversible covalent inhibitor with release of intact avibactam for most class A and C β-lactamases [214]. The enzymes appear to be slowly acylated and slowly deacylated, with no or only low-level hydrolysis of the inhibitor molecule. An exception to this was inhibition of KPC-2 which was rapidly acylated but slowly deacylated with hydrolysis of avibactam, so differences do exist. The release of intact avibactam in most cases however is thought to allow for recycling of the inhibitor by β-lactamases in the cell, leading to better inhibitory efficiency than BLIs like clavulanic acid or tazobactam that are hydrolyzed. Avibactam is a substantially more effective inhibitor of key β-lactamases like TEM-1, KPC-2, and AmpC from *P. aeruginosa* than clavulanic acid, sulbactam, or tazobactam but has limited coverage of class D enzymes, although it does cover OXA-48. Like previous BLIs, avibactam does not cover metallo-β-lactamases like NDM-1. Avibactam was introduced into the clinic very recently (2015), partnered with ceftazidime, and is in clinical trials for combination use with ceftaroline or aztreonam. The latter partnering with the monobactam aztreonam is meant to capitalize on the idea that monobactams are inherently stable to metallo-β-lactamases, and the combination should therefore cover strains expressing metallo-β-lactamases and/or serine β-lactamases. Although ceftazidime/avibactam has only been in clinical use a short while, resistance to this combination due to porin loss and upregulation of β-lactamase expression has been reported [215]. It has recently been suggested that ceftazidime/avibactam could be used in combination with aztreonam for coverage of metallo-β-lactamase/serine-β-lactamase-producing clinical isolates [216], but appropriate dosing would need to be established.

The success of avibactam has inspired efforts to identify next-generation DBO β-lactamase inhibitors. These include relebactam (MK7655), directed at some class A, including KPCs, and class C β-lactamases (currently in Phase III trials, in combination with imipenem [217]). Relebactam possesses a narrower spectrum than avibactam since it does not include class D β-lactamases such as OXA-48. Nacubactam (RG6080, OP0595), directed at class A and C β-lactamases, is currently in Phase I trials and is intended for combination with meropenem (Table 4.3). Some DBOs also possess intrinsic antibacterial activity and this warrants some discussion. They inhibit PBP2 in some Gram-negative pathogens, similar to the β-lactam mecillinam [218]. PBP2 inhibition can be synergistic with inhibition of other PBPs (i.e., with other β-lactams), as has been reported for nacubactam [218, 219], and this has been referred to as an “enhancer effect”

independent of BLI activity. Compounds that inhibit PBP2, such as mecillinam (amdinocillin) or nacubactam, select for mutants with reduced susceptibility in vitro at high frequency [219, 220]. There is a multiplicity of mutations that engender tolerance of PBP2 inhibition, generally related to the stringent or envelope stress responses [220], as well as stringent response-independent mechanisms [221]. Such mutations do not affect inhibition of PBP2 specifically; therefore the enhancer effect of DBOs is retained. Some intrinsically active DBOs may be considered as potent stand-alone antibacterials against Gram-negative pathogens, including *P. aeruginosa*, and this potential may be somewhat overlooked [222]. Interestingly, the gene encoding PBP2 was reported as nonessential in *P. aeruginosa* based on genetic deletion [223], suggesting that chemical inhibition of PBP2 is distinct from genetic deletion, possibly due to induction of a futile cell wall pathway cycle as described in [144], but this is not fully understood. At least one DBO with potent activity against *P. aeruginosa* demonstrated a high frequency of in vitro resistance selection [224]. It is not known if the intrinsic antibacterial activity of some PBP2-specific DBOs and their potential in vitro resistance profile will affect the clinical outcome when used in combination with a β-lactam. It has been proposed that mutants selected in vitro may not be fit enough to survive in the host [220], and it is possible that PBP2 inhibition would still provide efficacy against these mutants in vivo. Studies with potent antibacterial DBO molecules in relevant animal models to examine resistance potential in that context are needed to resolve this ongoing discussion.

Another DBO has recently been described (ETX2514 [204], Entasis) with broader inhibitory activity than other DBOs since it includes class D enzymes. Like nacubactam, ETX2514 has intrinsic antibacterial activity against some Gram-negative pathogens. ETX2514 is being paired with sulbactam (which has intrinsic antibacterial activity against *A. baumannii*) [217] for treatment of *A. baumannii* infections.

Another non-β-lactam BLI class is the boronic acid chemical scaffold. Boronic acid compounds were originally shown to inhibit serine proteases, and this observation was then extended to the serine β-lactamases. These inhibitors form a covalent reversible adduct between the boronate moiety and the catalytic serine of the β-lactamase. The most advanced of these is the cyclic boronate compound RPX7009 (Vaborbactam, The Medicines Company) which was the first of this class for which in vivo efficacy was demonstrated [218, 219]. Vaborbactam is active against class A carbapenemases (including KPCs), as well as other class A and class C β-lactamases [220, 221], but does not inhibit metallo-β-lactamases like NDM-1. A new drug application (NDA) has been filed for the combination of vaborbactam with meropenem for treatment of complicated urinary tract infections (cUTIs). Additional details on the inhibitors described above can be found in recent reviews [139, 210, 222].

The category of β-lactamases that has proven most challenging for the design of broad-spectrum inhibitors is the class B metallo-β-lactamases. To date, no inhibitor of these enzymes has reached the market. An alternative strategy to the design of a metallo-β-lactamase inhibitor is to exploit the fact that the monobactam aztreonam is intrinsically stable to metallo-β-lactamases and can therefore be partnered with

avibactam; this combination has the potential to cover strains expressing both metallo-β-lactamase and serine β-lactamases. This combination is currently undergoing Phase III clinical trials. More recently, an innovative approach was undertaken to design novel next-generation monobactams that are not significantly impacted by most serine β-lactamases while retaining their intrinsic stability to the metallo-β-lactamases. One of these, LYS228 [223], demonstrated excellent potency against MDR *Enterobacteriaceae*, including CRE [224, 225], and has entered Phase II clinical trials (Novartis). Significant effort has also been devoted to the discovery of therapeutically useful inhibitors of the class B metalloenzymes. This has lagged to some extent since class B enzymes have a different mechanism than serine β-lactamases, and it appears that the design of inhibitors capable of covering multiple clinically important class B enzymes is technically challenging. Inhibitors of class B enzymes would also need to be highly specific and avoid human metalloenzymes to avoid toxicity issues. While the prevalence of class B enzymes remained relatively low in the past and their contribution to worldwide carbapenem resistance was initially considered minimal, this viewpoint has changed in recent years with increased spread of class B enzymes like NDM-1 and their linkage with other resistance determinants. Recent efforts in the search for class B inhibitors include the discovery of the natural product aspergillomarasmine, which is active against NDM-1 and VIM-2 [226]. Novel bisthiazolidine (BTZ) inhibitors of class B enzymes have also recently been described [227]. Of most interest are reports from The Medicines Company on a new series of cyclic boronate compounds derived from RPX7009 (Table 4.3) with broad-spectrum carbapenemase activity including metallo-β-lactamases, in preclinical development [228].

4.2.2.4 Resistance to β-Lactamase Inhibitors

The implementation of β-lactamase inhibitors extended the clinical usefulness of several β-lactam antibiotics for decades; however the emergence of variant β-lactamases and other mechanisms has eroded their usefulness. One factor that impacts the effectiveness of BLIs is the expression level of β-lactamases and/or the number of β-lactamases being expressed in a given isolate. Even if a BLI is potent inhibitor of serine β-lactamases, this can be overwhelmed by high-level expression of one or multiple β-lactamase enzymes [229]. Overexpression can result from the β-lactamase gene residing on multicopy plasmids or via mutations in the promoter region causing high-level expression [230]. In particular, high-level expression of TEM-1 was an early mechanism identified that reduced susceptibility to amoxicillin-clavulanate [231, 232]. This can also be related to the induction of β-lactamase by certain BLIs. Clavulanic acid induces the expression of AmpC β-lactamase in bacteria where this enzyme is inducible. Since clavulanic acid is not a good inhibitor of class C enzymes, this induction can be antagonistic toward the partner antibiotic [233]. This is an issue in particular for the case of chromosomal inducible *ampC* (*P. aeruginosa*) or plasmid-borne inducible *ampC* such as DHA-1 in *K. pneumoniae*. In fact, antagonism by clavulanic acid is used as a diagnostic for the presence of

inducible *ampC* [234, 235]. Sulbactam and tazobactam do not have this induction effect and as such can be better options (e.g., tazobactam paired with piperacillin against *P. aeruginosa*). Active efflux of BLIs [20, 236] or changes in influx, possibly due to porin loss, may also serve to reduce their concentration relative to the β-lactamases in the cells, decreasing their effectiveness, although influx of compounds such as avibactam is not currently well understood [237]. Defects in porins OmpK35 and/or OpmK36 were, however, associated with decreased effectiveness of imipenem/relebactam and meropenem/RPX7009 in clinical isolates isolated from hospitals in New York [219, 238]. Efflux was implicated as an important mediator of resistance to ceftazidime/avibactam in *P. aeruginosa*, but this remains to be further explored [239]. Porin mutations combined with upregulated expression of plasmid-borne KPC-3 have been associated with clinical resistance to ceftazidime/avibactam in *K. pneumoniae* [215], and porin mutations were associated with ceftaroline/avibactam resistance in *E. cloacae* mutants selected in vitro [240].

Resistance to BLIs, mainly clavulanic acid, also resulted from the emergence of new β-lactamases that are resistant to the BLI. Very soon after the introduction of clavulanic acid into clinical use, such variants began to emerge, with the first reported being variants of the class A TEM enzyme that were resistant to clavulanic acid, found in *E. coli* clinical isolates [241, 242]. These TEM variants were altered at their Arg244 residues to either Cys or Ser [243]. This position had been shown earlier to be important for clavulanic acid inhibitory function, and so this clinical outcome might have been expected. These variants were initially designated inhibitor-resistant TEM (IRT-1 and IRT-2), but since then, 37 clavulanic acid-resistant TEM variants have been identified (cataloged at http://www.lahey.org/Studies/temtable.asp, functional group br), and the convention now is that these all have TEM numerical designations. These variants are found mainly in *E. coli* isolates but also occur in *Klebsiella* [244], *Proteus* [245], *Shigella* [246], and *Citrobacter* [247]. Inhibitor resistance can also be combined with amino acid substitutions conferring β-lactamase activity against oxyimino-β-lactams (ESBL), and these are referred to as complex mutant TEMs (CMT). Currently 11 of these variants have been described (http://www.lahey.org/Studies/temtable.asp, functional group ber). As well, seven inhibitor-resistant variants of the class A enzyme SHV have also been described (http://www.lahey.org/Studies/), with the most recent being SHV-107 found in a *K. pneumoniae* clinical isolate [248]. It should be noted that inhibitor-resistant β-lactamases generally refer to clavulanic acid, and these can also be resistant to sulbactam, but generally they remain susceptible to tazobactam [249–251]. Therefore these enzymes mainly affect amoxicillin/clavulanate, ticarcillin/clavulanate, or ampicillin/clavulanate but not piperacillin/tazobactam. However, in 2010, the emerging class A ESBL KPC-2 carbapenemase [252] was shown to also be resistant to clavulanic acid, sulbactam, and tazobactam, raising serious concerns [253].

Although ceftazidime/avibactam is active against KPC producers, providing effective treatments in the near term, the emergence of β-lactamase variants resistant to avibactam has already been reported [254]. In vitro selection studies demonstrated that variants of KPC-3 [254] or certain inhibitor-resistant SHV enzymes

(particularly the S130G variant) [255] were less susceptible to ceftazidime/avibactam. In the former study, the most frequently isolated variant of KPC-3 was Asp179Tyr, and the authors speculate this particular change may increase ceftazidime specificity rather than mediating resistance to avibactam per se. Interestingly, many of the alterations also appeared to impair the ability of the β-lactamase to hydrolyze carbapenems (reversal of ESBL), thereby increasing susceptibility of the bacteria to those agents. Consistent with these in vitro studies, plasmid-borne variants of KPC-3 have been described with reduced susceptibility to avibactam in *K. pneumoniae* clinical isolates [256]. These emerged within 10–19 days of ceftazidime/avibactam exposure. The KPC-3 variants found had either a D179Y/T243 M double substitution, D179Y single substitution, or V240G single substitution. Interestingly, these mutations also seemed to decrease the KPC-3 carbapenemase activity enough in some isolates to render them susceptible, and it has been suggested that agents like meropenem could be used to ameliorate to some extent the impact of such mutations. A large-scale analysis of the binding pockets of class A serine β-lactamases indicated that most would be susceptible to avibactam but some outliers were identified. In particular, PER-4 was shown to be highly resistant to avibactam [257], indicating the preexistence of class A β-lactamase variants in the clinic that are resistant to avibactam.

4.2.3 Quinolones

Quinolones and the related fluoroquinolones (Fig. 4.5) were introduced into clinical use in the 1960s and 1980s, respectively. First-generation quinolones (e.g., nalidixic acid) were restricted generally to treating urinary tract infections, because of suboptimal systemic distribution and somewhat limited activity. Second-generation fluoroquinolones (e.g., norfloxacin and ciprofloxacin) had improved tissue distribution and a broadened antibacterial spectrum, allowing for expanded use and perhaps overuse. Newer third- and fourth-generation fluoroquinolones (e.g., ofloxacin, lomefloxacin, levofloxacin, trovafloxacin, gatifloxacin, moxifloxacin, sparfloxacin) were focused mainly on improved Gram-positive and atypical (e.g., *Mycoplasma*, *Legionella*) coverage. Quinolone antibiotics act by inhibiting DNA gyrase and topoisomerase IV enzymes that control DNA topology and play essential roles in DNA replication, transcription, and recombination [258]. The DNA gyrase holoenzyme tetramer consists of two subunits each of GyrA and GyrB, which act to introduce negative superhelicity into DNA. This is required for initiation of replication, replication fork movement, and transcription [259]. The domain responsible for DNA strand passage resides on GyrA, whereas GyrB contains an ATPase domain. The topoisomerase IV tetrameric holoenzyme similarly consists of two subunits, each of ParC and ParE, and functions to relax both positive and negative supercoils and to direct decatenation (unlinking) of replicated chromosome copies to allow for chromosomal partitioning upon cell division. The DNA strand passing domain is located on ParC, and the ATPase activity is mediated by ParE. Both holoenzymes are

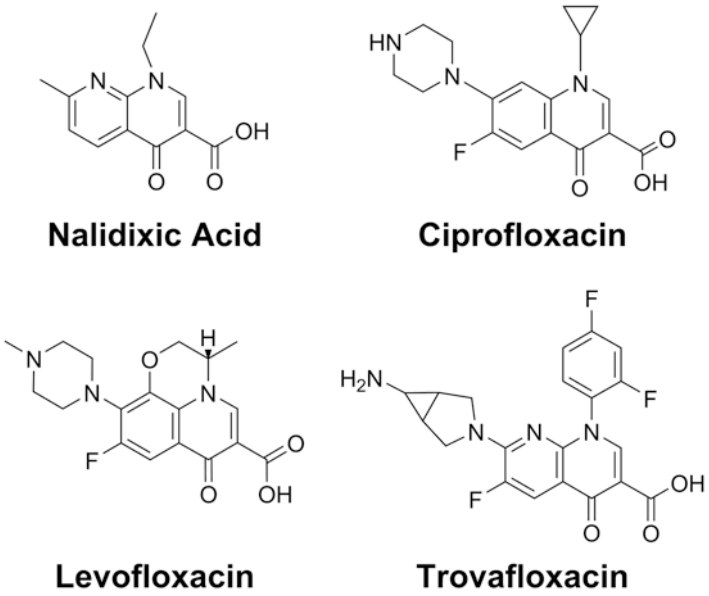

Fig. 4.5 Chemical structure of key quinolones. Nalidixic acid (first generation), ciprofloxacin (second generation), levofloxacin (third generation), and trovafloxacin (fourth generation)

type II topoisomerases that introduce double-stranded breaks in DNA and pass DNA strands/helices through each other via a transient "cleaved complex" where the enzyme, covalently linked to the DNA, serves as a bridge between the DNA ends, mediating strand breakage, strand passage, and resealing [259]. Although the exact mechanisms by which different quinolones kill bacteria have not been fully unraveled, their general mechanism involves forming reversible non-covalent complexes with the topoisomerases bound to DNA. This forms a drug-enzyme-DNA complex (ternary complex) that is trapped as the cleaved complex and ligation of the DNA ends is prevented [258]. Subsequent destabilization of the complex without rejoining the ends introduces double-stranded DNA breakage, fragmenting the genome and ultimately causing cell death [258, 260]. The trapped cleaved complexes also interfere with progression of replication forks, blocking DNA synthesis [261] and with transcription by blocking RNA polymerase [262] and disrupting the action of DNA helicases [263]. Additional mechanisms may contribute to cell killing in certain Gram-negatives. For example, a recent report detailing the transcriptomic interrogation of ciprofloxacin-treated *P. aeruginosa* implicated induction of a pyocin system in cell-killing activity [264] (See also Chaps. 16 and 20).

Since there is significant amino acid sequence homology between the GyrA and ParC and GyrB and ParE proteins, individual quinolone molecules can inhibit the activities of both enzymes, and most quinolones will inhibit both targets to varying degrees. Either topoisomerase can constitute the primary or secondary target of quinolones in different bacteria. Given their broad-spectrum and

excellent tissue penetration, fluoroquinolones are well suited to empiric therapy and became one of the most broadly used classes of antibiotic. Quinolones are also used fairly extensively in agriculture [265]. Given their widespread use, it is not surprising that resistance has emerged at a significant rate around the world. Resistance to fluoroquinolones in Gram-negative pathogens is mediated by several mechanisms, the most common being chromosomal mutations that alter the quinolone binding sites of the GyrA/B and ParC/E proteins. Additional mechanisms include chromosomal mutations that upregulate RND-mediated efflux or decrease compound penetration. Plasmid-based mechanisms also occur, including efflux, target protection, and compound modification. Each of these is discussed below, and the epidemiology of fluoroquinolone resistance is discussed in Chap. 10.

4.2.3.1 Target Mutations Conferring Quinolone Resistance

The most well-characterized mechanism conferring specific resistance to quinolones in both Gram-positive and Gram-negative bacteria is target alteration resulting from chromosomal mutations in the *gyrA* and/or *parC* genes, with mutations in *gyrB* and *parE* less frequently observed [266]. These changes occur in specific segments of the proteins referred to as their quinolone resistance determining regions (QRDRs). The GyrA QRDR consists of amino acids 67–106 and for ParC encompasses residues 63–102 (*E. coli* numbering). Both of these regions comprise quinolone-binding domains and are located near amino-terminal active-site tyrosines that interact covalently with transiently broken DNA [267–270]. Binding of quinolones to GyrA or ParC occurs via water-metal ion bridges between the hydroxyl of conserved serine or acidic amino acids within the QRDR and the oxygen of the quinolone amine group. Correspondingly the most frequently encountered resistance alterations are QRDR substitutions at Ser83 of GyrA or ParC, with the next most common being located at the Asp87 (acidic residue). Substitutions at Ser83 reduce quinolone binding but do not substantially impact gyrase function [271], whereas substitution at Asp87 decreases catalytic efficiency [272]. Although quinolones will usually engage one or the other topoisomerase preferentially, they still impact the secondary enzyme, and when mutations occur that reduce susceptibility of the primary target, alterations of the secondary target will usually also occur. For example, quinolones preferentially target GyrA in *E. coli*, and therefore changes at the Ser83 of GyrA are most commonly found [272]. Single substitutions generally cause modest changes in susceptibility to quinolones (Table 4.4), but over time additional substitutions can occur in GyrA and/or ParC which ultimately lead to high-level resistance [266, 272]. Alterations of the GyrB/ParE subunits are much less common but do occur [266].

Table 4.4 Summary of the impact of different resistance mechanisms on ciprofloxacin susceptibility of *E. coli* and *P. aeruginosa*

Organism and resistance mechanism	Fold change in ciprofloxacin MIC	References
E. coli		
gyrA	32–64	[268, 273]
gyrA + *parC*	128–2048	[273]
Efflux upregulation	4–8	[274]
qnr	32	[268]
aac(6′)-lb-cr	8	[268]
P. aeruginosa		
gyrA	8–16	[275]
gyrA + *parC*	256	[275]
Efflux upregulation	2–16	[275]
gyrA + *parC* + efflux upregulation	256–2048	[275]

4.2.3.2 Efflux and Reduced Compound Influx

The role of Gram-negative RND family efflux pumps in intrinsic and mutationally acquired resistance to antibiotics is covered in detail in Sect. 4.2.1 and in recent reviews [3]. The potent broad-spectrum activity of fluoroquinolones against even intrinsically resistant Gram-negative bacteria such as *P. aeruginosa* indicates that RND efflux does not mediate enough intrinsic resistance to some fluoroquinolones to limit their spectrum. This may relate to some extent to the hydrophilicity [3] as well as to the overall target potency and cidality of fluoroquinolones. However, fluoroquinolones are substrates of a wide range of RND family pumps (Table 4.1) including the AcrAB-TolC pump of *E. coli* and *Salmonella* spp., the AcrEF pumps of *E. coli* and *Salmonella enterica*, the CmeABC pump of *Campylobacter jejuni*, and MexAB-OprM, MexXY-OprM, MexCD-OprJ, and MexEF-OprN of *P. aeruginosa* [3]. Mutations in regulatory genes causing pump overexpression and decreased susceptibility can be readily selected by in vitro exposure to quinolones. However, in most cases overexpression of efflux pumps alone, in the absence of other mechanisms, affords only modest reductions in fluoroquinolone susceptibility (Table 4.4). Efflux pump overexpressing mutants are routinely found among clinical isolates [276]. Upregulation of the AcrAB-TolC pump in fluoroquinolone-resistant *E. coli* clinical isolates contributed to high-level fluoroquinolone resistance along with QRDR mutations [67, 277]. Similarly, AcrAB-TolC upregulation played a role in fluoroquinolone resistance in *K. pneumoniae* and *K. oxytoca* clinical isolates [278]. A *P. aeruginosa* clinical isolate with a mutation in *gyrB* and upregulated for MexAB-OprM emerged during ciprofloxacin monotherapy [279]. Since RND pumps have broad substrate ranges, selection of pump overexpression during previous treatment with various antibiotics will result in selection of pump upregulation which will affect fluoroquinolones and vice versa. RND family efflux pumps function in concert with the OM permeability barrier, and therefore any reduction in a compound's ability to cross the OM will have a corresponding enhancing effect on

efflux-mediated resistance. Fluoroquinolones cross the outer membrane either through water-filled porin channels or by diffusion through lipid domains in the outer membrane depending on the hydrophobicity of the quinolone [280]. Reduced porin levels have also been associated with fluoroquinolone resistance in *E. coli* [277, 281] and *S. enterica* [282] clinical isolates. Reduced porin levels often occur concomitantly with upregulation of efflux pumps [283] potentially linking reduced influx and increased efflux via a single mutation.

4.2.3.3 Plasmid-Mediated Quinolone Resistance (PMQR): Topoisomerase Protection, Quinolone Modification, and Efflux

Three different plasmid-borne quinolone resistance mechanisms have been described. These are topoisomerase protection (Qnr), quinolone modification, and efflux. Each is discussed below. The plasmid-borne quinolone resistance determinant *qnr* (now termed *qnrA1*) was first identified from a quinolone-resistant *K. pneumoniae* clinical isolate in 1998 [284]. Other *qnr* determinants were subsequently identified including *qnrB* [285] and *qnrS* [286], and over the years, the number has expanded to where there are currently seven families of Qnr proteins, identified from a range of organisms: QnrA, QnrB, QnrC, QnrD, QnrS, and QnrVC (cataloged at http://www.lahey.org/qnrStudies). The Qnr proteins are typified by having tandem repeats of a pentapeptide consensus sequence and as such are referred to as pentapeptide repeat proteins [287]. The mechanism of Qnr proteins is referred to as topoisomerase protection and involves binding of the Qnr protein to gyrase and topoisomerase subunits and the holoenzymes [288–290]. Binding is not dependent on DNA or ATP and likely occurs prior to establishment of the ternary complex, reducing quinolone interaction with the topoisomerases. More recent structural information indicated that Qnr can assume a rodlike structure resembling B-form DNA, suggesting it may compete with quinolones by binding in the gyrase QRDR or DNA-gate region [291]. Although Qnr proteins can bind to a number of subunits in vitro, they appear to mediate resistance to quinolones or other agents that bind the QRDR region of GyrA, but not to agents that target the ATPase function (e.g., GyrB) [292]. Qnr proteins, specifically, only cause a marginal shift in quinolone susceptibility similar to that of single-target mutations [284] (Table 4.4). There are wide dissemination of *qnr* plasmids in *Enterobacteriaceae* clinical isolates around the world [293–296] and significant diversity of plasmids that carry these genes (reviewed in [266]). In contrast they seem to be rare among nonfermenters such as *P. aeruginosa* and *A. baumannii*. Interestingly, *qnr* genes certainly predate the introduction of the synthetic quinolones into clinical use and are also found on the chromosomes of several bacteria [292]. It has been suggested that mobilization from the genome to small transmissible plasmids may have originated in Proteeae [297]. A final concern is that *qnr* genes typically reside on a range of plasmids that also encode other resistance markers, in particular extended-spectrum β-lactamases, such as SHVs and CTX-Ms, and AmpC-like enzymes such as DHA-1 (reviewed in [296]).

The second identified plasmid-borne resistance determinant was a bifunctional variant of the aminoglycoside-modifying enzyme *aac(6′)-1b* (see Sect. 4.2.5.1 on aminoglycoside-modifying enzymes) [298]. This variant, designated *aac(6′)-1b-cr*, differs from *aac(6′)-1b* by encoding two amino acid substitutions, Trp102Arg and Asp179Tyr. These differences allow the enzyme to bind and acetylate fluoroquinolones that have an amino nitrogen on the piperazinyl ring (e.g., ciprofloxacin and norfloxacin), thereby reducing their activity. Fluoroquinolones that have modifications on the piperazinyl structure (e.g., levofloxacin or moxifloxacin) are not affected. Importantly, this variant enzyme retains its aminoglycoside-modifying activity, thus creating a single protein that can affect two different classes of antibiotic. Like *qnr*, the *aac(6′)-1b-cr* gene is usually found in a cassette as part of an integron in multiresistance plasmids that encode β-lactamases or *qnr*, is disseminated worldwide, and can also be found on the chromosome of some bacteria (summarized in [298]).

The most recently identified class of plasmid-borne resistance determinants are genes encoding fluoroquinolone efflux pumps. The first of these was *oqxAB*, identified in *E. coli* isolates of agricultural origin and which encodes an RND family efflux pump with a broad substrate range [299–301]. This was later found in a range of human, animal, and environmental isolates [302–305]. Intriguingly, the *oqxAB* pump genes are found on the chromosome in *K. pneumoniae*, including drug-resistant human clinical isolates [306], and this appears to be the likely reservoir/origin of the plasmid-borne version [307]. Typical of chromosomally encoded RND family pumps, upregulation of OqxAB expression in *K. pneumoniae* requires mutations in the *oqxR* regulatory gene [308]; however expression from plasmid-borne *oqxAB* genes is constitutive, and therefore, this was the first report of a constitutively expressed, mobile plasmid-borne efflux pump [307]. The second was *qepA*, identified in 2008 an *E. coli* clinical isolate and which encodes a member of the major facilitator efflux pump superfamily [309]. Another variant *qepA2* has also been described [310]. Of concern, *qepA* genes often reside on mobile elements with genes encoding ribosomal methyltransferases which mediate resistance to aminoglycosides [311], again linking fluoroquinolone resistance with resistance to other antibiotic classes.

4.2.3.4 Interplay of Resistance Mechanisms

Efflux and possibly lowered porin levels reduce susceptibility to fluoroquinolones in Gram-negative clinical isolates. Efflux upregulation in isolation may however cause only modest shifts in susceptibility. In cases of higher-level resistance, mutations in the QRDR regions are also found along with upregulation of efflux. In many Gram-negative pathogens, such as *E. coli*, target-based resistance progresses from single mutations (e.g., encoding alteration at Ser83 of GyrA), which cause only small shifts in susceptibility, to accumulation of multiple target mutations leading to high-level resistance. The accumulation of mutations depends on stepwise enrichment of mutants, in turn depending in part on the level of quinolone being within the

mutant selection window, defined as being between the concentration required to block the growth of 99% of bacteria in culture (MIC_{99}) and the MIC of the least susceptible next step mutant, (termed the mutant prevention concentration (MPC) [312]. Since high-level resistance requires two or more target mutations, fluoroquinolone levels higher than the MPC can only select the simultaneous double mutants from a wild-type background at an extremely low frequency (approximately 10^{-12}). The relatively rapid emergence of target-based high-level resistance to fluoroquinolones in the clinic in organisms such as *P. aeruginosa* and *E. coli* and a general association with efflux suggest that a key role for efflux may be in enhancing survival of first step target mutants which then rapidly accumulate additional mutations conferring stable high-level resistance. Factors such as suboptimal exposure to drug can contribute to enrichment of earlier stage mutants, and in cases where certain target mutations confer clinical resistance levels only in conjunction with efflux, the presence of pumps would be very important to this process. A hollow fiber model used to simulate human drug treatment with *E. coli* lends support to this notion in that mutants with a two- to eightfold shift in susceptibility due to upregulation of AcrAB-TolC emerged first, followed by emergence of target mutations (single or double) [313]. The emergence of target mutations was also strongly delayed in a strain lacking AcrAB-TolC function. Efflux upregulation regressed after the emergence of target mutations suggesting that once target-based resistance was established, efflux upregulation may no longer be required and may revert back to wild-type expression.

In the case of *P. aeruginosa*, efflux has been shown to provide a significant contribution to establishing the intrinsic susceptibility to fluoroquinolones, which correspondingly enhances the ultimate levels of resistance caused by target QRDR mutations [314]. Upregulation of various pumps including MexAB-OprM, MexCD-OprJ, or MexEF-OprN can also provide substantial shifts in fluoroquinolone susceptibility without target mutations [314, 315]. The combination of target mutations and efflux in *P. aeruginosa* can mediate very high-level resistance [314]. Pump upregulation can occur at very high frequencies at lower compound levels since in many cases, this requires only loss-of-function mutations in regulatory genes like *mexR* (MexAB-OprM) or *nfxB* (MexCD-OprJ). Selection of resistance in *P. aeruginosa* in vitro at 4X MIC of levofloxacin occurred at 10^{-6}–10^{-7}, whereas the frequency at 4X MIC for an efflux-defective strain was $<10^{-11}$, indicating that selection of resistance in the absence of efflux even at relatively modest multiples of the MIC could be rare [314]. This suggests overall that efflux was even more of a factor in the emergence of target-based resistance in *P. aeruginosa*, consistent with the very rapid rise in fluoroquinolone resistance seen in *P. aeruginosa* in the United States after widespread fluoroquinolone use began, and the association of this with resistance to multiple antibiotics [316, 317]. The use of an efflux pump inhibitor to assess the prevalence of pump-mediated fluoroquinolone and multidrug resistance among *P. aeruginosa* clinical isolates also suggested a correlation between fluoroquinolone treatment and the co-emergence of target and pump-mediated multidrug resistance [276]. More recently, associations were seen in clinical isolates between target mutations and efflux pump upregulation; however the expression levels of

several pumps could not be correlated with higher-level resistance seen for some isolates that also harbored QRDR mutations, suggesting that other as yet unidentified factors may mediate higher-level resistance in some QRDR mutants [275]. Similar interplay between plasmid-borne resistance mechanisms and other mechanisms is likely also occurring, both in terms of determining susceptibility and in facilitating the emergence of high-level resistance. Qnr proteins only cause a marginal shift in quinolone susceptibility [284] (Table 4.4), but this is additive with target-based or other mechanisms and will contribute to the emergence of higher-level clinically relevant resistance [295]. Like *qnr*, the level of resistance conferred by *aac(6′)-1b-cr* alone was modest; however, more significant levels of resistance were observed when *aac(6′)-1b-cr* and *qnrA* were found together (Table 4.4). Furthermore, the presence of plasmid-borne *aac(6′)-1b-cr* in *E. coli* resulted in a greater recovery of resistant mutants during selection experiments with ciprofloxacin [298], essentially by widening the mutant selection window. This again highlights the interplay of determinants such as *aac(6′)-1-cr* and *qnrA* in the stepwise acquisition of clinically significant resistance [318]. An additional factor that may have contributed to the emergence of high-level fluoroquinolone resistance is that DNA damage and interference with DNA replication caused by quinolones induce the SOS response, leading to upregulation of error-prone DNA polymerases. Evolution of quinolone resistance in *E. coli* in vitro and in an animal model of infection was curtailed in mutants lacking the SOS response [319].

4.2.3.5 New Strategies for Targeting Type II Topoisomerases

Delafloxacin (Melinta Therapeutics) [320–322] is a new structurally unique anionic fluoroquinolone that was recently approved by FDA for the treatment of acute bacterial skin and skin structure infections (ABSSSI) and is in clinical trials for community-acquired pneumonia and complicated urinary tract infection. Delafloxacin is particularly potent against Gram-positive pathogens but is also active against several Gram-negative pathogens including *H. influenzae*, *Enterobacteriaceae* spp., and *P. aeruginosa*. Delafloxacin targets DNA gyrase and topoisomerase IV equally, which may reduce the emergence of resistance. Other efforts to discover novel agents that circumvent target-based or other fluoroquinolone resistance mechanisms in Gram-negative pathogens include the design of compounds referred to as novel bacterial type II topoisomerase inhibitors (NBTIs) that engage the GyrA/ParC targets via a mode of inhibition distinct from fluoroquinolones and that are not affected by QRDR mutations [133]. These compounds have activity against Gram-negative pathogens including *E. coli* and *P. aeruginosa.* These NBTIs also seem to benefit from balanced inhibition of both gyrase and topoisomerase IV targets, thereby requiring at least two target mutations in *E. coli* in order to observe decreased susceptibility [323]. Efforts to design novel inhibitors of the ATPase function of type II topoisomerases, in order to exploit DNA replication as a target but avoid QRDR-mediated resistance, have resulted in potent antibacterial compounds [324, 325]. Similarly, novel spiropyrimidinetrione agents with a mode

of action distinct from fluoroquinolones that may involve targeting GyrB (AZD0914, now ETX0914, Entasis Therapeutics) are in clinical trials and may find utility in treating infections due to Gram-positive and/or fastidious Gram-negative pathogens, such as *N. gonorrhoeae* [326–328]. Another class of novel inhibitors of GyrB, which bind to the TOPRIM domain and are not affected by fluoroquinolone resistance mutations, has recently been described [329]. A detailed discussion of non-quinolone inhibitors of topoisomerases is presented in Chap. 19.

4.2.4 Tetracyclines

Tetracyclines are bacteriostatic and prevent bacterial growth by binding to the ribosome, thereby blocking protein synthesis. They bind the A-site of the ribosomal 30S subunit which prevents the entrance of aminoacyl-tRNAs into the mRNA-ribosome complex, ultimately preventing incorporation of amino acids into the newly emerging polypeptide [330–332]. The ribosomal target is relatively conserved in bacteria, and tetracyclines can therefore have a broad spectrum of antibacterial activity, covering many Gram-positive, Gram-negative, anaerobic, and atypical pathogens. The original tetracycline, chlortetracycline (also referred to as Aureomycin), is a natural product produced by *Streptomyces aureofaciens* and was identified in the late 1940s by Benjamin Duggar at Lederle Laboratories [333]. Over time other natural examples were discovered, and routes for making semisynthetic tetracyclines were developed. The latter allowed detailed exploration of this chemical scaffold, leading to second-generation tetracyclines doxycycline and minocycline and culminating with third-generation tetracyclines omadacycline and the glycylcycline tigecycline [334] which has now been in clinical use for over 10 years. Tigecycline (Tygacil®, Pfizer Inc.) is approved in the United States and Europe for the treatment of complicated skin and intra-abdominal infections and in the United States for community-acquired bacterial pneumonia. More recently eravacycline (TP-434), a fully synthetic fluorocycline of the tetracycline class, has completed a Phase II study in complicated intra-abdominal infection (cIAI) and is currently undergoing Phase III studies in both cIAI and complicated urinary tract infection (cUTI) (www.clinicaltrials.gov) [335]. The latter two compounds are of particular interest in that they have a broader spectrum of antibacterial activity and largely evade the tetracycline-specific, acquired resistance mechanisms of MFS efflux and ribosomal protection [336], described below in Sects. 4.2.4.1 and 4.2.4.2. Examples of tetracycline chemical structures are shown in Fig. 4.6.

Tetracyclines have now been in use for several decades in human and veterinary medicine as well as in agriculture. Correspondingly, resistance to earlier-generation tetracyclines became fairly widespread some time ago [337–339]. There are two main tetracycline-specific mechanisms of resistance in Gram-negative pathogens: tetracycline-specific active efflux and ribosomal protection. Additional mechanisms are active-site rRNA mutations and tetracycline-modifying enzymes. Efflux by broad specificity RND family pumps (described in Sect. 4.2.1) also affects suscep-

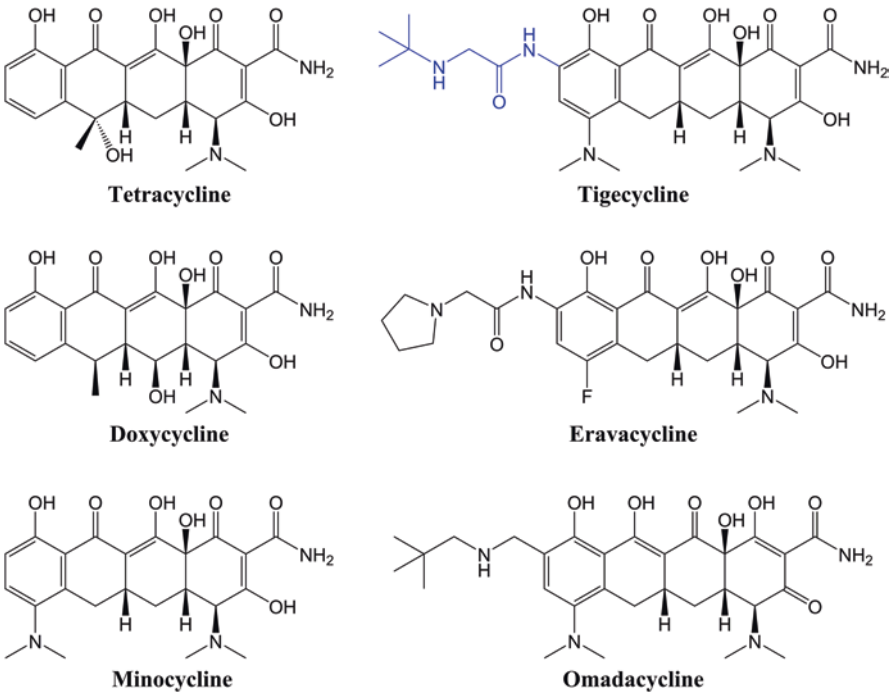

Fig. 4.6 Example of tetracyclines (left side) and glycylcyclines (right side). The key modification at the 9 position (*tert-butyl*-glycylamido) differentiating the glycylcycline scaffold is depicted in blue for tigecycline

tibility and contributes to defining intrinsic susceptibility to tetracyclines in different Gram-negative pathogens. Examples of tetracycline-specific resistance determinants are listed in Table 4.5 and are discussed below. To a large extent, the impacts of tetracycline-specific efflux and ribosomal protection have been circumvented by the third-generation compound tigecycline, currently in clinical use, and the fluorocycline eravacycline, and so these mechanisms have a comparatively more important effect on earlier-generation tetracyclines such as minocycline.

4.2.4.1 Efflux

Tetracycline-Specific MFS Family Efflux Pumps

There are many tetracycline-specific efflux pumps described for both Gram-positive and Gram-negative bacteria [340], and they function by actively extruding tetracycline from the cell and preventing accumulation to a level sufficient to fully inhibit the ribosome. Examples of pumps that are commonly found in Gram-negative pathogens are listed in Table 4.5, and an updated table of the distribution of these

Table 4.5 Examples of tetracycline-specific resistance determinants found in selected Gram-negative pathogens

Organism	Genetic determinant	Mechanism
Acinetobacter	*tet*(A) *tet*(B) *tet*(G) *tet*(H), *tet*(L), *tet*(39), *tet*(Y)	Efflux
	tet(M) *tet* (O) *tet*(W)	Ribosomal protection
Klebsiella	*tet*(A-E) *tet*(L)	Efflux
	tet(M) *tet* (S) *tet*(W)	Ribosomal protection
	tet(X)	Enzymatic modification
Enterobacter	*tet*(A-D), *tet*(G), *tet*(L), *tet*(39)	Efflux
	tet(M)	Ribosomal protection
	tet(X)	Enzymatic modification
Escherichia	*tet*(A-E), *tet*(G) *tet*(J) *tet*(L), *tet*(Y),	Efflux
	tet(M) *tet*(W)	Ribosomal protection
	tet(X)	Enzymatic modification
Haemophilus	*tet*(B)*tet*(K)	Efflux
	tet(M)	Ribosomal protection

Data extracted from https://faculty.washington.edu/marilynr/tetweb1.pdf and http://faculty.washington.edu/marilynr/tetweb2.pdf. These sites maintain a comprehensive list of the mechanisms and their distribution among Gram-negative bacteria, and the reader is directed there for additional details

genes among Gram-negatives is maintained at https://faculty.washington.edu/marilynr/tetweb1.pdf and http://faculty.washington.edu/marilynr/tetweb2.pdf. The TetA efflux pump is perhaps the most broadly distributed among important Gram-negative pathogens, and indeed plasmid-encoded TetA was the first bacterial antibiotic efflux pump identified in 1980 [341, 342]. Generally, tetracycline-specific efflux pumps genes reside on mobile genetic elements and are thus horizontally acquired resistance mechanisms. The genes encoding TetA and TetB efflux pumps were later identified in natural oxytetracycline-producing *Streptomyces* as mechanisms protecting the producer organisms from the effects of the tetracyclines they were producing [343] suggesting this is the likely original source of this resistance mechanism. Genes such as *tetA* are commonly used as antibiotic selection markers for genetic engineering in bacteria, highlighting the effectiveness with which they can confer resistance to first-generation tetracyclines. The genes encoding tetracycline-specific pumps are usually accompanied by the *tetR* gene, which encodes a tetracycline-responsive repressor that controls expression of the TetA efflux pump [344–347]. Unbound TetR functions as a repressor of *tetA* expression by binding to tandem operator sequences upstream of *tetA* as a homodimer and blocking expression [348]. Upon binding tetracycline, TetR dissociates from the DNA, allowing transcription to occur.

The tetracycline-specific transporters, typified by TetA, are located in the bacterial inner (cytoplasmic) membrane, and those found in Gram-negative bacteria are about 46 kDa in size and have 12 transmembrane spanning regions [341]. They belong to the major facilitator superfamily (MFS) and are tetracycline-H^+ antiport-

ers that operate through the exchange of a proton for the tetracycline molecule which drives transport of the tetracycline against a chemical concentration gradient, in this case from the cytoplasm across the inner membrane into the periplasmic space between the inner membrane and OM. Single-component pumps like TetA, located in the inner membrane, are generally thought to be more effective at extruding compounds from the cytoplasm than are pumps of the more broadly active RND family. The latter are generally thought to recognize compounds in the periplasm or when diffusing into the inner membrane. This is important, since in Gram-negative bacteria, the single-component pumps cannot extrude antibiotics completely out of the cell into the surrounding milieu but will deposit the compound into the periplasmic space between the inner and outer membranes. This can concentrate the tetracycline in the periplasm, from which it could diffuse back across the inner membrane into the cell in the absence of additional efflux across the OM. Consistent with this, higher levels of resistance mediated by pumps like TetA often require interplay with efflux across the OM by RND family pumps such as MexAB-OprM in *P. aeruginosa* or AcrAB-TolC in *E. coli* [6, 24]. These RND family pumps have a broad substrate specificity which includes tetracyclines, and the combined effect of specific single pump efflux from the cytosol and subsequent RND-mediated efflux from the periplasm to the outside of the bacterium can lead to high levels of resistance in some Gram-negative pathogens.

Although the TetA MFS family efflux pumps are currently widespread among clinical isolates, presumably driven by the extensive use of earlier-generation tetracyclines, there have been significant advancements in circumventing the impact of these pumps with each subsequent generation of tetracyclines. Understanding the emergence of clinical resistance to early generations of tetracyclines, along with improved understanding of tetracycline mechanism of action, was a key driving force for renewed interest in developing new tetracyclines that were not subject to this mechanism. Correspondingly, efforts leading to the identification of tigecycline were specifically directed toward achieving cellular activity against tetracycline-resistant bacteria [334, 349–351], including those expressing tetracycline efflux pumps. That this was achieved with tigecycline is shown by the potent antibacterial activity in broad susceptibility testing with resistant clinical isolates that supported clinical development as an "expanded-spectrum" antibiotic for treatment of multidrug-resistant Gram-negative infections (excluding *P. aeruginosa*) [349, 352–359]. Specifically showing that tigecycline circumvents resistance mediated by these pumps, expression of Tet(A), Tet(B), or Tet(X) in a susceptible *E. coli* strain background conferred very high levels of resistance to tetracyclines (MIC $\geq$ 128 μg/mL) but had a much smaller or no impact on the third-generation tigecycline (or eravacycline (TP-434)), depending on the pump [360]. Furthermore, no correlation was seen between the presence of tetracycline-specific efflux genes *tet(A)* to *tet(E)* and insusceptibility to tigecycline in strains of *Enterobacteriaceae* [353]. It should be noted though that some variation in amino acid residues important for recognition of tetracyclines has been reported for TetA proteins expressed from *tet(A)* genes residing on different genetic elements and this can have a modest effect on how much susceptibility is shifted when the pump is expressed [360, 361]. Whether this

portends the selection of mutations in tetracycline pump genes over time that increase recognition of tigecycline remains to be seen.

Efflux Mediated by RND Family Efflux Pumps

Tetracyclines are substrates of several RND family pumps [3] including AcrAB-TolC in *E. coli* and *K. pneumoniae* and the MexAB-OprM, MexXY-OprM, and MexCD-OprJ pumps in *P. aeruginosa*. RND efflux pumps are therefore important for determining the Gram-negative spectrum of these compounds. Second-generation compounds such as doxycycline and minocycline possess a broader antibacterial spectrum than tetracycline, most notably against *Acinetobacter*, *Burkholderia*, and *Stenotrophomonas*, but their activities against *P. aeruginosa* and most species of *Enterobacteriaceae* are still limited, in large part due to RND-mediated efflux [362]. Tigecycline has an expanded spectrum, covering a range of Gram-negative pathogens including *Enterobacteriaceae* and non-fermenters such as *Acinetobacter*, *Stenotrophomonas*, and *Burkholderia* [363]. Therefore basal-level RND-mediated efflux alone does not exclude these organisms from the spectrum of tigecycline. However, RND-mediated efflux is a factor excluding *P. aeruginosa* (MexAB-OprM, MexXY-OprM) [40] and *Proteus mirabilis* (AcrAB) [41] from the spectrum of tigecycline. Since tigecycline is a substrate of AcrAB-TolC present in many Gram-negative pathogens within the spectrum of tigecycline, RND-mediated efflux also posed a threat as a resistance mechanism, either via mutational upregulation of pump expression or indirectly by exacerbating other as yet unknown mechanisms. Supporting this, RND-mediated efflux has been implicated as a determinant of resistance in laboratory and clinical isolates of *Morganella morganii* [364], *K. pneumoniae* [42, 365, 366], *E. coli* [43], *Enterobacter cloacae* [367]*/E. aerogenes* [368], and *Salmonella enterica* [369]. As well, another RND family pump, OqxAB, may play a role in decreasing susceptibility to tigecycline in *K. pneumoniae* [366], and the AdeABC [61, 370, 371], AdeFGH [372, 373], and AdeIJK [374] efflux pumps have been associated with decreased susceptibility to tigecycline in *A. baumannii*.

4.2.4.2 Ribosomal Protection, Target Mutations, and Tetracycline-Modifying Enzymes

Resistance to some tetracyclines (first and second generation) can be caused by the action of ribosomal protection proteins (RPPs). Several of these proteins have been identified (e.g., Tet(B), Tet(O), Tet(M), Tet (S), Tet(Q), Tet(W)) [375] (Table 4.5), and a comprehensive update on the distribution of these determinants in Gram-negative bacteria is maintained at http://faculty.washington.edu/marilynr/tetweb2.pdf. The best studied of these proteins are Tet(O) and Tet(M) [375]. Plasmid-borne *tet*(O) was first identified in a *Campylobacter jejuni* clinical isolate [376] and later in *Campylobacter coli*. A similar gene, designated *otr*(A), was identified in the

tetracycline-producing organism *Streptomyces rimosus* [377], suggesting that, like tetracycline-specific efflux, ribosomal protection likely originated as a mechanism to protect the tetracycline-producing organisms and has spread on mobile genetic elements. Genes encoding Tet(M) and Tet(Q) also occur on mobile elements [338]. Ribosomal protection proteins are generally conserved GTPases that resemble the elongation factor EF-G (and to a lesser extent EF-Tu) [378, 379]. Early studies based on chemical probing and cryoelectron microscopy indicated that RPPs bind a similar site on the 50S ribosomal subunit as EF-G and subsequently cause the tetracycline to be released from the ribosome [375, 380–382]. It is thought that hydrolysis of GTP is not strictly necessary for causing the release of tetracycline from the ribosome but is required for RPP dissociation from the ribosome. Although the RPP-binding site is removed from the tetracycline-binding site on the 30S ribosomal mRNA, it was originally hypothesized that RPP binding caused an overall conformational shift in the ribosome sufficient to dislodge bound tetracycline [380] and stimulate binding of tRNA to the A-site which also reduced rebinding of tetracycline [379]. More recent cryoelectron microscopy and modeling of Tet(O) and Tet(M) bound to the 70S ribosome suggest that bound RPPs may also intrude directly into the binding site of tetracyclines located around residue C1054 of the 16SrRNA [383, 384]. Ribosomal protection confers resistance primarily to tetracycline, doxycycline, and minocycline, but as is the case for tetracycline-specific efflux, ribosomal protection has been circumvented by the third-generation tetracyclines including tigecycline, omadacycline, and eravacycline [360, 385, 386]. Target-based (active-site) resistance to tetracyclines is relatively rare but does occur. Mutations in the rRNA target were initially reported in the Gram-positive *Propionibacterium acnes* (G1058C) [387] and later in *Helicobacter pylori* [388, 389]. Resistance to tetracycline in *Neisseria gonorrhoeae* can be mediated by a mutation in *rpsJ*, encoding Val57Met substitution in the 30S ribosomal protein S10 [390]. This mechanism was later found in *K. pneumoniae* KPC-2-producing clinical isolates and associated with reduced susceptibility to tigecycline [391, 392].

The first identified tetracycline-modifying enzyme, TetX, was encoded on transposons isolated from *Bacteroides fragilis* [393], and several more have been identified since then (see Table 4.5). These enzymes are monooxygenases that act by hydroxylating the tetracycline, interfering with the tetracycline magnesium-chelating properties which are needed for ribosome binding [393, 394]. The hydroxylated tetracycline is also less stable and can then decompose. These enzymes interact with the central core of the tetracycline molecule, explaining why they can act on all tetracyclines including third-generation compounds [395, 396]. Nonetheless they appear to be less effective in conferring resistance to third-generation tetracyclines [360]. These enzymes have not emerged or spread as a major source of resistance yet, particularly to tigecycline, but should be monitored in the clinic.

4.2.4.3 Evasion of Tetracycline-Specific Resistance by Third-Generation Glycylcyclines (Tigecycline)

Third-generation tetracyclines were developed in direct response to the emergence and spread of resistance [350], and this effort led to the synthesis of a novel class of glycyl-substituted C9-aminotetracyclines that are referred to as glycylcyclines [334]. One of these, GAR-936, now tigecycline, bears a *t*-butyl amine substitution and is very potent against a broader range of Gram-positive and Gram-negative bacteria than tetracycline. Moreover, it evades the two main categories of acquired resistance to tetracycline, tetracycline-specific efflux and ribosomal protection. This is partly because tigecycline binds the ribosome with a much higher affinity (10–100-fold higher) than does tetracycline, and this is reflected in more potent inhibition of translation as measured using in vitro translation assays [397–399]. The basis for the improved affinity of tigecycline is an additional stacking interaction between the 9-t-butylglycylamido portion of tigecycline (C-9 moiety) and the C1054 nucleobase of the 16S rRNA [398]. There is also additional steric clash between tigecycline and the anticodon stem loop of the A-site tRNA compared to tetracycline, making it more effective in preventing tRNA entry into the A-site. This is likely important for the overall improved potency against a broader range of Gram-negatives than tetracycline (i.e., overcoming intrinsic resistance). The C-9 moiety also may enhance the target on-rate of tigecycline and sterically clash with important residues of the RPP TetM (within loop 3 of domain 4 of TetM) that interact with C1054, thus preventing TetM from dislodging tigecycline from the ribosome. Therefore the C-9 moiety itself is likely preventing TetM and other RPPs from conferring resistance rather than this being a function solely of higher tigecycline binding affinity [398]. As mentioned above, tigecycline also appears to escape recognition by tetracycline-specific efflux pumps, shown using TetB containing vesicles [400]. Tigecycline may also be less effective as an inducer of tetracycline efflux pump expression [401].

4.2.4.4 Mechanisms of Tigecycline Resistance Emerging in the Clinic

Resistance to early-generation tetracyclines in the clinic emerged rapidly after their introduction in the late 1940s, and the epidemiology of tetracycline resistance is described in Chap. 10. As described above, third-generation tetracyclines, exemplified by tigecycline, are able to largely overcome established resistance by circumventing tetracycline efflux and/or ribosomal protection. Tigecycline entered the clinic in 2005 (often used as a last line of defense in treating MDR isolates), and since it was refractory to the main resistance mechanisms, it was not clear what mechanisms of resistance would emerge in clinical use, although it seemed likely that RND efflux would play a role. The Tigecycline Evaluation and Surveillance Trial (TEST) is an ongoing global study to monitor in vitro susceptibility to tigecycline and other antibiotics in MDR isolates. The most recent report [130] examined isolates collected worldwide between 2004 and 2014 and found that rates of MDR

E. coli ranged from 4% in North America to 18% in Latin America. Approximately 94% of MDR *E. coli* were resistant to minocycline, but only 0.2% were resistant to tigecycline, the lowest rate for all antibiotics tested. Tigecycline-resistant *E. coli* isolates did not appear in this study until 2008; however eight resistant isolates have been identified between 2009 and 2014 across a wide geographic range that included one isolate from North America. For *K. pneumoniae*, rates of MDR were approximately 12%. Among those, the rate of tigecycline resistance was higher than seen for *E. coli*, at approximately 6%, although this was still the lowest of all the antibiotics tested. *A. baumannii* had the highest frequency of MDR, at 44% with some geographic areas having >50% MDR. The lowest rate of resistance among the MDR isolates was reported for minocycline (13%). Tigecycline resistance breakpoints are not established for *A. baumannii*, but tigecycline had a lower MIC_{90} than minocycline. The most recent report [130] concluded that tigecycline has remained active against most MDR isolates (excluding *P. aeruginosa* which has high intrinsic resistance), although this varies by geographical region. The acquisition of tigecycline resistance in small numbers of *E. coli* isolates over the course of the collection of these strains was observed and should be further monitored.

So far, decreased susceptibility to tigecycline in the clinic has been attributed to upregulation of RND family efflux pumps. For example, resistance has been correlated with upregulation of AcrAB-TolC expression in clinical isolates of *E. coli* [43, 402], *K. pneumoniae* [42, 365, 403], and *E. cloacae* [367] and of AdeABC in *A. baumannii* [44, 370]. Interestingly the *tetX* gene encoding enzymatic modification was detected in a clinical isolate of *A. baumannii* from China [44]. Decreased susceptibility in *Salmonella enterica* was attributed to the combined activity of a plasmid-borne *tet*(A) gene and mutation in *ramR*, presumably leading to RND efflux pump upregulation. There is not always a direct correlation between efflux and susceptibility however, and mechanisms of resistance to tigecycline may ultimately prove to be more complex as tigecycline is used longer in the clinic. For example, a recent study of tigecycline-resistant *A. baumannii* found involvement of AdeABC efflux but also uncovered a potential role for mutational disruption in the *trm* methyltransferase gene in resistance in clinical isolates [404]. Finally, in vitro tigecycline selection studies using strains harboring the well-characterized tetracycline resistance genes *tet(A)*, *tet(K)*, *tet(M)*, and *tet(X)* selected for mutations in these genes that increased the ability of the encoded proteins to act on tigecycline [405]. Since these genes are widespread in clinical isolates, it will be of interest to see if this occurs in the clinic going forward.

4.2.4.5 Novel Agents and New Approaches: Circumvention of Tetracycline-Specific Resistance Mechanisms in Third-Generation Tetracyclines (Glycylcyclines)

Current efforts in the search for next-generation tetracyclines are largely being done by Tetraphase Pharmaceuticals, specifically centered on the fully synthetic fluorocyclines eravacycline, TP-271, and TP-6076. Eravacycline (currently in Phase III)

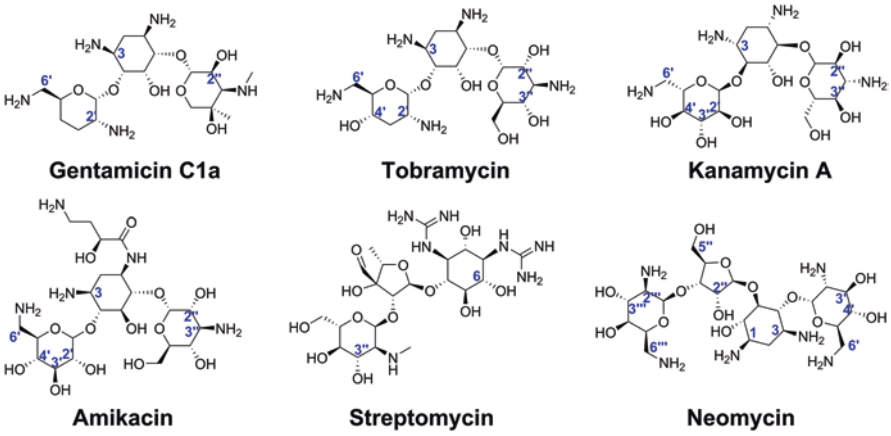

Fig. 4.7 Chemical structure of key aminoglycosides used in the clinic. The positions of covalent chemical modification by aminoglycoside-modifying enzymes are shown in blue

is generally more potent overall and has a slightly better spectrum than tigecycline, but also does not cover *P. aeruginosa*. TP-271 has a much more limited spectrum and is directed at the bacterial pathogens responsible for community-acquired pneumonia [406]. TP-6076 has potent activity against a range of pathogens including *A. baumannii* and carbapenem-resistant *Enterobacteriaceae* and has entered Phase I trials as of this writing. It was also selected for funding support from CARB-X (www.carb-x.org).

4.2.5 Aminoglycosides

Aminoglycosides are one of the major classes of antibiotics used in the clinic to treat Gram-negative bacillary infections. The most widely used aminoglycosides are tobramycin, gentamicin, and amikacin, mainly for the treatment of *P. aeruginosa* meningitis and pneumonia. Tobramycin is also used in two inhaled formulations (TOBI ® Podhaler ®, Novartis) for the treatment of chronic *P. aeruginosa* infections in cystic fibrosis patients (Fig. 4.7). Streptomycin, neomycin, and kanamycin are used for the treatment of infections caused by *E. coli*, *Proteus* species, *Enterobacter aerogenes*, *K. pneumoniae*, *Serratia marcescens*, and *Acinetobacter* species [407–410]. Aminoglycosides act by binding to bacterial ribosomes and therefore blocking bacterial protein synthesis. They mainly bind to the aminoacyl-tRNA recognition site (A-site) of the 16S ribosomal RNA of the 30S ribosome [331, 411–414]. This binding causes codon misreading and the corresponding introduction of incorrect amino acids in the growing polypeptide. This amino acid misincorporation causes rapid cell death [415]. All aminoglycosides are bactericidal and have a prolonged postantibiotic effect due to the extended time needed to

recover from protein synthesis inhibition [416–419]. Aminoglycosides have also been shown to act synergistically in vitro with other classes of antibacterials, in particular β-lactams [420–423]. These findings have encouraged the use of aminoglycoside in combination with β-lactams for the treatment of several infections in the clinic, especially for nosocomial infections caused by *P. aeruginosa* [410, 424–427]. In the past few years, retrospective studies looking at mortality outcomes of patients with carbapenem-resistant *Enterobacteriaceae* (CRE) have shown an improved outcome when aminoglycosides were used in combination therapy with β-lactams [428, 429] or tigecycline [430]. The clinical utility of aminoglycosides is imperiled by high rates of resistance often in conjunction with resistance determinants to other drugs used to treat Gram-negative infections [409, 431–436].

Aminoglycoside resistance in the clinic occurs via a number of different mechanisms: mutations altering the target (rRNA or ribosomal proteins), transport defects, efflux and, importantly, modifying enzymes that inactivate the drug [148, 409, 437]. Chromosomal mutations of the target are very rare in Gram-negative bacilli mainly due to the high number of copies of the 16S rRNA [409, 437]. Reports of target-site mutations in clinical isolates have been limited to *Mycobacterium* spp. [438, 439] and *Borrelia burgdorferi* [440], and so will not be addressed further. Each of the remaining mechanisms is discussed below, and the epidemiology of aminoglycoside resistance is discussed in Chap. 10.

4.2.5.1 Aminoglycoside-Modifying Enzymes

Aminoglycoside-modifying enzymes (AMEs) inactivate the drug by covalent chemical modification, reducing the binding affinity of aminoglycosides to their target. AMEs are the major resistance determinant in the clinic for this class of antibiotic and often encoded on plasmids harboring multiple resistant elements to multiple antibiotic classes [148, 409, 437, 441]. This presence on mobile genetic elements has enhanced the number of isozymes circulating in pathogenic and nonpathogenic bacteria. To date, well over 100 AMEs have been described and characterized from clinical isolates as well as from soil-dwelling bacteria that produce aminoglycosides [148, 442]. AMEs are divided into three major categories based on the specific chemical modification: *N*-acetylation (AACs), *O*-phosphorylation (AHPs), and *O*-adenylation (ANTs). These categories are further subdivided into classes based on their specific site of modification of the aminoglycoside. Variants of these are further subdivided using roman numerals, and in some cases, a letter is added when the same position is modified [442, 443]. Table 4.6 shows some of the major AMEs occurring in the clinic.

The aminoglycoside acetyltransferases (AACs) are the major class among these modifying enzymes. These enzymes are acetyl CoA-dependent, and they acetylate various amino groups found on the aminoglycoside structure [442, 443, 454, 455]. The most common AACs in Gram-negative bacteria are AAC(6′)-I, AAC(3)-IIa, and AAC(3)-I. Recently, a broadening in activity spectra for some of these enzymes has also been observed. AAC(6′)-Ib-cr has acquired the ability

Table 4.6 Major aminoglycoside-modifying enzymes present in clinical isolates and their resistance profile

Type	Enzymes	Resistance conferred	References
Aminoglycoside acetyltransferases (AACs)	AAC(6′)-I	Tobramycin, amikacin, netilmicin, dibekacin, sisomicin, kanamycin, isepamicin	[441, 444]
	AAC(3)-IIa	Tobramycin, gentamicin, netilmicin, dibekacin, sisomicin	[441, 445]
	AAC(3)-I	Gentamicin, sisomicin, fortimicin	[441, 446]
	AAC(6′)-Ib-cr	Kanamycin, amikacin and tobramycin, ciprofloxacin, and norfloxacin	[447, 448]
Aminoglycoside phosphotransferases (APHs)	APH (3′)-Ia	Kanamycin, neomycin, streptomycin, lividomycin, paromomycin, and ribostamycin	[409, 431–436, 441, 449, 450]
	APH (3″)-III	Kanamycin, neomycin, lividomycin, paromomycin, butirosin, and ribostamycin	[441, 451]
Aminoglycoside nucleotidyltransferases (ANTs)	ANT(2″)	Tobramycin, gentamicin, dibekacin, sisomicin, kanamycin	[441, 452]
	ANT(4′)	Tobramycin, amikacin, dibekacin, kanamycin, isepamicin	[441, 453]

to modify fluoroquinolones by acylation of the secondary amine of the piperazine ring of the antibiotic present on ciprofloxacin and norfloxacin but not levofloxacin [447, 448]. The aminoglycoside phosphotransferases (AHPs) and nucleotidyltransferases (ANTs) are both ATP-dependent enzymes. APHs phosphorylate the hydroxyl groups on the aminoglycoside similar to ATP-dependent kinases, sharing high similarity with serine-threonine eukaryotic kinases [442, 443]. ANTs utilize ATP as AMP donor that is added on the aminoglycoside hydroxyl groups. The major representatives of this class present in the clinic are ANT(2″) and ANT(4′) described in Table 4.6 [409]. Bifunctional enzymes with a broader spectrum of activity have been reported. ANT(3″)-Ii/AAC(6′)-IId that confers resistance to streptomycin, spectinomycin, and gentamicin has been isolated from *Serratia marcescens*, a human enteropathogen [456, 457]. Among the aminoglycoside-modifying enzyme genes, *aac*(6′)-Ib was the most prevalent (37.5% of isolates were positive), in a study looking at 200 Gram-negative bacilli resistant to aminoglycosides [409]. In another study from Spain of 330 aminoglycoside resistant *Enterobacteriaceae* isolates, the predominant resistance determinant was Aph(3″)-Ib (65.4% of isolates were positive) in accordance with the observed streptomycin resistance phenotype [409, 432].

4.2.5.2 Ribosomal Protection

Posttranscriptional methylation of 16S rRNA by aminoglycoside rRNA methyltransferases (RMTs) is an emerging resistance mechanism for this class of antibiotics. RMTs modify specific nucleotide residues (N7 position of nucleotide G1405 or N1 position of nucleotide A1408) of the 16S rRNA, thereby preventing aminoglycosides from binding to their target [408, 458]. This mechanism was originally identified and characterized in antibiotic-producing organisms as a self-protection mechanism [459] but has now been emerging in several important Gram-negative nosocomial pathogens [460]. In 2003, aminoglycoside rRNA methyltransferases were reported in *K. pneumoniae* (encoded by the *armA* gene) and *P. aeruginosa* (encoded by the *rmt*A gene) both conferring high-level resistance to 4,6-disubstituted deoxystreptamines [461, 462]. After the first identification of these genes, a series of plasmid-encoded RMTs have been identified (encoded by *rmtB1*, *rmtB2*, *rmtC*, *rmtD*, *rmtD2*, *rmtE*, *rmtF*, *rmtG*, and *rmtH*) in several clinical isolates [408]. In 2007 an aminoglycoside rRNA methyltransferase (encoded by the *npmA* gene) was reported from *E. coli* isolated in 2003 from the urine of an inpatient in a general hospital in Japan, which conferred resistance to 4,6- and 4,5-disubstituted 2-deoxystreptamines [463]. Further information on the class of enzyme including their origin and the impact on the use of aminoglycoside can be found in a review published in 2016 by Doi et al. [408]. Even though a low prevalence of this class of enzyme has been reported in clinical isolates, their ability to confer high-level pan aminoglycoside resistance in conjunction with their presence on mobile elements threatens the future use of aminoglycosides.

4.2.5.3 Decreased Permeability and Efflux

As described in Sect. 4.2.1, a major issue in Gram-negative bacilli is the inability of many drugs to penetrate the cell membrane. However, aminoglycosides are cationic and therefore can interact with negatively charged LPS to facilitate "self-promoted uptake" across the OM. This is followed by energy-dependent (electron transport-mediated) uptake across the inner membrane. Correspondingly, alterations in LPS or reductions in uptake across the inner membrane were proposed to play a role in reducing susceptibility. In the case of *P. aeruginosa*, this may involve aminoarabinose modification of the lipid A moiety of LPS, controlled by the PhoP-PhoQ two-component regulator pair. This system is a well-characterized determinant of resistance to the polymyxin class of antibiotics (see Sect. 4.2.6), but its involvement in aminoglycoside resistance is less well understood [464, 465]. Reduced expression of some oligopeptide transporters, such as OppA, may also reduce entry of aminoglycosides [437].

Efflux by RND family pumps has been shown to play a significant role in aminoglycoside extrusion and therefore resistance in several pathogens. In *E. coli*, the AcrAD pump has been shown to be able to efflux aminoglycosides [466, 467]. Other pumps involved in aminoglycoside efflux are AmrAB-OprA and

BpeAB-OprB in *Burkholderia pseudomallei* [466, 468], AdeABC in *A. baumannii* [76], and MexXY-OprM of *P. aeruginosa* [469, 470]. The MexXY-OprM efflux pump is unique among the complement of *P. aeruginosa* pumps in its ability to extrude aminoglycosides. It is also induced by agents inhibiting protein synthesis, contributing to both impermeability and adaptive aminoglycoside resistance [470, 471]. The latter refers to the induction of reversible resistance by exposure to aminoglycosides, which is now known to result largely from induction of MexXY and possibly by a concomitant upregulation of anaerobic respiration genes which may compromise aminoglycoside uptake across the inner membrane [472]. The regulation (inducibility) of MexXY expression involves the MexZ repressor and PA5471, a protein of unknown function, which sense disruptions of protein synthesis (translation) [52, 473]. More recent work showed the induction of MexXY by aminoglycosides also depends on the two-component system AmgRS [474]. This appears to be related to the role of AmgRS as a cell envelope stress response regulator and, in the case of aminoglycosides, in responding to incorporation of misfolded proteins in the inner membrane that results from aminoglycoside action on the ribosome. Mutations in *amgS* can also cause constitutive activation of MexXY expression. Mutations in genes encoding another two-component system ParRS, involved in resistance to polymyxins, also cause upregulation of MexXY and aminoglycoside resistance [75, 475]. It should be noted that although aminoglycoside-modifying enzymes are generally the most important resistance mechanism in Gram-negative bacteria, this does not appear to be the case in *P. aeruginosa* isolates recovered from CF patients. Since these patients tend to be colonized by strains common in the natural environment, there is less chance for these enzymes to accumulate, and therefore only a small percentage of CF isolates harbor aminoglycoside-modifying enzymes [476]. Therefore, efflux by MexXY, likely in conjunction with other mechanisms, is comparatively more important in this instance.

4.2.5.4 Biofilms

Growth in the biofilm mode is another barrier for the entry of aminoglycosides in bacteria, contributing to intrinsic and adaptive resistance to this class of antibiotics. Biofilms are defined as an intertwined community of bacteria adhering on a surface and surrounded by a self-produced matrix composed of extracellular DNA, proteins, and polysaccharides. Biofilms play a key role in chronic *P. aeruginosa* infections and have been associated with pulmonary infections in patients with CF where aminoglycosides and, in particular, tobramycin are routinely used [477, 478]. Therefore understanding the role of biofilms in relation to aminoglycoside resistance is of high importance. Subinhibitory concentrations of aminoglycosides, especially tobramycin, have been shown to induce biofilm formation in *P. aeruginosa* by the induction of the aminoglycoside response regulator (*arr*) gene. This gene is postulated to be involved in the regulation of cell surface adhesiveness and therefore contributes to biofilm-specific aminoglycoside resistance. Some studies

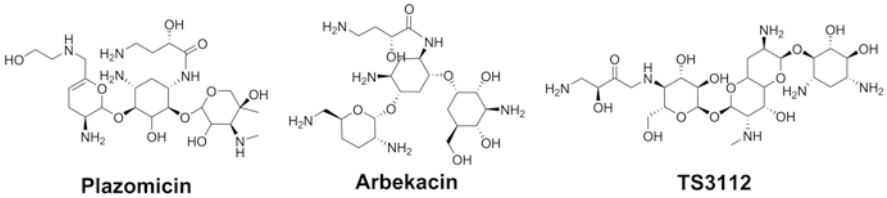

Fig. 4.8 Structure of novel aminoglycosides currently in clinical development (plazomicin and arbekacin) and preclinical characterization (TS3112)

have hypothesized the ability of the biofilm matrix to bind aminoglycosides and therefore drastically reduce their antibacterial activity [479, 480]. Several in vitro studies have shown co-dosing of an aminoglycoside with a cationic steroid antibiotic, CSA-13, or a cationic peptide, DJK-5, is effective in overcoming biofilm-mediated resistance [481, 482]. More study to understand the molecular mechanisms of biofilm induction by aminoglycosides as well as device strategies to inhibit biofilm formation are needed.

4.2.5.5 Novel Approaches and Treatment Strategies

Better understanding of aminoglycoside resistance, molecular mechanisms and dosing regimens to minimize toxicity, together with the increase of resistance in the clinic, has created interest in discovering new approaches or novel aminoglycosides aimed at overcoming resistance. One approach that has been pursued to extend the useful lifespan of aminoglycosides is the pursuit of aminoglycoside-modifying enzyme (AME) inhibitors that would prevent inactivation of aminoglycosides, similar to the successful combinations of β-lactamase inhibitors with β-lactams. Despite several three-dimensional structures of all representative members of the three classes of AMEs [483–492] and better understanding of their molecular mechanism of action, screening efforts and structure-based drug designs have not yet led to viable clinical candidates. Labby and Garneau-Tsodikova have reviewed several efforts aimed at identifying suitable AME inhibitors in a recent review [493].

The goal for new aminoglycosides has been mainly to improve their toxicity profile, avoid modification by key AMEs, and not be impacted by RMTs widely spread in clinical isolates. Arbekacin from Meiji Seika Pharma Co. and plazomicin from Achaogen are the front-runners currently under development (Fig. 4.8). Arbekacin is a broad-spectrum aminoglycoside active against Gram-positive bacteria, including methicillin-resistant *S. aureus*, and Gram-negative bacteria, such as *P. aeruginosa*, KPC-expressing *K. pneumoniae*, and ESBL-producing *E. coli* [494, 495]. Arbekacin is approved in Japan for the treatment of sepsis and pneumonia caused by MRSA. It is currently in Phase I clinical trials as an inhalation solution for the treatment of hospital-associated and ventilator-associated bacterial pneumonia (HABP/VABP) and under evaluation for treatment of patients with infections caused by multidrug-resistant organisms when treatment with other antibiotics can-

not be used [494, 495]. Arbekacin is stable to some of the most common APHs, ANTs, and AACs present in clinical isolates. Plazomicin, a sisomicin analog with potent activity against *Enterobacteriaceae* ($MIC_{90} \leq 2$ μg/mL), has recently completed Phase III trials in cUTI and in patients with serious bacterial infections due to CRE (http://www.achaogen.com/plazomicin/). Plazomicin, like its parent compound sisomicin, is resistant to several AMEs such as APH(3′)-III, APH(3′)-VI, and APH(3′)-VII and ANT(4′)). The addition of hydroxyl-aminobutyric acid substituent at the N-1 position and hydroxyethyl substituent at the 6′ position has rendered plazomicin resistant to AAC [3], ANT(2″), APH(2″), and AAC(6′) enzymes [409]. Plazomicin activity is abrogated by RMTs that are frequently present on mobile genetic elements that also carry β-lactamases like NDM-1 in *Enterobacteriaceae* [431, 434, 496]. This may turn out to be a liability for the clinical longevity of plazomicin against *Enterobacteriaceae*. Meiji Seika Pharma Co. recently reported a semisynthetic apramycin, named TS3112 (Fig. 4.8), which is active against Gram-positive and Gram-negative bacteria producing both AMEs and RMTs. TS3112 showed potent bactericidal activity in a murine thigh model of *K. pneumoniae* expressing RTMs [497]. TS3112 is currently in early-stage characterization, showing encouraging in vitro results.

New delivery strategies for aminoglycosides have been adopted in the past few years that provide higher local concentration at the infection site with a lower total amount of drug delivered, which reduces systemic exposure and safety liabilities of this class of drug. The best example of new delivery method for inhaled aminoglycoside is tobramycin inhalation powder (TOBI® Podhaler®), delivered via the T-326 inhaler (Novartis). Long-term safety studies in patients with CF have shown that it is well tolerated, there was no evidence of serum tobramycin accumulation with successive cycles, and no unexpected adverse events were observed. Further, the new powder delivery method improved compliance due to shorter administration time, convenience, and ease of use [498, 499]. Bayer Healthcare, in collaboration with Nektar, is currently developing BAY41-6551, a drug-device combination of a specially formulated amikacin. BAY41-6551 has recently completed Phase III as an adjunctive treatment for intubated and mechanically ventilated patients with Gram-negative pneumonia and showed bactericidal activity against most isolates tested with amikacin MICs ≤ 256 μg/mL [500–502].

Aminoglycosides are a key class of antibiotic used by physicians to treat serious infections caused by MDR Gram-negative and Gram-positive pathogens. Continued characterization of aminoglycoside resistance mechanisms may enable the design of resistance determinant inhibitors and/or new aminoglycosides capable of circumventing these mechanisms. Additionally, efforts aimed at optimization of dosing regimens and discovery of new delivery strategies should help to maintain and extend the clinical utility of this important antibiotic class.

4.2.6 Polymyxins

Polymyxins are an older class of cationic cyclic lipopeptide antibiotics that were introduced into clinical use in the 1950s (polymyxin B and colistin, also known as polymyxin E). However, the use of polymyxins declined sharply around the early 1970s due to concerns of toxicity [503, 504] and the availability of safer antibiotics. The mechanism by which polymyxins kill bacteria is not fully understood. One mechanistic step that is well established is an initial interaction of the cationic peptide with negative charges on the lipopolysaccharide (LPS) that forms the outer leaflet of the Gram-negative OM [505, 506]. This interaction occurs mainly via negatively charged phosphate residues located on lipid A and is required for the "self-promoted uptake" of polymyxins into the bacteria. Binding of polymyxin is thought to displace divalent cations (Mg^{2+}, Ca^{2+}) that cross-link adjacent LPS molecules, and this can disrupt to some extent the permeability barrier of the OM, which also increases uptake of the polymyxin. This is unlikely to be responsible entirely for cell killing. As discussed in more detail in Sect. 4.2.6.4 below, derivatives of polymyxin (e.g., polymyxin B nonapeptide) exist with dramatically reduced antibacterial activity that retain the OM disruption activity. Polymyxins may ultimately kill via mechanisms that include lysis of the inner membrane [505] generation of toxic hydroxyl radicals [507] and inhibition of respiration via targets such as type II NADH-quinone oxidoreductases (NDH-2) [508]. The interaction of polymyxins with the Gram-negative OM LPS has two main implications: first, that susceptibility to this class of compound can be decreased by restructuring LPS to reduce its negative charge (discussed below) and, second, that it limits the spectrum of polymyxins to some but not all Gram-negative pathogens. This includes the important Gram-negative ESKAPE pathogens *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *A. baumannii*. Polymyxins were reintroduced into the clinic in the early 2000s as a last-line therapeutic option to address the emergence of MDR and XDR in these pathogens. Not long after this reintroduction into clinical practice, emergence of colistin-resistant strains began to increase, prompting concern about their ongoing therapeutic usefulness, especially considering that no other options may exist in scenarios where colistin is being used. The main mechanism by which Gram-negative pathogens that are susceptible to polymyxins develop resistance is via alterations in their lipopolysaccharide that reduce its net negative charge, thereby reducing uptake of polymyxins. This can occur through selection of chromosomal mutations in genes involved in regulating LPS remodeling or by horizontal acquisition of plasmids harboring genes mediating this process. Some Gram-negative pathogens (e.g., *Burkholderia cepacia*, *Proteus mirabilis*, *Serratia marcescens*) are not susceptible to polymyxins (intrinsic resistance), because their LPS always possesses such modifications. These mechanisms are discussed in the sections below.

4.2.6.1 Mutationally Acquired Resistance Mediated by Reduction of Negative Charge Status of LPS

Gram-negative bacteria have a broad ability to remodel their OM. The best understood and most widespread mechanism that decreases susceptibility to polymyxins utilizes this capacity by modification of LPS to reduce its negative charge and, consequently, the initial binding step of cationic polymyxin to the cell surface. This is highly complex and varies among different strains but in general occurs in *Enterobacteriaceae* and *P. aeruginosa* via masking the negatively charged 4′-phosphates on lipid A, and to a lesser extent the 1 position phosphate or the 3-deoxy-D-manno-octulosonic acid (KDO), by addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N) [509]. Synthesis and transfer of L-Ara4N are mediated by the products of the *arn* locus (e.g., *arnBCADTEFpmrE* in *P. aeruginosa*, also known as *pmrHFIJKLME)*. This is perhaps the most common mechanism of reducing the negative charge of LPS described in these organisms. *Enterobacteriaceae* can also add phosphoethanolamine (pEtN) (mainly to the 1 position but also to other locations such as 4′-lipid A, KDO, or core oligosaccharide) via transferases such as PmrC. The contribution of pEtN to resistance appears to be smaller than that of L-Ara4N, but both clearly play a role, and both decorations can occur together. Recently, *P. aeruginosa* has also been shown to be able to modify its LPS with pEtN when zinc is present, under the regulation of the ColRS two-component regulatory system [510]. *A. baumannii* lacks an *arn* locus and therefore cannot carry out L-Ara4N modification but can undergo pEtN modification [526] or, as recently described, galactosamine modification [511]. Regulatory control of these modifications is highly complex and is often mediated by interrelated networks of two-component regulatory systems (TCSs). The PmrAB regulator pair controls L-Ara4 and/or pEtN modification and is widespread in Gram-negative pathogens. Similarly the PhoPQ system, also present in several organisms such as *S. enterica*, *E. coli*, *K. pneumoniae*, and *P. aeruginosa* (but absent in *A. baumannii*), is important for control of LPS modification and can be interconnected with the PmrAB system. For example, these two regulatory systems are interconnected in *S. enterica* and *E. coli* via the PmrD protein [512]. These systems can upregulate LPS modification (e.g., upregulate expression of the *arn* locus) in response to certain conditions such as magnesium limitation or exposure to polymyxin or other cationic peptides [513–515]. Although there is likely some adaptive change in susceptibility to polymyxins mediated by these systems upon drug exposure, or by exposure to cationic peptides in the host, resistance is generally mutationally acquired, via selection of mutations in the genes encoding these regulators, which leads to strong constitutive upregulation of LPS modification. Some mutations in these regulatory genes may also lead to stronger inducibility of the systems by the polymyxin [516]. Regulatory mutations can be selected in vitro and have also been associated with resistance in the clinical setting, including mutations found in *pmrA*/*pmrB* in *K. pneumoniae* [517–520], *P. aeruginosa* [516, 521, 522], and *A. baumannii* [523–527] and *phoP-phoQ* in *K. pneumoniae* [528–530] and *P. aeruginosa* [464, 522, 531]. *A. baumannii* lacks both *phoPQ* and an *arn* locus, so *pmrB* mutations are frequently found in this pathogen,

and these mutants will have pEtN modification through activation of the *pmrC* transferase gene located in the *pmrABC* locus [526]. However, mutations in *pmrAB* are not always found in colistin-resistant *A. baumannii* clinical isolates suggesting that mutations elsewhere on the chromosome can upregulate *pmrABC* [532].

The importance of the PmrAB and PhoPQ systems in controlling OM remodeling and resistance to polymyxins is well established; however a full understanding of these phenomena is still forthcoming. Recently in the case of *K. pneumoniae*, mutation of *mgrB*, which encodes a negative feedback regulator of the PhoPQ two-component system, was revealed as an important mediator of LPS modification and colistin resistance [533, 534]. Loss of MgrB function leads to constitutive activation of PhoPQ and LPS modification. This mechanism appears to be relatively widespread in *K. pneumoniae* clinical isolates [529, 535, 536] and can be mediated by insertion of genetic elements that may also carry other resistance genes such as β-lactamases [537]. The fact that colistin resistance can arise from any loss-of-function mutation of *mgrB* likely explains its relatively high prevelance among colistin-resistant *K. pneumoniae* isolates. Several additional TCSs involved in polymyxin resistance have been characterized more recently including the CrrAB (colistin resistance regulon) in *K. pneumoniae* [530]. Changes in CrrB result in upregulation of the PmrAB system via CrrR, which then upregulates *arn* genes and *pmrC*, leading to LPS modification and colistin resistance [538]. However, not all *K. pneumoniae* harbor the *crrAB* genes. In *P. aeruginosa*, three additional TCSs also known to be involved in modulating susceptibility to polymyxins have been described: ParRS [539], ColRS [540], and CprRS [541]. ParRS and CprRS participate in adaptive resistance to polymyxins by upregulating expression from the *arn* locus upon sensing polymyxins or other cationic peptides. ColRS and CprRS are required for high-level polymyxin resistance resulting from mutations in *phoPQ* [540]. These interactions appear complex, and mutational analysis also suggested that additional factors beyond L-Ara4N modification of lipid A could be involved in resistance in *P. aeruginosa*, but this remains to be fully elucidated [540]. Mutations in the *parRS* genes were subsequently shown to reduce susceptibility to multiple classes of antibiotic due to coordinately upregulating expression from the *mexXY* efflux pump genes and *arn* and downregulating expression of the *oprD* porin gene [75]. For additional details on polymyxin resistance mechanisms, see Jeannot et al. [542]. Another aspect of resistance relevant to colistin is the phenomenon of heteroresistance, which refers to the presence of a substantial stable resistant subpopulation in cultures of isolates that may score as susceptible to an antibiotic by standard susceptibility testing. Colistin heteroresistance has been described mainly in *K. pneumoniae* [528, 543, 544] and *A. baumannii* [545], and resistant subpopulations can harbor a range of resistance mutations [543]. Heteroresistant isolates can be recovered from patients with no prior treatment with polymyxins, and it is expected that the use of colistin could rapidly enrich for the resistant subpopulation leading to clinical failures. Heteroresistance can be missed by standard susceptibility tests, suggesting that the rates of, and potential for, selecting colistin resistance in the clinic may be underestimated. For more information on heteroresistance, see Chap. 9 in this volume. Finally, as mentioned above, a number of Gram-negative bacteria

are intrinsically resistant to polymyxins. These include *Burkholderia cepacia* complex, *Proteus*, *Serratia*, *Providentia*, and others. These organisms differ from susceptible strains in that their LPS is always constitutively modified with L-Ara4N or has other alterations affecting polymyxin binding.

4.2.6.2 Mutations Causing Loss or Reduction of LPS

Synthesis of LPS (in particular the lipid A portion) and assembly of the LPS-containing OM are essential for the growth and/or viability of most Gram-negative pathogens, but there are a few exceptions to this. *Neisseria meningitidis*, *Moraxella catarrhalis* [586, 587] and a subset of *A. baumannii* have been shown to tolerate loss of LPS biosynthesis. Indeed, this was uncovered in the case of *A. baumannii* during in vitro studies of colistin resistance, where mutations in genes involved in lipid A biosynthesis were directly selected from *A. baumannii* strain ATCC 19606 or other strains on polymyxin-containing medium [112, 546]. Point mutations were initially identified in the *lpxA*, *lpxC*, or *lpxD* genes [112] that encode enzymes catalyzing the first three steps of lipid A biosynthesis [25]. A follow-up experiment identified mutants where *lpxA* or *lpxC* were inactivated by insertion sequence ISAb11 [546], and subsequently an engineered mutant deleted for *lpxC* was reported (described in [547]), confirming that *lpxA* and *lpxC* are dispensable in *A. baumannii* ATCC 19606, at least under laboratory growth conditions. These mutants lack lipid A, the target of the initial interaction with polymyxins, and as such are highly resistant to polymyxins but are also highly susceptible to a range of other antibiotics due to loss of the protective lipid A-containing OM [112]. To date, it appears that this mechanism may be confined to mutations in genes encoding enzymes occurring early in the lipid A biosynthesis pathway since inactivation of steps occurring later in the lipid A biosynthetic pathway (e.g., LpxH [548] or LpxK [549]) causes toxic accumulation of lipid A synthetic pathway intermediates and is not tolerated. Furthermore, this mechanism does not apply across all *A. baumannii*, as only a subset appears to tolerate loss of lipid A biosynthesis (e.g., ATCC 19606). The reasons for this are not fully understood, but a recent study showed potentially compensatory transcriptomic changes in response to loss of *lpxA* [550], whereas others showed that expression of penicillin-binding protein (PBP)-1A rendered lipid A loss lethal in strains that could otherwise tolerate lipid A loss and that cells lacking both PBP 1A and lipid A had increased expression of lipoproteins on their surface that may compensate for lipid A loss [114]. Although lipid A loss and colistin resistance can be readily selected in vitro, it stimulates debate about its relevance in the clinic, both in terms of colistin resistance and, as discussed above in Sect. 4.2.1.5, with respect to the evaluation of novel antibacterial targets within the lipid A biosynthetic pathway (e.g., LpxC). This stems from the notion of whether *A. baumannii* lacking lipid A (LPS) can survive during infection and therefore could be selected during colistin treatment.

Since the Gram-negative OM provides protection from the host immune system, it is generally thought that loss of lipid A (OM) would render the cells unfit in the

host environment. Supporting this, colistin-resistant isolates with mutations in *lpxA*, *lpxC*, or *lpxD* [112] were highly attenuated in *C. elegans* and mouse models of infection [551], and an LpxC inhibitor that lacked in vitro antibacterial activity against *A. baumannii* was efficacious in a mouse model of infection [117]. Overall this is consistent with the very high detection of *pmr* mutations among colistin-resistant clinical isolates rather than loss of LPS and implies that total loss of lipid A may be more of an in vitro phenomenon. More recent analyses of colistin-resistant *A. baumannii* XDR clinical isolates identified mutations in *pmrA*, *lpxC*, and *lpxD* (and *lpsB*, involved in synthesis of the core region attached to lipid A and shown to be involved in intrinsic polymyxin resistance [552]) occurring together [524]. Isolates selected for further study (AC12 and AC30) produced considerably less LPS than either the laboratory strain ATCC 19606 or polymyxin-susceptible clinical isolates [553]. This suggests a possibility that mutations reducing, but not abolishing, lipid A biosynthesis may emerge over time in the clinic and that the combination of pEtN and/or galactosamine modification with reduced lipid A synthesis could conspire to decrease susceptibility. These isolates were generally drug resistant, suggesting that the reduction in lipid A may not be enough to severely compromise the OM permeability barrier, also allowing for their survival in the host. It is tempting to speculate that the mutation in *lpsB* may serve to further stabilize the reduced levels of modified lipid A core present in these cells and that other factors are likely involved in determining the overall susceptibility of *A. baumannii* to colistin [552, 554], including the level of lipid A acylation. A full understanding of this mechanism awaits further study. Whether this phenomenon can extend to other Gram-negative pathogens that strictly require LPS for growth or viability remains to be seen, but one recent study forcing the in vitro evolution of colistin resistance in *P. aeruginosa* using a morbidostat approach generated mutations in *pmr* genes and *lpxC* among others [555].

4.2.6.3 Plasmid-Mediated Modification of LPS

Mutations mediating resistance to colistin can occur fairly rapidly in some Gram-negative pathogens as described above, but there were initially no reports of horizontal transfer of mobile elements carrying genes mediating LPS modification and colistin resistance. This changed in 2015 with reporting of the plasmid-borne *mcr-1* gene encoding a pEtN transferase in *E. coli* strains in China and its distribution in strains isolated from raw meat, animals (pigs), and humans [556]. It was quickly established that: plasmid-borne *mcr-1* was widespread in many regions of the world; occurred in isolates from food animals, meat and vegetables, the environment, and humans; was found mainly in *E. coli* but also occurred in other bacteria; and was detected in isolate collections dating back to the 1980s [557, 558]. The first identification of the *mcr-1* gene in *E. coli* from a patient in the United States was reported in 2016 [559]. Additional *mcr-1.2* [560], *mcr-2* [561], and *mcr-3* [562] variants have now also been identified. The specific impact of the *mcr-1* gene in all four Gram-negative ESKAPE pathogens has very recently been reported. Mcr-1 mediates pEtN

modification in *E. coli*, *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa*; however it only seems to shift susceptibility in the first three [563]. Given that colistin was reintroduced into clinical use primarily as a last-line therapy for treating MDR Gram-negative infections, including carbapenem-resistant *Enterobacteriaceae* (CRE), the identification of a mobile element conferring colistin resistance raised immediate concern as to its potential dissemination into strains such as CREs, and indeed this has already occurred. For example, the *mcr-1.2* gene was originally found in a KPC carbapenemase-producing *K. pneumoniae* human clinical isolate of the clinically important ST512 lineage, isolated in Italy [560]. Two multidrug-resistant *K. pneumoniae* human clinical isolates were shown to harbor both the $bla_{ndm\text{-}5}$ metallo-β-lactamase and *mcr-1* genes [564]. *E. coli* isolates from food and human origin had both the $bla_{ndm\text{-}9}$ and *mcr-1* genes [565, 566], and an isolate from a human urinary tract infection in the United States had $bla_{ndm\text{-}5}$ and *mcr-1* [567]. The first death known to result from such an infection in the United States occurred in Nevada in 2016 and was attributed to an untreatable *K. pneumoniae* harboring an NDM metallo-β-lactamase and plasmid-borne *mcr-1* [568]. Although still generally of lower incidence worldwide [569], the spread of these untreatable strains is inevitable. If colistin therapy will continue to be used, vigilance in detection and surveillance of both carbapenem and colistin resistance and corresponding implementation of effective infection control and stewardship procedures are very important [570]. Furthermore, the need for new antibiotics to treat these infections has now become an extreme priority. Finally, the widespread distribution of *mcr-1* in food animals and food products is entirely consistent with the use of large amounts of colistin in agriculture [557] and therefore the dissemination of these antibiotics generally into the environment, particularly localized around farms. MCR-1 and variants are related to a resistance protein from natural producers of polymyxin and to another pEtN transferase, LptA from Neisseria [571, 572]. The evolutionary history remains to be fully understood in this case, but it is difficult not to speculate that this process was enhanced by extensive agricultural and veterinary use of colistin. Now that it has occurred, such continued use of colistin will continue to facilitate the maintenance and spread of *mcr-1*-containing strains, and so significant benefit may be derived from finding creative ways to address this issue (for more information on agricultural use of antibiotics, see Chap. 10 in this volume). It is interesting to note that since colistin was out of favor for some time in human clinical usage, the dissemination of *mcr-1*-containing strains into reservoirs, such as the human gut, and diversity of *mcr-1*-containing genetic elements may be underestimated [573].

4.2.6.4 Novel Approaches and New Agents

Current efforts in the area of novel polymyxins are aimed at the design of non-antibacterial polymyxin analogs for use as potentiators of currently used antibiotics or the design of new antibacterial analogs with reduced toxicity allowing for a higher therapeutic index or with increased antibacterial activity against emerging polymyxin-resistant isolates. Several of these efforts exploit the earlier finding that

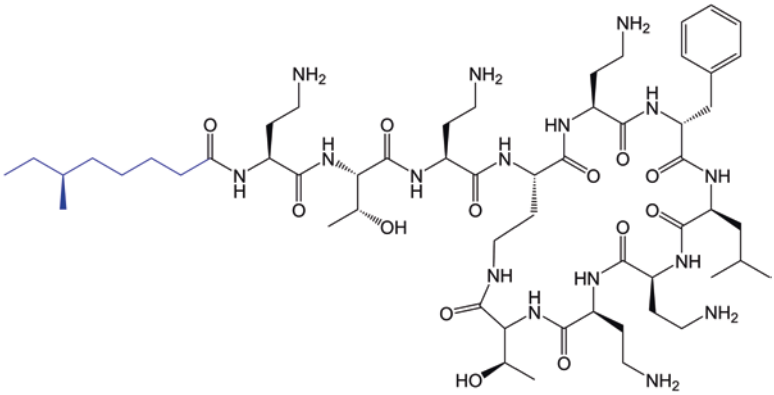

Fig. 4.9 Chemical structure of polymyxin B. Acyl chain depicted in blue is a key determinant of antibacterial activity but not outer membrane disruption activity

the N-terminal acyl chain of polymyxin B (Fig. 4.9) is involved in both antibacterial activity and toxicity.

A derivative of polymyxin B, polymyxin B nonapetide (PMBN), lacks this moiety and is less toxic and less potent as an antibacterial but retains the ability to interact with the bacterial OM and permeabilize cells. PMBN itself has been the subject of much interest and research over the years as a possible potentiating molecule for use in combination with other antibiotics, but the potential for unacceptable residual toxicity still exists. The number of positive charges on polymyxin has also been associated with toxicity. Northern Antibiotics/Spero has exploited this to design a PMBN derivative (SPR741) that contains an N-acetyl-threonine-D-serine side chain, thereby reducing the number of positive charges from five to three relative to PMBN [121]. This molecule does not have significant antibacterial activity and is reported to be less toxic but retains antibiotic potentiation activity in *E. coli*, *K. pneumoniae*, and *A. baumannii*, although it does not potentiate in *P. aeruginosa* [574–576]. As of this writing, SPR741 has entered Phase I clinical trials. Cubist Pharmaceuticals (now Merck) have designed a polymyxin decapeptide derivative containing a halo-aryl moiety at its N-terminus (CB-182804) to pursue a reduction in toxicity [577]. CB-182804 exhibited slightly lower antibacterial potency relative to polymyxin B but was efficacious in animal models of infection and is reported to have reduced toxicity. CB-182804 entered Phase I clinical trials but appears to be discontinued. Along the same lines, Pfizer reported a series of analogs replacing the N-terminal acyl chain with biaryl moieties and substituting the diamino-butyrate moiety at amino acid 3 with diamino-propionate. One of these, named 5 X, had slightly improved antibacterial activity and indications of reduced toxicity, but based on studies in dogs, the therapeutic index was not significantly better than polymyxin B [578]. Researchers at Monash University are exploring novel polymyxin lipopeptides to define structure activity relationships for gaining activity against colistin-resistant isolates [579] and have presented data on other less toxic polymyxin derivatives in conjunction with the Medicines Company [580]. Cantab

has also reported on the piperazine derivative that showed reduced cytotoxicity and improved in vivo efficacy over polymyxin B in *A. baumannii* and *P. aeruginosa* lung infection models. Additional details and chemical structures for these and other novel polymyxins can be found in the review by Brown and Dawson [581]. Finally, there is renewed interest in the octapeptin natural products which also interact with and traverse the Gram-negative OM but do so via a different mechanism and therefore may be active against polymyxin-resistant strains [582–584].

4.3 Concluding Remarks

The discovery of antibiotics, along with vaccines and improved concepts in hygiene, could be considered the greatest achievement in healthcare-related science in history. Unfortunately, decades of antibiotic use and perhaps misuse, in both medicine and agriculture, have enriched for resistant bacteria in the clinical setting, eroding the effectiveness of the antibiotics upon which we have relied and setting the stage for a potentially very different reality in medicine from what most of us had grown accustomed to. This is especially unfortunate since so much of medical practice, for example, surgery, has relied on antibiotics for success. As can be seen from the above discussions, antibacterial resistance is complex and multifactorial. However there are key mechanisms that affect susceptibility to certain classes, such as β-lactamases for β-lactams and AMEs for aminoglycosides, which may provide specific strategies for next-generation versions of these antibiotics that address those mechanisms. It is clear that no effort should be spared on these approaches for the near term and that new agents directed at previously unexploited novel targets should be aggressively pursued where they show promise. Hopefully the new awareness of the issue of antimicrobial resistance, and the various incentivizing efforts spawned from this, will be successful in moving us in the right direction to address this threat. Finally, even if new agents come along in the near term, it is imperative that complacency in antibiotic discovery never again sets in. New agents will likely be the last line of defense, and as such, when resistance to them emerges, the overall issue of untreatable infections would again be upon us.

Major Points

- Gram-negative pathogens have a unique additional asymmetric outer membrane (OM). This membrane establishes a significant permeability barrier (reduces influx) to toxic molecules including antibiotics. Mutations decreasing compound permeability can be selected under antibiotic exposure.
- Gram-negative pathogens have unique RND family efflux pumps that extrude most antibiotics and other toxic molecules; these work together with the OM to reduce intracellular compound accumulation. Upregulation of efflux pump expression, or changes in compound specificity, can be selected under antibiotic exposure.

- An understanding of the design of new compounds that accumulate sufficiently (overcome efflux) in Gram-negative bacteria is lacking.
- Mechanisms that cause resistance to specific antibiotics include mutations that alter the antibiotic target, acquisition of proteins that bind and protect the target, or acquisition of enzymes that modify antibiotics.
- Clinical resistance to specific classes of antibiotics often results from combinations of efflux and OM changes together with compound-specific mechanisms.
- Multidrug resistance arises from various combinations of all of the above.

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Chapter 5
Drug Resistance in Tuberculosis

Neil W. Schluger

5.1 Treatment of Tuberculosis and the Generation of Resistance

The problem of drug resistance in tuberculosis was apparent from the first experiment involving antibiotic treatment of this disease. Streptomycin, the first antibiotic with activity against *M. tuberculosis,* was discovered by Selman Waksman and Albert Schatz of Rutgers University in the 1940s. The first rigorous investigation of its use was conducted by the British Medical Research Council and reported in a paper in the *British Medical Journal* published in 1948 [1]. In that experiment, generally also acknowledged as the first randomized controlled trial ever to be published, 100 men were chosen to receive either bed rest, the standard of care at the time for tuberculosis (TB), or bed rest plus injections of streptomycin. The results were striking: they demonstrated that streptomycin was clearly effective in patients with respect to improvements in symptoms, chest radiographic findings, and results of sputum bacteriology (most of the study participants converted to negative sputum cultures within a few months after beginning streptomycin injections). However, after (often very soon after) converting to negative sputum cultures, essentially all the patients in the trial relapsed and again developed positive sputum cultures. In all these relapsed cases, cultures that had initially been susceptible to streptomycin had become resistant. That very first trial, which clearly established that antibiotic treatment of tuberculosis was effective, also demonstrated that drug resistance could easily emerge when the disease was treated with a single antibiotic for more than a few days. The need for multidrug regimens, in order to prevent the emergence of resistance during treatment of tuberculosis, was suggested [2]. Combination

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I. W. Fong et al. (eds.), *Antimicrobial Resistance in the 21st Century*, Emerging Infectious Diseases of the 21st Century, https://doi.org/10.1007/978-3-319-78538-7_5

treatment became possible in the early 1950s with the introduction of isoniazid, and the development of other so-called "first-line" drugs (rifampin, ethambutol, pyrazinamide) followed.

As noted above, isoniazid (INH) was initially introduced in the early 1950s and was recognized immediately as an effective antituberculosis drug [3–7]. Although the BMRC streptomycin trial had certainly brought the emergence of drug resistance to the fore, the highly active properties of INH led some to think that this drug could be used as a single agent in the treatment of TB disease in poor countries with limited resources. That would eliminate the need to obtain and use more expensive and complex agents such as streptomycin, which had to be given by injection. Trials in the late 1950s and early 1960s in Africa and India using INH monotherapy for TB showed a high rate of favorable responses, but also a very high rate—as high as 53%—of the development of INH resistance. This certainly (and perhaps predictably) underscored the findings in the initial streptomycin trial.

These early experiments clearly demonstrated the potential for drug-resistant strains of *M. tuberculosis* to emerge in a relatively short period of time following exposure to a single drug. Consequently, the use of single-drug regimens was discouraged and fell out of practice on a programmatic basis. On a worldwide level, relatively little attention was paid to the issue of drug resistance as a public health issue for the first several decades of the chemotherapy era.

By the 1980s, a short-course regimen consisting of isoniazid, rifampin, pyrazinamide, and ethambutol for 2 months followed by a continuation phase of isoniazid and rifampin for an additional 4 months (a 6-month course in total) had been shown to be effective in achieving cure in nearly all cases, and this became and has remained the standard regimen for treating tuberculosis around the world [8]. When administered correctly, this regimen should easily guard against the emergence of drug resistance.

5.2 Emergence and Recognition of Drug-Resistant Tuberculosis as a Public Health Issue

Aside from occasional cases of streptomycin-resistant tuberculosis (no longer a clinical problem since the development of effective multiagent regimens that did not include this drug) and a low background level of isoniazid monoresistant strains of *M. tuberculosis*, drug-resistant tuberculosis, and especially multidrug-resistant tuberculosis, MDR-TB—defined as tuberculosis caused by strains resistant to at least INH and rifampin—was not perceived as a significant global problem until cases started to accumulate in a few cities in the late 1980s and early 1990s. In fact, in the United States, a series of papers were published in the 1960s and 1970s indicating that drug resistance was becoming less and less of a problem [9–12]. That trend abruptly reversed in the late 1980s and early 1990s.

The emergence of MDR-TB was perhaps best described in a series of reports from New York City beginning in the early 1990s [13, 14]. This coincided with a

rapid rise in overall TB cases in the United States that began in the mid-1980s and peaked in 1992. This rise in cases has been attributed to several factors, including the emergence of the HIV epidemic; the deterioration and neglect of the public health tuberculosis control infrastructure; social conditions such as drug use, prison overcrowding, and homelessness; and poor infection control in hospitals and other congregate facilities.

In 1992, there were 3811 cases of tuberculosis in New York City (an incidence rate of 50/100,000), and of those roughly 12%, an astonishing percentage, had MDR-TB [15]. Patients infected with drug-resistant strains were more likely to have been HIV-infected and to have been previously treated for TB than patients with drug-susceptible strains. Several instances of nosocomial transmission of MDR-TB, usually among patients with HIV infection, were also documented using molecular epidemiology techniques, in several American cities. Extremely worrisome was the very high mortality rate associated with MDR-TB. In an early report from New York City, MDR was almost always a fatal disease, with reported mortality rates in excess of 80% [16].

Following these reports concerning the emergence of drug resistance in New York City, a landmark global survey was commissioned by the World Health Organization (WHO) and published in 1998 [17]. In many countries, particularly those with the highest burden of tuberculosis cases, resources were (and are still) not sufficient to perform drug susceptibility testing on all *M. tuberculosis* isolates. The global MDR-TB survey effort involved a network of 14 reference laboratories around the world and the development of a standardized methodology for defining and estimating the number of patients with MDR. The results of the first global MDR-TB survey were disturbing. Nearly 10% of patients with TB were infected with strains that had at least one type of drug resistance. Resistance to INH alone was found in 7.3% of cases. Although only 1.4% of the total cases in the survey were MDR, this distribution was far from uniform, as several hotspots were identified. In the original survey, prevalence rates of MDR-TB in Estonia and Latvia exceeded 10% among patients who had never been treated for TB. In patients previously treated, rates of MDR exceeded 10% in Argentina, Cuba, the Dominican Republic, England and Wales, Estonia, Latvia, Peru, Portugal, Puerto Rico, South Korea, Romania, Russia, Sierra Leone, and Spain. Over half of Latvian re-treatment patients were infected with MDR strains!

Subsequently, regular annual surveys of the prevalence of drug resistance have been published, and the resulting information has been included in the World Health Organization's annual Global TB Report [18]. A more complete, and extremely frightening, picture of drug-resistant TB around the world has emerged. Several countries of the former Soviet Union, including Russia, Belarus, Ukraine, Kazakhstan, and nearby Mongolia, all have rates of drug resistance that surpass 18% in previously untreated cases. In previously treated cases, rates exceed 50% in several of these countries.

In terms of sheer numbers, China contributes a large percentage of the world's drug-resistant cases, even though the percentage of MDR cases there is much lower than in Russia and the countries of the former Soviet Union.

As has been detailed in the WHO's annual Global TB Report, precise knowledge about the prevalence of drug-resistant tuberculosis is limited by the inability of many national TB control programs to conduct drug susceptibility testing for all diagnosed cases of TB [19]. This limitation is due to a combination of factors, including clinical diagnosis where culture methods are unavailable. Even when culturing is performed, comprehensive drug susceptibility testing is too complex and expensive in many resource-constrained settings. Testing has been a particular problem in Africa, where WHO maps indicate that no data is available for many of the countries. It is hoped that introduction of genotypic drug susceptibility testing, based either on nucleic acid amplification or whole-genome sequencing, will provide more robust and accessible drug susceptibility testing than traditional phenotypic testing using solid or liquid media.

5.3 Consequences of Drug Resistance in Tuberculosis

At the time of this writing, isoniazid monoresistance is quite common around the world, with a prevalence of above 10% in many regions [19]. Evidence of the clinical consequences of INH monoresistance is somewhat mixed. Some data suggest that patients with this pattern of resistance can be treated with the standard regimen for drug-susceptible tuberculosis and achieve cure and relapse rates similar to patients with fully susceptible TB. But other studies have suggested that the standard short-course regimen is associated with higher treatment failure and relapse rates when used for INH-monoresistant disease [20–23].

Rifampin monoresistance tends to be lower than with other agents. Many experts feel that this is because rifampin was introduced relatively late compared with the other first-line drugs and has never been used as a single agent to treat tuberculosis.

5.4 Patterns and Treatment of Drug-Resistant Tuberculosis

As noted above, isoniazid monoresistance is fairly common, though it has generally been thought that INH resistance alone does not portend a poor outcome when the standard regimen for treating drug-susceptible tuberculosis (2 months of isoniazid, rifampin, pyrazinamide, and rifampin, followed by 4 months of isoniazid and rifampin) is used [24, 25]. In recent years, as INH monoresistance has increased in prevalence, some clinicians have advocated adding or substituting a fluoroquinolone into the regimen [20–23, 26].

Streptomycin monoresistance is also reasonably common, but this resistance pattern is essentially of no practical consequence, as streptomycin is not part of the standard treatment regimen for drug-susceptible tuberculosis [27]. Rifampin monoresistance is less common; it is most often seen in patients with HIV infection who

have received intermittent high doses of rifampin [28–31]. As rifampin is the most active drug in the standard treatment regimen, treatment of rifampin monoresistance generally falls under the umbrella of treatment for multidrug-resistant tuberculosis.

Pyrazinamide (PZA) monoresistance has been somewhat difficult to detect because of the stringent acidic conditions under which PZA phenotypic testing must be performed. Thus, PZA monoresistance may be more common than previously thought [32]. Patients with PZA monoresistance require 9 months of treatment with INH and rifampin rather than the shorter course 6-month regimen.

The term polydrug resistance is used to describe patients who are infected with isolates that are resistant to isoniazid and one of the other first-line drugs (pyrazinamide, ethambutol, streptomycin) but not rifampin. Some data indicate that persons with polydrug resistance have worse outcomes than patients with fully drug-susceptible tuberculosis when treated with standard regimens, though all these data are retrospective. Optimal treatment regimens have yet to be defined for polyresistant strains of *M. tuberculosis.*

Multidrug-resistant tuberculosis (MDR) is generally treated with regimens that include one of the advanced fluoroquinolones (levofloxacin, moxifloxacin, or gatifloxacin) and an injectable agent (streptomycin, amikacin, kanamycin, or capreomycin). In carefully monitored settings, favorable outcomes for patients with MDR-TB can be achieved in 80–85% of cases using drug therapy alone [33].

The term pre-XDR-TB has come into use recently to describe isolates of *M. tuberculosis* that are resistant to INH, rifampin, and either a fluoroquinolone or one of the injectable drugs but not both. XDR-TB (extensively drug-resistant TB) indicates an isolate that is resistant to INH, rifampin, fluoroquinolones, and the injectables [34]. Both pre-XDR and XDR-TB are extremely difficult to treat successfully and require long duration of therapy with drugs that have a high rate of potentially serious adverse effects. Newer agents, such as bedaquiline, delamanid, and linezolid, may be useful in treating these infections, although the optimal dosing regimens with these newer drugs remain to be defined. In cases of XDR and pre-XDR-TB, surgery may be useful as an adjunctive therapy if the disease is localized.

5.5 Detection of Drug Resistance in *M. tuberculosis*

The detection and diagnosis of drug resistance in *M. tuberculosis* is a rapidly evolving field in which molecular approaches may dramatically change clinical practice.

For many years, detection of resistance was confined to demonstrating phenotypic resistance to growth inhibition with either solid or liquid antibiotic-containing medium. This type of drug susceptibility testing (DST) remains the gold standard against which all other techniques are measured [35]. Although approaches to DST on solid or liquid medium are generally well-standardized, different labs occasionally use different concentrations of drugs to define resistance, or they define

resistance as high-level (e.g., if a concentration of INH in the medium is 0.1 μgm/ml) or low-level resistance, if there is growth at an INH concentration of 0.1 μgm/ml but inhibition of growth at a concentration of 1.0 μgm/ml. Currently, the most widely used method of culture for *M. tuberculosis* is the broth-based non-radiometric mycobacterial growth indicator tube (MGIT) system, which is used in most labs in high-resource settings and in reference labs in many high-burden, low-resource settings.

The biggest change in the diagnosis of tuberculosis drug resistance came with the identification and understanding of molecular targets of the various antituberculosis drugs and the development of reliable, accurate, and robust methods for identifying, amplifying, and/or sequencing relevant regions of DNA in clinical samples [36–41]. The methods most useful in diagnosis of drug resistance in isolates of *M. tuberculosis* rely on nucleic acid amplification, most recently whole-genome sequencing (WGS).

A test called the line probe assay uses the polymerase chain reaction (PCR) to amplify regions of genes in *M. tuberculosis* DNA that are associated with drug resistance [42]. Prior to amplification, clinical samples must be treated and DNA extracted. These amplified segments of DNA are then hybridized with DNA fragments (probes) that have been immobilized on a strip of nitrocellulose. The captured, hybridized DNA fragments are detected by a colorimetric method that allows identification of wild-type and mutated regions of genes associated with drug resistance. The colorimetric assay is read by eye. Many mutations in many genes can be identified by the line probe assay, and it has been popular as a relatively low-cost approach to molecular detection of drug resistance in many parts of the world.

A significant advance in the detection of drug resistance occurred with the development of the PCR-based nucleic acid amplification GeneXpert system for tuberculosis diagnostics [43–45]. This is a highly automated system in which nucleic acid amplification of the critical segment of the gene associated with rifampin resistance is linked to molecular beacons that allow semiquantitative detection of the wild-type or mutated gene. This is a rapid (~2 h) and nearly completely self-enclosed system that can detect both the presence of *M. tuberculosis* in a clinical sample and the presence or absence of rifampin resistance. Most notably, very little sputum processing is required. A sputum sample is placed in a cartridge that is then placed in the GeneXpert machine. Every ensuing step is completely automated. The sensitivity and specificity of rifampin resistance detection, as compared with phenotypic resistance detection using bacterial culture systems, are very high, and GeneXpert has rapidly become a widely used technology, even in relatively low-resource settings. Over 20,000 units are in place globally, and roughly half of those machines are in South Africa [46–48]. The unit must be protected against extremes of temperature, and the cost of the individual cartridges is not inconsiderable. Several further iterations of the original GeneXpert are in development, including the Omni, a battery-operated, small portable unit that is meant to be taken into the field, the Ultra that has sensitivity that should approach that of bacterial culture, and a version

of Xpert that tests for resistance to a large number of drugs: isoniazid, rifampin, fluoroquinolones, and aminoglycosides.

As molecular biology techniques have become more automated and less expensive, the realistic prospect of using whole-genome sequencing (WGS) has emerged as a means for detecting drug resistance [49]. WGS in theory should be the most complete means of examining DNA sequences for mutations associated with drug resistance, whereas PCR-based methods have not, up to now, been able to interrogate all mutations in all genes associated with antibiotic resistance. WGS can examine the entire genome and in theory can detect any mutation known to be associated with drug resistance. Since this technique has an inherent error rate that could impair its robustness, the method will have to be evaluated in field trials to move forward.

5.6 New Developments in the Diagnosis of Tuberculosis Drug Resistance

It is worth exploring in some depth the role of genotypic detection of drug resistance in *M. tuberculosis* in light of developments in molecular biology, gene sequencing, and highly automated detection of mutations and single nucleotide polymorphisms that hold the promise for inexpensive, rapid, and accurate detection. As noted above, the Cepheid GeneXpert platform has now been used for several years to identify *M. tuberculosis* in sputum samples and for the rapid detection of rifampin resistance. Quite recently, a significant expansion of this platform holds the promise of rapid determination of drug susceptibility for a large number of antibiotics.

Xie and colleagues recently evaluated a rapid molecular drug susceptibility platform for detection of resistance to several anti-TB drugs [45]. This study provides some of the best data, using the most up-to-date methodology, for evaluating genotypic and phenotypic approaches to identifying drug-resistant strains of *M. tuberculosis*. Using a platform derived from the Cepheid MTB/RIF instrument, samples, obtained from patients in China and South Korea, were interrogated for the presence of mutations in several genes associated with antibiotic resistance (*katG*, *inhA*, *gyrA*, and *rrs*). In theory, this assay could detect resistance to isoniazid, fluoroquinolones, and the aminoglycosides kanamycin and amikacin. Results from the molecular assay were compared with phenotypic testing performed with the widely used BACTEC MGIT 960 system, using commonly employed breakpoint MICs for the determination of resistance: 0.1 μgm/ml for INH, 0.5 and 2.0 μgm/ml for moxifloxacin, 2 μgm/ml for ofloxacin, 1 μgm/ml for amikacin, and 2.5 μgm/ml for kanamycin. As a further check, sequencing of the target genes was carried out using the Sanger method on all the clinical isolates (aliquots of the same samples that were used for the phenotypic testing).

Using phenotypic testing as the gold standard, the investigational nucleic acid amplification platform had a sensitivity and specificity for detection of resistance to

drugs as follows: for isoniazid, 83.3% and 99.2%, respectively; for ofloxacin, 88.4% and 96.6%; for moxifloxacin at an MIC of 0.5 μgm/ml, 87.6% and 94.3%; for moxifloxacin at an MIC of 2.0 μgm/ml, 96.2% and 84%; for kanamycin 71.4% and 98.4%; and for amikacin, 70.7% and 99.6%. As compared to direct sequencing of resistance genes, the nucleic acid amplification method had the following sensitivity and specificity for individual drugs: for isoniazid, 98.1% and 100%, respectively; for fluoroquinolones, 95.8% and 100%; for kanamycin 92.7% and 99.6%; and for amikacin, 96.8% and 100%. There were 13 specimens out of 304 that had mutations that were not detected by the investigational assay.

These results indicate that the highly automated investigational platform, modeled after the current Cepheid MTB/RIF GeneXpert, is a highly accurate means of identifying mutations in genes that are associated with resistance to the drugs that define MDR-TB, pre-XDR-TB, and XDR-TB. As compared with phenotypic testing, specificity for drug resistance was quite good. Thus, a result from the investigational platform indicating drug resistance could be reliably used to exclude that drug from a therapeutic regimen in nearly every case. However, the sensitivity of the investigational platform was not quite as good as the specificity. It was excellent for high-level resistance to moxifloxacin, somewhat less reliable for low-level resistance to moxifloxacin, for ofloxacin, and for isoniazid, and probably unsatisfactory for amikacin and kanamycin. Looked at another way, the positive predictive value for determining drug resistance using the investigational platform was generally excellent, but the negative predictive value fell short of clinical desirability. If only the molecular testing were used to guide selection of an antibiotic regimen, as many as 20% of patients might be treated with drugs, such as aminoglycosides, that would not be clinically useful and could have serious adverse consequences, such as hearing loss.

The results described above underscore the discrepancies that are sometimes observed between phenotypic and genotypic drug susceptibility testing. These discrepancies point to at least two possible explanations. First, it is certainly possible that as yet unidentified mutations in genes not examined are responsible for drug resistance. Second, it is possible that there are limitations in the critical concentrations used to test drug susceptibility and that the phenotypic results obtained in the laboratory are in fact not entirely reflective of what might be achieved in clinical practice using standard dosing of the drugs studied in this experiment. At present, either of these two possibilities, or both, may be operative (see also Chap. 9 on heteroresistance).

The investigational nucleic acid platform returned results faster than would have been obtained using the WHO-recommended line probe assay, and it is more suitable for use in local labs, hospitals, and clinics. The line probe assay is generally confined to use in reference centers in most high-prevalence, low-resource settings. Since many of these settings already have experience using the MTB/RIF version of the platform, the only change would be in the cartridge that is used; the remaining hardware is identical. Also, for the purposes of the study, cultures using MGIT were highly controlled for quality; that level of quality might not be seen under field conditions, whereas the performance of the DNA amplification platform is fairly robust under most conditions.

It is unclear if, from a programmatic point of view, it is necessary to test initially for more than rifampin resistance. If an isolate is susceptible to rifampin, patients can be treated satisfactorily with a regimen for drug-susceptible TB, and further resistance testing is not likely to provide clinically meaningful information, as long as the background rates of resistance to isoniazid, pyrazinamide, and fluoroquinolones are known to be low.

5.7 Biological and Molecular Basis of Drug Resistance in *M. tuberculosis*

The following discussion focuses on the most clinically important antituberculosis drugs: isoniazid, rifampin, pyrazinamide, streptomycin and other injectables, fluoroquinolones, and the newer agent bedaquiline.

5.7.1 Isoniazid

Isoniazid, or isonicotinic acid hydrazide (INH), was one of the earliest antituberculosis drugs to be developed, coming into clinical use in the 1950s. Eventually, Winder and Collins demonstrated convincingly that INH worked by inhibiting synthesis of mycolic acids, the long-chain α-alkyl β-hydroxy fatty acids that are essential components of the mycobacterial cell wall [50, 51]. INH is a prodrug that is metabolized by the catalase-peroxidase enzyme KatG (encoded by the *katG* gene) and then binds to NAD to form an INH-NAD adduct as an intermediate form [52]. The INH-NAD adduct binds to and inhibits a reductase called InhA. InhA plays an important role in fatty acid synthesis leading to mycolic acid production; consequently, when its action is blocked by the binding of the INH-NAD adduct, cell wall synthesis is interrupted, thereby accounting for the anti-TB action of isoniazid.

Resistance to INH occurs more commonly than resistance for any other first-line agent used for the treatment of tuberculosis, and it can develop in a number of ways. First, initial activation of the prodrug can be inhibited by mutations in the catalase-peroxidase enzymes that are required to create the active form of the molecule. Thus, *katG* mutations cause INH resistance; they are found in a high percentage of clinical *M. tuberculosis* isolates, ranging from 30% to 90% of INH-resistant strains [53, 54]. Second, the inhibition of InhA by the binding of the INH-NAD adduct can be inhibited by mutations in the *InhA* gene, which can also lead to resistance to INH. The latter mechanism of resistance is observed less commonly than *katG* mutations, although it is nonetheless clinically significant [55]. Overall, mutations responsible for isoniazid resistance occur at a frequency of 10^{-5} to 10^{-6} bacilli, probably the highest frequency of naturally occurring resistance to any of the first-line antituberculosis drugs.

Although complete deletion of *katG* has been known to account for resistance to INH in clinical isolates, mutation within that gene is a much more common mode of resistance. Over 300 different mutations in *katG* have been identified. Most mutations, however, occur at codon S315, where each base (AGC) can be found mutated (mutants have Thr, Asn, Arg, Ile, Gly, or Leu residues). Interestingly, certain mutations are more likely associated with monoresistant strains, while others are more likely to be found in multidrug-resistant strains [56]. *InhA* mutations are less often the cause of INH resistance; they are often seen in cases of low- rather than high-level resistance, but they are clinically significant nonetheless [53].

Mutations in several other genes that are involved in the action of INH have been reported to cause resistance to isoniazid [57]. These include mutations in *furA* (a gene whose product regulates *katG* expression), *sigI* (a sigma factor that regulates *katG* expression), *and glf* (which encodes an NAD^+ – and flavin adenine dinucleotide-dependent UDP galactopyranose mutase). These seem to be less common or important clinically as mediators of isoniazid resistance. As will be discussed below, the relative contribution of these various mutations in clinical isolates is important for evaluating diagnostic tests that rely on genotypic rather than phenotypic approaches to identifying drug-resistant strains.

5.7.2 Rifampin and Other Rifamycins

Rifampin was first studied as a potential antituberculosis drug in the 1960s, and resistant strains were quickly identified. Rifampin is a potent, sterilizing drug, with MICs for susceptible strains generally less than 1.0 μgm/ml [58]. It is the cornerstone of all modern short-course regimens for the treatment of tuberculosis, and its introduction and use allowed treatment of tuberculosis to be reduced first to 9- and then to the current standard 6-month short-course regimen. The introduction of rifampin made the quite toxic injectable agents, notably streptomycin, obsolete in the vast majority of cases of drug-susceptible tuberculosis.

Unlike isoniazid, which has several genes that can be associated with the development of resistance, resistance to rifampin (and the other clinically used rifamycins, rifabutin, and rifapentine) is controlled almost exclusively by a single gene, *rpoB* [59, 60]. Rifampin interrupts RNA synthesis through binding to the beta subunit of RNA polymerase, and mutations in the gene that encodes that subunit, *rpoB*, prevent the binding of rifampin and allow RNA synthesis to continue unimpeded. The binding site of rifampin onto RpoB occurs upstream from its catalytic center, and elongation of the RNA chain cannot occur.

Mutations of *rpoB* occur at a natural frequency of 10^{-7}–10^{-8}, much less commonly than mutations associated with isoniazid resistance. Detection of rifampin resistance by genotypic methods has been aided by the fact that nearly all the *rpoB* mutations that are associated with resistance occur in a very small hotspot in the gene, in an 81-base-pair segment [59, 60]. It is felt that mutations in this hotspot region account for greater than 95% of clinical cases of rifampin resistance. Thus,

genotypic tests for rifampin resistance were among the first to come into widespread clinical use. This is particularly important given rifampin's importance in the treatment of tuberculosis. Testing for rifampin resistance alone allows one to exclude the possibility of MDR-TB (if *rpoB* is wild-type) or to alter therapy to include the use of potent second-line agents, such as injectable aminoglycosides and fluoroquinolones, if *rpoB* mutations are detected. In general, mutations in *rpoB* produce high-level resistance, requiring MICs of greater than 32 μgm/ml, a serum concentration not generally achievable with safe and well-tolerated dosing of rifampin. As noted above, all rifamycins have the same mechanism of action, so any detected mutation in *rpoB* should be taken as evidence for resistance. However, it has been noted that particular mutations, at codons 511, 516, 518, and 522, are associated with retention of susceptibility to rifabutin [61]. These mutations are less common, however.

Rifampin was the last of the so-called first-line drugs to be developed and to come into widespread clinical use. It was added to already effective regimens to allow treatment shortening, but it was never used alone. Likely because of this reason, rifampin monoresistance is substantially less common than multidrug resistance. Recent surveys bear this out. In Peru, a country where there had previously been a high prevalence of MDR-TB, only 2% of cases were found to have rifampin monoresistance [62]. Among a recently reported California cohort of HIV-infected patients (a group previously identified as being at higher risk of rifampin monoresistance), only 0.4% were found to have strains with this pattern of resistance [63]. MDR-TB was seen in only 1.5%. As if to underscore the effect of immunocompromise on the development of rifampin monoresistance (RMR), rates of RMR and MDR were sharply lower in the era of highly effective antiviral therapy than they were in the pre-HAART era. Although uncommon, in both the Peruvian and California cohorts, RMR strains were significantly associated with an increased risk of death, at least double that of patients infected with fully drug-susceptible strains. In the Western Cape province of South Africa, a region burdened by extraordinarily high rates of both tuberculosis and HIV infection, the number of cases of RMR-TB seems to be rising, as a recent report documents a tripling of the number of cases in a relatively short time frame. This seems to be recapitulating the experience of rifampin monoresistance in New York City [31, 64] from the early to mid-1990s, where a sudden rise in cases was noted among patients with HIV infection. At the time, the incidence of tuberculosis in the city had risen to 50/100,000, the equivalent of a medium-burden country, and roughly one-third of all persons with tuberculosis were also infected with HIV at a time when effective antiretroviral therapy was unavailable for most patients.

5.7.3 Pyrazinamide

Pyrazinamide (PZA) is another critical drug in modern short-course therapy [65]. After rifampin was introduced, it became possible, as noted above, to shorten therapy from 18 months to 9, using only INH and rifampin. By adding PZA for the first

2 months of therapy, the duration of treatment could be further shortened to a total of 6 months. PZA seems to kill a population of bacilli known as persisters, bacteria that are otherwise apparently impervious to the immediate action of other first-line drugs.

PZA has a complicated mechanism of action [66–68]. Most notably, it is active only at an acid pH of 5.5, and it has no activity at neutral pH. Even at pH 5.5, MICs for PZA are relatively high, in the range of 6–50 μgm/ml. As with INH, PZA is a prodrug that requires metabolism to an active form for its antibacterial effects to emerge. The active form of PZA is pyrazinoic acid (POA); the conversion of PZA to POA is under the control of the pyrazinamidase/nicotinamidase enzyme, which is encoded by the gene *pncA*. Interestingly, although nicotinamidase is a ubiquitous enzyme in prokaryotes, PZA has activity against no other bacteria aside from *M. tuberculosis.* POA apparently accumulates in the cell and disrupts the membrane potential of *M. tuberculosis.*

The usual function of nicotinamidase is to convert nicotinamide to nicotinic acid (i.e., niacin), which is then recycled to nicotinamide adenine dinucleotide (NAD) through a metabolic chain known as the Preiss-Handler pathway. In *M. tuberculosis,* the Preiss-Handler pathway is defective, so that nicotinic acid is secreted. Interestingly, a mycobacterial species closely related to *M. tuberculosis*, *M. bovis*, is lacking a key enzyme in the Preiss-Handler pathway (nicotinic acid phosphoribosyltransferase or PncB), and as a result *M. bovis* does not secrete niacin. This is the basis for the now largely outmoded niacin laboratory test for the speciation of mycobacteria. *M. bovis* strains all appear to have a point mutation from C to G in the *pncA* gene, rendering all *M. bovis* strains resistant to PZA.

The prodrug PZA is thought to enter the cell through passive diffusion and then is rapidly converted to POA via the mechanism described above. POA exits the cell both passively and by an efflux mechanism. In an acid extracellular environment, POA is further modified (protonated) outside the cell and reenters the tubercle bacillus where it accumulates. The introduction of protons into the cell by POA likely disrupts both the cell membranes and inhibits the function of vital enzymes.

Resistance to PZA is caused by mutations in the *pncA* gene, which converts the prodrug to POA. Apparently, no strain of *M. tuberculosis* is resistant to POA. The conversion of the prodrug to its active metabolite is thus the critical step in the action of the drug, and *pncA* mutations are the clinically relevant changes that generate PZA resistance. Most mutations in *pncA* are missense, although some nonsense mutations and mutations in the promoter region have been identified. Unlike the mutations in the short hotspot region of *rpoB* that cause resistance to rifampin, mutations in *pncA* are scattered throughout the gene, although there is some clustering at three different spots.

As noted above, identification of PZA-resistant strains of *M. tuberculosis* by traditional phenotypic drug-susceptibility testing is difficult due to the requirement for an acidic medium for drug activity. This has made precise estimates of the prevalence of clinically relevant PZA resistance difficult to obtain. This is discussed extensively in an excellent review by Zhang and Mitchison [66]. As mutations in *pncA* are now known to cause the vast majority (95%) of cases of clinical PZA

resistance, the use of genotypic testing has rapidly increased. *pncA* is 558 base pairs long, and mutations can occur all along the gene. Thus determining PZA resistance is a more difficult diagnostic problem than interrogating the 81-base pair hotspot region of *rpoB* that is responsible for rifampin resistance.

A recent review of global PZA resistance pooled a large number of studies to develop estimates of phenotypic and genotypic resistance [32]. Resistance was seen in every region of the globe. Allowing for the fact that there is probable ascertainment bias in deciding which strains to test for PZA resistance, results are nonetheless striking. By phenotypic testing, 16.2% of isolates from a worldwide collection were determined to be resistant. Geographically, this ranged from 11.4% of *M. tuberculosis* isolates in the European region to 21.9% in the Americas. Among strains at high risk for MDR, PZA resistance was found in 41.3% of patients, and in strains collected from people with MDR-TB, PZA resistance was found in a staggering 60.5%. In reviewing genotypic testing results, Whitfield and colleagues determined that 20.9% of a sample of 8651 reported cases showed resistance. As seen in previous reports of smaller sample sizes, the *pncA* mutations were diverse and widely scattered; 608 unique polymorphisms were noted at 397 positions in the gene. Although some polymorphisms were more common than others, the 20 most common accounted for only one-third of all isolates with phenotypic resistance.

These more recent estimates by Whitfield differ somewhat from a slightly older review published by Chang and colleagues [69]. They found that PZA resistance was present in 51% of multidrug-resistant strains of *M. tuberculosis* and in only 5% of non-MDR strains. The differences between these two reviews can be at least partially explained by sampling or ascertainment bias, as well as some differences in methodology of resistance detection. It is likely that in the future, more precise estimates of the prevalence of PZA resistance will be made as molecular testing becomes used more widely around the world.

5.7.4 Streptomycin and Other Injectable Drugs

As described above, the development of resistance to streptomycin in the 1948 landmark BMRC trial led to the initial recognition of mycobacterial drug resistance [1]. The near euphoria, which must have greeted both patients and physicians when the painful injections of this novel antibiotic led to marked clinical and radiographic improvement, was followed by the crushing disappointment of relapses with streptomycin-resistant organisms. The relatively rapid introduction of INH and para-aminosalicylic acid soon led to the development of combination chemotherapy regimens that greatly reduced the emergence resistance and established the principle that active tuberculosis could never be satisfactorily treated with a single antibiotic. Nor should one antibiotic ever be added to a failing drug regimen, as resistance to that single new drug would almost invariably emerge. Ultimately, the introduction of rifampin as the cornerstone of short-course therapy relegated streptomycin and the other injectables to the status of a second-line agents. However, as multidrug

resistance has emerged as a major problem in tuberculosis treatment and control around the world, these drugs have regained clinical importance.

Streptomycin belongs to the aminoglycoside class of antibiotics, as do two of the other injectables now commonly used, amikacin and kanamycin. A fourth injectable, capreomycin, is often assumed to be a member of this class, but in reality, it is a polypeptide having a different mechanism of action and of acquisition of resistance.

Streptomycin is a bactericidal drug that typically has MICs in the range of 2–4 μgm/ml [58]. Its mechanism of action is through the inhibition of protein synthesis. It binds to the 30S bacterial ribosomal subunit at ribosomal protein S12, thus leading to misreading of mRNA during translation [70]. Resistance to streptomycin is caused most often by amino acid substitutions in the S12 protein encoded by the *rps*L gene as well as by mutations in a gene, *rrs*, encoding a streptomycin-binding protein on the 16S ribosomal subunit [71–73]. Mutations in *rps*L are thought to be responsible for 50% of streptomycin resistance, and *rrs* mutations are considered responsible for 20% of resistance. The mutations in *rps*L and *rrs* are found at a relatively few loci, certainly as compared to the generation of PZA resistance by mutations in the *pncA* gene. In the 30% of strains of *M. tuberculosis* resistant to streptomycin that lack *rps*L or *rrs* mutations, other genes have been implicated. A mutation in a gene called *gid*B, which encodes a methyltransferase specific for 16S rRNA, has been found in as many as 20–30% of streptomycin-resistant cases [74].

Amikacin is a derivative of kanamycin. The mechanism of resistance to these drugs is the same, and it is related, but not identical, to the mechanism of streptomycin resistance [75, 76]. Amikacin and kanamycin inhibit protein synthesis by modifying the structure at 16S rRNA; mutations at a single *rrs* position, 1400, cause high-level resistance to both antibiotics.

As previously stated, capreomycin is a polypeptide, not an aminoglycoside, though it is often classified together with streptomycin, amikacin, and kanamycin because it, too, cannot be administered orally. Capreomycin also interferes with protein synthesis, but the target of its action seems to be an rRNA methyltransferase encoded by a gene called *tlyA* [77].

Because of the slightly different action mechanisms of the injectable drugs, resistance to one does not of necessity imply resistance to all. Isolates that are resistant to kanamycin can be assumed to be resistant to amikacin, but such isolates may still be susceptible to streptomycin or capreomycin, depending on the exact nature of the mutation causing resistance. Importantly, resistance to streptomycin does not necessarily imply resistance to amikacin or kanamycin.

Since streptomycin is the oldest antituberculosis antibiotic in clinical use, it is perhaps not surprising that resistance to it is fairly common, although isolated streptomycin resistance in the absence of any other drug resistance is of no real clinical significance in the vast majority of cases. Two recent reports, one from Cameroon and one from China, provide similar estimates for the prevalence of streptomycin resistance. Sidze and colleagues reported that in Cameroon, streptomycin resistance in the 1990s was common, with a prevalence of over 15% of cases [27]. Following this, the National TB Control Program in the country was reorganized to provide

better and more consistent care, and by 2011, among smear-positive patients, only 3.3% had streptomycin monoresistance. INH resistance was also lower than in many parts of the world, at 4.7%. Kanamycin resistance was very uncommon, reported in only 0.2% of strains tested, and MDR-TB was seen in only 1.1% of cases in the most recent survey. A recent report from Hunan province in China found streptomycin resistance in 20.5% of cases [78, 79]. Consistent with the discussion of resistance mechanism, resistance to capreomycin, amikacin, and kanamycin was found in only 2.3%, 1.2%, and 1.8% of isolates, respectively. Mutations in the *rrs* gene associated with streptomycin resistance were all in the 388–1084 bp region, whereas mutations in the gene associated with capreomycin, amikacin, or kanamycin resistance were in the 1158–1674 bp region, as expected.

5.7.5 Fluoroquinolones

Among the most important developments in tuberculosis therapeutics in the last several decades is the emergence of fluoroquinolones (FQ) as effective drugs for this disease. Because of their demonstrated in vitro efficacy against *M. tuberculosis,* widespread availability (perhaps too much so), and generally good safety and tolerability record, this class of drugs has rapidly established itself as a major weapon in the treatment of drug-resistant tuberculosis, often becoming the first choice of all the so-called second-line drugs [80]. In addition, there has been considerable interest in using fluoroquinolones in the treatment of drug-susceptible tuberculosis to shorten therapy below the 6-month short-course regimens that are currently the global standard [81–83]. While the proper formula for doing this has not yet been defined, there are many ongoing clinical trials that are seeking to determine the optimal use of fluoroquinolones in the treatment of drug-susceptible disease. In addition, this class of drugs is often used to substitute for isoniazid in cases of resistance or intolerance, although the evidence supporting the need for this is still incomplete.

The most commonly used fluoroquinolones for the treatment of tuberculosis have been ofloxacin, levofloxacin, and gatifloxacin, although most patients currently are treated with levofloxacin or moxifloxacin, as both are more potent in vitro than ofloxacin, and there have been concerns about episodes of dysglycemia expressed with the use of gatifloxacin.

The mechanism of action of fluoroquinolones against *M. tuberculosis* is the same as it is for all bacteria and involves enzymes known as DNA topoisomerases [84–86]. These enzymes maintain chromosome topology by regulating DNA supercoiling and helping to unlink tangles of DNA strands (catenanes) to facilitate replication and transcription. Fluoroquinolones exert their activity by trapping gyrase, the only type II topoisomerase in *M. tuberculosis*, on DNA such that the DNA is broken. The resulting complexes block DNA replication, and, in the bacterial species studied, chromosome fragmentation and accumulation of toxic reactive oxygen species occur. Both are expected to contribute to the lethal action of the fluoroquinolones.

DNA gyrase is a tetramer, consisting of 2 A and 2 B subunits (A2B2). The subunits have different functions and are encoded by two different genes, *gyrA* and *gyrB*. Mutations in *gyrA* and *gyrB* are associated with fluoroquinolone resistance. A conserved region in both genes, known as the quinolone resistance-determining region (QRDR), is generally the target region in both the *gyrA* (a 320 bp segment) and *gyrB* (a 375 bp segment) genes [87]. Quinolone resistance-causing mutations are generally clustered around a few particular codons in the *gyrA* and *gyrB genes,* and it is generally thought that two mutations in *gyrA* or mutations in both *gyrA* and *gyrB* are needed to develop high-level resistance in *M. tuberculosis*. Single mutations in *gyrB* are not generally associated with high-level resistance. Codons 90, 91, and 94 of *gyrA* are the most commonly mutated sites associated with resistance.

More than with the other antituberculosis drugs discussed so far, there has been considerable discussion and debate about whether DNA gyrase mutations are completely necessary or sufficient to cause phenotypic drug resistance to fluoroquinolones. This debate is assuming more and more importance as molecular techniques, such as whole-genome sequencing, are being increasingly used for detecting resistance. In some studies, the percentage of isolates of *M. tuberculosis* that are phenotypically resistant and also have gyrase mutations is extremely high, but in others it has been quite low. As Zhang and Yew pointed out in their review, the reasons for these discrepancies might be multiple [58]: There may be differences in methodology of genotyping from study to study; there may be differences in the MICs used to define drug resistance; or there may be other resistance mechanisms at work. Heteroresistance is also a common explanation.

A recent paper by Avalos and colleagues reviewed data from 46 studies reported from 18 countries involving nearly 4000 clinical isolates of *M. tuberculosis* [88]. Overall, 87% of isolates that were phenotypically resistant to moxifloxacin and 83% of isolates that were phenotypically resistant to ofloxacin contained *gyrA* mutations. That leaves a considerable number of isolates that are phenotypically resistant but that do not have identifiable *gyrA* mutations.

Most recently, Farhat and colleagues examined a cohort of 172 patients from Peru who had been treated with fluoroquinolones [89, 90]. In this study, baseline drug susceptibility testing was done using the method of proportions on 7H10 solid medium and, for PZA, in the broth-based BACTEC system. Isolates in which there was phenotypic fluoroquinolone resistance underwent genotypic analysis—sequencing of the *gyrA* and *gyrB* genes using molecular inversion probes. The authors found that there were significant discrepancies between phenotypic and genotypic resistance testing. A large percentage of *M. tuberculosis* isolates that were phenotypically resistant lacked specific *gyr* resistance mutations. In addition, mutations that were generally thought to be associated with high-level resistance were occasionally (3–4% of the time) found in isolates that were phenotypically drug susceptible. Importantly though, a consistent finding of the study was that mutations in gyrase that are associated with high-level resistance were strongly and consistently correlated with adverse outcomes, including treatment failure and death. In general, the correlation between poor outcome and resistance testing was stronger for genotypic resistance determinations than for phenotypic determinations.

Overall, the bulk of studies in this area indicates that *gyrA* mutations should almost always be taken to mean that there is high-level resistance to fluoroquinolones.

It is worth noting that the fluoroquinolones are among the most widely used antibiotics in the world for a host of bacterial infections, and they are available over the counter and without prescription in many countries. It is probably for this reason that the prevalence of fluoroquinolone resistance in *M. tuberculosis* strains is extraordinarily high in certain locations. Invariably, some patients with tuberculosis are being treated (either self-treated or under a physician's care) with fluoroquinolones as single agents for misdiagnosed pneumonia or bronchitis, a practice that is leading to the generation of FQ-resistant tuberculosis. A very recent multi-country survey of fluoroquinolone resistance in over 5000 tuberculosis patients found that the prevalence of resistance ranged from 0.5% to 12.4% for levofloxacin and 0.9% to 14.6% for moxifloxacin when using a breakpoint definition of resistance of MIC greater than 0.5 μgm/ml, with the highest rates of resistance occurring in Pakistan [91]. Using an MIC of 2 μgm/ml as a resistance breakpoint for moxifloxacin was uncommon, regardless of the country examined.

5.7.6 Bedaquiline

It seems appropriate to comment on the mechanism of action and the development of drug resistance related to the novel drug bedaquiline, as this agent is being increasingly studied and used in the treatment of multidrug-resistant tuberculosis. Moreover, there is interest in its use as part of a so-called novel, universal regimen that could be used with any case of tuberculosis, regardless of susceptibility testing results to conventional first-line agents.

Bedaquiline, formerly known as TMC207, belongs to a novel class of antituberculosis medications [92, 93]. It is an ATP synthase inhibitor that has little activity against most Gram-positive and Gram-negative bacteria, although it is quite active against *M. tuberculosis* and perhaps other mycobacterial species. This drug inactivates the F_1/F_0-ATP synthase of mycobacteria but not that of mammalian cells. Since this synthase is a critical enzyme in oxidative phosphorylation, interrupting its function leads to critical energy depletion and cell death. Bedaquiline exposure leads to rapid depletion of ATP in mycobacteria, although killing is delayed for several days after initial administration of the drug, perhaps because of activation of salvage pathways for energy sources in the cell. At any rate, increasing evidence suggests that this drug may be highly potent against *M. tuberculosis* when used clinically, particularly in regimens that do not contain rifampin, which actually may be a marked advantage in the treatment of MDR-TB. Since no other drug with this mechanism of action has been used in the treatment of tuberculosis, resistance to it should be rare, occurring only as emergence of resistance rather than as transmission of resistance. However, since the drug has started to find its way into clinical use (to date there have been no more than several thousand patients worldwide who have received it), reports of resistance have emerged [94].

Studies of the molecular basis of resistance focus on two genes. One, *atpE*, encodes the previously mentioned F_1/F_0 ATP synthase; mutations in this gene have been reported in about 30% of *M. tuberculosis* strains that have phenotypic bedaquiline resistance. Resistance through this mechanism seems to be mediated by abnormalities in the C subunit of the ATP synthase.

In the majority of reports, resistance to bedaquiline appears to be mediated by a gene called *rv0678*, which encodes a protein of the same name. All bedaquiline-resistant isolates identified in South Africa to date have had mutations in this gene. (Of note, mutations in this gene also confer resistance to clofazimine, a second-line drug that had long been relegated to the end of the therapeutic line, but which recently regained some prominence for the treatment of multidrug-resistant strains, particularly as a component of the Bangladesh regimen [95, 96].) The rv0678 protein encodes a transcriptional repressor of the genes encoding the MmpS5-MmpL5 efflux pump, and there is evidence that resistance via this mechanism can be overcome in part by the use of efflux pump inhibitors such as verapamil. However, the feasibility of such an approach in clinical practice is unclear, since high, toxic levels of verapamil may be needed to achieve this effect. The naturally occurring frequency of resistance to bedaquiline is on the order of 10^{-8}, similar to that of rifampin; thus resistance should, in general, emerge slowly. However, bedaquiline also has a very long tissue half-life, which may favor selection of resistant strains of *M. tuberculosis* in clinical use. There has not yet been enough experience with this drug to make confident statements about the likelihood of resistance emerging as a significant clinical issue.

5.7.7 Clofazimine

As noted above, clofazimine has long been relegated to the bottom of the list of second-line agents, primarily because of its side effect profile, which includes skin hyperpigmentation, which can be quite marked and bothersome to many patients, although it is generally said to be a reversible condition. Clofazimine is now a component of the 9-month Bangladesh regimen for the treatment of MDR-TB that has recently been endorsed for use in selected cases by the WHO [96]. Additionally, recent use of clofazimine in murine models of tuberculosis has encouraged reconsideration its potential as a treatment-shortening agent for patients with drug-susceptible tuberculosis [97].

Even some 50 years after its identification as a drug useful in the treatment of tuberculosis, the precise mechanism of clofazimine action remains obscure. There is some evidence that it exerts its effect through disruption of cellular redox cycling, and there is also evidence that the drug directly disrupts the cell membrane [95].

Clinical isolates that are resistant to clofazimine seem extremely rare, although resistant strains have been developed in this laboratory. Most of those laboratory strains have resistance mutations in the same *rv0678* gene that was described above as causing resistance to bedaquiline [95]. This gene is a transcriptional regulator

that represses expression of *mmpS5-mmpL5*, the gene that encodes an efflux pump of the same name. Interestingly, the *rv0678* locus *mmpS5-mmpL5* is absent in *M. leprae*, the causative agent of leprosy, and no strain of this mycobacterium has been found that is resistant to clofazimine. There have been other genes (*rv1979c* and *rv2535c*) that have been associated with clofazimine resistance, but the mechanisms for their involvement have not been identified. As expected, *Rv0678* mutations lead to cross-resistance with bedaquiline.

5.7.8 Multidrug Resistance Mechanisms

Although the majority of cases of antituberculosis drug resistance occur singly for each drug that is used to treat infection, in at least some cases, efflux pumps are associated with resistance to multiple drugs, as seen with cancer chemotherapy and which seems to occur at least in vitro in cases of cross-resistance to bedaquiline and clofazimine.

Recently, there has been evidence that efflux pumps may be playing a role in resistance to multiple drugs used to treat tuberculosis. As described by Almeida da Silva, there are five superfamilies of efflux pumps: the ATP-binding cassette (ABC), major facilitator superfamily (MFS), resistance nodulation division (RND), small multidrug resistance (SMR), and multidrug and toxic-compound extrusion (MATE) [98]. The ABC superfamily is of particular interest in *M. tuberculosis.* Roughly 2.5% of all genes in *M. tuberculosis* encode ABC transporters, and more than 12 efflux pump genes have been identified. Genes of other superfamilies have also been implicated in tuberculosis drug resistance. Several experiments have shown that MICs of antituberculosis drugs can be reduced by administration of efflux pump inhibitors such as verapamil. This suggests strongly that these pumps can play a role in at least some forms of drug resistance.

5.8 Treatment of Drug-Resistant Tuberculosis

At the outset, it is important to state that there is no well-defined regimen for the treatment of most forms of drug-resistant tuberculosis, whether monoresistant, polyresistant, multidrug resistant, pre-extensively drug resistant, or extensively drug resistant. Treatment recommendations for all of these forms of resistance rely on expert opinion based on experience, observational cohorts, small case series, and extrapolations from clinical trials early in the antibiotic era when drugs such as rifampin were not yet available. Newer drugs that have become available in the last few years, such as delamanid, bedaquiline, fluoroquinolones, and linezolid, have also been studied mostly as single agents added to optimized background regimens rather than as part of novel regimens tested in randomized controlled trials.

In cases of isoniazid-monoresistant tuberculosis, most experts recommend that the standard regimen may be used and that it is unnecessary to add additional drugs, such as fluoroquinolones, to compensate for the loss of INH. Thus, a regimen of rifampin, pyrazinamide, and ethambutol could be used for a total of 6 months, without the addition of a quinolone [80].

On the other hand, in most cases of rifampin monoresistance (a much less common form of resistance), most experts recommend treating the patient as if he/she had multidrug resistance.

The treatment of multidrug-resistant tuberculosis depends to a great degree of the availability of drug susceptibility testing and of a steady supply of the second-line agents that will be required to construct the best possible regimen. If drug susceptibility testing results are available, then a regimen should be designed using those results as a guide, with the inclusion at all times of at least three drugs in the regimen to which the isolate is susceptible. Two of the three drugs in the regimen should be from the fluoroquinolone and injectable groups of antibiotics. If however, drug susceptibility testing is not routinely (or immediately) available, regimens must be constructed on programmatic grounds, taking into account prevailing drug susceptibility patterns in the community as determined by periodic surveys using regional, national, or supernational reference laboratories.

At present, there are two main approaches to the design of a regimen for multidrug-resistant tuberculosis: the WHO-recommended approach to designing an MDR regimen [80] and the so-called Bangladesh regimen [96]. The WHO assigns drugs to categories based on their mechanism of action, like efficacy and safety, and tolerability profiles. Drugs are then selected from the different categories until a regimen is constructed. Ideally, such a regimen will consist of a fluoroquinolone, an injectable (either amikacin or kanamycin, or capreomycin), pyrazinamide, and ethambutol. Overall, the WHO guidelines state that in patients with MDR-TB (or rifampin monoresistant TB), a regimen with at least five effective TB medicines during the intensive phase is recommended, including pyrazinamide and four core second-line TB medicines—one chosen from group A (levofloxacin, moxifloxacin, or gatifloxacin), one from group B (amikacin, capreomycin, kanamycin, or streptomycin), and at least two from group C (ethionamide or prothionamide, cycloserine or terizidone, linezolid or clofazimine). If the minimum number of effective TB medicines cannot be composed as given above, an agent from group D2 (bedaquiline, delamanid) and other agents from group D3 (p-aminosalicylic acid, imipenem-cilastatin, meropenem, amoxicillin clavulanate, or thiacetazone) may be added to bring the total to five. In patients with MDR-TB, it is recommended that the regimen be further strengthened with high-dose isoniazid and/or ethambutol. The total duration of treatment with the regimen is 18–24 months.

As the WHO guidelines themselves state, these recommendations are not made with a high degree of confidence or support from controlled clinical trials. Many experts would favor using bedaquiline (a WHO group D drug) instead of any of the drugs in group C if bedaquiline can be obtained because of its reported efficacy and side effect profile, which seem favorable as compared to the group C drugs.

The Bangladesh regimen is a 9-month program that consists of an intensive phase of 4 months (extended up to a maximum of 6 months in cases where sputum smear conversion does not occur) of gatifloxacin (or moxifloxacin), kanamycin, prothionamide, clofazimine, high-dose isoniazid, pyrazinamide, and ethambutol. The intensive phase is followed by a continuation phase of 5 months with gatifloxacin (or moxifloxacin), clofazimine, pyrazinamide, and ethambutol. This regimen is restricted in the WHO guidelines to patients who have not been previously treated and in whom the prospect of resistance to fluoroquinolones and aminoglycosides is felt to be very low. In addition, the regimen is not recommended for use in pregnant women with tuberculosis.

Although the Bangladesh regimen is appreciably shorter than the standard WHO approach to the treatment of MDR-TB, it has uncertainties and limitations. First, as noted above, the only available data concerning its use and success come from two reports of cohorts rather than from randomized, controlled trials. Second, the number of patients who are actually eligible for this regimen is a matter of debate, as many experts have argued that at most 25% of patients with MDR-TB would meet the criteria that WHO has set for its use.

Very recently, preliminary results from a clinical trial comparing the Bangladesh regimen with the standard longer course WHO-recommended regimen were reported in abstract form [99]. The crude favorable outcome rate with the standard, longer regimen was 80.6%, and for the Bangladesh, regimen was 78.1%. The trial was designed as a non-inferiority trial, and the lower bound of the confidence interval for the Bangladesh regimen arm included the non-inferiority margin. Thus, a claim of non-inferiority for the Bangladesh regimen could not be supported by the findings (that does not mean that the regimen is inferior). In addition, there was a trend toward worse outcomes using the Bangladesh regimen in patients with HIV infection that was not appreciated in patients without HIV infection.

A novel regimen for patients with MDR- or XDR-TB is being studied in the Nix-TB regimen, sponsored by the Global Alliance for TB Drug Development [100]. In this open-label, single-arm observational trial, patients are being treated with bedaquiline, linezolid, and Pa-824, an oxazolidinone that is being developed by the Global Alliance. Preliminary results from this study have been reported in abstract form and are promising in terms of sputum culture conversion status, although adverse effects have required careful patient management.

We note that none of the strategies consider the importance of pharmacokinetic overlap. For combination therapy to severely restrict the emergence of resistance, at least two agents must be at concentrations above their MICs. If a regimen contains an agent with a half-life that is much longer than that of other members of the regimen, the long half-life compound will be present at the equivalent of monotherapy. Resistance to that compound is likely to emerge during long treatment periods. Conversely, good pharmacokinetic overlap is likely to contribute to little emergence of resistance. An example of the latter was seen in a very small trial in which resistance emerged more of with rifapentine than with rifampin when in combination with isoniazid (ref. is Andrew Vernon *et al., The Lancet* 353: 1843–1847).

5.9 Conclusions

The problem of drug-resistant tuberculosis, particularly MDR- and XDR-TB, is one that threatens to upset and reverse decades of progress in tuberculosis control around the world [101]. Delays in diagnosis are leading to prolonged periods of infectiousness with strains of *M. tuberculosis* that are difficult to treat under the best of circumstances and that generally are concentrated in countries and regions that lack access to the most modern tools for drug susceptibility testing and newer, more potent antibiotics. In order to reverse this trend, a greater sense of urgency is needed, and resources must be devoted to strengthening basic TB control program activities, to implementation of novel diagnostics that allow drug susceptibility testing for first- and second-line drugs, and to assuring that drugs needed for the treatment of MDR- and XDR-TB are available to patients who need them. Without that kind of sustained effort, decades of progress in tuberculosis control will be reversed.

Major Points

- Tuberculosis is a serious, often fatal, airborne disease if untreated or untreatable.
- Drug resistance emerges so readily within individual patients that tuberculosis serves as a paradigm for implementing combination therapy.
- Most of the antituberculosis drugs are preferentially active with *M. tuberculosis*; thus long treatment times do not contribute to resistance in other pathogens (fluoroquinolones are an exception).
- Effective treatment regimens exist, even for MDR-TB; however, the necessary infrastructure is often lacking in resource-challenged countries, which then become “breeding grounds” for new drug-resistant strains.
- The increasing prevalence of resistance requires that new antituberculosis agents be developed and administered with sufficient resources and evidence-based knowledge to stem what is becoming a global healthcare crisis.

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Chapter 6
Anaerobic Bacteria: Antimicrobial Susceptibility Testing and Resistance Patterns

Audrey N. Schuetz

6.1 Introduction

Anaerobic bacterial pathogens cause serious infections, particularly in the hospital environment. Among the better known anaerobic bacteria are *Bacteroides fragilis* associated with intra-abdominal abscesses, clostridia recovered from skin and soft tissue infections, *Fusobacterium necrophorum* leading to Lemierre disease, and *Clostridium difficile* causing disease in critically ill patients. Antibiotics are a mainstay of treatment protocols, and, as with aerobic pathogens, resistance is becoming a problem [1]. Anaerobic pathogens are generally more difficult to culture than aerobic bacteria, and most antimicrobials have been developed using aerobic assays. Thus, our ability to manage populations of anaerobic microbes lags far behind control methods for aerobic bacteria. To understand some of the problems, it is necessary to define terms and methods that are often glossed over in discussions of aerobic pathogens.

A suitable starting point is with the terms "susceptible (S)," "intermediate (I)," and "resistant (R)." These are "interpretive categories" used to explain the results obtained from in vitro antimicrobial susceptibility testing of microorganisms isolated from infected patients. In a general sense, they predict a patient's response to treatment with a particular antimicrobial agent. An isolate that tests resistant to an antimicrobial is highly unlikely to respond to treatment with that antimicrobial, while an isolate that tests susceptible is likely to respond clinically. However, even when an isolate is "susceptible" to an antimicrobial agent in vitro, the ultimate outcome in the patient is very dependent on the condition of the patient and the site of the infection.

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I. W. Fong et al. (eds.), *Antimicrobial Resistance in the 21st Century*, Emerging Infectious Diseases of the 21st Century, https://doi.org/10.1007/978-3-319-78538-7_6

Interpretive categories are interrelated with minimal inhibitory concentrations (MICs) and "breakpoints" (also known as clinical breakpoints). An MIC is the lowest concentration of an antimicrobial agent that prevents the growth of a microorganism as measured with in vitro susceptibility tests. "Breakpoints" are the MIC values that categorize an isolate as S, I, or R to a specific antimicrobial agent.

The process used to establish breakpoints involves examination of three types of data:

MIC distribution data, pharmacokinetic/pharmacodynamic (PK/PD) data, and clinical outcome data. For MIC distribution data, the MICs determined for a large number of randomly selected isolates (i.e., at least 100 isolates) are plotted as a distribution curve or graph (Fig. 6.1). The MIC distribution curve may demonstrate a unimodal or bimodal pattern depending on the antimicrobial and organism. In general, isolates with lower MICs are less likely to carry microbial resistance mechanisms, and isolates with higher MICs (e.g., on the right of the curve) are more likely to express resistance mechanisms.

In addition to the MIC distribution curve, PK/PD data must be obtained by examining the levels of antimicrobial agent attained in the patient and the manner in which the antimicrobial agent acts on the infecting organisms. Finally, clinical outcome data are based on how well the patient does when treated for an infection due to a specific organism having a specific MIC (Fig. 6.2). All these data are gathered and used to set breakpoints. S and R interpretive categories are fairly straightforward; however, the meaning of the intermediate category is complex. This category includes isolates having MICs between the susceptible and resistant populations such that the MIC is usually below attainable blood and tissue levels but for which good clinical response rates may be lower than for isolates with MICs in the susceptible range.

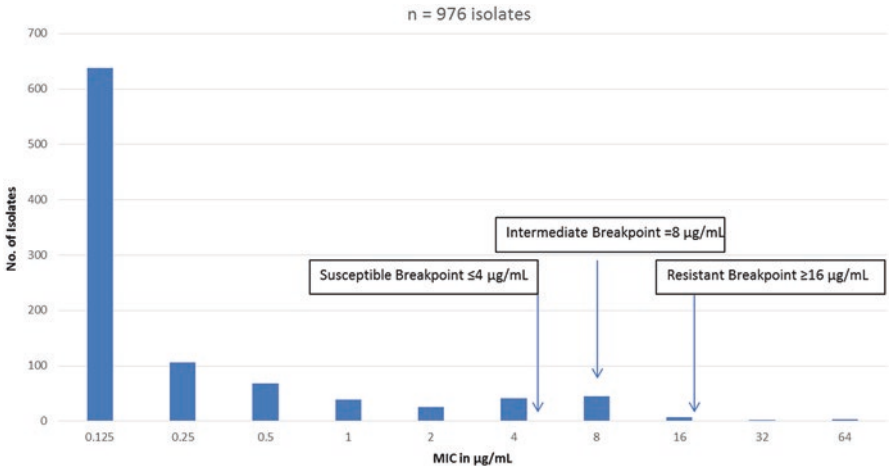

Fig. 6.1 Minimal inhibitory concentration (MIC) distribution curve of meropenem for 976 *Bacteroides fragilis* isolates with Clinical and Laboratory Standards Institute (CLSI) breakpoints noted

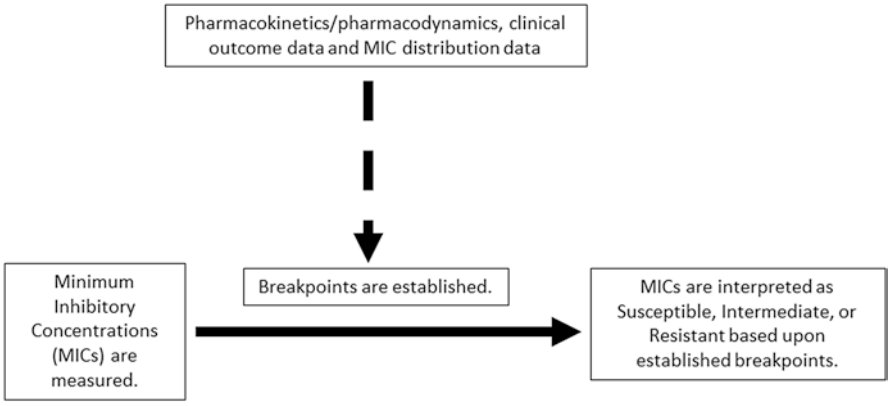

Fig. 6.2 Graphic display of the general process by which breakpoints are determined

Committees of experts who are members of breakpoint-setting organizations examine available data and determine breakpoints. In the USA, the Clinical and Laboratory Standards Institute (CLSI) sets breakpoints. In Europe, the European Committee for Antimicrobial Susceptibility Testing (EUCAST) sets breakpoints. These committees do not always agree on the breakpoints, especially when the distribution of MIC values is indistinct or when the clinical treatments (e.g., treatment dosages) differ between the patient populations examined.

Information collected from clinical laboratories can be used to determine the prevalence of resistance for a particular pathogen, antimicrobial agent, and region (resistance is fundamentally a local phenomenon). Data from such resistance surveys (e.g., antibiograms) guide clinicians in their choices of antimicrobial for empirical therapy (i.e., treatment either without culturing the pathogen or when the organism identification is known but the MIC has not yet been measured). When surveys are carried out in the same location over a period of years, they can provide insight into how quickly resistance is increasing or whether changes in antimicrobial exposure are lowering the prevalence of resistance. Many assumptions underlie such longitudinal studies, including unchanging breakpoints, stable patient populations, and comparable sampling from 1 year to the next. Consequently, the methods underlying antimicrobial susceptibility testing (AST), the determination of breakpoints, and whether the breakpoint committees agree are crucial to interpreting survey data. These factors are sometimes problematic with anaerobic pathogens. Nevertheless, important statements have emerged concerning these bacteria. I begin this chapter with a discussion of AST. A discussion of antimicrobial resistance mechanisms of major groups of anaerobic pathogens then follows.

6.2 Antimicrobial Susceptibility Testing of Anaerobes

With the increasing use of MALDI-TOF MS (matrix-assisted laser desorption/ionization-time of flight mass spectrometry) by clinical laboratories to identify anaerobic bacteria, accurate species identification is becoming increasingly available. As compared to the limited identifications of anaerobes in the past by phenotypically or biochemically based methods, MALDI-TOF MS offers faster and more specific identification. An expected result is that increasing attention is being given to anaerobes in their role in human infections. Although the data are only anecdotal, it is believed by some that AST is being requested more often by clinicians. In the past, anaerobic pathogens were often reported only with broad terms such as "Gram-positive anaerobe," while now they are often being reported using genus and species names. Thus, anaerobe susceptibility testing and reporting not only continue to remain relevant, but they are also likely to increase in demand as clinical microbiology laboratories improve technologically.

Empirical information regarding antimicrobial susceptibility can be particularly helpful for clinicians treating a patient with an infection due to an anaerobe. In contrast to many aerobic bacteria, anaerobes tend to be slow-growing, and pathogen identification is often obtained well before antimicrobial susceptibility test results. In addition, anaerobes are often present in polymicrobial aerobic/anaerobic infections or mixed with other anaerobes. Thus, it may be difficult to obtain pure cultures for AST. In such cases, it is helpful to know the predicted anaerobic susceptibility patterns based on information about strains circulating in the local region.

Individual hospitals and healthcare systems perform periodic surveillance of anaerobes and publish the results as susceptibility rates (antibiograms). CLSI guidance M39 *Analysis and Presentation of Cumulative Antimicrobial Susceptibility Test Data*, which is currently under revision, provides recommendations for the proper collection, analysis, and presentation of antimicrobial susceptibility test data [2]. If a clinical laboratory cannot perform AST for anaerobes, it may be helpful for the laboratory and local clinicians to refer to the anaerobe antibiogram data published in the CLSI M100 and CLSI M11 documents [3, 4]. Changing trends in antimicrobial resistance over time, based on the antibiogram data, have been published by members of the CLSI [5]. It is important to recognize that the data presented in the anaerobe antibiogram from CLSI are derived from isolates obtained globally and may not be applicable to isolates within a given hospital or region.

Analysis and comparison of published reports of anaerobic antimicrobial susceptibility patterns is complex for several reasons. First, many different methods are used for susceptibility testing of anaerobes; different testing methods can lead not only to different MICs but also to different interpretive categories (e.g., susceptible or resistant). There are two reference methods for anaerobic susceptibility testing accepted by breakpoint-setting organizations: broth microdilution for the *B. fragilis* group of bacteria and agar dilution for all anaerobes, including the *B. fragilis* group. In the broth microdilution assay, a series of wells in a microtiter plate, containing dilutions of the test antimicrobial and bacterial culture, are incubated to determine

the lowest drug concentration that blocks visible bacterial growth. In the agar dilution assay, the antimicrobial is placed in agar on which a dilute bacterial culture at a standardized concentration is applied (a set of plates is prepared in which the antimicrobial concentration is varied). The drug concentration that inhibits colony formation after suitable incubation is taken as the MIC. Another method that is used by clinical laboratories is called agar gradient diffusion. Examples of this commercially available method include the Etest (bioMérieux, Durham, NC) and the MTS strip (Liofilchem, Italy). Plastic or paper strips are impregnated on one face with concentrations of an antimicrobial in a gradient fashion. When the strip is placed on an agar plate that has been inoculated with a standardized number of bacteria, the antimicrobial agent diffuses onto the agar and inhibits bacterial growth where concentrations are above the MIC. The MIC is read from a scale printed on the strip – the point at which the ellipse of bacterial growth meets the strip is taken as MIC. Results from agar gradient diffusion assays may not always correlate well with the gold standards of broth microdilution and agar dilution, but these commercial strips are widely used due to their ease of use [6]. Although appropriate for use with aerobic organisms, disk diffusion testing (placement of antibiotic-impregnated disks onto an agar plate in which a bacterial culture grows) is not suggested by the CLSI as a test method for anaerobes due to inaccurate results and poor correlation with the agar dilution method [3]. However, some research groups are currently trying to develop EUCAST disk diffusion breakpoints for testing of the *B. fragilis* group organisms [7]. Unfortunately, MIC results from these various AST methods do not always correlate well with each other. Thus, knowledge of the testing method used to obtain a particular data set is important when assessing the literature or reviewing the MIC of a particular isolate. Moreover, the assays can be difficult to adapt for routine testing by clinical microbiology laboratories. The important point is that published reports using anaerobic AST may vary in resistance rates simply from differences among the methods used to determine the MIC.

AST for anaerobes is still an unsettled situation. For example, CLSI recommends agar dilution as the testing method for anaerobes, but few clinical laboratories use this methodology [3]. Use of broth microdilution is limited to the *B. fragilis* group. CLSI is now undertaking studies to reevaluate whether broth microdilution can be performed for anaerobes other than the *B. fragilis* group, but results for other anaerobes, such as *C. difficile,* do not look promising [8]. Moreover, broth microdilution itself can be difficult to perform, and restricting it to only one group of anaerobic organisms limits its utility for clinical laboratories where many types of anaerobes other than solely members of the *B. fragilis* group are isolated.

When considering the published literature, careful attention must be placed on the breakpoints applied to the datasets, because breakpoints can differ between breakpoint-setting organizations (see Table 6.1 below for examples of differing breakpoints for CLSI and EUCAST). Thus, when comparing resistance prevalence between regions, one may expect a higher prevalence of resistance reported for ertapenem if the EUCAST resistant breakpoint is applied rather than the CLSI breakpoint, which for this drug is three doubling dilutions higher. The reason for the

Table 6.1 Examples of current resistant breakpoint differences for EUCAST and CLSI

Antimicrobial	Organism(s)	EUCAST resistant breakpoint	CLSI resistant breakpoint
Penicillin	Gram-positive and Gram-negative anaerobes	>0.5 μg/mL	≥2 μg/mL
Piperacillin-tazobactam	Gram-positive and Gram-negative anaerobes	>16/4 μg/mL	≥128/4 μg/mL
Ertapenem	Gram-positive and Gram-negative anaerobes	>1 μg/mL	≥16 μg/mL
Metronidazole	Gram-positive and Gram-negative anaerobes, except *C. difficile*	>4 μg/mL	≥32 μg/mL

different breakpoints is due in part to the different testing methodologies used in the data gathered for setting breakpoints and to differences in interpretation of breakpoint data.

Updates in bacterial taxonomy have also contributed to confusion in interpretation of previous reports. Some anaerobes that had previously been grouped within a particular species are now grouped in a different genus (e.g., *Peptoniphilus harei* was previously listed within the *Peptostreptococcus* genus). Other anaerobes have been newly described (e.g., *Murdochiella asaccharolytica*), and it is uncertain where they would have fallen in prior classification schemes [9, 10]. Taxonomic refinements are ongoing for anaerobes and will continue to require close attention until new data sets are accumulated.

Finally, the increasing use of nucleic acid and proteomic methods to identify anaerobic bacteria has resulted in more accurate differentiation and characterization of isolates. Thus, more anaerobes are identified to the genus or species level as compared to previous practices that occasionally reported the organism according to a morphologic group (e.g., anaerobic Gram-positive cocci). As a result, more accurate AST results are published on anaerobes when attributed to particular bacterial species.

In summary, surveys of resistance trends among anaerobes vary in their quality and size. Resistance patterns differ by geographic region, and some resistance rates have changed significantly over time. It is unclear how much of the differences among regions is due to true geographic variation, to methodologic testing variation, or to application of different breakpoints. Patterns of resistance that deserve close attention due to rising rates include:

- Resistance to the β-lactam-β-lactamase inhibitor combinations among the *B. fragilis* group.
- Increasing clindamycin resistance among all anaerobes.
- Metronidazole resistance is no longer limited to the *B. fragilis* group, as it now includes Gram-positive cocci and bacilli.
- Resistance of *Clostridium* to vancomycin.

Moreover, many anaerobes are frequently isolated from polymicrobial infections; sometimes as many as eight or more different organisms are cultured from the site

of infection. The importance of performing AST on anaerobes from mixed infections can be questionable.

Below I consider several groups of anaerobes and the types of antimicrobial resistance they display. As with aerobic bacteria, anaerobes have developed multiple mechanisms that lead to antimicrobial resistance. These resistance factors include genes located in the bacterial chromosome or within mobile genetic elements (plasmids or transposons) leading to production of enzymes that degrade antimicrobials, as well as to alterations in antimicrobial targets.

6.3 Anaerobic Cocci: Gram-Positive and Gram-Negative

6.3.1 Clinical Disease and Taxonomic Changes

Gram-positive anaerobic cocci comprise approximately one-fourth of all anaerobic isolates cultivated from human infections [11]. This group of anaerobes is part of the normal flora of the human mouth and upper respiratory tract, but it also comprises normal flora of the gastrointestinal tract, female genitourinary tract, and skin [11]. Oral infections due to Gram-positive anaerobic cocci are usually polymicrobial in nature and often are associated with a breach in the mucosal barrier or an immunocompromised state. These anaerobes are often implicated in cases of periodontitis, gingivitis, and abscesses in and around the oropharynx, mouth, and neck. Gram-positive anaerobic cocci are also associated with anaerobic infections of the lower respiratory tract, such as empyema or aspiration pneumonia [11]. Cases of gynecological and obstetrical sepsis may be due to Gram-positive anaerobic cocci [12]. Skin and soft tissue abscesses due to Gram-positive anaerobic cocci are usually located in the neck or perigenital area due to the preponderance of these anaerobes as normal flora in these anatomic sites. Finally, Gram-positive anaerobic cocci are highly associated with diabetic foot infections [11]. They are less commonly causes of bloodstream infections or intra-abdominal infections as compared to *B. fragilis* or other Gram-negative anaerobic bacilli.

Anaerobic Gram-negative cocci include *Veillonella* and *Megasphaera*, among others. *Veillonella* is the most commonly recovered anaerobic Gram-negative coccus in the clinical microbiology laboratory. It is found as normal flora in the upper respiratory and gastrointestinal tracts and may cause disease in these anatomic sites when defenses are compromised. *Veillonella* is often recovered as part of a mixed culture; its virulence capabilities are poorly understood [10].

More than any other group of anaerobes, the anaerobic Gram-positive cocci have undergone major taxonomic updates within the past 20 years [10]. For example, there are now over 13 different genera of anaerobic bacteria that were previously classified within the genus *Peptostreptococcus,* which formerly was one of the largest anaerobic groupings [9, 10]. Now only four species remain in the *Peptostreptococcus* genus: *P. anaerobius*, *P. canis, P. russellii,* and *P. stomatis*.

Taxonomic updates and the increased ability to identify organisms more accurately over time must be taken into account when interpreting AST resistance trends and patterns over different geographic regions.

6.3.2 Resistance Patterns

Anaerobic Gram-positive cocci are generally considered susceptible to most anaerobic antimicrobials including penicillin, piperacillin-tazobactam, meropenem, and metronidazole. In most cases, infections with these anaerobic cocci are polymicrobial: other anaerobes and aerobes are often present. Thus, providing AST results to clinicians for anaerobic Gram-positive cocci may not be as critical in guiding the choice of antimicrobial therapy as for the more resistant anaerobic Gram-negative bacteria, such as *B. fragilis*. For polymicrobial anaerobic and aerobic infections, the empiric antimicrobial chosen by clinicians tends to be broad-spectrum, thereby covering both aerobes and anaerobes. Although it is likely that broad-spectrum antimicrobials do cover anaerobic Gram-positive cocci, this is not always the case. If an anaerobic coccus is isolated in pure culture from a sterile source and is thought to be contributing to infection, performance of AST should be considered.

The most common Gram-positive anaerobic cocci associated with human infections include *Finegoldia magna*, *Parvimonas micra*, *Peptoniphilus harei*, and *Peptoniphilus asaccharolyticus* [13–15]. In general, many antimicrobials are considered to be effective against these bacteria. Strains of *P. asaccharolyticus*, *F. magna*, and *P. micra* are usually susceptible to the penicillins. A resistance survey by Brazier and colleagues reported approximately 7% resistance to penicillin and clindamycin for Gram-positive anaerobic cocci and 3.5% resistance to amoxicillin-clavulanate [16]. β-Lactam-β-lactamase inhibitor combinations and cephalosporins are usually active [16, 17]. Carbapenems and metronidazole are highly active [17]. In contrast, the prevalence of clindamycin resistance among the Gram-positive cocci approaches 30% in some surveys [18]. In another resistance survey that included 113 Gram-positive anaerobic cocci, the highest rates of resistance for all tested antimicrobials was to tetracycline (42%) [16].

Among the Gram-positive anaerobic cocci, *F. magna* stands out for its high rates of resistance to penicillin and clindamycin. The sites of infection most commonly affected by *F. magna* include skin and soft issue as well as bone and joints [10]. *F. magna* also carries virulence factors that appear to be involved in bacterial pathogenesis, among which are host cell adherence factors [19]. Resistance rates of *F. magna* range between 10% and 20% for antimicrobials such as penicillin and clindamycin [13, 16, 20, 21]. For example, Wren reported 16% and 8% resistance to penicillin for *F. magna* and *P. micra*, respectively [14]. In another survey, the rate of clindamycin resistance was 13% for 98 isolates of *F. magna* as compared to 1% for *Peptostreptococcus anaerobius* ($n = 92$ isolates tested) and <1% for *Parvimonas micra* ($n = 146$) [22]. Although much lower resistance rates to metronidazole are seen for *F. magna* relative to clindamycin, significant metronidazole resistance rates

of 3% have been noted in some large surveys [22]. Although penicillin susceptibility rates for *Peptoniphilus asaccharolyticus* and *Anaerococcus prevotii* are similar to those of other anaerobic Gram-positive cocci, high MIC_{90} (e.g., MIC of 90% of the isolates) values for clindamycin of >32 μg/mL have been noted with these species [17, 23].

Peptostreptococcus anaerobius and *P. stomatis* are taxonomically distinct from other anaerobic Gram-positive cocci. These species are generally associated with female genital tract and intra-abdominal infections [10]. Resistance of *P. anaerobius* to penicillin was notably high (7%) as compared to <1% for the other anaerobic Gram-positive cocci assessed in one resistance survey [16]. Antimicrobial susceptibility comparisons between *P. anaerobius* and *P. stomatis* demonstrate higher resistance rates of the former to amoxicillin-clavulanate and consistently higher MIC_{50} and MIC_{90} values for many other antimicrobials [24]. Thus, species identification can be an important guide for therapy.

Peptoniphilus harei is another Gram-positive anaerobic coccus isolated from infections of skin and soft tissue. It generally demonstrates low MICs to a variety of antimicrobials [25]. This species can be difficult to identify phenotypically, since it resembles *P. asaccharolyticus* biochemically, and in the past it has likely been misidentified as *P. asaccharolyticus* [25]. Thus, past reports of AST results of *P. asaccharolyticus* may have been limited by misidentification.

In general, *Veillonella* spp. are highly susceptible to several of the anti-anaerobic agents, such as penicillins, β-lactam-β-lactamase inhibitor combinations, carbapenems, clindamycin, and metronidazole. Indeed, no resistance to amoxicillin, amoxicillin-clavulanate, clindamycin, or metronidazole was found in a recent survey of *Veillonella* in the Netherlands [26].

6.3.3 Mechanisms of Resistance

While mechanisms of antimicrobial resistance among the anaerobic cocci have not been extensively studied, Reig and colleagues postulated that penicillin-binding proteins may account for the elevated MICs to penicillin that are occasionally seen in *Veillonella* spp., because β-lactamases were not detected in penicillin-resistant *Veillonella* isolates [27]. Likewise, penicillin resistance in Gram-positive cocci is thought to be due to penicillin-binding proteins [28]. Resistance of anaerobes to clindamycin is due to methylation of 23S rRNA at the site of drug action in the 50S subunit of ribosomes [29]. The nitroimidazole *nimB* gene has been detected in a large number of anaerobic Gram-positive cocci, but it is not always associated with metronidazole resistance [30]. In a study by Theron and colleagues, the *nimB* gene was present in 19/21 metronidazole-susceptible strains of Gram-positive cocci. Thus, the presence of the *nimB* gene is not sufficient in and of itself for expression of metronidazole resistance.

6.4 Gram-Positive, Non-Spore-Forming Bacilli

6.4.1 Clinical Disease and Taxonomic Changes

The "*Eubacterium*" group, *Actinomyces*, *Cutibacterium*, *Propionibacterium*, *Lactobacillus*, *Eggerthella lenta*, and *Bifidobacterium* are included in this group of bacteria. These bacteria are commensals of the skin and the mucocutaneous surfaces of the oral cavity, the gastrointestinal tract, and the urogenital tract. Anaerobic Gram-positive, non-spore-forming bacilli typically are associated with infections of the head and neck that originate from the oral cavity [31]. Infections are frequently polymicrobial in origin. Intra-abdominal and urogenital infections may develop due to a break in the mucosal barrier. *Actinomyces* are particularly common oral colonizers; infections may result from untreated dental caries, thus leading to cervicofacial lesions and abscesses. Pulmonary actinomycosis may result from aspiration of oral contents into the lungs. Pelvic actinomycosis has been associated with the use of intrauterine contraception devices (IUDs). *Cutibacterium* species cause a variety of infections, such as endocarditis, central nervous system (CNS) infections (notably CNS shunt infections), and osteomyelitis [32]. Specifically, *C. acnes* is associated with foreign-body infections of joints (particularly the shoulder joint due to the large number of *C. acnes* normally present around the axillary area) [31]. Clinical infections most commonly reported with *Lactobacillus* spp. include bacteremia and endocarditis. The mouth is believed to be the route of entry of lactobacilli into the bloodstream in patients with dental caries [33]. Reports of *Lactobacillus* bacteremia associated with ingestion of probiotics containing lactobacilli have been published [34]. *Eubacterium* spp. are present as normal flora in the oral mucosa and can cause oral infections. *Eggerthella lenta* causes disease in the intra-abdominal cavity [35]. *Bifidobacterium* spp. colonize the gut, but the most common clinical disease due to this genus is dental caries. Other organisms included in this group of Gram-positive, non-spore-forming anaerobic bacilli are *Mobiluncus* and *Atopobium*; they are not frequently recovered in the clinical microbiology laboratory.

The propionibacteria have recently undergone significant taxonomic changes. Traditionally, species within the genus *Propionibacterium* have been informally classified as either cutaneous or classic propionibacteria [36]. The "cutaneous group" included *P. acnes*, *P. avidum*, and *P. granulosum*. A new member of the cutaneous group, tentatively named *P. humerusii*, has been reported, but the work has not yet been formally published [37]. The "classic group" of propionibacteria include *P. freudenreichii* and *P. propionicum,* as well as other species, such as those isolated from dairy products. In 2016, in an effort to address the issue of the taxonomically challenging *P. propionicum*, Scholz and Kilian regrouped the propionibacteria into three novel genera, *Acidipropionibacterium*, *Cutibacterium*, and *Pseudopropionibacterium*, in addition to an amended description of the genus *Propionibacterium* [38]. The new genus *Cutibacterium* now contains the cutaneous species formerly known as *P. acnes*, *P. avidum*, *P. granulosum*, and

P. humerusii. *Pseudopropionibacterium propionicum* is the sole member of its genus. The former "classic propionibacteria" group has been divided into the genera *Acidipropionibacterium* and *Propionibacterium*. These name changes must be taken into account when assessing reports regarding changes in susceptibility patterns of the propionibacteria over time.

6.4.2 Resistance Patterns

The anaerobic non-spore-forming bacilli are usually susceptible to the β-lactams, including the penicillins, cephalosporins, cephamycins, carbapenems, and β-lactam-β-lactamase inhibitor combinations [39–41]. *Eggerthella lenta* (formerly *Eubacterium lentum*) is an exception: MICs of third-generation cephalosporins are elevated with this species. Currently, no CLSI clinical breakpoints exist for vancomycin with anaerobes; however, this agent shows good in vitro activity against the "*Eubacterium*" group, *Cutibacterium*, *Propionibacterium*, and *Actinomyces* [42]. Many of the non-spore-forming Gram-positive anaerobic bacilli, including *Actinomyces*, *Bifidobacterium*, *Cutibacterium*, and some *Lactobacillus* species, are intrinsically resistant to metronidazole. Occasionally, non-spore-forming Gram-positive bacilli demonstrate resistance to clindamycin [41]. For example, surveys have shown approximately 7% resistance of *C. acnes* to clindamycin [5]. Telavancin and moxifloxacin have shown good activity against many of the non-spore-forming Gram-positive anaerobic bacilli [43, 44]. In summary, resistance to metronidazole is typical for the anaerobic non-spore-forming Gram-positive bacilli, with most isolates being susceptible to the β-lactams.

Most lactobacilli that grow well in ambient air are intrinsically vancomycin resistant. Vancomycin-resistant lactobacilli include *Lactobacillus casei*, *L. rhamnosus*, *L. plantarum*, *L. salivarius*, and *L. fermentum*. In contrast, the majority of lactobacilli that grow only anaerobically demonstrate low MICs to vancomycin. Among these are bacteria such as the *L. acidophilus* group, *L. crispatus*, *L. gasseri*, *L. johnsonii*, and *L. jensenii*. In general, lactobacilli that demonstrate aerobic growth should be tested for susceptibility aerobically as outlined in the CLSI M45 document guidelines for infrequently isolated or fastidious bacteria [45]. *Lactobacillus* spp. are generally reported as susceptible to clindamycin but with AST results that may vary according to species (e.g., *L. fermentum* modal imipenem MIC is ≤0.03 μg/mL [8/12 isolates]; *L. rhamnosus* imipenem MICs ranged from 0.25 to 4 μg/mL [modal MIC 2 μg/mL]) [46]. Likewise, AST results differ according to the cephalosporin tested (e.g., modal ceftriaxone MIC for *L. rhamnosus* isolates was ≥256 μg/mL [17/22 isolates], while modal cefuroxime MIC for the same strains was 4 μg/mL [15/22 isolates]). Notably, most surveys of *Lactobacillus* susceptibility have either not addressed incubation conditions (e.g., aerobic vs. anaerobic) or have used microaerobic atmospheres. Data on resistance mechanisms among anaerobic lactobacilli are rare.

6.5 Gram-Positive, Spore-Forming Bacilli

6.5.1 Clostridioides difficile

6.5.1.1 Clinical Disease and Taxonomic Changes

Clostridium difficile has recently been renamed as *Clostridioides difficile* [47]. *C. difficile* is currently the most commonly reported pathogen causing healthcare-associated infections in US hospitals. *C. difficile* is also an important agent of diarrheal illness in outpatients. Gastrointestinal disease associated with *C. difficile* usually presents with a range of clinical findings, from diarrhea to pseudomembranous colitis or even toxic megacolon. Disease is due to the ingestion of spores from the environment, as the spores are resistant to alcohol-based gels and to many disinfectants used in hospitals. When spores are transmitted by the fecal-oral route, they can then germinate in the intestinal tract and produce toxins. The most significant risk factor for acquisition of *C. difficile* infection (CDI) is antibiotic exposure, presumably due to reduction of competing organisms. Although clindamycin and broad-spectrum β-lactam antimicrobials are most often implicated in CDI, any antimicrobial may lead to acquisition of CDI.

6.5.1.2 *C. difficile* Resistance Patterns

The most common antimicrobials used to treat CDI are metronidazole and vancomycin. These agents are effective for most cases of CDI, although isolates with elevated MICs to these antimicrobials have been reported [48]. It is important to note, however, that the existing clinical breakpoints for *C. difficile* are based on systemic infections that utilize systemic rather than intra-luminal (intraintestinal) pharmacokinetics and pharmacodynamics of the antimicrobials. For example, the levels of vancomycin orally administered to treat CDI are very high intraluminally in the intestine (e.g., higher than 1000 μg/mL in feces), which is well above the MIC resistant breakpoint of >2 μg/mL [49].

Antimicrobial resistance of *C. difficile* is important to monitor, not only for patient treatment but also for the epidemiologic associations with certain ribotypes. For example, the emergence of the hypervirulent *C. difficile* strain 027/BI/NAP has been associated with consumption of fluoroquinolones [50]. Even though fluoroquinolones are not used clinically to treat *C. difficile* infection, antimicrobial pressure from widespread use of these agents is believed to have allowed the spread of this fluoroquinolone-resistant, hypervirulent strain.

Resistance of *C. difficile* to metronidazole is reported to be relatively infrequent in most areas of the world, but this conclusion depends upon which breakpoints are used to interpret the data [51]. CLSI metronidazole breakpoints for *C. difficile* are susceptible ≤8 μg/mL, intermediate 16 μg/mL, and resistant ≥32 μg/mL. EUCAST

breakpoints are lower: susceptible ≤2 μg/mL and resistant >2 μg/mL. In a US-based survey of 925 isolates obtained in 2011 and 2012, 2.6% displayed resistance to metronidazole based on the EUCAST breakpoint, while no isolate was resistant based on the higher CLSI breakpoint [52].

Elevated MICs of *C. difficile* to vancomycin have also been reported. Most studies have applied the EUCAST vancomycin breakpoint (susceptible ≤2 μg/mL; resistant >2 μg/mL), since CLSI has not set a clinical breakpoint for vancomycin with anaerobes. Surveys have generally reported vancomycin resistance below 5%, but higher percentages have been reported from smaller studies [52, 53]. Elevated MICs to rifampin (>16 μg/mL) have also been reported, ranging from 8% to over 50% in different regions [53, 54]. There are no EUCAST or CLSI breakpoints for rifampin. Fidaxomicin is also a common alternative agent used to treat antibiotic-refractory CDI or recurrent CDI. Decreased susceptibility to fidaxomicin is currently rare, although one *C. difficile* isolate has been reported to have an MIC of 16 μg/mL. This isolate was discovered in a clinical trial of fidaxomicin from a case of recurrent CDI [55].

C. difficile has shown resistance to a variety of antimicrobials that include clindamycin (8–100%), cephalosporins (51%), erythromycin (13–100%), and fluoroquinolones (47%), based on 30 AST studies published between 2012 and 2015 [51]. Among the cephamycins, resistance to cefoxitin and cefotetan is common, with at least 80% resistance in tested isolates [51]. In North America, ribotype 027 is resistant to multiple antimicrobials, including rifampin, clindamycin, and moxifloxacin [54]. Another hypervirulent strain, ribotype 078, also shows resistance to ciprofloxacin, moxifloxacin, erythromycin, and imipenem [56]. The most relevant statement is that elevated MICs to both metronidazole and vancomycin have been reported among *C. difficile*, while elevated MICs to fidaxomicin are relatively rare.

6.5.1.3 *C. difficile* Resistance Mechanisms

Resistance in *C. difficile* is associated with mobile genetic elements and chromosomal mutations [57]. Mobile genetic elements comprise a large proportion of the *C. difficile* genome – approximately 11% [57]. Clindamycin and erythromycin resistance is mediated by transposons, which are associated with the *ermB* gene and MLS_B-inducible resistance [51]. Resistance to the β-lactam antibiotics is due to both penicillin-binding proteins and β-lactamase production [51]. *Cfr* gene expression leads to linezolid resistance, while *tet(M)* expression leads to tetracycline resistance [58]. Resistance to metronidazole and vancomycin are believed to be due to alterations in targets or changes in the metabolic pathways of the organism [51]. Mutations in the *rpoB* gene are associated with reduced susceptibility to fidaxomicin and rifampin [59, 60].

6.5.2 *Clostridia Other Than* C. difficile

6.5.2.1 Clinical Disease

Clostridia are commonly found in the environment in nature. Disease due to *Clostridium* spp. other than *C. difficile* may be exogenous or endogenous in origin. Exogenous clostridial infections include diseases such as tetanus due to *C. tetani*, foodborne botulism due to *C. botulinum,* and wound infections leading to gangrene or myonecrosis due to a variety of clostridia. Endogenous infections associated with clostridia are usually due to breaches in the mucosal barrier (e.g., mucosa of the intestine or respiratory tract) that lead to abscesses, lung empyemas, aspiration pneumonia, or intra-abdominal infections. Risk factors for clostridial disease include surgical procedures, dirty wounds, malignancy, and diabetes mellitus [61]. Most clostridia isolated in the clinical microbiology laboratory are obtained from bloodstream infections, wounds, or intra-abdominal sources. *C. septicum* and *C. perfringens* are two of the most common clostridia that cause bacteremia. In a Canadian study of *Clostridium*-associated bacteremia, the most common species identified was *C. perfringens* (58/138, 42%), followed by *C. septicum* (19/138; 14%) [62]. Since *C. septicum* is commonly associated with occult gastrointestinal malignancy, the isolation of this bacterium from blood should prompt a study of the gastrointestinal tract. Other clostridial species isolated in cases of bacteremia include *C. tertium* and *C. sordellii*. The most common clostridia associated with skin and soft tissue infections include *C. perfringens*, *C. septicum*, *C. histolyticum*, and *C. sordellii*. Spontaneous myonecrosis is often associated with *C. septicum*, while traumatic gas gangrene is usually associated with *C. perfringens*. *C. sordellii* is known for its historical association with gynecologic infections, especially medically induced abortions. Antimicrobial susceptibility patterns of these species are listed below.

6.5.2.2 Resistance Patterns

Many of the most common species of non-*C. difficile* clostridia isolated from human infections are highly susceptible to antimicrobials having anti-anaerobic activity (e.g., metronidazole, clindamycin, carbapenems, and piperacillin-tazobactam). Among the non-*C. difficile* clostridia species, the "RIC" group – namely, *C. ramosum*, *C. innocuum*, and *C. clostridioforme* – demonstrates the highest resistance rates. *C. clostridioforme* produces β-lactamase and thus is resistant to several β-lactam antibiotics but is susceptible to vancomycin [63, 64]. *C. innocuum* is resistant to cefoxitin and cefotetan, and it displays high MICs (8–32 μg/mL) to vancomycin [65]. Fortunately, *C. innocuum* is susceptible to metronidazole. Finally, *C. ramosum* also demonstrates high vancomycin MICs. Many species of clostridia have shown resistance to clindamycin, including *C. perfringens*, *C. ramosum*, *C. tertium*, and *C. sporogenes* [66]. In general, clindamycin resistance among clostridia ranges from 10% to 20%, but it varies by species [4]. Metronidazole,

carbapenems, and β-lactam-β-lactamase inhibitor combinations have maintained activity against most clostridial species [66, 67]. Resistance to metronidazole is likewise low, while resistance to moxifloxacin averages around 20% in some surveys [5].

C. perfringens isolates are highly susceptible to penicillin and clindamycin. However, recent surveys, such as that by Hastey et al., demonstrate rising resistance of *C. perfringens* over time to clindamycin, from no resistance noted in 2007–2009 to 7% resistance noted in 2010–2012 [5]. In another clostridia resistance survey of blood isolates from 2000 to 2006, 8/58 (14%) *C. perfringens* isolates were resistant to clindamycin [62]. All *C. perfringens* isolates in that study were susceptible to penicillin and metronidazole.

6.5.2.3 Mechanisms of Resistance

The "RIC" group of clostridia, as well as *C. butyricum*, produces β-lactamases and rarely penicillin-binding proteins that confer resistance to the β-lactams, including the cephamycins and cephalosporins [29, 66]. The β-lactamases are chromosomally encoded and are released extracellularly [29]. Resistance to clindamycin is caused by methylation of the 23S ribosomal RNA subunit, leading to modification of the site of drug action [29].

6.6 Gram-Negative Anaerobic Bacilli

6.6.1 Bacteroides fragilis *Group*

6.6.1.1 Clinical Disease and Taxonomic Changes

The *B. fragilis* group has historically been comprised of a variety of *Bacteroides* spp. including *B. fragilis*, *B. thetaiotaomicron*, *B. ovatus*, and *B. vulgatus*. The *B. fragilis* group was originally conceptualized as the particular bile-resistant *Bacteroides* species. However, this grouping is "informal," in that it is not formally recognized as a taxonomic group. Therefore, some authors refer to the *B. fragilis* group as the "former *B. fragilis* group" and prefer to refer to the specific species of *Bacteroides* instead of aggregating together the various species. There are other *Bacteroides* species, such as *B. caccae*, that are not grouped with the *B. fragilis* group. Likewise, *Parabacteroides distasonis*, previously known as *Bacteroides distasonis,* had been placed within the *B. fragilis* group. Therefore, it is discussed with the *B. fragilis* group below.

Bacteroides spp. are some of the most frequently encountered anaerobes in the clinical microbiology laboratory and are among the most virulent. *Bacteroides* spp. are also typically more resistant than other anaerobes to antimicrobial agents. They are the most common anaerobes isolated from blood, abscesses (particularly intra-

abdominal), bone, and skin lesions [68]. Although usually recovered from abscesses as mixtures with other anaerobes and aerobes, *Bacteroides* spp. are usually recovered from blood as the only pathogen present.

6.6.1.2 Resistance Patterns

Of the *Bacteroides* species, *B. fragilis* is generally one of the most susceptible to antimicrobials. A susceptibility survey of various *B. fragilis* group isolates collected from ten US medical centers from 1997 to 2004 demonstrated differences in the prevalence of susceptibility across various species [69]. Compared to other members of the *B. fragilis* group, *B. ovatus* and *B. thetaiotaomicron* displayed higher MICs to ertapenem [69]. *B. vulgatus*, *P. distasonis*, and *B. thetaiotaomicron* also displayed higher MICs than *B. fragilis* to piperacillin-tazobactam. *P. distasonis* demonstrates high MICs to many of the β-lactams, excluding carbapenems. *B. thetaiotaomicron* is also known for higher rates of resistance to several antimicrobials as compared to other members of the former *B. fragilis* group. Decreasing susceptibility of the *B. fragilis* group to these frequently used antimicrobials is concerning.

β-Lactam-β-lactamase inhibitor combinations are highly active against many strains of the former *B. fragilis* group, with the exception of *P. distasonis* [4]. Recent worldwide studies have reported increasing resistance to ampicillin-sulbactam and piperacillin-tazobactam in *Bacteroides* species [70]. One survey of 1580 isolates demonstrated a significant increase in resistance to piperacillin-tazobactam from 2% in 2007–2009 to 7% in 2010–2012 [5]. During this time period, susceptibility to ampicillin-sulbactam also significantly decreased, from 86% in 2007–2009 to 82% in 2010–2012.

Cefoxitin and cefotetan are generally active against members of the former *B. fragilis* group. Although some surveys demonstrate slight decreases in resistance of the *B. fragilis* group to cefoxitin, other surveys show that rates of resistance to cefoxitin and cefotetan are rising in some areas of the world [5, 71], implying that susceptibility testing should be performed if these antimicrobials are to be used for therapy. Broad-spectrum cephalosporins show poor activity against the *B. fragilis* group organisms [72].

Carbapenems are also generally active against members of the *B. fragilis* group. Most studies report susceptibility rates of ≥95% and usually well above 99% susceptibility [69]. However, carbapenem resistance is increasing, albeit slowly, from zero resistance noted in 2007–2009 to 1% resistance in 2010–2012 [5]. Seifert et al. reported 95% carbapenem susceptibility for *B. fragilis* group isolates associated with intra-abdominal infections in a multicenter German study [73].

Resistance to clindamycin and moxifloxacin appears to be increasing. Clindamycin resistance is increasing worldwide and has reached approximately 40% for *Bacteroides group* isolates [4]. Rates of susceptibility to moxifloxacin are variable among species, with approximately 31% and 43% of *B. fragilis* and *B. ovatus* isolates demonstrating resistance, respectively [69]. In another study, Snydman et al. reported an overall resistance rate of >80% for moxifloxacin for

B. fragilis group isolates [74]. *Bacteroides* isolates appear to be acquiring resistance to moxifloxacin, as demonstrated by a multicenter European surveillance study in which susceptibility rates to moxifloxacin decreased between 2003 and 2009 from 91 to 86.4% [71].

Metronidazole-resistant strains of *B. fragilis* group isolates are rare, but they have been reported. In 2011, Sherwood and colleagues published a case report concerning a multidrug resistant strain of *B. fragilis* from a leg wound [75]. The isolate possessed the *nim* gene, which was located on a plasmid and conferred resistance to metronidazole. The isolate did, however, display low MICs to moxifloxacin and linezolid. Overall, approximately 95–98% of *B. fragilis* isolates are susceptible to metronidazole [5].

6.6.1.3 Mechanisms of Resistance

Close to 97% of all *Bacteroides* isolates are resistant to penicillin and ampicillin, principally due to β-lactamase production. However, penicillin-binding proteins can also be expressed by strains [76]. Penicillin and cephalosporin resistance is mediated primarily by *cepA* or *cfxA* [77]. A chromosomal cephalosporinase is encoded by *cepA*, which leads to cephalosporin and aminopenicillin resistance. However, isolates expressing *cepA* may remain susceptible to piperacillin and β-lactam-β-lactamase inhibitor combinations. Porin losses can also lead to increases in β-lactam MICs, including MICs to the β-lactam-β-lactamase inhibitor combinations, as demonstrated by one group which reported amoxicillin-clavulanate resistance in a *B. thetaiotaomicron* strain [78]. The *cfxA* gene encodes a broad-spectrum β-lactamase that is responsible for loss of susceptibility to cefoxitin and several other β-lactam drugs [79, 80]. Carbapenem resistance is often mediated by a zinc metallo-β-lactamase, encoded by *cfiA,* that confers resistance to β-lactams and β-lactam-β-lactamase inhibitor combinations [75]. *cfiA* in *Bacteroides* is silent unless an insertion sequence element activates it [81]. A report from Turkey noted 10% resistance to carbapenems in *B. fragilis* group isolates, many of which were confirmed to carry *cfiA* [82]. Resistance to clindamycin is mediated by *erm*, which is located on transferable plasmids [83]. Metronidazole resistance is linked to *nim* (nitroimidazole reductase). Expression of this gene leads to reduction of the nitrate moiety of metronidazole to an amino derivative, which decreases effectiveness of the antibiotic. However, the mere presence of *nim* is not predictive of metronidazole resistance, as *nim* can be found in metronidazole-susceptible strains. Of 206 *B. fragilis* isolates in one study, *nim* was detected in 24%, and metronidazole MICs of the *nim*-positive isolates ranged from 1.5 (susceptible) to >256 (resistant) μg/mL [84]. Although many *nim*-positive *Bacteroides* strains do not show a loss of susceptibility, they can be induced to express high MICs to metronidazole by subinhibitory concentrations of the antibiotic [85]. Resistance to moxifloxacin is mediated by *gyrA* mutations and efflux pumps. In summary, there are many different types of resistance genes that may be carried and expressed by *B. fragilis* group isolates.

6.6.2 *Gram-Negative Anaerobic Bacilli Other Than the* B. fragilis *Group*

6.6.2.1 Clinical Disease

Gram-negative anaerobic bacilli other than the *B. fragilis* group include *Porphyromonas*, *Prevotella*, *Fusobacterium*, and *Bilophila wadsworthia*, among others. *Porphyromonas* spp. are known components of oral flora and may cause significant oral and periodontal disease. *Porphyromonas* may also be isolated from intra-abdominal sites and the female genital tract. *Prevotella* spp. are also predominant in the oral cavity. *Prevotella* may be implicated in human bite wounds and periodontal infections. The two most commonly recovered fusobacteria include *Fusobacterium nucleatum* and *F. necrophorum*. Lemierre syndrome (e.g., septic thrombophlebitis of the internal jugular vein) is most often caused by *F. necrophorum,* but it may be caused by other anaerobes or aerobes. *Fusobacterium* spp. are also recovered from blood in cases of sepsis, and they are associated with abscesses in other sites of the body. *B. wadsworthia* is a significant cause of polymicrobial intra-abdominal infections.

6.6.2.2 Resistance Patterns

Prevotella and *Porphyromonas* are more susceptible to antimicrobials used to treat anaerobic infections than the *B. fragilis* group. *Prevotella* tends to be less susceptible to a variety of antimicrobials than *Porphyromonas*. Resistance to penicillin and ampicillin is found in approximately three-fourths of *Prevotella* isolates [86]. In a New Zealand surveillance study covering 1999 to 2003, susceptibility rates among 45 isolates of *Prevotella* spp. showed only 18% susceptibility to penicillin but 96% susceptibility to clindamycin [86]. Fewer than 5% of *Prevotella* are resistant to cefoxitin. Liu and colleagues reported a small decrease in susceptibility of *Prevotella* spp. over time to imipenem; their isolates demonstrated 100% susceptibility in 2002 but decreased to 94% in 2006 [87].

Antimicrobial surveillance studies of clinically significant *Porphyromonas* spp. are rare. Authors of one study used the Etest method to assess susceptibility of a variety of anaerobes and reported 94% susceptibility for 45 *Porphyromonas* isolates to penicillin and 97% susceptibility to cefoxitin [88]. In addition, *Porphyromonas* demonstrated 100% susceptibility to ampicillin-sulbactam, imipenem, clindamycin, and metronidazole. Production of β-lactamase in *Porphyromonas* is rare. *Prevotella* and *Porphyromonas* are almost uniformly susceptible to carbapenems, metronidazole, and tigecycline.

Resistance among fusobacteria is unusual, and susceptibility rates may vary by species. Penicillin resistance among *Fusobacterium* spp. is relatively rare. In a Taiwanese survey including 36 *Fusobacterium* isolates, only 4 (11%) were positive for β-lactamase [87]. All *Fusobacterium* spp. were susceptible to metronidazole,

piperacillin-tazobactam, and ertapenem, while resistance to moxifloxacin was demonstrated in approximately 20% of isolates. While *F. mortiferum* may be resistant to cephalosporins, over 90% of *F. nucleatum* and *F. necrophorum* isolates are susceptible to cephalosporins and cephamycins [22].

Campylobacter gracilis is susceptible to most antimicrobial agents, including β-lactam-β-lactamase inhibitor combinations, cefoxitin, and clindamycin [89]. *Sutterella wadsworthensis* may be resistant to clindamycin, piperacillin, or metronidazole [42]. *Bilophila wadsworthia* is usually resistant to penicillin, but it is highly susceptible to other antibiotics, such as amoxicillin-clavulanate, clindamycin, carbapenems, and metronidazole, that are used to treat anaerobic infections.

6.6.2.3 Mechanisms of Resistance

Most *Prevotella* spp. are resistant to penicillin and ampicillin due to β-lactamase production. When penicillin resistance is present in *Porphyromonas*, *Fusobacterium*, or *B. wadsworthia*, it is usually as a result of β-lactamases [90]. Most β-lactamases in the Gram-negative anaerobic bacilli other than the *B. fragilis* group are penicillinases, although cephalosporinases have been described in *Prevotella* spp. [29].

6.7 Concluding Remarks

Although anaerobic bacteria cause serious diseases, they have not received as much attention as their aerobic counterparts, in part because they are more difficult to cultivate. In addition, anaerobes are often present in polymicrobial aerobic/anaerobic infections or mixed with other anaerobes; therefore, their exact role in contributing toward infection can sometimes be difficult to ascertain. Some antimicrobials are effective against both aerobic and anaerobic bacteria, but certain antimicrobials, such as metronidazole, are only effective against anaerobes. Since many of the anaerobes tend to grow slowly in culture, susceptibility testing is slow. For anaerobes, pathogen identification is often obtained well before antimicrobial susceptibility test results are available. Thus, the anaerobe antibiogram can be particularly useful in guiding appropriate empirical choice of antimicrobial. It has been shown that AST results vary according to the type of assay used; thus, future anaerobic antimicrobial surveys should clearly present the methodologic method of testing and the breakpoint cutoff applied. Clear delineation of data will aid data interpretation. Ideally, authors should also present AST data on different species separately, rather than lumping together groups of morphologically similar organisms (e.g., anaerobic cocci).

In addition to the technical issues discussed above, aerobic and anaerobic pathogens may differ in virulence. Such activity is not reflected in susceptibility (MIC) measurements due to the nature of the assays, but it is likely to be clinically impor-

tant for clearing infection. It has been proposed that with aerobic bacteria, reactive oxygen species contribute to the lethal activity of aerobes (see Chap. 20). These toxic molecules are not expected to be present in anaerobes. Thus, as resistance emerges to carbapenems and metronidazole, for example, it may be necessary to develop more agents that are specifically lethal for anaerobes. Such agents could have additional utility against both aerobic and anaerobic organisms.

Major Points

- With the increasing use of MALDI-TOF MS to identify anaerobic bacteria in clinical microbiology laboratories, more accurate species identification and antimicrobial susceptibility results are becoming available. More accurate differentiation and characterization of strains provide more meaningful AST results.
- When reviewing anaerobic AST data, it is important to be aware of the method of AST utilized in generating the data and the specific breakpoints applied, since various methods and breakpoints affect interpretation of data.
- A unified AST testing approach for clinical laboratories needs to be developed for anaerobes. For example, broth microdilution testing is recommended only for the *B. fragilis* group. Agar dilution testing can be performed on all anaerobes, including the *B. fragilis* group.
- It is important to recognize that the data presented in the anaerobe antibiogram from CLSI M100 document [4] are derived from isolates obtained globally and may not be applicable to isolates within a given hospital or region.
- Taxonomic name changes must be taken into account when assessing antimicrobial susceptibility testing results of many anaerobes including the anaerobic Gram-positive cocci, the propionibacteria, and the clostridia.
- *Bacteroides* spp. are some of the most frequently encountered anaerobes in the clinical microbiology laboratory and are also among the most virulent. They are typically more resistant than other anaerobes to antimicrobial agents.
- Certain patterns of resistance in anaerobes that are on the rise include resistance to the β-lactam-β-lactamase inhibitor combinations among the *B. fragilis* group, increasing clindamycin resistance among all anaerobes, and resistance of *Clostridium* to vancomycin. In addition, metronidazole resistance is no longer limited to the *B. fragilis* group, as it now includes Gram-positive cocci and bacilli. Although the prevalence of resistance to certain antimicrobials is rising, effective antimicrobials are generally available.

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Chapter 7
Clinical Significance and Biologic Basis of HIV Drug Resistance

Rodger D. MacArthur

7.1 Introduction

Antiretroviral resistance limits the initial and subsequent activity of antiretrovirals, as well as limits the duration of their usefulness. Worse, some antiretroviral-selected drug mutations limit the activity of one or more other antiretrovirals within the same class, or even all antiretrovirals within a particular class. An understanding of the topic is still important, despite the overall decline in circulating drug resistance-associated mutations (DRAMs). For instance, some drugs and classes are substantially less likely to be limited by the development of resistance or retain activity in an environment of previously selected DRAMs. Thus, even the current Department of Health and Human Services (DHHS) guidelines [1] recommend different classes of antiretrovirals for initial use in the setting of uncertain medication adherence, chaotic lifestyles, etc.

Knowledge accumulated after three decades of studying antiretroviral resistance, at least arguably, has been primarily responsible for the decline in circulating DRAMs over the last 15 years. In 2000, 15–20% of HIV isolates from various North American and European cohorts showed resistance to at least one class of antiretrovirals; that figure is around 5–10% today. Unfortunately, circulating DRAMS to key antiretrovirals in low- and middle-income countries are substantially higher, typically exceeding 15–20% [2, 3]. This chapter will summarize much of the relevant antiretroviral resistance data, especially information that is still relevant today. As such, it will serve as a useful resource for clinicians caring for HIV-infected persons today, as well as provide an historical framework to the field.

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I. W. Fong et al. (eds.), *Antimicrobial Resistance in the 21st Century*, Emerging Infectious Diseases of the 21st Century, https://doi.org/10.1007/978-3-319-78538-7_7

7.2 General Principals

Mutations in various structural HIV proteins are random events. They occur as long as the virus is replicating. These two statements result in Key Concept 1: the only way to prevent HIV viral resistance from occurring is to shut down viral replication completely. This concept and other key concepts are summarized in Table 7.1. There are two corollaries to this concept: (a) If the virus is not replicating, it is not mutating; and (b) if the virus is replicating, whether in the presence or absence of antiretroviral therapy (ART), it is mutating. The rate of mutation development in various structural HIV proteins is impressive. For instance, it is estimated that in the absence of antiretroviral therapy, every single possible mutation in every single position of, say, the 99 amino acid protease, occurs once each day. Of course, many of these mutations are "dead-end" mutations that are not compatible with viral existence. Other mutations result in a viral "quasispecies" that is replication-competent but has a lower replicative capacity (i.e., is less "fit") than "wild-type" virus. Due to limitations in typically used commercial resistance assays, these less-fit subpopulations are not detected until their numbers exceed 10–20% of the total viral pool. However, in the presence of drug, they can become the dominant population, as Fig. 7.1 shows. In addition, when using more sensitive assays that can detect these mutated quasispecies down to a level of 1%, it has been shown that, when present, they adversely affect treatment options [4]. Even very low levels of viral replication, occurring in the presence of drug, will result in the accumulation of drug-limiting resistance mutations.

Factors that affect the likelihood of selecting for drug-limiting resistance mutations include (a) adherence to the antiretroviral regimen, (b) the potency of the antiretroviral drugs, (c) the drug's intrinsic barrier to resistance, and (d) the duration of time that antiretroviral drug levels exceed zero but are lower than the level needed to achieve maximal viral suppression. Intermediate rates of adherence (e.g., 20–80% of drug taken) typically are associated with the greatest risk for selecting drug-limiting resistance mutations. Lower adherence rates (e.g., below 20%) usually result in drug levels too low to exert any selective pressure on the virus, and higher

Table 7.1 Key concepts concerning HIV resistance and antiretrovirals

The only way to prevent HIV viral resistance from occurring is to shut down viral replication completely
If a drug, or combination of drugs, is unable to completely shut down viral replication, the higher the drug levels, the greater the selective pressure on the virus to mutate
If a drug has no activity, there will be no selective pressure on the virus and no further mutations will occur
The longer drug levels exceed zero but are lower than the level needed to completely shut down viral replication, the greater is the selective pressure on the virus to mutate
Failure to detect mutations on resistance testing does not rule out their presence (at low levels) in previously antiretroviral-treated persons

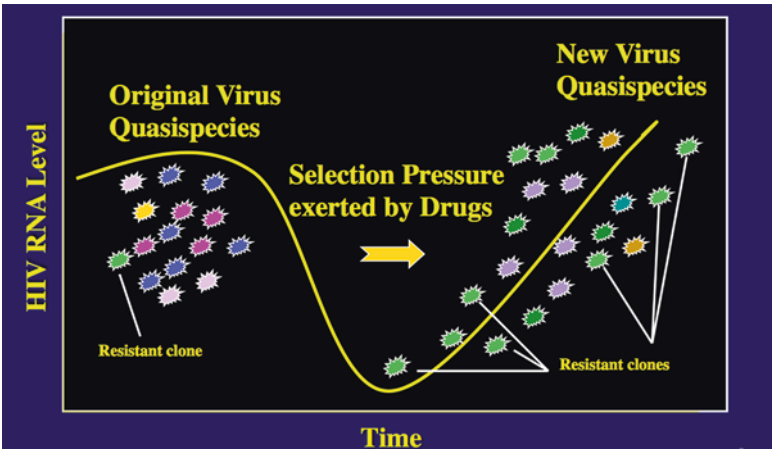

Fig. 7.1 Evolution of a new dominant quasispecies

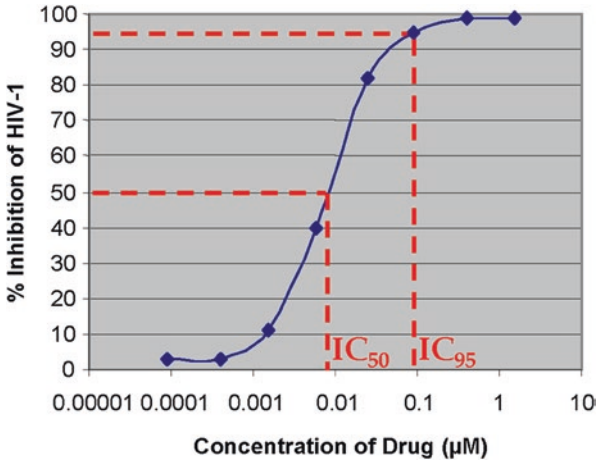

Fig. 7.2 In vitro measurement of drug potency

adherence rates usually result in drug levels high enough to maximally suppress viral replication. On the other hand, factors such as poor absorption of the drugs, or poor bioavailability, will change the dynamics such that higher levels of adherence, resulting in higher but suboptimal drug levels, will lead to substantial selective pressure on the virus to mutate. Similarly, lower-potency antiretrovirals, or lower-potency combinations of antiretrovirals, have the potential to exert substantial selective pressure on the virus if maximal suppression of replication does not occur. In vitro, potency of an antiretroviral drug is expressed as percent inhibition of HIV for any given drug concentration (Fig. 7.2). Clinically, potency of an antiretroviral drug is expressed as the log reduction in HIV RNA level when that drug is used

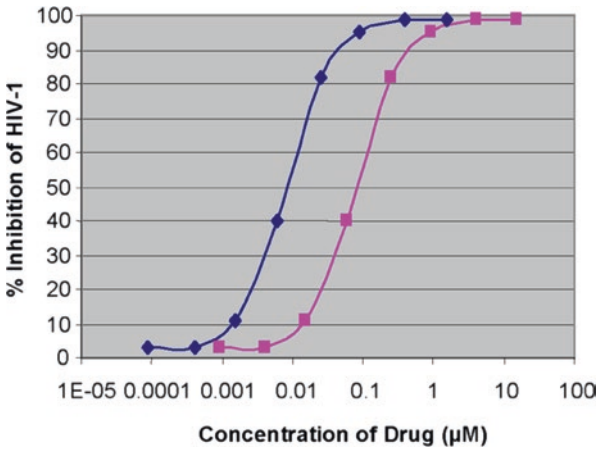

Fig. 7.3 A tenfold increase in resistance as a result of mutation(s)

alone. Different drugs and different classes have different intrinsic potencies. Log reductions in HIV RNA level for each drug contribute to the potency of the entire regimen in an additive fashion: the anticipated potency of any particular antiretroviral regimen is the sum of the potencies (in log HIV RNA reduction) of the individual components. Several key concepts follow: Key Concept 2 – if a drug or combination of drugs is unable to completely shut down viral replication, the higher the drug levels, the greater the selective pressure on the virus to mutate. Key Concept 3: if a drug has no activity, there will be no selective pressure on the virus, and no further mutations will occur. Key Concept 4: the longer drug levels exceed zero but are lower than the level needed to completely shut down viral replication, the greater is the selective pressure on the virus to mutate.

Mutations that occur as a result of selective pressure of a drug have the effect of increasing the amount of drug necessary to achieve the same degree of killing (Fig. 7.3). That is, graphically, they shift the "kill curve" to the right. If the shift results, for instance, in ten times more drug necessary to get the same amount of killing, this effect often is referred to as a tenfold increase in resistance to a particular drug. These mutations may have no effect on other antiretrovirals of the same class, or they may have the same effect, a greater effect, or a lesser effect. In some situations (discussed later), these mutations may even shift the kill curve to the left for another drug in the same class, effectively resulting in increased activity of that drug. This phenomenon is referred to as "hypersusceptibility" of HIV to the drug with the left-shifted curve.

If the initial mutation that occurs as a result of selective pressure of a particular drug is not sufficient to eliminate all activity of that drug, additional mutations will accumulate in the presence of that drug until that drug (and typically many others in the same antiretroviral class) no longer has any activity against the virus. In some circumstances, these subsequent mutations will not decrease the activity of the offending drug but rather restore fitness (replicative capacity) to the virus.

In these cases, the mutations are referred to as "secondary" or "compensatory" mutations. Note that not all mutations result in a viral quasispecies with lower fitness than wild type. In that case, these mutated quasispecies will co-circulate with wild type and not be "overgrown" (outcompeted) by wild-type virus. Even when there is overgrowth of wild-type virus, such that a mutated quasispecies is not detected on resistance testing after discontinuation of the offending drug, the mutated quasispecies typically will "reappear" on reintroduction of the drug. In other words, the mutated quasispecies has been "archived" but not eliminated from the total viral pool. Key Concept 5 follows: failure to detect mutations on resistance testing does not rule out their presence (at low levels) in previously antiretroviral-treated persons.

7.3 The Language of Resistance

There are three HIV enzymes that are the main targets for antiretrovirals: reverse transcriptase, protease, and integrase. Reverse transcriptase is a 560 amino acid (AA) heterodimer comprised of p51 and p66 subunits. The p51 subunit contains the first 450 amino acids; the p66 subunit contains the rest [5]. Most resistance assays stop sequencing around AA 250 or so, as most of the relevant mutations occur from AA 41 through AA 219. Protease is composed of two non-covalently associated, structurally identical monomers of 99 amino acids each [5]. Integrase is a 288 AA dimer comprised of 3 domains: the N-terminal domain, the catalytic core domain, and the C-terminal domain. The catalytic core contains the triad of AAs comprising the D,D-35-E motif, made up of aspartic acid at positions 64 and 116 and glutamine at position 152. Mutations at any of these positions essentially abolish all catalytic activity of integrase [6]. As such, these mutations occur uncommonly. Most of the relevant mutations selected by exposure to the integrase strand transfer inhibitor class of antiretrovirals occur in the catalytic core domain, from AA 74 through AA 230.

By convention, mutations at any position in these enzymes are listed as the one-letter AA code that is expected at a particular numbered position, followed by the one-letter AA code of the mutated quasispecies. So, for instance, the very commonly occurring valine-for-methionine mutation at position 184 of reverse transcriptase is listed as M184V. Often, multiple mutations can be seen (detected) at each position, including position 184 of reverse transcriptase. The convention is to list these mutations separated by a slash (e.g., M184V/I). Not every detectable mutation at a given position results in the same loss of activity to a particular drug. In addition, because three nitrogenous bases encode each amino acid, and multiple triads can code for the same amino acid, reverting (or back reverting) to wild type from a mutated quasispecies often involves going through an intermediate form [7]. So, for instance, the zidovudine-selected mutation, T215Y, tends to mutate to T215S prior to reverting to wild type. Typically, the back revertants (e.g., T215S) tend to be fitter than the original mutated version. Finally, mutations such as T215Y that

Table 7.2 One-letter amino acid codes and corresponding DNA codons

Alanine	A	GCT, GCC, GCA, GCG
Arginine	R	CGT, CGC, CGA, CGG, AGA, AGG
Asparagine	N	AAT, AAC
Aspartic acid	D	GAT, GAC
Cysteine	C	TGT, TGC
Glutamic acid	E	GAA, GAG
Glutamine	Q	CAA, CAG
Glycine	G	GGT, GGC, GGA, GGG
Histidine	H	CAT, CAC
Isoleucine	I	ATT, ATC, ATA
Leucine	L	CTT, CTC, CTA, CTG, TTA, TTG
Lysine	K	AAA, AAG
Methionine	M	ATG
Phenylalanine	F	TTT, TTC
Proline	P	CCT, CCC, CCA, CCG
Serine	S	TCT, TCC, TCA, TCG, AGT, AGC
Threonine	T	ACT, ACC, ACA, ACG
Tryptophan	W	TGG
Tyrosine	Y	TAT, TAC
Valine	V	GTT, GTC, GTA, GTG

require double-nucleotide mutations (e.g., $215T_{(ACC)}$ to $215Y_{(TAC)}$) often are slower to develop than mutations that require only a single nucleotide change [7]. Table 7.2 lists the one-letter amino acid code and the three nitrogenous base sequences for each amino acid.

7.4 Key Mutations in Reverse Transcriptase

Two classes of antiretrovirals, the nucleoside reverse transcriptase inhibitors (NRTIs) and the non-nucleoside reverse transcriptase inhibitors (NNRTIs), target reverse transcriptase. The NRTIs, also known as nucleoside and nucleotide analogues, are analogues of thymidine (zidovudine and stavudine), adenosine (didanosine, tenofovir), cytidine (lamivudine, zalcitabine, emtricitabine), and guanosine (abacavir). These drugs serve as "chain terminators" when they are incorporated into the viral DNA during HIV synthesis in newly infected cells. No other base can be added to viral DNA once an analogue has been incorporated. These antiretrovirals are considered "1-log" drugs, as they each lower HIV RNA levels by that amount. Mutations selected by these drugs typically are drug-specific, as indicated in Table 7.3. With monotherapy, some of these mutations develop over many months (e.g., T215Y/F), others over a few weeks (M184V). Some of the listed mutations in

Table 7.3 Mutations selected by nucleoside and nucleotide reverse transcriptase inhibitors

Zidovudine	M41L, D67N, K70R, L210W, T215Y/F, K219Q/E
Stavudine	M41L, D67N, K70R, L210W, T215Y/F, K219Q/E
Didanosine	K65R, **L74V***
Tenofovir	K65R
Zalcitabine	K65R, **T69D***, L74V, M184V
Lamivudine	M184V
Emtricitabine	M184V
Abacavir	**K65R***, L74V, Y115F, M184V

Note: The most common mutations selected by some drugs are indicated by *

Table 7.3 are never selected during combination therapy. For instance, the M184V mutation is not selected when abacavir is used in combination with zidovudine, likely due to the hypersusceptibility that M184V imparts to zidovudine. The extent of resistance conferred to a particular drug by a particular mutation often depends on both the drug and the mutation. So, for instance, the M184V mutation confers high-level resistance to lamivudine and emtricitabine, hypersusceptibility to zidovudine, tenofovir, and stavudine, and only partial resistance to abacavir. On the other hand, the mutations that are selected by both zidovudine and stavudine, referred to as "thymidine analogue mutations (TAMs)," confer some degree of resistance to all of the NRTIs.

There are some mutations that are associated with properties not limited to one or a few of the NRTIs. Foremost among these is the M184V mutation. This mutation, while having the effect on individual drugs described above, also results in a viral quasispecies that has substantial loss in fitness. This phenomenon, first described by Mark Wainberg and colleagues in Montreal [8], results clinically in a sustained 0.5 log reduction in HIV RNA; at the same time the virus has developed up to 1000-fold resistance to either lamivudine or emtricitabine [9]. In addition, this mutation results in a viral quasispecies that has greater "fidelity," meaning that it is more difficult for the virus to revert to wild type [10].

Other mutations of note that are selected by exposure to the NRTIs are (1) Q151M (along with accessory mutations at positions 62, 75, 77, and 116) which conveys high-level resistance to all NRTIs; (2) 69 insertion complex, which refers to amino acid insertions between codons 67 and 70 and conveys resistance to all NRTIs; (3) 70 deletion complex, which refers to amino acid deletions between codons 67 and 70 and conveys resistance to all NRTIs; and (4) E40F, E44D/A, and V118I are referred to as "accessory" mutations that are seen occasionally in association with the TAMs and contribute to the extent of resistance conferred by the TAMs.

Mutations, in general, impart some degree of structural change to the relevant enzyme. In the case of NRTI-limiting mutations, the resulting structural changes to

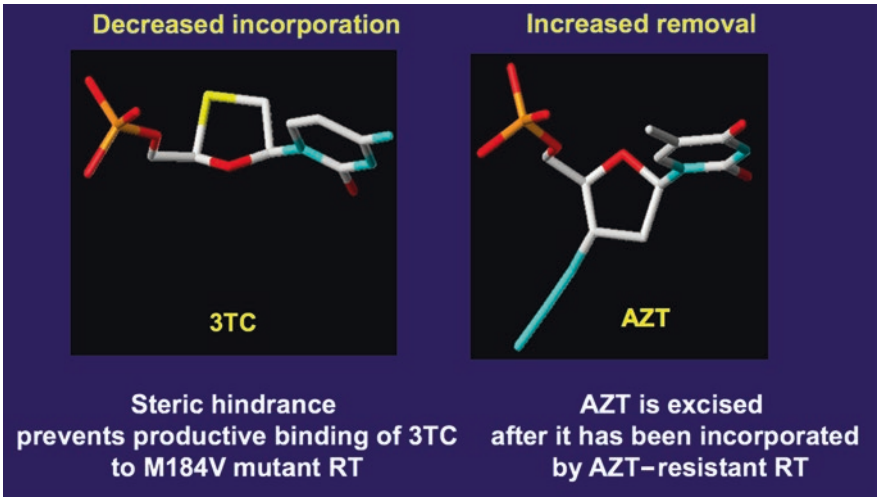

Fig. 7.4 Mechanisms of resistance to NRTIs

reverse transcriptase have one of two effects: they prevent the nucleoside analogue from binding, due to steric hindrance, or they facilitate excision of the already incorporated analogue (Fig. 7.4). The excision activity of HIV reverse transcriptase catalyzes the 3′-terminal residue of the analogue to an acceptor substrate, most likely ATP [11]. Some mutations, such as K65R, have the effect of decreasing binding/incorporation for several of the NRTIs, while at the same time having a variable effect on the stability of the incorporated drug. So, for instance, the effect of K65R is to make the virus hypersusceptible to zidovudine, susceptible to stavudine and abacavir, and more resistant to didanosine and tenofovir.

The NNRTI class of antiretrovirals also inhibit HIV reverse transcriptase, although the mechanism is not as straightforward as with the NRTIs. Unlike the NRTIs, the NNRTIs are structurally quite diverse. However, all of them bind allosterically to reverse transcriptase in the same general region, albeit to different amino acids. The NNRTI-binding pocket of reverse transcriptase is hydrophobic, located about 10 angstroms from the catalytic site in the "palm" region of the p66 subunit [12]. Binding of the NNRTIs induces conformational changes that inhibit the catalytic activities of reverse transcriptase. Drugs in this class are more potent than the NRTIs, typically able to decrease HIV RNA levels by 2–4 logs or more. However, single-point mutations at positions 103 and 181 (K103N, Y181C) developed quickly in the presence of the "first generation" of NNRTIs, nevirapine, delavirdine, and efavirenz [13]. These mutations alter the size, shape, and polarity of the NNRTI-binding pocket, thereby reducing access to the pocket by those NNRTIs [12]. Interestingly, although not clinically relevant due to the lack of current use of zidovudine, it was shown that the addition of zidovudine to nevirapine changed the mutation selection pattern from predominantly Y181C to K103N [14]. The "second-generation" NNRTIs, etravirine and rilpivirine, retain good activity against HIV in

Table 7.4 Other important key mutations

K103N	Most common mutation selected by efavirenz. Confers high-level resistance to efavirenz, nevirapine, and delavirdine. Does not limit the activity of etravirine or rilpivirine
Y181C	More commonly selected by nevirapine and delavirdine than efavirenz [13]. Confers high-level resistance to efavirenz, nevirapine, and delavirdine and intermediate-level resistance to etravirine and rilpivirine
V90I, E138A/K, M230L	Selected by, and limits the activity of, etravirine and rilpivirine
L33F, I84V	Multi-protease inhibitor mutations, resulting in substantial reductions in, or elimination of, activity of all of the protease inhibitors
Q148H/R/K, N155H	Major mutations for both raltegravir and elvitegravir

the presence of the K103N mutation but have reduced activity in the presence of the Y181C/I/V mutation. Other mutations that limit the activity of etravirine and rilpivirine include V90I, E138A/K, and M230L. The effect of these mutations on the NNRTIs is summarized in Table 7.4.

7.5 Key Mutations in Protease

The protease inhibitor class of antiretrovirals, when "boosted" with the cytochrome p450 inhibitors ritonavir or cobicistat, is the most potent of all of the classes. The drugs in this class inhibit a key enzyme (protease) in the replicative cycle of HIV. This enzyme cleaves unnecessary pieces of viral protein just prior to maturity of the virus, thereby allowing the virus to fold into its compact, infectious, shape/form. The class is the least susceptible to the development of resistance mutations, when combined with one of the boosters. Even monotherapy (with a booster) results in sustained HIV RNA decreases of 4–6 logs. It is the only class for which monotherapy can be considered, although it is not recommended to do so routinely. It is extraordinarily rare for any mutations to develop in previously antiretroviral-naïve individuals when on a standard multidrug combination. Mutations have developed, rarely, in persons on lopinavir and atazanavir, for instance, when each is boosted with ritonavir. Much of our knowledge of the virologic consequences of the development of mutations in this class comes from 20 years ago before the use of a booster was common. For instance, unboosted indinavir selected for mutations slowly and retained antiviral activity even in the presence of several mutations. On the other hand, by the time additional mutations developed, HIV protease typically was resistant to all of the drugs in the class (unboosted). From a historical perspective, mutations that developed at key binding locations in protease resulted in lengthening of the bond between the enzyme and the inhibitor, as well as other structural changes. For instance, the L33F multidrug resistance mutation [15] has

been shown to reduce flexibility of protease in the 30s and 80s loops [16]. As a result of early mutational changes in protease, the enzyme typically loses a substantial degree of fitness. Over time, additional mutations develop away from the binding site that have the effect of restoring fitness by "tightening" the structure of the enzyme

Certain other unboosted protease inhibitor-selected mutations are now of passing interest only, as these drugs are not used either at all or in the absence of a booster. So, for instance, the PI nelfinavir selected for the D30N mutation, which substantially limited the activity of that drug but had no effect on other PIs. The atazanavir-selected I50L mutation resulted in high-level resistance to atazanavir but had no effect on the activity of amprenavir. Similarly, the I50V amprenavir-selected mutation resulted in high-level resistance to amprenavir but had no effect on the activity of atazanavir. As with the NRTI and NNRTI classes, there were a number of other "signature" mutations (e.g., G48V and L90M for saquinavir) that often would reliably indicate the previous drug exposure history of the virus.

7.6 Key Mutations in Integrase

The integrase strand transfer inhibitor (INSTI) class also is extremely potent, resulting in sustained reductions in HIV RNA of 4–5 logs. However, the class is more susceptible to the development of drug-limiting resistance mutations than is the PI class. For instance, in the setting of prior virologic failure on an NRTI-based regimen, a switch from a boosted PI regimen (lopinavir-ritonavir) to a raltegravir-based regimen was associated with a substantial virologic failure rate in the raltegravir arm, despite participants having an HIV RNA level < 400 copies/ml at the time of the switch [17].

In addition to raltegravir, there are two other currently available integrase strand transfer inhibitors: elvitegravir, which must be boosted (and coformulated) with cobicistat, and dolutegravir. All of these drugs work at the last step of integration, by inhibiting insertion of viral DNA into host chromosomal DNA (strand transfer step). There is substantial overlap in the mutations that are selected by, and limit the activity of, raltegravir and elvitegravir. These include T66A/I/K, E92Q, E138K/A/T, G140S/A/C, Y143R/C/H (raltegravir only), and, especially, Q148H/R/K and N155H. These mutations are located near the catalytic core domain of integrase, which is where the INSTIs bind [18]. These mutations have the effect of excluding binding of the INSTIs but at the expense of reduced enzymatic fitness. As might be anticipated, a number of accessory mutations often are seen in association with the major mutations. These secondary mutations have the effect of restoring fitness to the virus.

Dolutegravir, the third INSTI currently available, is the most resilient and least susceptible to the development of resistance of the three. In fact, mutations which substantially limit the activity of both raltegravir and elvitegravir typically have little impact on the activity of dolutegravir. However, mutations do develop, espe-

cially when the drug is used as monotherapy in an "off-label," not recommended, fashion. In addition, combinations of mutations at positions 138, 140, 148, and 155 result in substantial loss of activity of the drug, which may be overcome by doubling the dose.

7.7 Mutations from Other Classes

Enfuvirtide is the only drug in the fusion inhibitor class of antiretrovirals. It also is the only antiretroviral which must be administered by injection. Relatively little is known about the development of resistance to this drug and even less about the impact of specific mutations. Furthermore, routine resistance testing is not available, and the use of the drug is limited to the heavily treatment-experienced group of patients, those defined by mutations in multiple other classes, and comprising at most 5% of the total HIV-infected population. Enfuvirtide binds to the HR1 domain of GP41, after the envelope protein has bound to the CD4 receptor of the cell [19]. Binding of enfuvirtide prevents the conformational changes that are needed for membrane fusion. While mutations in the envelope gene at positions 36–43 have been detected in viruses from patients on enfuvirtide, it is not clear what impact any single or multiple mutations has on drug activity. In fact, most clinical trials using resistance as an entry criterion default to "ever use" of enfuvirtide to arbitrarily declare that HIV-1 likely is resistant to that drug.

Maraviroc is the only antiretroviral that targets a human protein, the CCR5 co-receptor. The drug has good activity in both B- and non-B-subtypes of HIV-1, as long as the individual is CCR5-tropic and not dual- (CCR5, CXCR4) or mixed-tropic. The main resistance mechanism involves utilization of maraviroc-bound CCR5 co-receptors for entry, as a result of multiple mutations in the V3 loop of GP120 [20]. Another way HIV can enter in the presence of maraviroc is by co-receptor switching (i.e., utilizing CXCR4). Co-receptor switching occurs infrequently, as it results in substantially reduced fitness and efficiency of entry, as a result of multiple mutations in GP160 [20]. There is no cross-resistance with enfuvirtide. Like enfuvirtide, maraviroc is used infrequently at the present time. Early assays used to detect minority variants that were dual- or mixed-tropic were not sensitive enough to exclude this population of patients from entry into clinical trials.

7.8 Mutation Pathways

Mutation "pathways" have been well-described for the thymidine analogue drugs zidovudine and stavudine, as well as for the INSTI raltegravir. Pathways refer to the accumulation of specific mutations based on the previous development of other mutations. Little is known about the factors that "force" the virus down one pathway

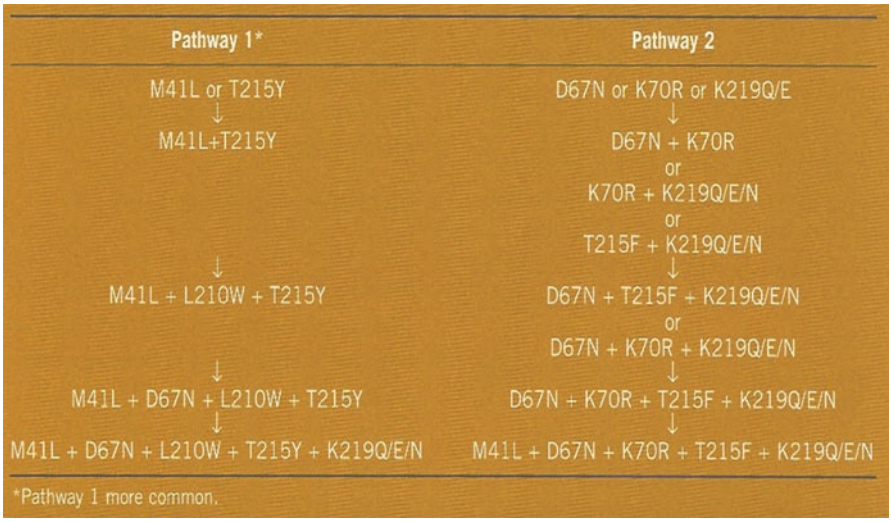

Fig. 7.5 Zidovudine pathways

or another, but fitness likely plays a role. The two thymidine analogue pathways are shown in Fig. 7.5. While the mutations seen at the full expression of each pathway overlap, the process by which they accumulate differs between the pathways. In addition, it has been observed that the mutation L210W is always found in association with T215Y, a mutation that is not present in the "second" TAM pathway. Note that the first TAM pathway, involving the mutations L210W and T215Y, is the more common of the two and results in a virus more resistant to all of the NRTIs.

Even less is known about the 2–3 raltegravir pathways, the N155H pathway, the Q148H/R/K pathway, and the Y143R/C/H pathway. The Q148H/R/K pathway is associated with the least reduction in viral fitness. Minority variants that exist at the time of drug initiation may play a role in determining the specific pathway the virus takes to accumulate mutations.

7.9 Resistance Tests and Algorithms

There are two main types of HIV resistance tests: genotypic tests, based on sequencing and determining the specific amino acids present at each position in key enzymes, and phenotypic tests, which test for the degree of killing of the isolated virus in the presence of different antiretrovirals. Genotypic tests are an indirect measure of resistance, based primarily on assigning a "likelihood of failure" score to a particular mutation or set of mutations. Phenotyping is a direct measure of a drug's activity in killing the mutated or unmutated virus. Genotyping is quicker, more readily available (e.g., not limited to commercial laboratories), and less expensive.

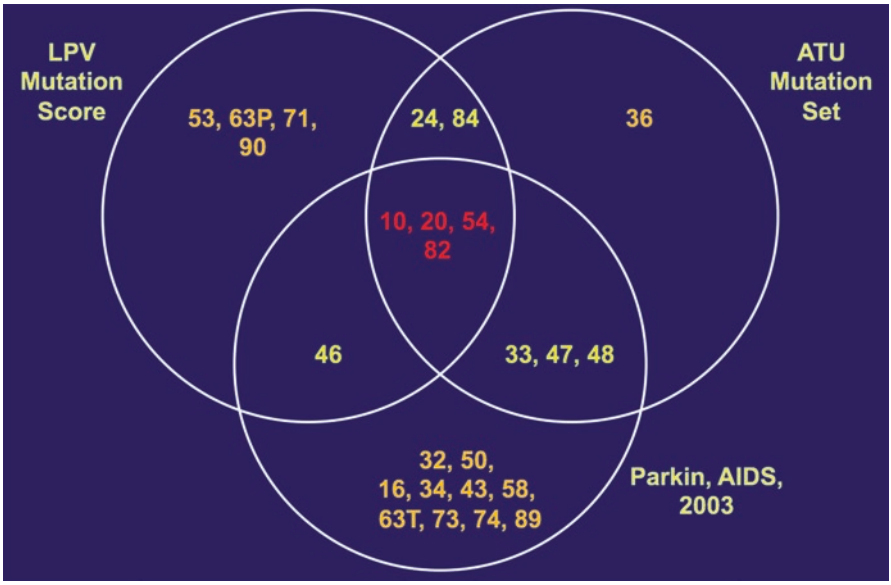

Fig. 7.6 Key mutations predicting lopinavir/ritonavir resistance

However, a certain amount of "faith" needs to be placed in the algorithm-based interpretation of mutations inherent in this approach.

The earliest algorithms that purported to predict resistance to antiretrovirals were relatively primitive, often relying on the total number of mutations detected. In other words, the more mutations present, the more likely was the virus to be deemed resistant to a particular drug. Even though it was known relatively early that, for instance, the M184V mutation in reverse transcriptase resulted in high-level resistance to lamivudine and emtricitabine, and the K103N mutation had the same effect on the first generation of NNRTIs, a simple count predominated the early efforts at predicting resistance to the PIs. An early refinement to this approach involved pattern recognition and the identification of "key" mutations. Predicting resistance to the ritonavir-boosted PI lopinavir is an excellent example of how this approach progressed. Figure 7.6 illustrates the identified key mutations from three different algorithms [21–23]. All were reasonably effective at predicting lack of efficacy, but the three had only about 1/3 of their mutations in common. Call these the "really key" mutations, but it wasn't until "weights" were assigned to all mutations in each algorithm that the algorithms became substantially predictive of drug activity [24]. A similar approach for the NNRTI etravirine is shown in Fig. 7.7. Around that same time (circa 2007), multiple algorithms were "competed" to see which was best at predicting resistance to particular PIs [25]. As a result of these competitions, it became apparent that large databases had a huge advantage over much smaller databases.

The Virco™ Vircotype (or virtual phenotype) report (no longer available) took this approach one step further, by correlating over 350,000 genotype specimens

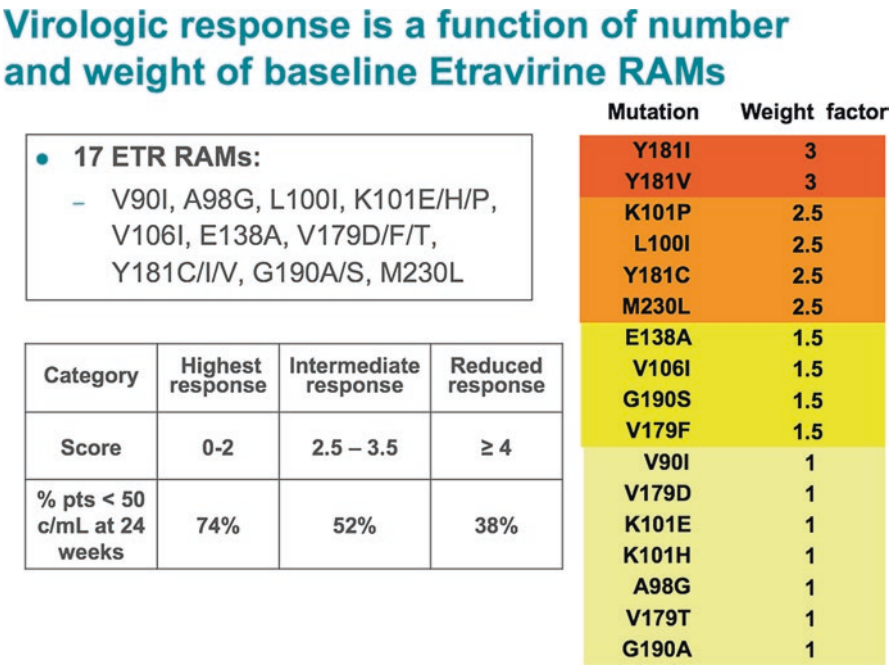

Fig. 7.7 Algorithm-based determinations

with over 90,000 paired phenotype specimens and assigning weights to each singlet and pair of mutations associated with different antiretrovirals [26]. By doing so, Virco™ was able to show that a single mutation (i.e., I84V) that substantially reduced the activity of, for instance, tipranavir, behaved substantially differently in combination with the L10F mutation. Today, as the cost of phenotyping has dropped in North America and Europe, genotype and phenotype tests often are run together. When genotype tests are run without a phenotype, the reported results typically are substantiated by a large database of isolates.

7.10 Clinical Issues of Relevance

The history of treating HIV-infected persons with antiretrovirals has benefitted from three decades of research on resistance issues specific to these drugs. Treatment guidelines [1] have evolved as we have acquired a better understanding of the development and prevention of resistance to antiretrovirals. For instance, prior to 2005, the DHHS guidelines recommended baseline resistance testing only in the acutely or recently infected individual. This recommendation was based on the belief that drug-selected resistance mutations would be overgrown by (revert to) wild-type virus in the absence of continuous selective pressure of antiretroviral therapy. That

belief was shown to be incorrect, by an article documenting the persistence of mutations in multiple classes in individuals likely infected years earlier [27]. Unfortunately, the HIV RNA and resistance assays that we take for granted in North America and Europe are not available in much of the rest of the world. As a result, resistance continues to develop at an alarming rate in individuals still clinically well who remain on failing antiretroviral regimens [28].

Finally, it is worth noting two excellent sources of current information about the effect of mutations on various antiretrovirals: the Stanford University HIV database [29] and the annually updated IAS-USA list of mutations by class [30].

Major Points

- Understand basic principles of antiretroviral resistance
- Learn key drug- and class-related resistance mutations
- Apply basic science-related principles of drug resitance to clinical applications

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Chapter 8
Resistance of Herpesviruses to Antiviral Agents

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8.1 Antiviral Agents for Herpesvirus Infection

Three antiviral agents and a prodrug are currently available for the systemic treatment of human cytomegalovirus (HCMV) infections. Ganciclovir (GCV, Cytovene®, Roche) is a deoxyguanosine analogue and was the first drug to be approved in 1988. Since then, it has remained the first-line treatment for HCMV infections in immunocompromised patients. Upon entry in HCMV-infected cells, GCV is selectively phosphorylated by a viral phosphotransferase (the product of the UL97 gene, pUL97). Subsequently, cellular kinases convert GCV-monophosphate into GCV-triphosphate, which acts as a potent inhibitor of the HCMV DNA polymerase (pol) by competing with deoxyguanosine triphosphate on the enzyme binding site (Fig. 8.1). Ganciclovir is also incorporated into the viral DNA where it slows down and eventually stops chain elongation [15, 22, 213]. Ganciclovir formulations are available for intravenous (IV) or oral administration as well as intravitreal injections for the treatment of HCMV diseases in immunocompromised patients. Due to its poor bioavailability (~6%), efforts were made to develop a prodrug of GCV. Valganciclovir (VGCV, Valcyte®, Roche) is a L-valyl ester formulation of GCV exhibiting about ten times the bioavailability of GCV following oral administration [175].

The other two compounds approved for systemic treatment of HCMV infections are also potent inhibitors of the viral DNA pol. However, due to their toxicity

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I. W. Fong et al. (eds.), *Antimicrobial Resistance in the 21st Century*, Emerging Infectious Diseases of the 21st Century, https://doi.org/10.1007/978-3-319-78538-7_8

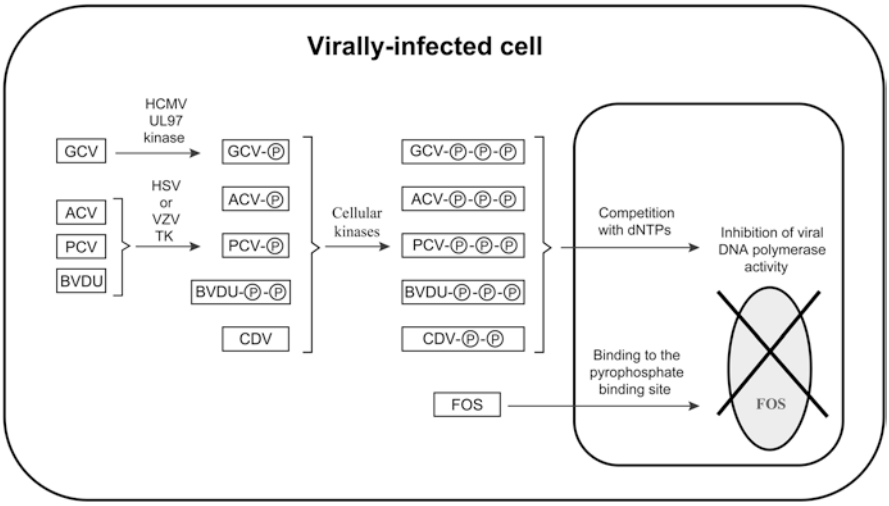

Fig. 8.1 Mechanisms of action of the different classes of antiviral agents. The nucleoside analogues such as ganciclovir (GCV), acyclovir (ACV), and penciclovir (PCV) must be first phosphorylated by the UL97 protein kinase or viral thymidine kinase (TK) and then by cellular kinases to be converted into their active forms. The cyclic nucleoside analogue brivudine (BVDU) is converted into monophosphate and diphosphate forms by the viral TK followed by an additional phosphorylation by cellular kinase. The acyclic nucleoside phosphonate derivatives such as cidofovir (CDV) must be phosphorylated by cellular kinases only to be active. The resulting triphosphate forms compete with deoxynucleotide triphosphates (dNTPs) to inhibit viral replication. The pyrophosphate analogue foscarnet (FOS) directly inhibits the activity of the viral DNA polymerase. *Key*: Ⓟ represents one phosphate group

profiles and absence of oral formulation, they are usually reserved for patients failing or not tolerating GCV therapy. Cidofovir (CDV, Vistide®, Gilead Sciences) is a nucleotide analogue of cytidine (also called acyclic nucleoside phosphonate) that only requires activation (phosphorylation) by cellular but not viral enzymes to exert its antiviral activity [61]. The diphosphate form of CDV is a competitive inhibitor of HCMV DNA pol, and it may act as a DNA chain terminator when two consecutive incorporations into DNA occur (Fig. 8.1). The IV formulation of CDV is indicated for the treatment of HCMV retinitis in patients with AIDS and is also occasionally used in transplant recipients. Foscarnet (FOS, Foscavir®, AstraZeneca), a pyrophosphate analogue, differs from the two previous antivirals both by its mechanism of action and by the fact that it does not require any activation step to exert its antiviral activity. Foscarnet binds to and blocks the pyrophosphate binding site on the viral polymerase, thus preventing incorporation of incoming deoxynucleotide triphosphate (dNTP) into viral DNA (Fig. 8.1) [59]. The IV formulation of FOS is indicated for the treatment of HCMV retinitis in individuals with AIDS and for GCV-resistant HCMV infections in immunocompromised patients.

In addition to the treatment of established HCMV disease, antivirals have also been used to prevent symptomatic episodes, especially in transplant recipients. The

first strategy, defined as prophylaxis, consists of administering an antiviral to patients during the first 3–6 months after transplantation. This strategy is employed in recipients of solid organ transplants (SOT) but not in hematopoietic stem cell transplants (HSCT) due to the marrow toxicity of ganciclovir. However, the occurrence of late-onset HCMV disease which is associated with high rates of graft loss [7] and mortality [146] is an important issue after discontinuing prophylaxis. The second strategy, referred to as "preemptive therapy," consists of using short courses of antivirals only for high-risk patients based on evidence of active viral replication (e.g., detection of early HCMV antigens such as the pp65 protein or sufficient amounts of viral DNA/mRNA in the blood) [24]. The advantages of preemptive therapy include a lower rate of delayed occurrence of HCMV disease and less toxicity [203]. However, patients are more prone to recurrent episodes of DNAemia, and the indirect effects of HCMV infection on graft and patient survival may not be prevented. These preventive strategies have shown efficacy in preventing HCMV disease in both SOT and HSCT patients [25, 95, 118, 151, 171–173].

Antiviral agents currently licensed for the treatment of herpes simplex virus (HSV) and varicella-zoster virus (VZV) infections include acyclovir (ACV, Zovirax®, GlaxoSmithKline) and its L-valyl-ester prodrug valacyclovir (VACV, Valtrex®, GlaxoSmithKline), famciclovir (FCV, Famvir®, Novartis) which is the L-valyl-ester prodrug of penciclovir (PCV), and FOS. Acyclovir and PCV are deoxyguanosine analogues that must be phosphorylated by the thymidine kinase (TK) of HSV and VZV and then by cellular kinases to exert their antiviral activity [22, 98]. The triphosphate forms are competitive inhibitors of the viral DNA pol of HSV and VZV (Fig. 8.1). In addition, incorporation of ACV-triphosphate into the replicating viral DNA chain stops synthesis [15]. Oral ACV, VACV, and FCV are used for short-term therapy of primary and recurrent HSV infections (particularly genital herpes), long-term suppressive therapy of recurrent genital herpes, as well as treatment of herpes zoster. The IV formulation of ACV is indicated for the management of severe HSV (including encephalitis and neonatal herpes) and VZV infections. Topical formulations of ACV and PCV (Denavir®, Novartis) are used for the treatment of herpes labialis and keratitis. Brivudine or bromovinyldeoxyuridine (BVDU) is an analogue of thymidine that is converted into monophosphate and diphosphate metabolites by the TK of HSV-1 and VZV and then in its triphosphate form by cellular kinase. BVDU triphosphate competes with dNTPs and also acts by terminating DNA chain elongation. Brivudine was also shown to inhibit the activity of thymidylate synthase of VZV [113]. In some European countries, BVDU is approved for the treatment of herpes zoster in immunocompetent adults [71]. The pyrophosphate analogue FOS is usually indicated for ACV- or PCV-resistant HSV or VZV infections [185–187]. Topical and IV formulations of CDV may be used "off label" in the treatment of nucleoside analogue- and/or FOS-resistant HSV infections [225].

8.2 Human Cytomegalovirus Resistance

8.2.1 Phenotypic and Genotypic Assays to Evaluate HCMV Drug Susceptibility

Two different albeit complementary approaches have been developed to assess HCMV drug resistance. In the phenotypic method, the virus is grown in the presence of various concentrations of an antiviral in order to determine the concentration of drug that will inhibit a percentage (most commonly 50%) of viral growth in cell culture. In this assay, a standardized viral inoculum is inoculated in different wells. The virus is then allowed to grow for a few days (typically 7–10 days) in the presence of serial drug dilutions before staining the cells. The number of viral plaques per concentration is first determined. Then, the percentage of viral growth, as compared to a control well without antiviral, is plotted against drug concentrations to determine the concentration that will inhibit the growth of 50% of viral plaques (50% effective concentration or EC_{50}). Even though recent efforts have been made to standardize this assay [139], the inter-assay and interlaboratory variability is still problematic. In addition to the relative subjectivity of this method, there are some differences in the cutoff values defining drug resistance depending on the laboratory. Similar assays, either based on detection of HCMV DNA by hybridization [68] or quantitative PCR [200] or detection of specific HCMV antigens by ELISA [217], flow cytometry [131, 158], immunofluorescence [219] or immunoperoxidase [100], or real-time cell analysis [180], have also been developed. EC_{50} cutoff values defining resistance are still a matter of debate although a value of 6 μM is most often used for GCV. Altogether, phenotypic assays are time-consuming, unstandardized, and subject to possible selection bias introduced during viral growth of mixed viral populations in cell culture [104, 111] and may lack sensitivity to detect low-level resistance or minor resistant subpopulations [54, 104].

In contrast to phenotypic assays, which directly measure drug susceptibility of viral isolates, genotypic assays detect the presence of viral mutations known to be associated with drug resistance. Those assays are based on DNA sequencing of viral genes (UL97 and UL54) that are the sites of HCMV resistance to antivirals. One of the advantages of these assays is that they can be performed directly on clinical specimens [28, 234, 235], thus reducing considerably the time required to obtain results. By omitting the need to grow the virus, such methods also minimize the risks of introducing a selection bias. The limited number of UL97 mutations responsible for GCV resistance has allowed the development of rapid assays to detect their presence in clinical samples [49, 112]. Indeed, approximately 70% of GCV-resistant clinical isolates contain mutations in one of three UL97 codons (460, 594, and 595) [29]. However, due to reports of resistance mutations at other codons, DNA sequence of the entire UL97 region involved in GCV resistance should be determined for a comprehensive analysis, and this approach is the current standard (Fig. 8.2).

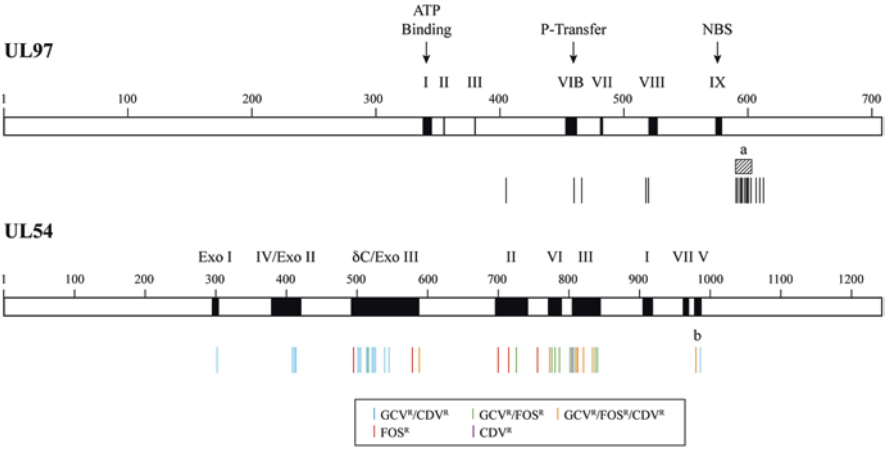

Fig. 8.2 Confirmed mutations associated with resistance to ganciclovir in the UL97 gene and to ganciclovir (GCV^R), foscarnet (FOS^R), and/or cidofovir (CDV^R) in the UL54 gene of clinical HCMV isolates. In UL97 gene, the ATP-binding site, the phosphate transfer (P-transfer) domain, the nucleoside-binding site (NBS), and some regions conserved among the protein kinase family (i.e., I, II, III, VIB, VII, VIII, and IX) are represented by the black boxes. In UL54 gene, conserved regions among the *Herpesviridae* DNA polymerases are represented by the black boxes. The roman numbers (I to VII) and δ-region C corresponding to each of these regions are indicated above the boxes. Conserved motifs (Exo I, Exo II, and Exo III) in the exonuclease domain are also indicated above the boxes. Scale represents nucleotide positions in each gene. Bars (|) indicate amino acid substitutions associated with antiviral drug resistance. [a]shaded area corresponds to the codon 590–603 region in UL97 gene where different amino acid deletions conferring ganciclovir resistance were identified (i.e., deletions 591–594, 591–607, 595, 595–603, 600, and 601–603). [b]amino acid deletion 981–982 in UL54 gene that confers resistance to all three antivirals

For genotypic analysis of UL54 DNA polymerase mutations, DNA sequencing is required due to the large number of mutations reported within all conserved regions of this gene [103] (Fig. 8.2). Genotypic approaches are fast, but their interpretation is not always straightforward, i.e., discriminating between mutations associated with natural polymorphisms [31, 52, 94, 152, 154] from those related to drug resistance. In order to prove that a new mutation is associated with drug resistance, recombinant viruses need to be generated using either overlapping cosmid/plasmid inserts [62] or by marker transfer experiments of the mutated gene in a wild-type [10, 11, 50] or genetically engineered [54, 55] virus prior to testing of this mutant virus in phenotypic assays. The introduction of a reporter gene (luciferase) in a permissive cell line [105] or directly in a recombinant virus [56, 79] should accelerate the phenotypic testing of new mutants by allowing an automated and more objective evaluation of viral replication.

8.2.2 *Clinical Significance, Incidence, and Risk Factors for Drug-Resistant HCMV Infections*

Drug-resistant strains first emerged as a significant problem in patients with AIDS being treated for HCMV retinitis. Numerous studies have documented the emergence of drug-resistant HCMV strains (detected by phenotypic or genotypic methods) and their correlation with progressive or recurrent HCMV disease (mainly retinitis) during therapy [10, 50, 51, 75, 204, 206, 234, 235]. The first study to evaluate the prevalence of GCV resistance in AIDS patients was conducted by evaluating the excretion of GCV-resistant strains in the urine of 31 patients with AIDS treated with IV GCV for HCMV retinitis.

In this study, no resistant isolates were recovered in patients treated for ≤3 months, whereas 38% of those excreting the virus in their urine after >3 months of GCV had a resistant isolate, representing 8% of the entire cohort of patients [73]. Since then, larger studies have evaluated the temporal emergence of GCV-resistant strains using either phenotypic [120] or genotypic [29] assays. In all studies, GCV resistance (defined by an EC_{50} value ≥6 μM) at the initiation of treatment was a rare event (≤2.7% of tested strains). Phenotypic evaluation of blood or urine isolates from 95 patients treated with GCV (mostly intravenous) for HCMV retinitis revealed that 7, 12, 27, and 27% of patients excreted a GCV-resistant strain after, respectively, 3, 6, 9, and 12 months of drug exposure [120]. In a more recent study of 148 AIDS patients treated for HCMV retinitis with oral VGCV, the presence of GCV resistance mutations was detected in 2, 7, 9, and 13% of patients after 3, 6, 9, and 12 months of therapy, respectively [29]. The lower incidence of GCV resistance in the latter study despite the use of sensitive genotypic methods might be explained by differences in the treatment of the underlying HIV disease in the study population, notably improved antiretroviral therapy. Due to their less frequent use in clinic, fewer data have been reported on the temporal emergence of FOS- and CDV-resistant HCMV strains in HIV-infected individuals. One small study found an incidence of phenotypic resistance to FOS of 9, 26, 37, and 37% after 3, 6, 9, and 12 months of therapy using an EC_{50} cutoff value of 400 μM [121], whereas another one reported rates of 13, 24, and 37% after 6, 9, and 12 months using an EC_{50} cutoff value of 600 μM [231]. The data on CDV resistance (EC_{50} value ≥2–4 μM) are even more limited, but they seem to indicate a resistance rate similar to what has been observed with GCV and FOS [121].

Proposed risk factors for the development of HCMV resistance in this patient population include inadequate tissue drug concentrations due to poor tissue penetration (e.g., the eyes) or poor bioavailability (e.g., oral GCV), a sustained and profound immunosuppression status (CD4 counts <50 cells/μl), frequent discontinuation of treatment due to toxicity, and a high pre-therapy HCMV load [77, 168].

HCMV resistance to GCV appears to be an emerging problem in SOT recipients and has been associated with an increased number of asymptomatic and symptomatic viremic episodes, earlier onset of HCMV disease, graft loss, and an increased risk of death [21, 144]. Due to the different HCMV preventive strategies and

immunosuppressive regimens in use at different centers and considering the heterogeneity of the transplant populations, it has been difficult to precisely evaluate the temporal emergence of HCMV resistance in that setting. Lung transplant recipients appear to have the highest incidence of HCMV resistance development with rates of 3.6–9% after median cumulative GCV exposures ranging from 79 to 100 days [137, 145, 153]. In two of those studies, the incidence of resistance increased from 15.8% to 27% in D+/R– (HCMV antibody-positive donor with HCMV-negative recipient) patients [145, 153] and occurred as a late complication, i.e., a median of 4.4 months after transplantation [145]. As opposed to what has been reported in lung transplant recipients, the incidence of GCV resistance in other SOT populations has been much lower and almost exclusively seen in D+/R– patients [144, 153]. More specifically, Lurain and colleagues studied two cohorts of SOT patients including heart, liver, and kidney recipients at two US centers [153]. Phenotypic evaluation for HCMV resistance prompted by either clinical suspicion or positive blood cultures indicated that rates of resistance were generally low (e.g., <0.5%) at one center and varied from 2.2% to 5.6% at another center depending on the transplanted organ. Another retrospective study by Limaye and colleagues evaluated 240 SOT patients including 67 D+/R– patients but excluded lung transplant recipients [144]. In their cohort, GCV-resistant HCMV disease developed only in D+/R– patients, with resistance rates of 7% in these patients. HCMV resistance was more frequently seen among recipients of kidney/pancreas or pancreas alone (21%) than among kidney (5%) or liver (0%) recipients. Of note, cases of GCV-resistant HCMV infections occurred at a median of 10 months after transplantation with a median total drug exposure of 194 days (129 days of oral GCV) including two to three treatment courses for HCMV disease per patient. Importantly, GCV-resistant HCMV infections accounted for 20% of HCMV diseases that occurred during the first year after transplantation [144]. In a retrospective analysis published in 2008, Eid and colleagues reported a similar rate of GCV resistance in D+/R- recipients of SOT (other than lung) who received VGCV prophylaxis [82].

Boivin and colleagues reported the first prospective study evaluating the emergence of GCV resistance in SOT recipients [30]. In this study, molecular methods were used to assess the emergence of UL97 and UL54 mutations associated with GCV resistance in D+/R– patients (175 liver, 120 kidney, 56 heart, 11 kidney/pancreas, and 2 liver/kidney recipients) receiving HCMV prophylaxis with either oral GCV (1 g TID) or oral VGCV (900 mg OD). Among 301 evaluable patients, the incidence of GCV resistance at the end of the prophylactic period (day 100 post-transplant) was very low in both arms (0% and 1.9% for the VGCV and oral GCV arms, respectively). During the first year following transplantation, GCV resistance-associated mutations were found in none compared to 6.1% of patients at the time of suspected HCMV disease after receiving VGCV and oral GCV prophylaxis, respectively. Of note, however, no lung transplant and a small number of kidney/pancreas recipients were included in this trial, which might explain at least partly the low emergence of GCV resistance in this study as compared to previous ones. Interestingly, several studies have shown that detection of known GCV resistance mutations is not always associated with adverse clinical consequences in non-lung

transplants in contrast to more immunosuppressed lung transplants [30, 32]. Documented risk factors for the emergence of GCV resistance in SOT patients include the lack of HCMV-specific immunity (as encountered in the D+/R– group) [14, 17], lung or kidney/pancreas transplantation, longer drug exposure (prophylaxis > preemptive therapy), suboptimal plasma or tissue drug concentrations (as seen with oral GCV), potent immunosuppressive regimens, a high HCMV viral load, and frequent episodes of HCMV disease [21, 144, 145].

Limited data from small-scale studies suggest that the incidence of GCV resistance in the bone marrow transplant (BMT)/HSCT population might not be as high as observed in SOT recipients and AIDS patients, perhaps because of the more limited immunosuppression exposure and the greater use of preemptive antiviral strategies. In a study published by Gilbert et al., molecular methods were used to detect the presence of the most common UL97 mutations associated with GCV resistance in blood samples of HSCT patients selected on the basis of having a positive HCMV PCR despite ≥14 days of preemptive IV GCV or a second viremic episode within the first 98 days after transplantation [102]. No UL97 mutations associated with GCV resistance were detected in this cohort of 50 patients (10 of them fulfilling the above criteria for genotypic testing) [102]. However, this was a small study, and resistance would be unlikely after just a short period of preemptive treatment. In another study designed to evaluate risk factors and outcomes associated with rising HCMV antigenemia levels during the first 2–4 weeks of preemptive therapy, Nichols and colleagues prospectively evaluated 119 HSCT patients receiving preemptive GCV or FOS therapy following a positive pp65 antigenemia test [167]. Among these subjects, 47 (39%) exhibited a significant rise in antigenemia levels despite antiviral administration, and 15 had at least one isolate available for susceptibility testing. Only one GCV-resistant isolate was identified in a patient who received 4 weeks of GCV therapy [167]. In contrast, Erice et al. reported genotypic or phenotypic evidences of infection with a GCV-resistant HCMV strain in two of five selected patients who had received GCV for a median of 58 days [87]. However, all five patients had also received ACV prophylaxis for a median of 47 days which could have predisposed to the selection of a GCV-resistant HCMV strain [160]. Of note, the impact of prior ACV in selecting for GCV resistance has not been confirmed by another group [74]. Springer et al. also reported two HSCT patients who developed persistent and severe drug-resistant HCMV infections, including one virus with a DNA polymerase mutation conferring multidrug resistance [209]. Even though short courses of GCV therapy are not usually complicated by emerging resistance in adult BMT patients, Eckle et al. reported that the situation might differ in pediatric patients receiving T-cell-depleted unrelated transplants [81]. In their study of 42 such patients, 3 showed genotypic evidences of GCV resistance, followed by the excretion of a resistant strain after 30–93 days of GCV exposure. Of note, in the same study, none of the 37 patients who underwent a similar procedure, but who received their transplant from a mismatched related donor, developed GCV resistance [81]. Rapid emergence of GCV resistance was also documented in four of five children with congenital immunodeficiency disorders who underwent T-cell-depleted BMT [236]. In those patients, genotypic evidence of

GCV resistance was demonstrated after only 7–24 days (median 10 days) of cumulative GCV therapy. Finally, the emergence of GCV-resistant strains has been recently associated with previously uncommon central nervous system HCMV disease and retinitis occurring late after HSCT [111, 237].

8.2.3 Role of HCMV UL97 and UL54 Mutations in Drug-Resistant Clinical Strains

Ganciclovir resistance is mediated by mutations in one or both of the following genes – UL97 and UL54. UL97 is responsible for phosphorylating ganciclovir, and UL54 is the DNA polymerase gene. When resistance develops, it is usually initiated by mutations in UL97 and later mutation(s) in UL54 ensue. Cumulative results obtained in three studies that have documented the emergence of UL97 mutations in clinical isolates [123, 153] or in blood samples [29] from 61 AIDS and SOT patients are in general agreement with the proposed frequency of UL97 mutations based on characterization of 76 independent UL97 mutants gathered in a single laboratory over years [54]. Those data suggest that mutations A594V (30–34.5%), L595S (20–24%), M460V (11.5–14.5%), and H520Q (5–11.5%) represent the most frequent UL97 mutations present in GCV-resistant mutants (Fig. 8.2) [76, 106]. Other frequent UL97 mutations associated with resistance include C592G and C603W. Based on marker transfer experiments, mutations M460V (7× increase in resistance) [49] and C603W (8×) [50], deletion of codons 595–603 (8.4×) [53], H520Q (10×) [112], L595S (4.9–11.5×) [49, 54], A594V (10.7×) [49], and C607Y (12.5×) [12], and deletion of codon 595 (13.3×) [10] appear to be associated with the highest increase in GCV resistance over the parental strain, whereas mutations C592G, A594T, and E596G and deletion of codon 600 seem to confer only modest decreases in susceptibility [54]. Interestingly, analysis of GCV-phosphorylating activity of mutated UL97 genes expressed in a recombinant vaccinia virus expression system would have predicted mutations H520Q and M460V to confer the greatest decrease in GCV susceptibility [13].

Among the most frequent DNA pol mutations associated with drug resistance, there are V715M, V781I, and L802M conferring FOS resistance and F412C, L501I/F, and P522S conferring GCV/CDV resistance (Fig. 8.2) [76, 106]. Mutation A809V conferring GCV/FOS resistance has also been reported with some frequency. Importantly, some mutations (E756K, V812L, and del981–982) have been associated with resistance to all three antivirals, but these mutations are rarely encountered [55, 62]. With regard to the levels of resistance, mutations L501I and K513N and deletion of codons 981–982 have been associated with a six- to eight-fold decrease in GCV susceptibility [55, 62, 63] and mutations F412C/V, K513N, and A987G with a 10–18-fold decrease in CDV susceptibility [50, 62, 63], whereas mutations D588N, V715M, E756K, L802M, and T821I seem to confer a 5.5–21-fold increased resistance to FOS [11, 50, 55, 62, 164]. A few UL54 mutations have

been studied in marker transfer experiments for their effect on viral fitness. Among those, mutations T700A and V715M (conserved region II) [11], K513N (δ-region C) [63], and D301N (Exo I motif) [55] were shown to significantly reduce the yield of progeny virus in cell culture supernatants, whereas some others (D413E, T503I, L516R, and E756K/D) were only associated with a modest attenuation of viral replication [55]. In the case of HCMV DNA pol mutants selected during GCV therapy, it should be noted that UL97 mutations have been generally shown to emerge first and to confer a low level of resistance (EC_{50} < 30 μM), whereas subsequent emergence of UL54 mutations usually leads to a high level of drug resistance (EC_{50} > 30 μM) [86, 122, 205]. Most clinical isolates resistant to GCV have mutations in UL97 only. However, isolated UL54 mutations have been reported occasionally in clinical HCMV strains [31].

8.2.4 Management of Infections Caused by Drug-Resistant HCMV Strains

HCMV resistance to antivirals should be suspected in patients failing treatment who have been exposed to an antiviral for substantial periods of time (typically >3–4 months of treatment in AIDS patients and after 2–3 months or more of prophylaxis or treatment in transplant recipients), especially if some risk factors are present (i.e., D+/R– SOT, lung or kidney/pancreas transplant, AIDS patients with CD4 counts <50 cells/μl). Resistance should be suspected in pediatric patients with shorter periods of drug exposure if they had T-cell depletion. Clinical resistance is more likely if active viral replication (high or increasing levels of DNAemia/antigenemia or viremia) persists or recurs despite maximum IV doses of the antivirals [145, 166]. On the other hand, rising CMV DNA or antigenemia levels during the first 2–3 weeks of antiviral therapy in HSCT recipients have not been associated with antiviral resistance but rather with host and other transplant-related factors [101, 166]. Whenever antiviral resistance is suspected, phenotypic and/or genotypic investigation for resistance should be undertaken. As discussed above, genotypic methods are fast and more convenient and provide useful information for selection of an alternative treatment. However, identification of mutations of unknown significance remains problematic and may require phenotypic assays for validation. Furthermore, genotypic assays do not quantitate the degree of resistance while phenotypic assays do. The choice of the sample to analyze may also have some importance. Some studies have reported that there is a good correlation between genotypes detected in the eyes and the blood (93.5%) [116] or between blood and urine isolates (87.5%) [122] of AIDS patients with HCMV retinitis. However, there have been at least some reports of resistant HCMV strains restricted to specific body compartments [81, 148]. This suggests that resistance assessment based solely on only one bodily fluid or tissue may be misleading in some cases.

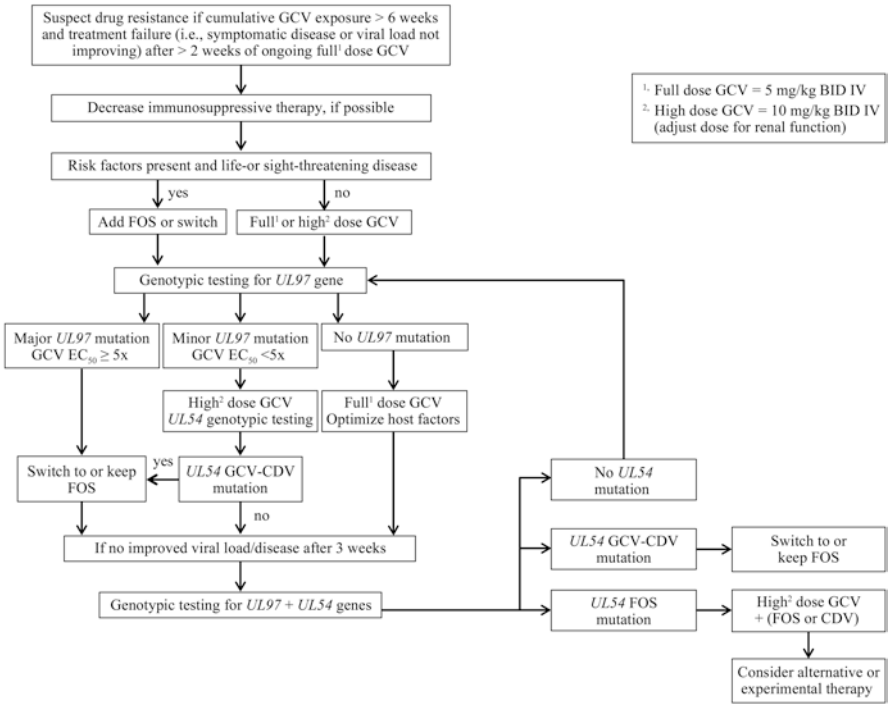

Fig. 8.3 Suggested algorithm for the management of suspected drug-resistant HCMV infections in solid organ transplant recipients. *Key*: GCV ganciclovir, FOS foscarnet, CDV cidofovir, BID twice a day, IV intravenous, EC_{50}, concentration of antiviral that reduces HCMV replication in cultured cells by 50% compared to control (without drug) determined in phenotypic assay. (Adapted from [134])

As mentioned, resistance is more likely when stable or rising viral loads (especially DNAemia levels) or persistence of clinical symptoms are observed more than 2–3 weeks after initiating appropriate full-dose IV antiviral therapy. In this context, clinical decisions on disease management should be based on genotypic analysis of UL97 and UL54 genes, the patient's immune status (e.g., high-risk D+/R– recipients, lung transplant recipients), and disease severity (i.e., sight- or life-threatening conditions) [77, 145] (Fig. 8.3). Despite the limitation mentioned above, genotypic resistance testing is more practical and rapid (results in 72–96 h) than phenotypic assays. Thus, rescue therapy should be ideally based on results of the genotypic assays. In centers where genotypic testing is unavailable or performed infrequently, initial management should avoid the use of drugs with similar pathways of resistance. For instance, patients failing GCV should be given FOS in the absence of any sequencing data because of the possibility of UL54 mutations that could confer resistance to both GCV and CDV. On the other hand, if UL97 and UL54 sequencing data are available and indicate that only UL97 mutations are present, then CDV therapy can be attempted. Other empiric options for patients failing GCV therapy

could consist in re-inducing the patient with higher than normal doses of GCV (up to 10 mg/kg IV BID) or combination therapy with reduced doses of GCV and FOS [165, 208] although these strategies are associated with significant toxicity and can be clinically risky in patients with life- or sight-threatening diseases. Leflunomide, an anti-inflammatory compound, which appears to inhibit viral capsid assembly, has not been systemically evaluated but is the subject of successful case reports [182]. When GCV resistance is encountered, discontinuation of the drug and the use of foscarnet alone may hasten return to "wild," i.e., sensitive virus [78]. Whenever possible, improvement of the patient's immune status (i.e., reduction of immunosuppressive regimen in transplant patients or aggressive antiretroviral therapy in AIDS patients) should also be considered. HCMV viral load should be carefully monitored (once weekly) to quantitate a response to the change in therapy.

8.3 Herpes Simplex Virus and Varicella-Zoster Virus Resistance

8.3.1 Phenotypic and Genotypic Assays to Evaluate HSV and VZV Drug Susceptibility

Four mechanisms are involved in HSV resistance to ACV and lead to different phenotypes: (i) a complete deficiency in viral TK activity (TK-deficient); (ii) a decreased production of viral TK (TK low producer); (iii) a viral TK protein with altered substrate specificity (TK altered), i.e., the enzyme is able to phosphorylate thymidine, the natural substrate, but does not phosphorylate ACV; and finally (iv) a viral DNA pol with altered substrate specificity (DNA pol altered). Alteration or absence of the TK protein is the most frequent mechanism seen in the clinic, probably because TK is not essential for viral replication in most tissues and cultured cells [99, 162]. Thymidine kinase mutants resistant to ACV usually exhibit a reduction in fitness and neurovirulence. In animal models, TK low producers show some reduction in pathogenicity compared with wild-type strains but are generally able to reactivate. In contrast, TK-deficient mutants demonstrate impaired pathogenicity as well as a lower efficiency to establish latency in sensory ganglia and a poor reactivation compared with wild-type strains. However, it has been suggested that ultralow levels of TK enzyme activity could be sufficient to allow reactivation [18]. Mutants with altered DNA pol activity exhibit different degrees of neurovirulence attenuation in animals [2, 67].

The TK phenotype can be determined by the selective incorporation of iododeoxycytidine and thymidine into infected cells using plaque autoradiography [156]. However, it can be difficult to evaluate residual TK activity in HSV isolates of immunocompromised patients in whom heterogeneous populations (TK-competent/TK-deficient) may coexist [99, 191, 223]. Nonisotopic methods have been developed using ADP-Glo kinase assay [39, 174] and DiviTum assay based on

bromodeoxyuridine as substrate [193, 194] to evaluate ACV phosphorylation and thymidine kinase activity, respectively. However, both methods do not distinguish between the three different TK phenotypes. More interestingly, thymidine kinase functionality was assessed by measuring monophosphate forms of both ACV and thymidine by high-pressure liquid chromatography with diode-array detection [155]. Although such enzymatic assays do not allow the determination of the resistance levels conferred by a specific TK mutation, they may facilitate the discrimination between resistance-associated mutations and polymorphic alterations.

Only approximately 5–10% of ACV-resistant HSV strains have polymerase mutations. Resistance to FOS and to CDV is conferred by specific mutations within the viral DNA pol which is the ultimate target of all current antiviral drugs. Depending on the locus of the pol mutation, there may or may not be cross-resistance between ACV, FOS, and CDV [20]. At the present time, no simple enzymatic assay has been described to rapidly assess the DNA pol activity of herpesviruses.

Levels of drug resistance (EC_{50} values) are best measured by cell-based (phenotypic) assays. Such assays are more practical in the case of HSV (and to some extent VZV) than for HCMV considering the more rapid replication kinetics of the former viruses. The gold standard phenotypic method to determine the susceptibility of HSV isolates to antiviral drugs is the plaque reduction assay in Vero cells that is approved as a standard protocol by the Clinical and Laboratory Standards Institute (Wayne, PA) [215]. Herpes simplex virus resistance cutoffs to ACV have varied in the literature from 4.4 μM to 13.2 μM according to the method selected, i.e., plaque reduction or dye uptake assays and various other factors (although a cutoff of 2 μg/ml or 8.8 μM is mostly used with the plaque reduction assay) [44, 65, 84, 89, 188]. Due to this variability, a susceptibility index is said to be a better measure of viral resistance. The ratio of the EC_{50} of the patient's isolate should be at least three times or greater than the EC_{50} of a known, sensitive HSV control [190].

Susceptibility of VZV to ACV can be tested in plaque reduction assays using fibroblastic cell lines such as MRC-5 [93]. The use of the plaque reduction assay is limited by the low rate of VZV isolation from vesicle samples (from 20% to 43%) and its slow growth in cell culture (typically 5–6 days) [195]. The end point for detecting resistance is a susceptibility index greater than or equal to four, i.e., the test strain has an EC_{50} greater than four times that of a control, known sensitive strain, e.g., the Oka strain. Regarding absolute values, three resistant strains from a single series had mean ACV EC_{50} values of 85 μM vs 3.3 μM for the Oka strain [189].

An alternative to phenotypic assays is genotyping by sequence analysis. For a comprehensive genotypic analysis, the whole TK gene and the conserved regions of the DNA pol gene of HSV or VZV should be sequenced because of the large number of TK mutations (substitutions, deletions, and additions) as well as DNA pol mutations associated with drug resistance [163]. The development of fast and efficient methods for detecting viral mutant sequences directly in clinical specimens by next-generation sequencing [128] should improve the evaluation of heterogeneity and temporal changes that occur in populations of drug-resistant viruses during antiviral therapy [4]. As some degree of inter-strain variability exists in these genes,

mutations conferring drug resistance must be discriminated from natural polymorphisms. Different systems can be used to generate recombinant HSV or VZV and evaluate their phenotype of drug resistance, such as the transfection of a set of overlapping cosmids and plasmids allowing rapid site-directed mutagenesis [20, 202] and the cloning of the viral genome coupled with a reporter gene expressing a fluorescent protein into a bacterial artificial chromosome [36, 37]. Compilations of confirmed drug resistance mutations and natural polymorphisms in the TK and DNA pol genes of HSV [196] and VZV [177] are described in several reviews.

8.3.2 Clinical Significance, Incidence, and Risk Factors for Drug-Resistant HSV and VZV Infections

Antiviral drugs against herpesviruses provide some of the best examples of effective and selective antiviral therapy. However, drug-resistant viruses have been rapidly selected in the laboratory and also identified in the clinic. Contrasting with HCMV resistance data, no extensive survey has been performed to evaluate the rate of emergence of drug-resistant HSV isolates according to the duration of antiviral therapy. Such study would be a difficult task considering that oral and topical ACV formulations are widely used.

In immunocompetent hosts, HSV resistance to ACV is not a clinically important problem. Studies have shown that 0.1–0.6% of HSV isolates recovered from untreated, prophylaxed, or treated immunocompetent subjects harbor a resistant phenotype to ACV ($EC_{50} \geq 8.8$ μM) as assessed by a plaque reduction assay, and this seems to reflect the natural occurrence of TK-deficient mutants in a viral population [8, 9, 33, 60, 69, 91, 159, 211, 232]. Except for a few notable cases [133, 136, 214], the occasional recovery of ACV-resistant HSV-2 from immunocompetent hosts has not been associated with clinical failure and proved to be transient [110, 232]. A higher prevalence (6.4%) of ACV-resistant HSV-1 isolates has been reported in immunocompetent patients with recurrent herpetic keratitis [80], and some of these cases were clinically refractory to ACV therapy [41, 125, 170, 224]. The lower immune surveillance in the cornea, which is an immune-privileged site, could explain the rapid selection of drug-resistant viruses [6]. Herpes simplex virus strains resistant to ACV are more often isolated in immunocompromised hosts, and such isolates have been associated with persistent and/or disseminated diseases [26, 47, 60, 84, 89, 114, 162, 184, 228]. In the few clinical surveys reported, the rate of ACV-resistant HSV isolates has varied from 4.3% to 14% among all immunocompromised groups [60, 69, 72, 84, 143, 169, 181, 211]. The prevalence of ACV resistance has ranged from 3.5% to 7% in HIV-positive patients in several studies [60, 84, 143, 149, 181, 239]. It is estimated that 6.5% of HSV isolates obtained from patients with cancer were resistant to ACV compared to 10% from heart or lung transplant recipients [60] and 5–14% from other SOT recipients [84]. Of note, high resistance rates have been reported in HSCT recipients, ranging from 4.1% to 10.9%

[47, 69, 85, 96, 162, 229, 233]. In another study, 8% of allogeneic cell transplant recipients demonstrated persistent HSV excretion despite ACV therapy, whereas 5% of HSV isolates showed significant level of ACV resistance in vitro [42]. Morfin and Thouvenot reported that patients receiving either autologous or allogenic bone marrow have a similar incidence, i.e., 9%, of HSV infection, but resistance only occurred in allogenic transplants, reaching a prevalence of 30% [163]. The severity of immunosuppression and the prolonged use of ACV are considered two important factors for the development of drug resistance. The importance of the severity of immunosuppression is underscored by Langston et al. who studied adult patients undergoing lymphocyte-depleted hematopoietic progenitor cell transplant from HLA-matched family donors [140]. All seven evaluable HSV-1 or HSV-2 seropositive patients reactivated at a median of 40 days posttransplant, and the five strains tested were all resistant to ACV. Furthermore, FOS resistance developed rapidly in the three patients treated with this drug [140]. Importantly, the prevalence of ACV-resistant HSV isolates has remained stable in immunocompromised patients over the past decades [69, 211], and there has been no unequivocal evidence of transmission of a resistant HSV strain from person to person.

The emergence of VZV isolates resistant to ACV has not been described in immunocompetent individuals with primary VZV infection or herpes zoster, except for one case report of a patient with an ACV-resistant VZV keratitis [109]. Acyclovir-resistant VZV isolates in the clinic have been mainly found in AIDS patients with low CD4 cell counts who presented with atypical, disseminated, or relapsing zoster lesions [27, 161, 189, 216, 226]. Cases of resistance to ACV have also been described in SOT and HSCT recipients as well as in hemato-oncological patients with VZV reactivations unresponsive to therapy. In these patients, VZV infections not responding to ACV therapy persist in the form of chronic skin lesions and are associated with significant morbidity and mortality due to visceral dissemination. An unusual verrucous form of VZV infection caused by ACV-resistant mutants has also been described in some patients [38, 66]. Two cases of immunocompromised children presenting herpes zoster due to the Oka vaccine strain and who developed chronic, disseminated drug-resistant VZV infections following ACV therapy have been reported [38, 142]. However, the prevalence of ACV-resistant cases in these different populations is unknown because only case reports have been published so far. It was reported that 27% of hemato-oncological patients, including HSCT recipients, with persistent VZV infections had mutations probably associated with ACV resistance [222].

Only a few FOS-resistant HSV ($EC_{50} \geq 330$ μM or ≥ 100 μg/ml and at least a threefold increase in EC_{50} value compared with the parental susceptible strain) have been reported in the clinic [64, 119, 199]. Nine FOS-resistant HSV clinical isolates from HIV-infected subjects in whom ACV and FOS therapy sequentially failed have been described [19, 199]. A few reports have described the emergence of VZV strains resistant to FOS in immunocompromised patients [16, 92, 186, 226, 227].

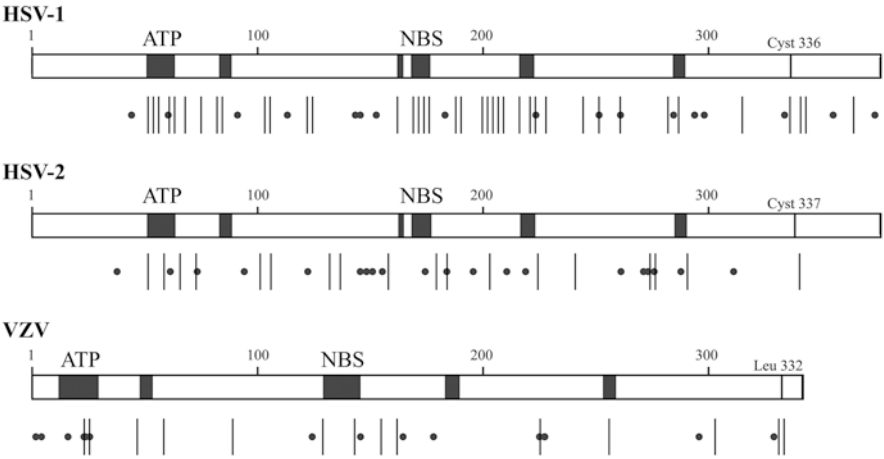

Fig. 8.4 Confirmed mutations associated with acyclovir resistance in the UL23 and ORF36 gene of clinical HSV-1, HSV-2, and VZV isolates. Conserved regions among the thymidine kinases of *Herpesviridae* including the ATP-binding site (ATP) and the nucleoside-binding site (NBS) are represented by the black boxes. Scale represents nucleotide positions in the gene. Bars (|) indicate amino acid substitutions, whereas dots (●) represent nucleotide additions and/or deletions that confer resistance to acyclovir

8.3.3 Role of Viral TK and DNA Polymerase Mutations in Drug-Resistant Clinical Strains

Mutations in the TK of HSV (encoded by the UL23 gene) leading to ACV resistance consist of either additions or deletions in homopolymer runs of Gs and Cs associated with a premature stop codon or single nucleotide substitutions in conserved and non-conserved regions of the gene (Fig. 8.4) [176, 177]. Each mechanism accounts for approximately 50% of ACV-resistant phenotypes in the clinic [99, 162]. However, recent studies reported an increased proportion of additions/deletions which accounted for 62% [97] or even 80% [40] of TK gene mutations. Nucleotide substitutions are scattered within the TK gene including the three catalytic sites of the enzyme (ATP-binding site, nucleoside-binding site, and a.a. 336) [70]. Albeit rarely seen in clinic, most mutations in the DNA polymerase of HSV (encoded by the UL30 gene) conferring drug resistance are located in the conserved regions of the enzyme, most specifically in regions II, VI, III, and VII (Fig. 8.5) which are directly or indirectly involved in the recognition and binding of nucleotides or pyrophosphate as well as in catalysis [176, 177]. The greatest clusters of mutations in the DNA pol enzyme have been found in conserved regions II and III. Most FOS-resistant clinical HSV isolates contain single-base substitutions in conserved regions II, VI, III, or VII and in a non-conserved region (between I and VII) of the DNA pol. Some of these isolates retain susceptibility or borderline levels of susceptibility to ACV and CDV. However, some mutations, in particular in regions II (V715G and S724N) and VII (Y941H) of the DNA pol, can confer resistance to both ACV and

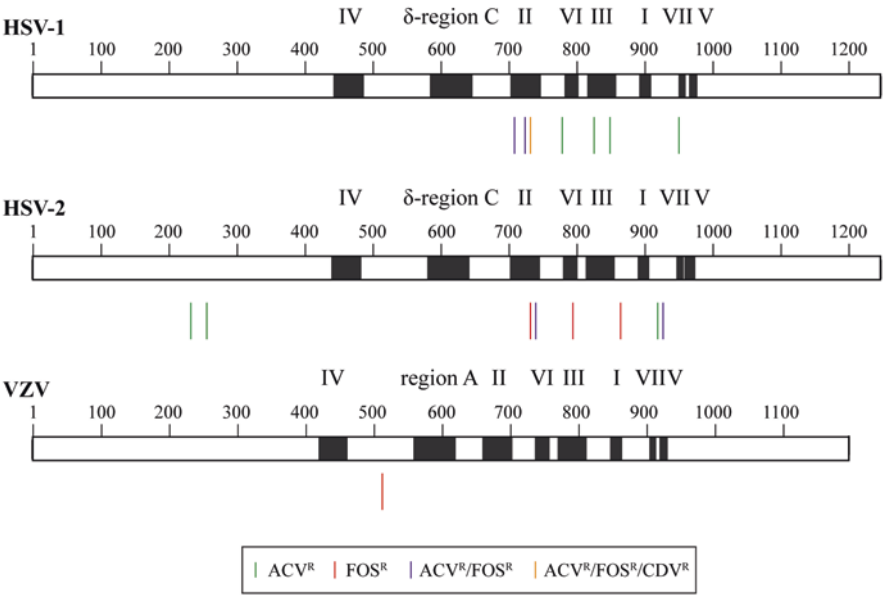

Fig. 8.5 Confirmed mutations associated with resistance to acyclovir (ACV^R), foscarnet (FOS^R), and/or cidofovir (CDV^R) in the UL30 and ORF28 genes of clinical HSV-1, HSV-2, and VZV isolates. Conserved regions among the *Herpesviridae* DNA polymerases are represented by the black boxes. The roman numbers (I to VII) and δ-region C or region A corresponding to each of these regions are indicated above the boxes. Scale represents nucleotide positions in the gene. Colored bars (|) indicate amino acid substitutions

FOS. Mutations associated with CDV resistance mapped in DNA pol regions II (R700M), VI (L773M), III (G841C and G850I), and VII (Y941H) and in δ-region C located in the Exo III motif (V573M).

A recent study of Topalis et al. showed that mutations conferring drug resistance in DNA pol of HCMV are mostly detected in the 3′-5′ exonuclease domain (60.6%) and to a lower extent in the palm (18.2%), fingers (16.7%), and thumb (4.6%) domains, whereas those identified in DNA pol of HSV are mainly located in the palm (25.0%), fingers (25.0%), and thumb (21.5%) domains with a lower proportion of mutations being found in the 3′-5′ exonuclease domain (27.3%) [220]. The different distribution of mutations in DNA pol domains may reflect various mechanisms of drug resistance. Mutations conferring resistance to nucleoside analogues located within conserved regions of the pol domain might reduce the binding of the inhibitor or the incorporation of the active drug into growing DNA [117]. It has been suggested that mutations conferring resistance to nucleoside analogues located in the exonuclease domain might enhance the rate of excision of the incorporated drug [55]. However, a recent study demonstrated that mutant HCMV with reduced exonuclease activity might efficiently synthetize DNA in the absence of drug excision [46]. It has been proposed that resistance of HSV-1 and HCMV to FOS may

result from subtle conformational changes in the DNA pol that adopts a more open conformation to which the drug binds with a lower affinity [178, 218, 238].

In VZV clinical isolates, resistance to ACV is mostly associated with mutations in the viral TK (encoded by the ORF36 gene) and, less frequently, with mutations in the viral DNA pol (encoded by the ORF28 gene) [177, 179]. The string of six cytosines located at codons 493–498 of the TK gene emerged as a hot spot for the insertion or deletion of nucleotides involved in ACV resistance (Fig. 8.4) [3, 27, 161, 222]. Deletions of nucleotides that result in frameshift reading leading to a stop codon at position 231 are often detected in ACV-resistant VZV clinical isolates [161]. In addition, nonsynonymous nucleotide substitutions conferring resistance to ACV are widely dispersed in the TK gene. However, these amino acid changes occur more frequently in the ATP-binding and nucleoside-binding sites of the TK enzyme [27, 93, 161, 189, 192, 197, 216]. A few reports have described ACV-resistant and/or FOS-resistant VZV clinical isolates with mutations in the DNA pol gene (Fig. 8.5) [127, 192, 226]. The amino acid substitutions are mainly found in the catalytic site and in the conserved regions of the DNA pol and may confer cross-resistance to ACV and FOS [177, 179]. The TK and DNA pol genes of VZV are highly conserved compared with those of HSVs, and only a few natural polymorphisms have been identified in these genes [192].

8.3.4 Management of Infections Caused by Drug-Resistant HSV and VZV Strains

With the emergence of ACV-resistant HSV infections observed in patients with AIDS and other immunocompromised hosts, several studies have examined the utility of alternative antiviral agents and treatment regimens. Standard doses of oral ACV have no clinical benefit if the HSV isolate is resistant to ACV in vitro. Most ACV-resistant strains isolated from immunocompromised patients are TK-deficient and are therefore also resistant to PCV and its prodrug FCV. The persistence of active lesions due to HSV for more than 7–10 days after initiation of high-dose oral ACV, VACV, or FCV therapy without apparent decrease in size, an atypical appearance, or the emergence of satellite lesions is suggestive of treatment failure (Fig. 8.6). In the presence of suspected or confirmed resistance to ACV, the initial options are either to switch to high-dose oral VACV or IV ACV (10 mg/kg of body weight every 8 h adjusted for renal function). Acyclovir-resistant HSV strains remain usually susceptible in vitro to vidarabine (a purine nucleoside analogue), which is phosphorylated without TK and appears to interfere with the early steps of viral DNA synthesis, and to FOS, which does not require phosphorylation for activity. Studies have confirmed that FOS is superior to vidarabine in the treatment of these TK-deficient, drug-resistant HSV infections [43, 88, 187]. If lesions do not begin to respond to high-dose oral VACV or IV ACV within 5–7 days, a switch to IV FOS (40 mg/kg every 8 h with reduction in dose for renal dysfunction) should be

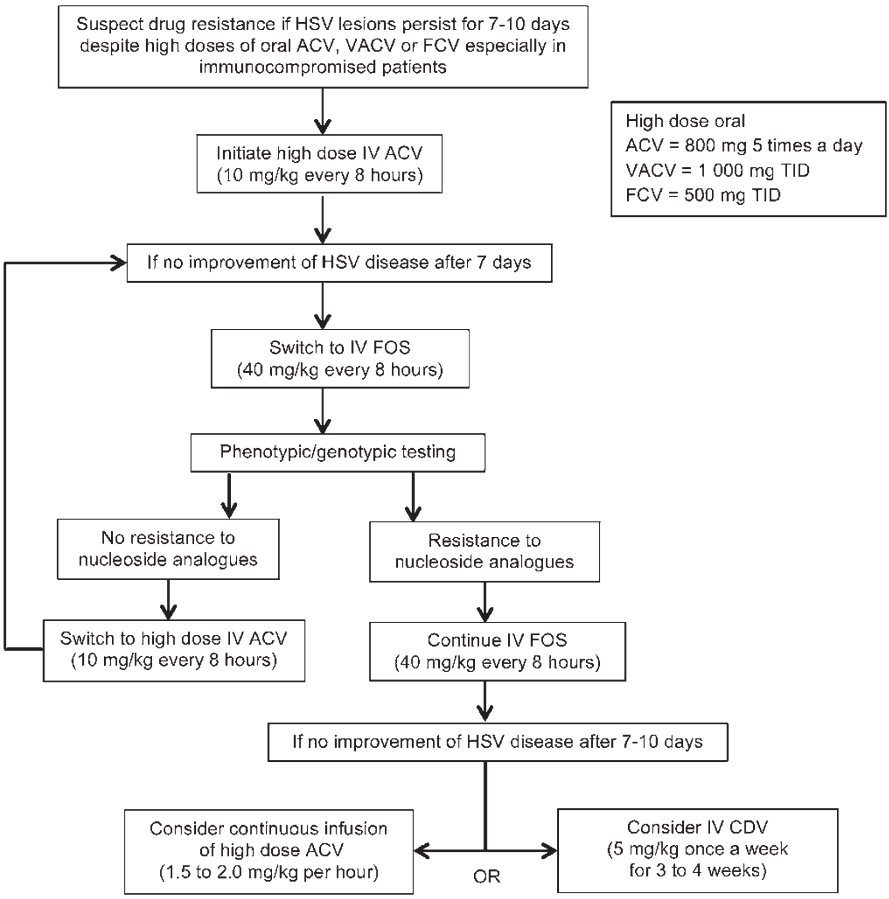

Fig. 8.6 Proposed algorithm for the management of suspected nucleoside analogue-resistant HSV infections. *Key*: ACV acyclovir, VACV valacyclovir, FCV famciclovir, FOS foscarnet, CDV cidofovir, TID thrice a day, IV intravenous

considered. In parallel, isolates from the lesions should be submitted for susceptibility testing and/or genotypic analysis of the TK gene. If there is still no improvement of HSV disease after 7–10 days, continuous infusion of high-dose ACV at a dosage of 1.5–2.0 mg/kg/h for 6 weeks could be initiated, as it is a well-tolerated option for severe ACV-resistant or multidrug-resistant HSV infections [83, 132]. Cidofovir (5 mg/kg once a week for 3–4 weeks) could be considered, as it has shown some efficacy in the treatment of progressive multidrug-resistant mucocutaneous HSV infection in immunocompromised patients [150, 207], but it is not approved for this indication. Although topical formulations of FOS [126] and CDV [138, 183] were effective in the treatment of mucocutaneous infections not responding to ACV and could avoid the adverse effects associated with their IV administration, they are not commercially available. A topical formulation containing 5% imiquimod, an

immunomodulatory drug, was effective in the treatment of recurrent and severe mucocutaneous lesions due to ACV-resistant HSV-2 isolates in HIV-infected individuals [141]. Ophthalmic ointment containing 3% vidarabine is indicated for the treatment of acute keratoconjunctivitis and recurrent epithelial keratitis due to HSV. A final option for topical therapy is trifluorothymidine (a fluorinated deoxyuridine analogue that inhibits thymidylate synthase) as an ophthalmic solution which may be applied to the affected area three to four times a day until the lesion is completely healed [48, 130].

As with many opportunistic infections in AIDS patients, there is a high incidence of recurrent HSV disease after successful treatment of drug-resistant HSV. Some (but not all) relapses in this setting have been due to drug-resistant strains, suggesting that these mutant viruses are capable of causing latency in the immunocompromised host. Chronic prophylaxis with daily ACV, VACV, FCV, or FOS can be considered in patients who have been treated successfully for drug-resistant HSV, although there are no data to confirm efficacy in this setting. Foscarnet-resistant strains of HSV have been reported, raising concerns over the possible selection for multidrug-resistant HSV with suppressive therapy [199, 207].

Drug-resistant VZV strains have been identified in patients with AIDS, SOT, HSCT, and hemato-oncological patients. These patients may present with atypical-appearing cutaneous lesions that shed VZV intermittently despite ongoing high-dose antiviral therapy. Visceral dissemination of the infection could also lead to significant morbidity and mortality in these patients. The persistence of clinical signs of VZV infections for more than 10–14 days after initiation of high-dose oral ACV is suggestive of treatment failure, and it should lead to alternate therapy depending on the clinical severity of the disease [1]. Strains have been isolated from patients previously treated with ACV for recurrent VZV or HSV infection, and these strains may be resistant to ACV, VACV, and FCV by deficiency of the TK enzyme [27, 124, 161]. Genotypic testing of the TK gene could be performed in samples from vesicular fluids, biopsy of mucocutaneous lesions, or other body compartments when necessary [35]. Foscarnet has been shown to be effective in small studies conducted mainly in HIV-infected individuals [34, 186] and some oncology patients [38, 66, 142], but, as with HSV, cross-resistance between ACV and FOS may occur due to viral DNA polymerase mutations [186]. The IV dosage recommended for FOS is 60 mg/kg three times daily for at least 10 days or until complete lesion healing is observed [1]. Clinical experience with the use of CDV in the treatment of drug-resistant VZV diseases is very limited [198].

8.4 Conclusions and Future Directions

All currently available antiviral agents target the viral DNA pol. The development of new anti-herpetic compounds with different mechanisms of action and with adequate safety profile is urgently needed. Some promising compounds are currently in clinical trials. The orally bioavailable lipid ester prodrug of CDV (hexadecyloxypropyl-cidofovir; brincidofovir) could avoid the dose-limiting toxicity of the parent drug and

provide a safe alternative for nucleoside analogue-resistant herpesviruses in immunocompromised patients [115]. Treatment with oral brincidofovir significantly reduced the incidence of HCMV infections in HSCT recipients in a phase II study [157]. Diarrhea was a dose-limiting adverse event in this population at a dose of 200 mg twice weekly. Maribavir is a competitive inhibitor of the UL97 kinase [23]. Mutations selected in vitro with maribavir often map to the UL97 gene; low-level resistance mutations are also detected in the UL27 gene and seem to be the result of a loss of UL97 kinase activity. Mutations in the UL97 gene conferring resistance to maribavir are generally distinct from those described in GCV-resistant strains, and some have been detected outside the conserved kinase domains [57]. Thus, maribavir retains activity against most GCV-resistant HCMV mutants. However, some mutations, such as mutation F342S which is located in the p-loop, confer cross-resistance to GCV and maribavir [58]. The emergence of resistance to this drug has been reported in some clinical cases [201, 212]. Letermovir targets the terminase complex of HCMV and interferes with viral DNA concatemer maturation [107, 147]. Accordingly, mutations conferring resistance to letermovir map to the UL56 gene encoding the HCMV terminase [108]. Successful treatment of a multidrug-resistant HCMV infection with letermovir has been reported in a lung transplant recipient [129]. Preemptive treatment of HCMV infection with letermovir was effective in kidney transplant recipients [210]. Moreover, prophylaxis with letermovir was effective in reducing the incidence of HCMV infection in HSCT recipients [45]. Pritelivir, a potent orally bioavailable helicase-primase inhibitor, reduced the rate of genital HSV-2 shedding and days with lesions in a phase II trial [230]. A humanized monoclonal antibody was shown to be effective for immunotherapy of severe HSV infections, including those caused by multidrug-resistant isolates, in immunocompromised mice and warrants further clinical developments [135]. The bicyclic nucleoside analogue FV-100 and carboxylic nucleoside analogue valomaciclovir were well tolerated and effective for the treatment of herpes zoster in phase II trials [5, 221]. Novel classes of antiviral agents targeting the ribonucleotide reductase, the helicase-primase complex, and the process of viral DNA encapsidation are at earlier stages of development [90].

Major Points

- Resistance of herpesviruses to antiviral drugs is mostly detected in immunocompromised patients but it is increasingly recognized in immunocompetent individuals with herpetic keratitis.
- Genotypic testing is more frequently used for the detection of antiviral drug resistance in herpesvirus infections.
- Interpretation of genotypic testing requires a database linking amino acid changes to mutations associated with natural polymorphisms or drug resistance.
- Algorithms are proposed for the management of infections caused by drug-resistant herpesvirus strains.
- Novel antiviral agents acting on viral targets other than the viral DNA polymerase are in development for the treatment of herpesvirus infections.

Acknowledgments This study was supported by a Foundation Grant from the Canadian Institutes of Health Research (grant no. 148361 to G.B.). G.B. is the holder of the Canada research chair on emerging viruses and antiviral resistance.

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Chapter 9
Heteroresistance: A Harbinger of Future Resistance

Karl Drlica, Bo Shopsin, and Xilin Zhao

9.1 Introduction

Heteroresistance is a condition in which a microbial population contains subpopulations whose minimal inhibitory concentration (MIC) is above the resistance breakpoint, while the bulk population MIC is below that breakpoint. Since heteroresistant infections usually respond favorably to antimicrobial treatment, largely due to effective host defense systems, heteroresistance has often been seen as a minor problem for treating individual patients. However, when heteroresistance is considered as an intermediate state in the evolution to resistance, it is a warning sign – a window through which we can see the future.

Emergence of resistance is important for individual patients with three diseases: tuberculosis, malaria, and HIV disease. On a global basis, these diseases are top-ranked in terms of mortality. However, in industrialized countries, individual patients are more troubled by horizontal transmission of resistance, especially with

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I. W. Fong et al. (eds.), *Antimicrobial Resistance in the 21st Century*, Emerging Infectious Diseases of the 21st Century, https://doi.org/10.1007/978-3-319-78538-7_9

opportunistic infections caused by bacteria such as *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae* [1, 2]. For these organisms, the de novo emergence of resistance in individual patients is thought to occur rarely or to have little clinical consequence. In the absence of immediate consequences, little incentive has existed to implement dosing strategies for restricting the emergence of resistance [3]. That perspective may soon change as heteroresistance becomes increasingly common with many different opportunistic pathogens.

The present chapter begins with a brief overview of heteroresistance by considering detection methods, types of genetic changes involved in heteroresistance, and general resistance features of pathogen categories. We then consider the phenomenon in two phylogenetically distant pathogens, *Mycobacterium tuberculosis* and methicillin-resistant *S. aureus* (MRSA). These two organisms, both of which pose serious antimicrobial resistance problems, serve to illustrate how the path to resistance depends on the pathogen, the drug, the fitness of pathogen variants, and the epidemiology of infection. With *M. tuberculosis,* many of the genetic changes associated with heteroresistance are the same as those causing complete resistance. Thus, DNA-based detection methods are practical. With MRSA, we see a situation in which fitness costs limit the evolution of vancomycin resistance to an intermediate state called VISA (vancomycin-intermediate *S. aureus*). Multiple evolutionary pathways to VISA exist, which makes the development of DNA tests challenging. Examination of these two pathogens may eventually lead to an understanding of factors that determine the outcome of host-pathogen-antimicrobial encounters. Moreover, the resulting framework should help us predict failure of particular therapeutic interventions. We conclude the chapter by surveying other pathogens for which heteroresistance is beginning to threaten standard surveillance and diagnostic procedures. In sum, heteroresistance is an under-reported phenomenon that will become increasingly important as we move deeper into the era of antimicrobial resistance. Readers interested in an earlier review of heteroresistance are referred to Ref. [4].

9.2 Overview of Heteroresistance

9.2.1 Detection of Heteroresistance

Heteroresistance manifests itself in several ways. The most graphic is the growth of bacterial colonies within a zone of inhibition created when an antimicrobial diffuses from a central source on agar that had been covered with bacteria prior to incubation (for example, see Fig. 1 in Ref. [7]). If the colonies in the inhibition zone test positive for antimicrobial resistance using assays that measure minimal inhibitory concentration (MIC), the overall population is said to be heteroresistant. When those resistant colonies continue to test resistant following multiple rounds of growth in or on drug-free medium, the heteroresistance is said to be stable. Many examples exist in which the resistance phenotype is lost during subculturing in the absence of drug. Those situations are called unstable heteroresistance. The “colonies within the inhibition zone” is the easiest method for detecting heteroresistance and is

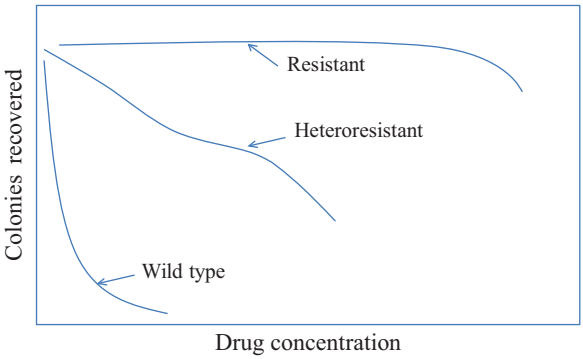

Fig. 9.1 Population analysis profile. A bacterial culture or specimen is applied to a series of agar plates containing different concentrations of the test antimicrobial. After incubation to allow colony formation, colonies are counted and plotted for each drug concentration. A resistant culture is unaffected by the drug until concentrations are very high, while a fully susceptible culture (wild type) exhibits a sharp drop in colony recovery at MIC. Results for a heteroresistant culture containing a variety of subpopulations having reduced susceptibility are depicted. (Data for hVISA can be seen in Refs [5, 6])

commonly used as an initial screen with samples that would otherwise be scored as susceptible by diagnostic laboratories.

With some pathogens, discordance in susceptibility testing indicates heteroresistance. For example, with *M. tuberculosis,* DNA tests may indicate the presence of mutations associated with drug resistance, while drug susceptibility testing (determination of MIC) indicates that the isolate is in the drug-susceptible category. Discordance can also occur between liquid-growth and agar-plate tests. In both situations the discordance arises from assay sensitivity differences.

The gold standard for establishing heteroresistance is detection of "resistant" subpopulations in a population analysis profile (PAP, Ref. [8]). For this assay, a series of agar plates is prepared such that each plate contains a different concentration of drug. A large number of cells, generally $>10^6$, are applied to each agar plate, and after incubation at the appropriate growth temperature (usually 37 °C), the number of colonies is scored. A fully susceptible pathogen isolate will exhibit a sharp drop in colony number when the drug concentration in the agar reaches MIC. In contrast, a heteroresistant isolate will show colonies at concentrations above MIC. The resulting plot of colony number versus drug concentration is the population analysis profile (Fig. 9.1). The area under the curve (AUC) generated by the PAP provides an integrated description of the heteroresistant subpopulations; normalization to a reference strain lacking detectible heteroresistance provides a single number for comparing the heteroresistance status of pathogen samples.

Although PAP can be readily applied to any pathogen that forms colonies on solid medium, including mycobacteria [9, 10] and fungi [11], the method is very labor intensive. Thus, it is generally used only for research purposes or to confirm the presence of heteroresistance in a clinical setting. For research it is important to recognize that incubation time can be a factor if the antimicrobial induces resis-

tance: in the case of fluoroquinolones, the number of colonies increases dramatically over the course of 2 weeks with rapidly growing bacterial species [12].

An important issue for all detection methods is how the patient specimen is handled (material directly collected from a patient is called a specimen). When specimens are examined without subculturing, the percent heteroresistance reflects pathogen subpopulations at a particular location within a given patient at a particular time. Such specifications are important with diseases such as tuberculosis, because considerable heterogeneity exists within a patient (discussed below).

Frequently a specimen is plated on agar prior to testing the predominant colonies for drug susceptibility. Clonal expansion of those colonies generates a sample called an isolate. When an isolate tests positive for heteroresistance, heterogeneity could have been produced during expansion of the culture. Such isolates would have an elevated propensity to *generate* heteroresistance. For example, a gene amplification might occur more frequently in such an isolate, or a mobile resistance element might be lost from some cells in the population. The percent of isolates showing heteroresistance reflects the prevalence of patients having a heteroresistance-prone infection. In contrast, direct examination of specimens reflects both the resistant subpopulation within an individual patient and the prevalence of patients harboring heteroresistance-prone clones.

9.2.2 Types of Heteroresistance

Antimicrobial heteroresistance represents a point along the evolutionary path that pathogens take toward complete resistance or, in some cases, the loss of a resistance element that exerts an excessive fitness cost in the absence of antimicrobial. The path varies considerably among pathogen and antimicrobial species [4]. In some cases, multiple paths exist. A major distinction among heteroresistance types concerns their origin. In one type, heterogeneity arises from coinfection with multiple, dissimilar infecting strains. Such a situation may be common with tuberculosis due to spread of disease from one person to another that leads to superinfection (discussed below). Alternatively, diversity can evolve along clonal lines; this is the usual scenario when superinfection is rare. Clonal heteroresistance, in turn, has two forms. One is derived from infection by a single pathogen cell followed by clonal expansion; the other derives from infection by multiple cells followed by clonal expansions.

Another major distinction is whether the diversity is genetically stable. Fitness is an important consideration, as some resistance features are maintained in the population only when antimicrobial pressure is present, while others persist through multiple passages in drug-free medium. In Fig. 9.2 we illustrate common types of clonal heteroresistance.

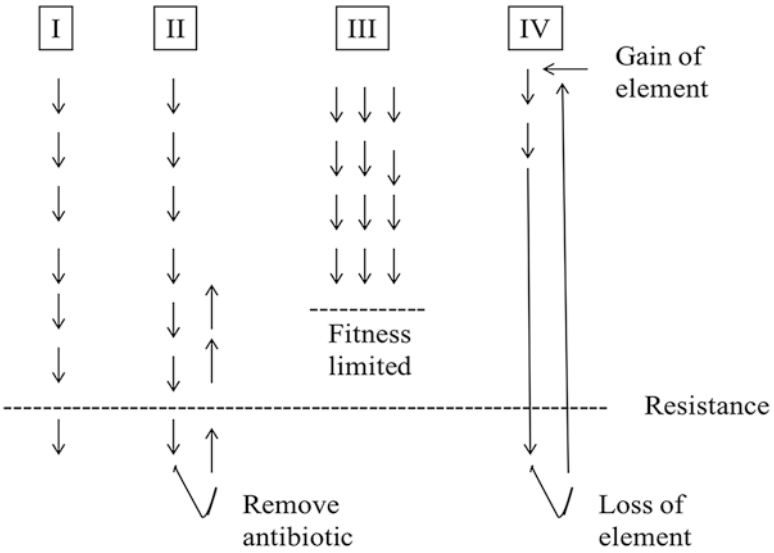

Fig. 9.2 Common types of clonal heteroresistance. Four general themes are shown schematically. Type I represents the acquisition of a resistance mutation, either spontaneously or by horizontal transfer, that is maintained in the population. Resistant subpopulations are enriched with each antimicrobial challenge until resistant cells dominate the population. Examples are fluoroquinolone and rifampicin resistance in *M. tuberculosis*. In Type II heteroresistance, antimicrobial pressure is needed to maintain the resistant phenotype as it is enriched. When drug pressure is removed or relaxed, susceptible members of the population regain dominance. An example of this type of heteroresistance is represented by gene amplification in *S. enterica*. Type III illustrates a situation in which multiple pathways lower susceptibility and also reduce pathogen fitness. Fitness problems can limit the loss of susceptibility to a state called intermediate resistance. An example of Type III heteroresistance is seen with vancomycin-intermediate *S. aureus*. In Type IV heteroresistance, a resistance determinant enters the population by horizontal transfer and is rapidly enriched due to continuing horizontal transfer. If the element is unstable, it can be lost when antimicrobial pressure is reduced. Heteroresistance is seen as a balance of resistance acquisition, loss, and antimicrobial pressure. An example of Type IV heteroresistance is methicillin-resistant *S. aureus*

9.2.3 Heteroresistance and Antimicrobial Tolerance

Tolerance is a situation in which pathogen growth (reproduction) is blocked by an antimicrobial, but the pathogen is not killed. In contrast, resistant pathogens reproduce in the presence of the antimicrobial, and susceptible ones die. As with resistance, genes are involved in some types of tolerance [13]. Phenotypic tolerance derives from environmental conditions that block antimicrobial lethality. For example, some β-lactams and first-generation quinolones fail to kill *E. coli* in cultures that have been grown to stationary phase. The clinical danger from tolerant pathogens is population outgrowth following removal of the antimicrobial; in contrast, heteroresistant pathogens are dangerous even during treatment. Tolerance, or persistence as it is sometimes called, is particularly problematic with tuberculosis – it is estimated that a third of the global human population is infected with *M. tuberculosis* in a tolerant, asymptomatic state called latency. For additional discussion of tolerance see Chap. 13.

9.2.4 Pathogen Types Displaying Heteroresistance

Although a single cell can acquire resistance in a single step, pathogen populations generally require amplification of the resistant cell. During selective amplification, the population will be heteroresistant. Thus, heteroresistance may be a general aspect of the emergence of resistance.

Among infections exhibiting heteroresistance are those caused by commensal bacteria that occasionally act as pathogens. Important examples include MRSA, vancomycin-resistant *Enterococcus* (VRE), *Acinetobacter baumannii*, *Escherichia coli*, and *Klebsiella pneumoniae*. Repeated antimicrobial exposure, sometimes aimed at other bacterial species, results in subpopulations of resistant mutants. These mutant subpopulations can be enriched during treatment and thereby restrict therapeutic options when the microbes cause infection. *E. coli*, a common inhabitant of the human digestive tract, serves as an example. Fluoroquinolone treatment for a variety of reasons unrelated to *E. coli* populations can selectively enrich fluoroquinolone-resistant *E. coli* in the digestive tract. If these organisms contaminate the urinary tract, they can cause fluoroquinolone-resistant urinary infection, which is now a global problem [14].

Heteroresistance is also associated with pathogens for which infection is required for transmission. Among these obligate pathogens are *M. tuberculosis* and the human immunodeficiency virus (HIV). Spontaneously resistant mutants emerge readily, which makes every treated patient at risk for developing a resistant infection. As a result, the standard of care involves the use of multiple antimicrobials. With tuberculosis, the antimicrobials are administered daily by healthcare workers to assure adherence to treatment protocols. In the next section, we consider heteroresistance in *M. tuberculosis* as an example of emerging resistance with an obligate pathogen.

9.3 Heteroresistance with *Mycobacterium tuberculosis*

9.3.1 Emergence of Resistance in Individual Tuberculosis Patients

With individual patients, monotherapy for tuberculosis often leads to the emergence of resistance and treatment failure [15–17]. Host defense systems appear unable to readily clear *M. tuberculosis*, and thus some tuberculosis patients harbor large numbers of pathogen cells (on the order of 10^9) [18, 19]. This feature, coupled with the early finding that cell cultures contain resistant mutants at a high frequency (about 10^{-6} for isoniazid and 10^{-8} for rifampicin and streptomycin [17, 20–22]), led to the idea that monotherapy simply enriches existing mutant subpopulations. More recent measurement of mutation rate, which avoids the jackpot effects of frequency assays, suggests that mutation rate for cultured *M. tuberculosis* is similar to that of other

bacteria (discussed by McGrath et al. [23]). Thus, lack of immune clearance, which is especially obvious with patients coinfected with HIV, results in heavy bacterial burden; the bacterial load is probably a key factor in the emergence of resistance rather than an abnormally high mutation rate. With *M. tuberculosis*, mutagenesis can be induced by DNA damage [24], which likely contributes to the mutagenic effect of some antimicrobials. As expected, combination therapy largely overcomes the rapid emergence of resistance [25].

Enrichment of mutant subpopulations is further favored by the long treatment time required to achieve cure. During infection, *M. tuberculosis* is thought to remodel its metabolism such that part of the bacterial population enters a drug-tolerant, semi-quiescent state known as dormancy (persistence). When this state is modeled in the laboratory, dormant bacteria are difficult to eradicate [26]. In addition, infection occurs in diverse compartments [27, 28], some of which may not be readily accessible to active compounds [28]; moreover, some cells may be non-culturable but viable. Consequently, antibiotic treatment must be maintained for many months to be effective. When adherence to treatment is poor, drug exposure becomes intermittent, which allows cycles of population expansion followed by selective reductions. These cycles also occur with many other pathogens but usually in different patients rather than in a single individual. The easily observed emergence of resistance with *M. tuberculosis* has made tuberculosis a paradigm for understanding the process.

Awareness of tuberculosis heteroresistance is high in part because PCR-based detection of resistant subpopulations is straightforward: resistance arises from point mutations in an otherwise highly conserved pathogen genome (reviewed in [23]). Moreover, the need for rapid diagnosis has been a high priority, which has led to the widespread application of DNA-based methods. These tests now show that 10–20% of patients in localities of high tuberculosis incidence have infections containing diverse subpopulations. Heterogeneity is seen with both HIV-positive and HIV-negative patients [29], and it is detected with a variety of genes, including those that encode resistance to ethambutol [30–33], isoniazid [32–34], rifampicin [34], fluoroquinolones [32, 35], streptomycin [33], pyrazinamide [32], and amikacin [36]. Thus, *M. tuberculosis* heteroresistance within individual patients is common to many antimicrobials.

9.3.2 Two Forms of Heterogeneity

DNA analyses of *M. tuberculosis* specimens reveal two general types of heterogeneity. In one form, bacterial isolates contain subpopulations having very different DNA fingerprints (IS*6110* RFLP or VNTR patterns) [37]. This form of heteroresistance is generally attributed to mixed infections arising from superinfection, or perhaps coinfection if the initial inoculum contained multiple bacterial cells having mixed genotypes [38–40]. Mixed-clone infections tend to occur in localities where tuberculosis burden is high and resistant disease is common. In an example from

Tashkent, Uzbekistan [34], sputum samples from 35 patients were examined by culture-based, drug-susceptibility testing and by a variety of DNA-based methods. Seven of the 35 samples contained susceptible cells mixed with cells resistant to isoniazid, rifampicin, or both. By DNA analysis, five of the seven heteroresistant isolates were shown to contain different strains, which indicated mixed infection due to coinfection or superinfection. Three of the mixed infections were newly diagnosed in patients who had not been treated; thus, continuous antimicrobial pressure is not required to observe mixed infection. These cases of mixed infection, which derived from dissemination of resistant *M. tuberculosis*, have been taken as evidence for inadequate infection control (isolation of patients, controlled air flow, etc.). Mixed infection is likely due to multiple factors.

In two heteroresistant cases from the Tashkent collection, the resistant subpopulations and the major, susceptible population had very similar DNA fingerprints [34]. Although the study did not show identical fingerprints and although it lacked the whole-genome sequencing or epidemiological information required to establish a de novo origin for the major and minor populations, clonal relation is the most likely explanation. Inadequate treatment and poor adherence to therapy regimens, rather than lax infection control, are the likely causes of this type of heteroresistance.

The experience of an HIV-positive Italian tuberculosis patient, who failed to adhere to treatment protocols for more than a decade, is best explained by evolution to resistance within an individual host. The patient first exhibited a fully susceptible infection that appeared to respond to therapy [41]. But after 3 years, the dominant isolate exhibited resistance to rifampicin and streptomycin. A subsequent sample contained a mixture of streptomycin-resistant and streptomycin-susceptible cells. The original streptomycin-resistance marker was later replaced by a different allele that became fixed in the patient. Eventually the strain, which had the same DNA fingerprint throughout, became resistant to rifampicin, streptomycin, isoniazid, and pyrazinamide. Had the patient lived to continue treatment with other agents, his pathogen population could have acquired even more resistance markers: some isolates from New York City have nine different resistance markers [42].

M. tuberculosis heterogeneity is not restricted to drug-resistance markers. For example, in a collection of largely pan-susceptible specimens from Bangladesh [37], ten colonies were examined from each of 97 samples. When DNA analyses (spoligotyping and IS*6110* RFLP tests) were applied, most samples had identical DNA patterns for all ten replicate colonies. However, with eight specimens, replicate colonies contained similar but nonidentical DNA fingerprints. That result was taken as evidence for clonal heterogeneity. Only two specimens had DNA fingerprints that were distinct enough for the samples to be from mixed-clone infections. A similar finding of mixed infections has been reported for samples collected in Georgia [43].

9.3.3 Dynamics of Clonal Evolution

A study from South Africa illustrates the complex dynamics of clonal heteroresistance [32]. The subjects of the study suffered from multidrug-resistant tuberculosis (MDR-TB) that had persisted through more than 12 months of treatment. Since the overall prevalence of MDR-TB in the community was low (0.3% in new patients, 1.7% in previously treated patients), clonal heterogeneity was more likely to occur than mixed infections. Indeed, when sputum samples from 13 HIV-negative MDR-TB patients were examined at 2-week intervals, no evidence was found for superinfection: all carried bacteria having a single IS*6110* RFLP type and spoligotype pattern. Nucleotide sequence analysis for eight resistance genes (*katG, inhA* = isoniazid; *pncA* = pyrazinamide*; embB* = ethambutol; *rrs* = amikacin, kanamycin; *rpsL* = streptomycin; *gyrA* = fluoroquinolone; *rpoB* = rifampicin) showed that several of the infections changed resistance patterns over the course of sampling.

One patient in the South African study [32] was examined for mutations in *katG, embB*, and *gyrA* during 56 weeks of therapy. At the beginning of sampling, all three genes were wild type, but at weeks 4 and 6, the *katG* marker was scored as resistant. Subsequent samples showed that it returned to wild type. The *embB* marker converted to resistant by 6 weeks, and it remained resistant throughout the observation period. The *gyrA* gene showed a mixture of alleles at week 6, and in subsequent samples transient changes were observed among several *gyrA* resistance forms, often mixed with wild-type alleles. Even after 48 weeks, *gyrA* was a mixture of resistant (D94C) and wild-type alleles. By week 52 a different *gyrA* allele (D94G) had emerged as the dominant form.

Specimens from two other patients [32] also contained different alleles of genes involved in drug resistance. For example, one patient evolved a mixture of wild-type and resistant alleles for *pncA*, changes in *gyrA* alleles over time, and a mixture of *rrs* alleles at the beginning of sampling that later saw one allele emerge as dominant. Another patient began with wild-type *gyrA* that after 36 weeks changed to resistant. But after 48 weeks, *gyrA* returned to wild type. Wild-type *pncA* also persisted until week 36, and then it shifted to resistant for the remainder of the study (week 52). The *katG* gene began as wild type, but after week 6 it was resistant, except for one sample at week 30 that was wild type. Overall, these fluctuations in drug-resistance markers illustrate the dynamic and varied nature of clonal heteroresistance when sputum samples are the source of information.

Examination of lung tissue provides an explanation for the allelic diversity: heteroresistance measured in sputum samples arises at least in part from independent clonal evolution in different regions of the lung. When surgical samples were examined from three HIV-negative patients having undergone long-term therapy, DNA IS*6110* fingerprints were the same for bacteria from different regions of the lung; thus, the isolates from individual patients appeared to be clonally related [27]. One patient had a streptomycin-resistant strain in an open lesion, while wild-type cells were detected in a closed granuloma. Wild-type cells were also recovered from

sputum. Bacteria from a second patient carried two different *gyrA* resistance alleles when isolated from open lesions, while wild-type *gyrA* alleles were obtained from sputum and from two closed lesions. The third patient produced three types of *M. tuberculosis*: (1) cells from apparently normal lung tissue had wild-type genes *for katG, embB,* and *rrs*, (2) cells from sputum and four pathological sites had *katG* and *embB* resistance markers but wild-type *rrs*, and (3) another pathological site yielded bacteria with resistance for all three genes. These observations, plus similar findings in another study [44] and autopsies [45], led to the conclusion that evolution occurs independently in different lung compartments and that wild-type cells can survive treatment. The results of sputum-based analyses probably reflect opening of granulomas and release of bacteria at different times during infection. Thus, analysis of a single sputum sample may not accurately reflect the diversity of bacterial populations in the infection.

9.3.4 Consequences of Heteroresistance

In the early days of tuberculosis chemotherapy, diagnostic criteria were set up to avoid mistakenly identifying a susceptible strain as resistant, because to do so would deprive a patient of a useful treatment. For example, with the agar proportion method, a specimen is considered to be resistant only if at least 1% of the colonies are resistant [22] (when resistance is identified at the 1% level, enrichment to full resistance requires only seven generations of selective growth, roughly *1 week* for *M. tuberculosis*). We conclude that 1% heteroresistance is a late stage in the evolution to resistance. Such an infection can still be treated with combination therapy, but close monitoring and treatment adjustment are required to avoid conditions that enrich mutant subpopulations.

Failure to recognize resistant subpopulations leads to inappropriate treatment, the expansion of those subpopulations, and eventually full resistance [46]. In one example, infection with *M. tuberculosis* was scored as susceptible at the time of diagnosis, but after 3 months of first-line therapy, MDR tuberculosis was diagnosed by drug susceptibility testing [38]. Retrospective analysis, using strain-specific PCR-based methods, showed that an MDR subpopulation had been present throughout treatment [38].

An added complication is that interruption of treatment can lead to reemergence of susceptible *M. tuberculosis*. In an example from South Africa, the susceptible subpopulation was not eradicated, even by 17 months of therapy; at treatment interruption, susceptible bacteria repopulated the infection [38]. In another example, an MDR infection was treated with second-line agents, and after 3 months of treatment, the infection was judged fully susceptible [38]. Reduced antibiotic pressure, as may occur with second-line agents, allowed the susceptible strain to become dominant. In such cases, treatment needs to be reassessed periodically, and perhaps first-line therapy needs to be continued with MDR strains even after applying second-line agents. To maintain adequate therapy while resistance markers are

changing requires rapid and accurate diagnostic methods. Below we consider the development of genetic (DNA-based) assays.

9.3.5 Detecting Heteroresistance Using DNA-Based Methods

Although *M. tuberculosis* population heterogeneity had been known for many years from phage-typing of *M. tuberculosis* subpopulations [47, 48], it was recognized as an important phenomenon only after molecular diagnostic methods emerged. When PCR was used to amplify specific regions of *M. tuberculosis* DNA encoding proteins associated with resistance and the amplified fragments were separated by gel electrophoresis, the size distribution characteristic of both susceptible and resistant alleles was observed from a single bacterial specimen [33]. Heteroresistance was then used to explain the occasional discordance between results from drug-susceptibility testing and DNA-based methods: the DNA tests indicated resistance, but only susceptibility was detected following the bacterial outgrowth required for susceptibility testing. A fitness advantage among the susceptible bacteria was thought to allow them to dominate during outgrowth [49].

The various DNA-based tools differ in sensitivity (Table 9.1). For example, Sanger DNA sequencing of PCR products reported 15% of isolates as heteroresistant, while with the same samples deep sequencing found almost 40% heteroresistance [35]. When heteroresistance is greater than a few percent, current hybridization methods are sufficiently sensitive. Unfortunately, PCR-based diagnostic methods encounter a specificity problem when subpopulations are below 1%, because templates from the major bacterial population can generate false-positive, variant signals due to mis-priming, mis-incorporation, and mis-hybridization.

As pointed out above, sensitivity to 1% is unlikely to be adequate for monitoring the emergence of resistance, because 1% is considered fully resistant for that marker if the equivalent of monotherapy is employed. Moreover, a negative result cannot rule out heteroresistance. In essence, current genetic diagnostics can give false-negative results. We conclude that other methods are needed to detect heteroresistance at levels low enough to allow successful intervention.

Work in cancer biology is driving new, DNA-based tests for heteroresistance – a priority in the cancer field is detection of a small number of transformed cells within a large background of normal cells. One approach is called digital PCR [57]. In this method, the sample is diluted into a series of wells in a multi-well microfluidic plate such that only a single molecule of mutant DNA is expected to be present in a given well (most wells will contain only wild-type DNA). Amplification of DNA in the wells produces a digital readout: either the presence or absence of mutant DNA. The fraction of total wells scoring positive estimates the percent of the sample containing mutant DNA. In principle, the sensitivity of this method is limited only by the number of wells assayed. Digital PCR has been applied to *M. tuberculosis* isolates by mixing wild-type cells with *M. tuberculosis* containing resistance mutations in *katG, rpoB, gyrA*, and *rrs*. The method reliably detects heteroresistance at a ratio of

Table 9.1 Sensitivity of DNA-based detection methods for heteroresistance

Method	Resistance Gene(s)[a]	Size of detectable sub-population	Reference
Sanger sequencing	*katG, fqn, rif, rrs* *katG* *rif* *fqn*[b]	28–60% 50% 50% 15%	[35, 50, 51, 52]
Melting curve	*inh*	40%	[53]
Sloppy molecular beacons	*rif* *fqn*	40% 5–10%	[54, 55]
qPCR bacteriophage	ns	10%	[50]
qPCR	*katG, fqn, rif, rrs*	10%	[50]
Line probe	*katG* *rif*	5% 5%,1–70%[c]	[51] [51, 56]
iPLEX	*amk*	0.5%	[36]
Digital PCR	*katG, fqn, rif, rrs*	0.1%	[50]

[a]Abbreviations: *ns* not stated, *amk* amikacin, *fqn* fluoroquinolone, *katG* isoniazid, inh various isoniazid markers, *rif* rifampicin, *rrs* aminoglycosides

[b]Fluoroquinolone resistance was the only marker in the population; deep sequencing identified 38% heteroresistant

[c]Depends on allele

1 mutant per 1000 wild-type cells [50], which is about 10-times more sensitive than previous PCR-based methods. For digital PCR to achieve this sensitivity with sputum samples, the samples must contain more than 1000 *M. tuberculosis* cells per ml (bacillary content varies among sputum samples, but it can exceed one million cfu [58–60]).

Another approach, single-nucleotide primer extension, is used to incorporate a nucleotide having a distinctive mass modification that can be identified by mass spectroscopy [61]. The method, called iPLEX Gold, has the advantage of detecting multiple resistance alleles in the same reaction mixture. In one application of the method, a reconstruction experiment detected one amikacin-resistant cell per 200 wild-type cells [36].

A third strategy is called pyrophosphorolysis-activated polymerization [62–67]. In this method, a primer containing a dideoxyribonucleotide at its 3′ terminus (noted as P*) is hybridized to the test DNA at the preselected mutation site. Removal of the dideoxyribonucleotide by pyrophosphorolysis, which is highly specific for perfect hybridization of the primer, is required for extension of the primer by DNA polymerase. Primer extension then amplifies the signal for real-time detection by fluorescent probes. When a P* primer is used that contains the complement of the *mutant* sequence, the polymerization assay is expected to detect mutant alleles at a frequency as low as 10^{-8} of wild-type DNA, a level that approaches background (spontaneous) mutation frequency. To our knowledge, the pyrophosphorolysis-activated polymerization method has not been applied to detection of heteroresistant *M. tuberculosis*.

A fourth strategy, which is also derived from cancer diagnosis, uses what are called SuperSelective primers for real-time PCR assays [68]. In this system, a DNA primer is designed in which one region hybridizes strongly to a portion of the target DNA being queried. This anchor region is separated from a detector region, the "foot", by a long region expected to mispair with the target and thus form a loop. The foot is designed to hybridize only with the mutant sequence in the target. By adjusting the length of the loop and the foot, conditions can be obtained in which hybridization only occurs with mutant DNA. The resulting hybrid then primes real-time PCR. The system can detect multiple mutations in the same reaction tube by using fluorophores having different colors to discriminate the amplification products. This method has not yet been applied to diagnosis of heteroresistance.

A fifth strategy is based on CRISPR, a bacterial system that recognizes and destroys foreign nucleic acids. The underlying idea is as follows. A DNA sample from the pathogen is transcribed in vitro and incubated with the Cas13a protein system plus a quenched, fluorescently labeled reporter RNA. Recognition of the target RNA by Cas13a, which is designed to occur only if the resistance mutation is present, will cause collateral damage in the reporter RNA, eliminate the quenching, and generate a fluorescent signal. This method, which has been dubbed SHERLOCK [69], has single-molecule sensitivity, similar to droplet digital PCR and quantitative PCR (qPCR). Moreover, it has point-of-care diagnostic features. To our knowledge SHERLOCK has not been applied to detection of heteroresistant *M. tuberculosis.* However, the CRISPR system has been modified to function in this pathogen [70].

A general problem associated with PCR-based diagnosis of resistant bacterial subpopulations is laboratory contamination by amplicons present in the laboratory from previous tests. Published estimates of laboratory cross-contamination using open-tube methods are presently almost 4% [38, 40]. Although closed-tube methods exist [53, 71, 72], current closed-tube methods require refinement to be sensitive enough for heteroresistance detection.

9.4 Heteroresistance with *Staphylococcus aureus*

9.4.1 Methicillin Heteroresistance

Heteroresistance with *S. aureus*, which has been known for many years [73], is not routinely detected by standard susceptibility testing (MIC determination). Such determinations typically examine only 10^4 to 10^5 cells, and the frequency of resistant subpopulations is generally below 10^{-5}. However, when susceptibility testing uses a large number of cells, on the order of 10^7 to 10^{10}, subpopulations having reduced susceptibility can be seen. For example, heterogeneity is a distinctive feature of methicillin resistance due to the presence of a mobile chromosomal element called *SCCmec* [6, 74]. *SCCmec* elements, which vary in size, contain a gene, *mecA,* that encodes a low-affinity penicillin-binding protein (PBP2' or PBP2a). PBP2' is a transpeptidase [75] that allows *S. aureus* to form cell walls in the presence of

methicillin. Clinical isolates carrying *mecA* usually show moderate-level heterogenous resistance to all β-lactams [6]. However, subpopulations emerge in which resistance levels are high. Indeed, repeated β-lactam challenge leads to homo-resistant *S. aureus*, sometimes, but not always, due to enhanced *mecA* expression [6, 74]. Most homogenous, high-level resistance strains revert to heterogeneity, although some laboratory isolates, such as strain COL, demonstrate stable high-level resistance. Overall, the evolution of methicillin heteroresistance is a classic example of antimicrobial resistance emerging in an opportunistic pathogen.

Early work identified a chromosomal mutation, *chr**, as being important for high-level methicillin resistance [74]. Whole-genome sequencing and genetic reconstruction experiments subsequently showed that at least one type of *chr** is a substitution in the β subunit of RNA polymerase that, along with *mecA,* confers high-level resistance to methicillin [76]. The molecular basis for *rpoB* action on *mecA* is unknown, but it is likely to be important, because RNA polymerase substitutions are also involved in intermediate resistance to vancomycin [77]. One speculation is that the RNA polymerase variants alter the expression of genes that protect from antimicrobial activity.

One of the more relevant examples of *S. aureus* heteroresistance concerns ceftaroline, a cephalosporin (β-lactam) that shows activity against MRSA and vancomycin-intermediate *S. aureus* (VISA, discussed below). In one study, a collection of 57 isolates contained 12 heteroresistant members, some of which also exhibited reduced susceptibility to vancomycin, daptomycin, or linezolid [78]. We conclude that controlling MRSA with new β-lactams is likely to be difficult.

9.4.2 Vancomycin-Intermediate Heteroresistance

MRSA infection is commonly treated with the glycopeptide vancomycin. The result has been the emergence of an intermediate level of resistance (VISA, which is distinct from the rare, *vanA*-mediated, fully vancomycin-resistant *S. aureus*). VISA is associated with a poorly defined thickening of the bacterial cell wall that reduces the uptake of vancomycin [79]. Other features associated with VISA are excess peptidoglycan production, low fitness manifested by reversion toward susceptibility during growth in vitro [80], and attenuated virulence in animal models of infection [81–84]. VISA probably represents the end stage of evolution from heteroresistant strains (hVISA) in which subpopulations slightly elevate the overall MIC of an MRSA isolate. Since clinical isolates of MRSA are heteroresistant due to instability of the *SCCmec* elements [85], hVISA emerging during vancomycin treatment can be co-heteroresistant (heteroresistant for two or more antimicrobials).

With both hVISA and VISA, *S. aureus* populations exhibit considerable heterogeneity in their susceptibility to vancomycin (see examples in Ref. [5]). To better detect hVISA, the breakpoint for full susceptibility was lowered to an MIC of 2 μg/ml [86]. In some samples, hVISA cells are abundant enough to raise vancomycin

MIC to the high end of the susceptible range (MIC = 0.5 to <2 μg/ml; with most antimicrobial-pathogen combinations, heteroresistance has no effect on MIC because the subpopulations are small). Thus, a slightly elevated MIC can indicate hVISA, but a more sensitive test is needed.

PAP (population analysis profile) determination can definitively identify hVISA, but the method is too labor intensive for routine clinical use. Consequently, efforts have shifted toward other agar-plate screens [87]. Developing DNA tests is still challenging, because multiple genetic pathways lead to VISA. For example, when seven successive samples of MRSA were obtained from a single patient and analyzed by vancomycin-PAP, a clear evolution from susceptible to hVISA to VISA was observed [88]. Whole-genome sequencing then identified six mutations that generated five distinct genetic profiles that correlated with evolution along three pathways involving cell wall biology.

In another whole-genome sequencing study, a VISA strain was compared to a closely related, susceptible isolate. Several gene differences were found, and wild-type alleles were introduced into the VISA strain to regain susceptibility and thereby identify genes involved in VISA [79, 89]. Among these were the GraSR and VraSR two-component systems that contribute to the evolution of VISA by upregulating cell wall synthesis (for additional detail, see Chap. 15). Other associated alterations, listed in Ref. [77], include a substitution in RNA polymerase (H481Y/L/N) that also confers rifampicin resistance (whether rifampicin resistance is acquired before VISA is not known). We conclude that unlike the situation with *M. tuberculosis* heteroresistance, a simple DNA-based diagnostic for hVISA is not likely to be available soon.

The clinical importance of VISA and hVISA can be assessed by surveillance studies. VISA represents a few percent of MRSA recovered from serious infections, and hVISA prevalence is 4 to 5 times higher [90, 91]**.** Apparently, the genetic changes that create VISA have fitness costs that trap most *S. aureus* in the intermediate-resistance state. Since antimicrobial resistance is inherently a local phenomenon, variation in prevalence is expected (assay methods also differ, which adds to variation). For example, surveys from Asia (excluding China) indicate a prevalence for hVISA of a few percent [92], but a report from Taiwan places it at about 10% (2012–2013; Ref. [91]). A hospital study from Turkey also reported a high prevalence of hVISA in blood isolates (almost 14%, Ref. [93]). In a troubling study from Michigan (cited in Ref. [94]), the prevalence of hVISA in blood isolates increased from 2% (1987–1993) to 8% (2003–2007), and in a multicenter US study, the prevalence increased from 0.4% in 2009 to 1.2% in 2011. Thus, hVISA dissemination within and between hospitals is taken seriously; it may require special consideration by infection control departments to limit transmission from patients colonized by or infected with hVISA.

hVISA surveillance data also suggest that treatment changes are needed. Although VISA is associated with treatment failure, reports on hVISA are mixed. For example, a Michigan study of MRSA bacteremia found that hVISA was not clearly associated with treatment failure [90]. However, in another study, hVISA

correlated with a doubling in mortality from pneumonia, relative to vancomycin-susceptible *S. aureus* (treatment failure was also higher, but not significantly so [95]). Although a clear statement about outcome from hVISA cannot be made, hVISA remains important as a likely precursor to VISA.

Studies of hVISA are also contributing to a test for restricting the emergence of resistance. One approach is to increase antimicrobial dose. We pointed out that resistant mutants are selectively enriched when drug concentrations fall inside a specific concentration range called the mutant selection window; keeping drug concentrations above that window should restrict amplification of mutant subpopulations [3, 96]. The increasing problem of hVISA and VISA with serious infections led to a medical commission concluding from pharmacokinetic studies that dosing to generate vancomycin AUC/MIC >400 h should control most serious MRSA infections [97]. It was argued that measuring this pharmacodynamic parameter was not practical for routine clinical use, but as a surrogate goal, the recommendation was to maintain a minimum serum concentration between 15 and 20 μg/ml [97]. The commission did not know the upper boundary of the selection window. Several years later, that boundary was measured and found to be 19 μg/ml (over 400 MRSA isolates were examined, Ref. [98]). Thus, the proposed vancomycin target level for favorable clinical outcome (15–20 μg/ml) fit with the value needed to restrict the emergence of new resistant mutants. Clinical studies, again in Michigan [99], reported increases in the minimum serum concentration of vancomycin from 10 μg/ml in 2002–2003 to 19.7 in 2010–2012. During that time, the prevalence of hVISA dropped from 9.7% to 2.1%. This vancomycin work with heteroresistance is the first example for convergence between efforts to achieve favorable patient outcome and efforts to restrict the emergence of resistance.

9.5 Other Pathogens Displaying Heteroresistance

Consideration of heteroresistance with *M. tuberculosis* and *S. aureus* provides an introduction to two important features. First, some resistance mutations, such as gyrase-mediated resistance to fluoroquinolones, have little fitness cost and are readily enriched; in contrast, high fitness cost, as seen with VISA, limits the evolution to a state of intermediate vancomycin resistance, at least in nature. Second, it is straightforward to develop a DNA-based diagnostic to query a limited number of mutations associated with antibiotic resistance, as with *M. tuberculosis*; however, design is difficult when numerous mutations are associated with drug resistance, as with VISA. Applying these ideas to heteroresistance with other pathogens has not been done, since much less is known. Nevertheless, heteroresistance is clearly a widespread phenomenon (Table 9.2). Below we list recent work that establishes the potential importance of heteroresistance.

Table 9.2 Selected examples of heteroresistance

Pathogen species	Antimicrobial	Prevalence	Locality	Reference
Acinetobacter baumannii	Carbapenem	Ns[a]	Greece	[7, 100]
Acinetobacter baumannii	Cephalosporin, Penicillins	Case study	Taiwan	[101]
Acinetobacter baumannii	Colistin	Case study;	S. Korea, Argentina	[102, 103]
Candida glabrata	Fluconazole	58%	Israel	[11]
Clostridium difficile	Metronidazole	29%	Spain	[104]
Corynebacterium striatum	Daptomycin	Case study	USA	[105]
Escherichia coli	Cefepime	22%	China	[106]
Escherichia coli	Carbapenem	34%	China	[107]
Haemophilus influenzae	Imipenem	37%	Switzerland	[108]
Helicobacter pylori	Several[b]	48%	Tunisia	[109]
Klebsiella pneumoniae	Carbapenem	Ns	Spain, Greece	[110, 111]
Klebsiella pneumoniae	Colistin	75%	Greece	[112]
Mycobacterium tuberculosis	Fluoroquinolone	23%	China	[113]
Pseudomonas aeruginosa	Carbapenem	24; 19%	Greece, China	[114, 115]
Salmonella enterica	Colistin	Laboratory	Na[d]	[116]
Staphylococcus aureus	Ceftaroline	21%	USA	[78]
Staphylococcus aureus	Vancomycin-intermediate	10%	Taiwan	[91]
Streptococcus pneumoniae	Penicillin	44%	Multinational	[117]

[a]*Ns* no surveillance
[b]Multiple infection
[c]Not applicable

9.5.1 Gram-Negative Bacteria

Acinetobacter baumannii has become an important source of opportunistic nosocomial infection, largely due to widespread multidrug resistance. Indeed, isolates have been reported that are resistant to all commonly used antimicrobials. Heteroresistance in *A. baumannii* is well known, having been observed in carbapenem Etest analyses more than a decade ago [7]. Individual carbapenems may differ in the genes involved in resistance, since for one carbapenem (meropenem), heteroresistance persists during subculturing on drug-free agar, while that stability is not seen with another (imipenem) [100]. *A. baumannii* also displays heteroresistance to cephalosporins and penicillins [101]. Population analysis profiles for these β-lactams can be complex, as illustrated by a report in which PAP showed colony numbers dropping at low concentrations of cefepime and climbing at high concentrations [101]. This phenomenon is not yet understood.

Heteroresistance to colistin, an agent of last resort, is also seen among isolates of *A. baumannii* [102, 103]. In a survey performed at an Argentine hospital, heteroresistance doubled (46–95%) from 2004 to 2012, a period in which colistin consumption increased by more than fourfold [103]. Colistin resistance in Argentina tends to be unstable, and the increase in heteroresistance did not presage an increase in resistance [103]. Nevertheless, the widespread occurrence of heteroresistance with *A. baumannii* does not bode well for antimicrobial success with this pathogen.

Escherichia coli is a common inhabitant of the human digestive tract that is becoming a serious urinary pathogen as multidrug-resistant forms become more prevalent. In a study that examined more than 300 isolates for cephalosporin (cefepime) heteroresistance, almost a quarter displayed colony growth inside the zone of inhibition on agar plates [105]. In two-thirds of the cases, the patients had received prior treatment with a cephalosporin. These observations are consistent with a model in which antimicrobial pressure enriches mutant subpopulations. *E. coli* also causes septicemia, and invasive *E. coli* has exhibited clonally diverse, carbapenem heteroresistance [107]. In one case, examination of consecutive samples from the same patient showed a gradual shift of the *E. coli* subpopulation profile (PAP) to higher carbapenem concentrations and eventually to complete resistance. Such data establish heteroresistance as an intermediate step along the evolutionary climb toward complete carbapenem resistance, at least for *E. coli*. To our knowledge, the contribution of plasmid-mediated resistance, which is common, has not been addressed.

Haemophilus influenzae is an opportunistic pathogen that colonizes the human airway. Resistance to β-lactams is commonly due to plasmid-mediated β-lactamases and altered penicillin-binding protein-3 [108]. While imipenem resistance is rare, heteroresistant *H. influenza*e isolates have been described [108]. In one report, PAP revealed heteroresistance in 46/124 isolates that had an intermediate Etest MIC. With *H. influenzae*, β-lactam heteroresistance arises from multiple genetic and biochemical factors, which will make DNA testing a challenge.

Helicobacter pylori causes a chronic infection of the human gastric mucosa that is thought to be central to peptic ulcer disease, chronic gastritis, and gastric cancer. Extensive use of antimicrobials has led to loss of antimicrobial susceptibility among isolates of *H. pylori*. Clinical testing of gastric biopsies is complicated by the heterogeneous distribution of *H. pylori* in the stomach. In a survey of 66 patients in which isolates were obtained from two distinct gastric regions, 15% exhibited infection of clonal origin in which the isolate from one compartment was susceptible to the antibiotics tested, while the sample from the other compartment was resistant to at least one of four agents (clarithromycin, metronidazole, levofloxacin, and rifabutin) [118]. In this situation, simply labeling an infection as heteroresistant would have obscured the compartmentalization associated with *H. pylori*.

Since transmission of *H. pylori* is common and since infection persists for long times, heteroresistant infections may arise from multiple superinfection. The frequency of multiple infection may be less common in industrialized countries, as indicated by a comparison of isolates from university hospitals in France and Tunisia [109]. For 21 isolates examined from each country, multiple infection was observed 10-times more often with Tunisian patients than with French ones (clonal heteroresistance was similar

for the two countries). While the reasons for differences in heteroresistance are complex, these data show that clinicians in developing countries should be watchful for multiple infections that might impact susceptibility testing.

Klebsiella pneumoniae causes serious diseases, such as pneumonia, meningitis, and urinary infections. Since *K. pneumoniae* inhabits the human digestive tract, it readily disseminates in hospitals by fecal contamination. Thus, when multidrug-resistant *K. pneumoniae* strains develop resistance to carbapenems, they become a major nosocomial problem. Low reproducibility of MIC tests for carbapenems, followed by population analysis profiling, led to the conclusion that *K. pneumoniae* heteroresistance is overlooked by automated susceptibility testing [110]. Heteroresistance appears to arise from drug-induced expression of carbapenemases, since heteroresistance to meropenem is lost when drug pressure is withdrawn [111].

As the prevalence of resistance to the major antimicrobials mounts, colistin is being used to treat *K. pneumoniae* infections. The result has been a sharp increase in colistin resistance. For example, in one Greek hospital, resistance to colistin rose from 0% in 2007, to 8% in 2008, and 24% in 2009 [112]. When PAP was performed on a small set of patient isolates, heteroresistance to colistin was observed in 12/16 isolates that had been deemed susceptible by standard MIC assays [112]. With *K. pneumoniae*, colistin heteroresistance is associated with the PhoPQ regulatory system [119], as pointed out below for *E. cloacae*. The PhoPQ system alters the lipopolysaccharide of cell surfaces (the negative charge on lipid A is reduced, thereby lowering the affinity for colistin, a cationic peptide). Colistin monotherapy is contraindicated for serious disease caused by *K. pneumoniae*.

Pseudomonas aeruginosa is an opportunistic pathogen that is particularly problematic for patients suffering from cystic fibrosis. Antimicrobial resistance with *P. aeruginosa* is mediated by multiple efflux systems and production of β-lactamases. In a study from Greece, 27% of presumably susceptible isolates exhibited stable carbapenem heteroresistance [114]. This result may be common for *P. aeruginosa*, as a similar finding was reported from China [115]. With *P. aeruginosa,* it may be necessary to perform heteroresistance testing on many isolates, since automated methods do not reliably detect heteroresistance.

Salmonella enterica serovar Typhimurium is noted for causing outbreaks of food poisoning. Since isolates that exhibit multidrug resistance are associated with increased mortality and morbidity, colistin is being considered for treatment of *S. enterica*-associated diseases. A study of laboratory-generated colistin heteroresistance with *S. enterica* revealed a correlation between heteroresistance and a moderate gene dosage of *pmrD*, a gene that upregulates proteins that modify lipid A and thereby lower susceptibility to colistin [116]. Successive passages in the presence of colistin increased amplification of *pmrD*, while the number of amplified copies declined when cells were passaged on drug-free medium. A similar phenomenon may have contributed to tetracycline heteroresistance in a clinical isolate [116]. Antimicrobial resistance arising from gene amplification has also been observed with *M. tuberculosis* [120], suggesting that it may underlie heteroresistance in a variety of pathogens.

9.5.2 Gram-Positive Bacteria

Clostridium difficile causes serious diarrhea, especially in nosocomial settings where antibiotic resistance plays an important role in driving outbreaks. *C. difficile* is an anaerobic pathogen that is frequently treated with metronidazole. In a Spanish study [104], almost 30% of *C. difficile* samples showed metronidazole heteroresistance when examined for colony formation within inhibition zones on agar plates. Thus, a major treatment option for this opportunistic pathogen is threatened by resistance.

Corynebacterium striatum is a commensal skin inhabitant that occasionally causes infection. A case was reported [105] in which a patient with a *C. striatum* infection, treated with daptomycin, developed endocarditis. *C. striatum* was recovered from the patient, and after plating for an Etest, colonies formed within the zone of inhibition. Bacteria from those colonies, when purified and retested, had very high MICs for daptomycin, while the bulk of the culture was daptomycin susceptible. These data show that daptomycin is subject to heteroresistance issues.

Enterobacter cloacae is a nosocomial pathogen that causes a wide range of infections, largely in the very young and the elderly. The pathogen is readily distributed within hospitals on medical devices and via hospital workers. Due to multidrug resistance, colistin is being used in the hospital setting. Colistin heteroresistance is readily detected by colonies in the zone of inhibition during susceptibility testing on agar, but examination of individual colonies shows that resistance is lost upon subculturing on drug-free agar [121]. During infection of mice with the heteroresistant isolate, the fraction of resistant cells increased even in the absence of colistin. This enrichment was due to a portion of the innate immune response exerted by macrophages: heteroresistance rendered *E. cloacae* refractory to colistin if administered after infection was established, but experimental depletion of macrophages maintained colistin susceptibility [121]. Thus, host functions can expand the effect of heteroresistance. Such data emphasize that automated susceptibility testing can be misleading.

Transcriptional analysis revealed increased expression of PhoQ in the transiently resistant strain of *E. cloacae* (for additional detail, see Chap. 15). PhoQ expression leads to a modification of membrane lipid A, which then restricts the action of colistin. How the innate immune system stimulates expression PhoQ is not yet known.

Streptococcus pneumoniae is responsible for roughly half of all pneumonia cases. Since *S. pneumoniae* is commonly carried in the nasopharynx of young children and since children are treated with many antibiotics, resistance is expected to be a problem. Penicillin has been used extensively to treat infections caused by *S. pneumoniae,* and penicillin heteroresistance has been reported [117]. In an effort to expand the number of useful antibiotic agents for *S. pneumoniae*-related infections, a Swiss study examined *S. pneumoniae* isolates for heteroresistance to fosfomycin [122]. Even though fosphomycin is not currently used for treatment, 10 of 11 isolates exhibited fosfomycin-heteroresistance. These data, which indicate that fosfomycin resistance may emerge quickly, show that heteroresistance can be used as a way discriminate against certain new antimicrobials.

9.5.3 *Invasive Fungus*

Candida glabrata is an important fungal pathogen that can be lethal to immunocompromised patients. Fluconazole, a common antifungal agent, readily enriches stable heteroresistant strains of *C. glabrata* [11]. It is likely that many genes are involved in heteroresistance, since population analysis profiles showed a wide distribution. As with bacterial pathogens, heteroresistance in *C. glabrata* is not readily detected by standard drug susceptibility testing; consequently, some isolates may be misclassified as susceptible. To assess the relevance of fluconazole heteroresistance, mice were infected with *C. glabrata* and treated with fluconazole. Persistent infection was observed four times as often with a highly heteroresistant isolate. Thus, heteroresistance in disease caused by *C. glabrata* is likely to be clinically important.

9.6 Concluding Remarks

Efforts to control the expansion of resistance by reducing antimicrobial consumption have met with mixed results (e.g., [123, 124]), and heteroresistance is becoming widespread (Table 9.2). A preemptive attack on heteroresistance may slow the emergence of resistance. In the case of tuberculosis, that entails identifying heteroresistant infections and then adjusting treatment protocols. In the case of MRSA, it requires treating infections with higher vancomycin concentrations. With many other pathogens, detection of heteroresistance needs to be improved (automated susceptibility testing currently fails to detect heteroresistance); then treatment protocols need to be modified to block further mutant enrichment. A central problem is that raising doses to suppress evolution to resistance is likely to increase toxic side effects. Thus, strategies that may be good for the community as a whole may be harmful to some individual patients. A long-term solution requires more research focus on chemical adjuvants that will increase antimicrobial lethality to allow nontoxic, anti-mutant dosing.

Major Points

- Antimicrobial heteroresistance derives from a variety of phenomena ranging from subpopulations of stable, fully resistant mutants to reversible, antimicrobial-mediated induction or amplification of protective genes.
- Heteroresistance is common: it has been observed in many different pathogenic bacterial species and found in almost 25% of patient isolates
- Heteroresistance can evolve to full drug resistance.
- The importance of heteroresistance has been underappreciated, because infections containing heteroresistant pathogen populations can often be treated successfully.

- Detection provides an opportunity to adjust antimicrobial treatment to slow the evolution of heteroresistant populations into populations exhibiting complete drug resistance.
- DNA-based methods can be used to detect heteroresistance when specific genetic alterations are known to be responsible for reduced susceptibility; methods developed for cancer diagnostics may apply to detection of *M. tuberculosis* heteroresistance.

Acknowledgments We thank the following for helpful discussions and critical comments: Veronique Dartois, Dorothy Fallows, Marila Gennaro, Ben Gold, Barry Kreiswirth, Richard Pine, and George Zhanel. The authors' work was supported by NIH grants 1DP20D007423, 1R01AI073491, 1R21A03781, and 1R01AI87671.

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Part II
Biology of Resistance

Chapter 10
Epidemiology of Bacterial Resistance

Patricia A. Bradford

10.1 Introduction

Bacterial pathogens have developed resistance to antibacterial agents via multiple routes. When any given pathogen mutates and becomes resistant, it can rapidly result in immeasurable resistant daughter cells. Mutants that develop following exposure to antibiotics favor mechanisms that confer resistance with the least cost to fitness, that is, the strains that are least burdened by their resistance will survive. This enhanced survival may also include increased virulence. Antimicrobial resistance complicates the treatment for bacterial infections, resulting dosing with multiple antibiotics, prolonged courses of therapy, and excess hospitalizations. The Centers for Disease Control and Prevention (CDC) published their first report on antibiotic resistance in the USA in 2013, regarding the continued threat in the treatment of bacterial infections [1]. In this report, the CDC estimated that at least two million people acquired serious infections from antibiotic-resistant pathogens and that at least 23,000 deaths in the USA could be attributed to infectious caused by these organisms. It is important to understand not only the mechanisms by which bacteria become resistant but also how resistance spreads from organism to organism and then from person to person. By understanding the epidemiology of resistance, we can then learn how to address it with infection control and/or new therapies. This chapter will examine the epidemiology of resistance by looking at the mechanisms by which resistance spreads, examining the molecular methods used for tracking resistance in bacterial pathogens, and reviewing some instances of successful resistance dissemination within the hospital and in some populations of people within the community.

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I. W. Fong et al. (eds.), *Antimicrobial Resistance in the 21st Century*, Emerging Infectious Diseases of the 21st Century, https://doi.org/10.1007/978-3-319-78538-7_10

10.2 Development of Resistance

Bacterial resistance to antibiotics can result via three main pathways: modification of the bacterial target for the antibacterial, decreased intracellular concentrations due to reduced permeability and efflux, or enzymatic inactivation of the drug. In some cases, all members of a given species might be resistant to a particular antibiotic. For example, all isolates of the Gram-negative non-fermenter *Stenotrophomonas maltophilia* express a chromosomally encoded metallo-β-lactamase. Therefore, resistance to imipenem and other carbapenems is a diagnostic tool for identifying this organism. Alternatively, resistance can develop in previously susceptible organisms through genetic mutation or by acquisition of foreign DNA encoding resistance genes. The specific mechanisms that affect various classes of antibiotics are discussed in other chapters. The discussion here will focus on the selection and spread of resistance when it occurs.

10.2.1 Selection of Bacterial Pathogens with Innate Resistance

The use of antibacterial drugs disrupts the microbiome of the patient being treated. In turn, the hospital unit or other groups of people in close proximity such as in daycare centers or in long-term care facilities can be affected. As a consequence, an entire species of bacterial pathogen might be selected with antibiotic pressure due to natural resistance occurring in that species. For example, the increased role of enterococci as opportunist pathogens in the 1980s and 1990s correlated with the introduction and increased usage of fluoroquinolones and cephalosporins, as these organisms are inherently resistant to those agents [2]. Similarly, the increasing incidence of coagulase-negative staphylococci and α-hemolytic streptococci in hematology patients, especially those who have indwelling central lines, correlated with the increased use of fluoroquinolones in these patients [3]. Among Gram-negative pathogens, *Acinetobacter baumannii* and *S. maltophilia* have become increasingly prevalent in many intensive care units (ICUs) following the increased usage of carbapenems, especially among patients with mechanical ventilation [4, 5]. *S. maltophilia* has a naturally occurring metallo-β-lactamase that renders it resistant to carbapenems, and *A. baumannii* is often resistant to all antibacterials except trimethoprim-sulfamethoxazole. The introduction of each of these new therapies has led to the unexpected consequence of shifting the etiology of some of the common hospital-based infections to species that are naturally more resistant than the pathogens they replaced.

10.2.2 Resistance by Mutation

As bacteria grow, the DNA (both the chromosome and plasmids) is replicated through a process that is highly prone to errors in base incorporation. These errors, leading to base substitutions, occur randomly, at a frequency of approximately 10^{-9} per gene [6]. Even one amino acid substitution can greatly alter the functionality of a gene. For example, the substitution of serine for glycine at residue 238 in the SHV-1 β-lactamase led to the first extended-spectrum β-lactamase (ESBL), SHV-2, that conferred resistance to expanded spectrum cephalosporins [7]. In addition to these random point mutations, replication errors may lead to deletions or insertions of small pieces of genes. Each of these mutations may result in the altered interaction of antibacterial agents with the bacterium through changes of the drug target, enzymatic inactivation of the drug inactivation, or changes in the efflux or uptake inactivated by the importation of insertion sequences, such as the case with the *ccrA* gene expressing a metallo-β-lactamase in *Bacteroides fragilis* that is only expressed only if an insertion sequence has inserted upstream of this structural gene [8]. Exposure to antibiotics does not cause the mutations but rather selects for strains that have pre-existing mutations that allow the bacterial cell to survive in the presence of the antibiotic.

Most mutations occurring in the drug target or in an antibiotic-modifying enzyme affect only a single antibacterial class. However, mutations also occur in genes encoding outer membrane porin proteins that allow penetration through the outer membrane by passive diffusion, or efflux systems that expel out of the cell multiple antibiotic classes as well as other cell toxins such as dyes can greatly impact the susceptibility of a bacterial cell to the antibiotic [9]. The maintenance of a mutation in a bacterial pathogen causing antibiotic resistance is completely dependent upon whether or not that mutation affects the fitness or virulence of that organism. If resistant mutants emerge at high frequency and are still able to replicate and cause disease, they can gain a foothold in the bacterial population that is further selected through continued use of the drug [10]. There have been several antimicrobials introduced in the 1980s and 1990s that had reduced utility following mutational resistance in certain species. Resistance to fluoroquinolones among staphylococci rapidly emerged by the upregulation of NorA-mediated efflux [11]. Another example was the use of imipenem that led to the selection of *P. aeruginosa* that have lost the OprD porin, which provides carbapenem-specific pores through the outer membrane [9]. Interestingly, the recent development of resistance to linezolid due modification of the domain V of 23S rRNA (the binding site for linezolid) in *Staphylococcus aureus* and *Enterococcus* spp. has not led to widespread resistance among clinical isolates [12, 13].

10.2.3 Acquired Resistance by DNA Transfer

DNA transfer among bacteria primarily occurs via plasmids, some of which are self-transmissible, in that they carry genes to initiate the direct transfer to another bacterium. Many plasmids are large and are able to accommodate multiple resistance genes. These large transferrable plasmids are the ideal vector for the dissemination of resistance genes. Within plasmids, resistance genes are often carried by transposons, which can transfer determinants between plasmids, or transport them into and out of the chromosome [14]. In addition, resistant bacteria often contain integrons that have the capability to acquire and express resistance determinants behind a single promoter. They are widely distributed among Gram-negative bacteria and are found within plasmids and transposons [14]. Very diverse resistance determinants have been found in integrons, including genes conferring target-based resistance to trimethoprim and fosfomycin, efflux-mediated quinolone resistance, and metallo-β-lactamase-mediated carbapenem resistance [15–17]. Mechanisms of transferrable resistance are presented in detail in Chap. 11.

The dissemination of plasmids, transposons, and integrons among bacterial pathogens has resulted in "gene epidemics" [10]. The TEM-1 plasmid-mediated β-lactamase was first described in 1965 in an *Escherichia coli* isolate from a patient in Greece but has since spread globally to multiple species. It has been found in up to 60% of clinical isolates of *Enterobacteriaceae*, to a few *Pseudomonas aeruginosa*, and up to 50% of *Haemophilus influenzae* and *Neisseria gonorrhoeae* isolates [18]. There are probably multiple factors that determine whether or not a mobilized gene will spread widely, but these are not well understood. For example, TEM-2 β-lactamase differs from TEM-1 by only a single amino acid substitution and provides an identical spectrum of resistance. It is also found on similar kinds of plasmids and transposons. However, the β-lactamase TEM-2 is at least tenfold less prevalent than TEM-1 in every region [18].

Many of the resistance determinants now found on plasmids, integrons, and transposons are believed to have originated in the chromosomes of other bacterial species, a phenomenon that has been well-documented in plasmid-mediated β-lactamases. The SHV-type β-lactamases are derived from the chromosomal β-lactamases of *Klebsiella pneumoniae*; plasmid-encoded AmpC enzymes expressed in *K. pneumoniae* and *E. coli* are nearly identical to chromosomal AmpC genes found in *E. cloacae* (ACT-1, MIR-1), *Citrobacter freundii* (CMY-type), *Hafnia alvei* (ACC-1), *Morganella morganii* (DHA-1), and the very successful cefotaxime-hydrolyzing CTX-M-type ESBLs from *Kluyvera* spp. [7, 19–21]. In addition, many aminoglycoside-modifying enzymes found in pathogenic bacteria were determined to have originated in environmental species of *Acinetobacter* [22, 23]. Many genes that are responsible for resistance to antibiotics that are natural products have migrated from the antibiotic-producing organisms (mostly streptomycetes), which have developed and retained these genes in order protect themselves against their own by-products. For example, the *erm* determinants that methylate 23S rRNA block binding of macrolides, lincosamides, and group B streptogramins to the target

ribosome are thought to have originated with the producing organism, *Saccharopolyspora erythraea*. Most plasmids, integrons, and transposons now carry multiple resistance genes conferring resistance to antibacterials of many different drug classes. Selection for any one of these resistance determinants will concurrently select for all of the resistance genes contained on this plasmid.

A few bacterial genera, such as α-hemolytic *Streptococcus* spp., *Neisseria* spp., and *Haemophilus* spp., are naturally transformable and can absorb and incorporate fragments of DNA that have been released by lysed organisms in close proximity, resulting in the creation of "mosaic" genes [24]. Mosaic gene formation is primarily responsible for penicillin resistance in *Streptococcus pneumoniae* [25].

10.3 Methods for Tracking Resistance

Typing systems for the epidemiological study of bacterial pathogens are based on the observation that, although different isolates of the same genus and species share microbiological, biochemical, serological, and physiological characteristics that distinguish them from other species, they also have detectable genetic differences that make discrimination at the intraspecies level possible [26]. In many circumstances, intraspecies variability is very high among unrelated isolates, and therefore, it is easily detectable. However, when dealing with human disease, several species of bacterial pathogens share overlapping niches and are subjected to identical environmental selective pressures. Molecular genetic studies of bacterial populations have demonstrated that there is some degree of homogeneity between pathogenic and environmental strains and making genetic differentiation relatively more complicated [27]. Consequently, one must understand that different typing methods give different, sometimes somewhat contradictory information that should be viewed as a totality of information for an examination of the phylogenetic and epidemiological relationships between pathogens. Molecular typing methods that utilize the genetic structure of bacterial pathogens have been used to address many different problems such as the study of genomic organization and evolution. In the context of bacterial resistance, they are now being used for the identification of patterns of infection and sources of transmission, the epidemiological surveillance of infectious diseases, and outbreak investigations [28].

10.3.1 Phenotypic Typing Methods

10.3.1.1 Antibiogram

Susceptibility testing can be performed with a number of antimicrobial agents, including drugs and antiseptic agents, to determine patterns of resistance on a macro level for most microbial species. Resistance breakpoints that are used clinically for

the detection of acquired resistance determinants may not coincide with therapeutic breakpoints used in the clinical microbiology laboratory. In addition, minimal inhibitory concentration (MIC) values are more informative than qualitative resistance patterns. However, discrimination is dependent on the diversity and relative prevalence of detectable resistance in the isolates in question. One drawback to using the antibiogram for epidemiology is that the stability of resistance pattern can be insufficient for use as a clonal marker, because resistance determinants may be encoded on plasmids or resistance genes may be expressed under control of complex regulatory systems [29–31]. The antibiogram is often the most valuable first-line typing methods in clinical laboratories that can quickly be used to assess the prevalence of resistance or the appearance of an outbreak strain. However, the integrity of data used to generate the antibiogram is crucial and is dependent upon the methods used for determining susceptibility. Many automated systems use short dilution ranges that surround the breakpoint for a given drug and may not provide enough information to discriminate between strains [32]. Nevertheless, the generation of an antibiogram has the advantage of being technically easy to use and interpret, even in small and resource-limited laboratories. It is relatively low-cost test suitable for testing large numbers of isolates and relies on routine clinical practice. Good reproducibility allows its use for definitive typing if a standard method such as MIC or disk diffusion as well as a standard set of marker antibiotics are utilized [28].

10.3.1.2 Serotyping

Traditional serotyping is applicable to single bacterial genus or species by using a defined set of polyclonal or monoclonal antibodies that detect specific surface antigens on the bacterial cell surface. The discrimination and frequency of cross-reactions of serotyping schemes are variable according to the specificity of reagents [28]. It is considered to be accurate and definitive, but only moderately discriminatory and requires the availability of high quality antisera [33]. In recent years, molecular serotyping assays have been developed that utilize DNA microarrays to detect sequences that encode various serovars of a bacterial pathogen. This has been applied to typing of the O antigen of *Salmonella* spp. [34, 35]. In addition, gene sequencing has been used to detect flagellin genes in *Campylobacter* spp. (*flaA*), capsular proteins in *S. pneumoniae* (*cps*), and M protein in Group A streptococci (*emm*) [36–38]. These arrays and sequencing schemes have been shown to have comparable results to traditional serotyping [33].

10.3.2 Molecular Typing Methods

Different high resolution molecular-based procedures have been used to detect the unique features of each individual organism. As a result, guidelines and some interpretive criteria have been proposed in an attempt to standardize what constitutes the "same strain" [27, 39].

10.3.2.1 Plasmid Analysis

The profile of the number and size of bacterial plasmids, some of which carry antimicrobial resistance determinants, can be used to determine the relatedness of strains during an epidemiologic investigation especially when combined with the utilization of restriction endonucleases to generate a restriction fragment length polymorphism (RFLP) analysis [40, 41]. However, plasmids that can be transferred even to other strains, including those of different bacterial species, are often unstable and may be lost or new ones acquired spontaneously. This makes plasmid fingerprinting somewhat difficult to reproduce [26]. Because of this variability in plasmid content, the use of plasmid profiling has been found to be insufficient for use as a clonal marker in some studies [29, 31, 41]. It is best combined with other genomic typing methods (at the chromosome level) to distinguish between spread of a clone and that of a plasmid [28].

10.3.2.2 Ribotyping

For ribotyping, chromosomal DNA is cleaved with a frequently cutting restriction endonuclease such as EcoRI or HindIII followed by conventional gel electrophoresis that resolves fragments from 50 to 0.5 kb. This is then followed by Southern blot hybridization with a probe that detects rRNA genes (*rrn*) [42, 43]. Because of the multiple copies of rRNA that are carried by most bacterial pathogens, this results in a pattern of 5–15 fragments [44]. The level of discrimination achieved with ribotyping varies depending on the bacterial species and the restriction enzyme used, but is typically low [45]. However, this can be improved with the use of a second restriction enzyme. Ribotyping has been used to determine whether pretreatment and posttreatment isolates of ESBL-producing *Enterobacteriaceae* were the same strain [46]. Ribotyping was also used to track an outbreak of *Clostridium difficile* with reduced susceptibility to vancomycin in a long-term care facility in Israel [47]. At least one automated system for performing ribotyping has been developed, which provides consistent data that can be compared across studies (Riboprinter® System, Qualicon). Using this system, ribotyping identified strains of methicillin-resistant *S. aureus* (MRSA) that were genotypically related to community-associated strains (CA-MRSA) isolated from Phase 3 clinical trials for complicated skin and skin structure and complicated intraabdominal infections [48].

A somewhat different approach to ribotyping from the RFLP-based method described above uses PCR to amplify the intergenic region between the genes encoding 16S and 23S rRNA. Most organisms contain more than one copy of the rRNA operon; therefore, the size of the intergenic region varies both within the same strain and between strains [49]. The amplified fragments are often separated with capillary gel electrophoresis [50]. This method is very reproducible, but the discriminatory power is moderate [33]. PCR-ribotyping can be applied to any organism, but in practice, it is mainly used for tracking and subtyping *C. difficile* [50].

10.3.2.3 Polymerase Chain Reaction (PCR) Fingerprinting

Several amplification techniques using PCR have been proposed as bacterial typing systems. The various PCR-based fingerprinting methods may involve the entire genome by the use of either arbitrary primers or primer pairs directed at the short sequences lying between repeat motifs in the bacterial genome [28, 51]. They are universal typing methods that can be applied to most bacterial species and exhibit a high level of discrimination between strains [51]. Major advantages of these techniques include flexibility, technical simplicity, wide availability of equipment and reagents, and same-day results [28].

RAPD/AP-PCR

One such PCR fingerprinting technique is the random amplification of polymorphic DNA with arbitrarily primed PCR (RAPD/AP-PCR). With this method, small genomic fragments are amplified using short primers (usually <14 bp) with random sequences that are hybridized under low stringency [52, 53]. Under these conditions, the primers will bind to both matching sequences and those regions with a few mismatches. The result is the amplification of numerous fragments of various sizes, which is unique to each strain. This technique is slightly less discriminatory than PFGE and is sometimes difficult to standardize because of the low-stringency PCR conditions [33]. RAPD fingerprinting has been used to detect the emergence of highly virulent strains of *Helicobacter pylori* following incomplete therapy [54]. It has also been used to track nosocomial outbreaks of CA-MRSA in China and Iran [55, 56].

Rep-PCR

Subtyping of strains based on the amplification of the region between interspersed repetitive loci in the DNA is called repetitive-element PCR (Rep-PCR). These duplicative sequences are present in many copies in most bacterial pathogens. Depending on the proximity of these sequences, they may or may not be amplified by PCR, resulting in a different sized amplification fragments in each strain [33]. Several different repetitive elements have been identified, but the ones most often used are extragenic palindromic sequences that are 33–40 bp in length, enterobacterial repetitive intergenic consensus sequences (124–127 bp), and BOX elements (154 bp, containing three subunits present in varying combinations) [57, 58]. Rep-PCR performed by manual methods is not always reproducible; therefore, it is difficult to compare results across different laboratories [33]. An automated platform for performing Rep-PCR has been developed (DiversiLab™ System, bioMérieux) that has standardized the method and improved the reproducibility [59]. Rep-PCR has recently been used to characterize the isolates of an outbreak of *A. baumannii* in Iran [60]. It was also able to track the dominance of CTX-M-type ESBLs among *Enterobacteriaceae* from environmental samples in Australia [61].

VNTR/MLVA Analysis

The intergenic regions of many bacterial genomes contain repetitive DNA sequences that are highly variable with regard to the number or structure of these repeat units. These variable-number tandem repeats (VNTR) allow for the differentiation between strains using a PCR-based method [62]. When multiple regions of these repeat sequences are analyzed, VNTR analysis is sometimes also referred to as multiple locus variable analysis (MLVA). The speed at which these intergenic regions change and evolve is also highly variable, making the various loci important for determining the evolutionary clock for the strains. This adds to the discriminatory power of this tool [33]. MLVA was famously applied during the 2001 bioterrorism-associated anthrax outbreak in the USA [63]. *Bacillus anthracis* is notoriously difficult to type using most molecular strain typing methods because there is very little variability among strains. We used MLVA to subtype 135 isolates from infected patients, powders from mail sources, and environmental samples. Subtyping of *B. anthracis* allowed anthrax cases to be linked to environmental specimens and powders and provided information about potential sources. MLVA was able to not only determine that all of the samples involved in the terror attack were identical to each other but that they were distinguishable from other Ames strains that had been isolated from other sources [63]. VNTR/MLVA has been used for multiple other applications. VNTR analysis is considered to be the gold standard for molecular typing of *Mycobacterium tuberculosis* [64]. It was also shown to be superior to Rep-PCR for strain discrimination when typing *Mycobacterium bovis* [65]. In addition, VNTR analysis was used to characterize an outbreak of linezolid-resistant isolates of enterococci in Polish hospitals [66].

10.3.2.4 Pulsed-Field Gel Electrophoresis (PFGE)

PFGE analysis (also known as macrorestriction) can simplify the fingerprint of bacterial pathogens by utilizing restriction endonucleases that infrequently cut the chromosomal DNA into a relatively small number of large fragments (20 kb to >1 Mb) [33]. PFGE uses a current that pulses in alternating directions to separate and resolve significantly larger fragments of DNA than is possible using constant field gel electrophoresis. In addition, shearing of these large fragments is avoided by stabilizing the DNA by embedding samples into an agarose plug [67]. PFGE can be applied to isolates of most species, although the restriction enzymes used may be specific to a particular organism [29]. The profiles generated by PFGE are highly reproducible [26]. One limitation for using PFGE to track resistance is that it is very labor intensive and can take up to 4 days to complete. Therefore, it cannot be used in a rapid response to an outbreak.

Interpretive criteria for chromosomal DNA macrorestriction patterns produced by PFGE have been proposed and guidelines applied successfully for different bacterial organisms [39]. Strains are considered to be unrelated if there are three or more bands (number and/or size of fragments) differing between two isolates. This

standardization has allowed for not only the use of PFGE for comparing isolates at a local level but also across many sites. The Centers for Disease Control (CDC) developed PulseNet in 1996, which is a national laboratory network that uses PFGE to detect thousands of local and multistate outbreaks of foodborne illnesses https://www.cdc.gov/pulsenet/index.html [68]. PFGE has been applied in many different scenarios for tracking resistance, including carbapenem-resistant *K. pneumoniae* in China [69], following NDM-1 among *A. baumannii* in Israel [70], and determining nosocomial transmission of MRSA in Malaysia [71].

10.3.2.5 Multilocus Sequence Typing

Multilocus sequence typing (MLST) utilizes nucleotide sequencing to detect variation due to mutations or recombination in fragments of five to ten housekeeping genes. Even a single point mutation difference between genes is considered to be a new allele [33]. The allele types for each of the housekeeping genes are used together to determine the sequence type (ST). MLST results can be analyzed using clustering software that can compare the genetic relatedness of strains belonging to different ST. Isolates with a high degree of similarity (e.g., differing by only one allele) may be placed into clonal complexes [72]. One advantage to MLST over PFGE is that the nucleotide sequence data is unambiguous and is not subject to variations in experimental technique. MLST data can be shared and tracked across laboratories via several websites such as http://pubmlst.org and http://www.mlst.net. In recent years, this has been increasingly replaced by whole genome sequencing with examination of the various loci [73, 74]. In addition, other new technologies such as the determination of base composition using electrospray ionization-mass spectrometry have been used [75].

MLST has been used extensively to track and monitor the spread of resistance, and several ST types have been noted that are highly associated with resistance mechanisms and have disseminated widely. *E. coli* sequence type 131 (ST131) has been identified as the predominant lineage among extraintestinal pathogenic *E. coli* and has been attributed to the rapid increase in antimicrobial resistance in that pathogen [76]. The global dissemination of this sequence type has contributed immensely to the worldwide emergence of fluoroquinolone-resistant and CTX-M-type β-lactamase-producing *E. coli* [76–78]. Surveillance studies have shown that the prevalence of ST131 comprises up to 30% of all *E. coli* clinical isolates, up to 80% of fluoroquinolone-resistant isolates, and up to 60% of ESBL-producing isolates [79].

K. pneumoniae ST258 is a prototype of a high-risk clone and has been largely responsible for the global spread of carbapenem resistance among the *Enterobacteriaceae* [80]. In particular, this ST type is highly associated with the spread of the KPC-2 carbapenemase [81]. In a global survey of KPC-producing *K. pneumoniae*, Peirano et al. found that 290 of 522 (55.6%) isolates from 19 different countries belonged to ST258 [81]. These isolates were evenly divided between two subclone groups, and bla_{KPC} was encoded on IncFIIK2-like plasmids in the majority

of the strains. A large outbreak of KPC-producing *K. pneumoniae* in Warsaw, Poland, was also determined to be caused by strains belonging to ST258 [82].

10.3.2.6 Whole Genome Sequencing

In recent years, next generation sequencing technology has become an easy and cost-effective method for performing molecular epidemiology by sequencing the entire genome of pathogens of interest. The strain relatedness of VRE isolated during three outbreaks in a hospital in Sweden was investigated to determine how WGS would compare to PFGE and MLST. The whole genome sequencing (WGS) data was analyzed using the average nucleotide identity analysis. PFGE analysis of the isolates confirmed what was already known by the clinical epidemiological investigation: that three outbreaks had occurred. However, there was no indication of further strain relatedness, or if there was a larger cluster. In contrast, the WGS analysis could clearly distinguish six clusters among the isolates [74]. WGS was also used to investigate a prolonged outbreak of KPC-producing *K. pneumoniae* and *E. cloacae* in a burn unit in the USA. WGS revealed that this outbreak, which seemed epidemiologically unrelated, was in fact genetically linked. The outbreak was primarily maintained by a clonal expansion of *E. cloacae* sequence ST114 that contained multiple resistance determinants including $bla_{\text{KPC-3}}$ that was transmitted via plasmids containing an identical Tn4401b [83].

Looking at the genome for any differences between strains can be overwhelming with the amount of data that this generates. Therefore, it is often more useful to focus on a subset of conserved genes in the bacterial species. Using this approach, carbapenemase-producing *K. pneumoniae* isolates from two distinct outbreaks that occurred in Switzerland in 2013 and 2015 were analyzed. The analysis correctly identified the two clusters of strains from the two outbreaks and differentiated these from *K. pneumoniae* that were unrelated to the outbreak [84]. Many of the previously described typing methods that utilize PCR and sequencing to detect differences in strains can now be done by WGS. The PulseNet International network conducts global laboratory-based surveillance for foodborne illnesses. PulseNet relies on MLST typing to track outbreaks of many pathogens. Previously, the MLST was done by PCR and sequencing; however, they have now transitioned into the standardized use of WGS to perform this subtyping [73, 85]. WGS was recently used to track an outbreak of carbapenem-resistant *K. pneumoniae* expressing OXA-232 to a contaminated duodenoscope in a California hospital [86].

10.4 Patterns of Resistance

Resistance patterns among bacterial pathogens should be measured and monitored at many levels. At one level, the epidemiology of resistance is extremely local, and unique patterns of resistant pathogens can be noted between different wards of the

same hospital. In the USA, a study found that the incidence of MRSA, VRE, ceftazidime-resistant *E. cloacae* and *P. aeruginosa*, and imipenem-resistant *P. aeruginosa* was two times higher in ICU patients than in patients in general wards or outpatients at the same hospitals [87]. Likewise, in Europe, the prevalence of MRSA was noted to be higher in ICUs than in the general patient population [88]. Most outbreaks and cluster cases of resistant pathogens involve a few patients in a single unit. The prevalence of resistance is often highest in units where the most debilitated patients are located. These units are also often where antibacterial usage is the greatest, resulting in a constant selective pressure for resistant strains [10].

The epidemiology of resistance can also vary greatly depending on region. In North America, resistance rates are generally higher in the USA than in Canada. The prevalence of MRSA among hospitalized adults in Canada was 22–28% but was 42–45% in the USA [89, 90]. In Europe, an extreme variation in the prevalence of resistance between countries is noted in that there is a very low incidence in the Scandinavian countries and very high percentages in the Mediterranean countries. The incidence of MRSA among *S. aureus* ranges from <1% in Sweden to 5–9% in Austria;10–28% in the UK and France; 25–49% in Portugal, Italy, and Greece; and a high of 50–>75% in Ukraine [91]. Very high prevalence of resistance has been noted in Asia and Latin America. Prevalence of penicillin-resistant *S. pneumoniae* (PRSP) in pediatric patients was 91.3% in Taiwan, 85.8% in Korea, and 70.4% in Vietnam, compared to <1%–5% in the UK and Scandinavian countries [91, 92]. Differences in the prevalence of specific pathogens are likely due to a combination of factors including variations in medical care and antibiotic prescribing habits.

Strains with similar resistance phenotypes to organisms causing outbreaks and epidemics have been found in the same hospitals or patient groups; however, they fail to spread [10]. The reason for the success of a particular resistant clone is not well understood. However, some potential factors may include (i) increased adherence or virulence mechanisms; (ii) retention of the fitness of the bacterium in the presence of the resistance genes; (iii) greater tolerance of desiccation, thus remaining longer in the environment outside of the human body; and (iv) resistance to disinfectants. A few of these outbreak strains have been studied in detail with regard to virulence factors contributing to their proliferation. In one study, one of the major subunits of a new fimbrial protein KPF-28 that aids adherence to the gut mucosa was found to be co-localized on a plasmid that also encoded the ESBL SHV-4 in serotype K25 *K. pneumoniae* strains that were circulating in France and Belgium in the 1990s [93].

10.5 The Increase and Spread of Resistance

Resistant strains have now disseminated globally. In part, this is due to increased international travel and open immigration. Several examples of the spread of resistant strains are discussed in the sections below.

10.5.1 MRSA

Shortly after methicillin (then called Celbenin) was introduced into clinical use in the 1950s in the UK, there were reports of clinical isolates of *S. aureus* that were resistant to methicillin [94]. *S. aureus* strains became methicillin-resistant by the acquisition and expression of PBP 2a, a low-affinity PBP not native to *S. aureus* and encoded by *mecA* [95]. The *mecA* gene confers resistance to all β-lactam antibiotics except for the anti-MRSA cephalosporins ceftaroline and ceftobiprole that can bind to PBP 2a [96]. The *mecA* gene is located within a mobile element in the *S. aureus* chromosome known as the staphylococcal chromosomal cassette (SCC*mec*) region. Numerous different *mec* regions (SCC*mec* I to XI) have been described [97–99]. However, transfer of the *mec* region between staphylococcal strains has never been documented.

Humans are a natural reservoir for *S. aureus*, including MRSA, with asymptomatic colonization of the nasopharynx, perineum, or skin being commonplace. This often occurs shortly after birth and may be transient throughout one's lifetime [100]. Transmission usually occurs by direct contact to a colonized carrier. In adults, the incidence of MRSA carriage is 25–50% for the general public. A higher incidence is observed in injection drug users, persons with insulin-dependent diabetes, patients with dermatologic conditions, and patients with long-term indwelling intravascular catheters. It is thought that healthcare workers may be an important reservoir for MRSA [101]. Young children tend to have higher colonization rates, most likely due to frequent contact with bodily secretions from other children [102]. Epidemic strains have spread through entire hospitals and even cities. The stability of the *mecA* genetic environments differ between various clones of *S. aureus* clones, suggesting a potential explanation for the limited lineages within which the resistance determinant has been found [103]. In 2011, MRSA of both human and animal origin isolates from Europe which were found to have a divergent *mecA* homologue termed *mecC* were first reported [104]. Isolates harboring *mecC* can be detected by susceptibility testing but not by commercial assays targeting *mecA* or PBP 2a.

Multilocus enzyme electrophoresis and other molecular population genetic techniques were used to determine the extent of *mec* distribution among phylogenetic lineages of the species and genetic relationships among MRSA strains isolated from 1961 to 1992 from various geographic regions [105]. This study found that the *mec* gene was harbored by many divergent phylogenetic lineages representing wide chromosomal diversity in the *S. aureus*. The conclusions were that multiple episodes of horizontal transfer and recombination contributed to the spread of this resistance determinant in natural populations of bacteria [105]. However, there can be regional difference in the spread. This study also identified a single multilocus enzyme genotype among MRSA isolates recovered in the UK, Denmark, Switzerland, Egypt, and Uganda, from the 1960s, which indicated that MRSA isolates recovered from those countries at that time were progeny of a single ancestral cell that had probably recently acquired the *mec* determinant.

Within a few years after that first occurrence, hospital outbreaks caused by MRSA occurred in Europe. By the mid-1970s, MRSA were recognized as an important hospital infection control problem in the USA, and subsequently, these organisms have now achieved global distribution [27]. In a study of bacteremic patients infected with *S. aureus* in England and Wales, the incidence of MRSA remained low (1–3%) for the first years of the study (1989–1993), but then the percentage of MRSA rapidly increased, reaching 42% by the year 2000 [106]. This increase coincided with the emergence of two new epidemic strains, (designated EMRSA) 15 and 16, which now comprise 95% of all *S. aureus* bacteremias in England and Wales [107]. Presently, many industrialized countries report that MRSA comprises at least 25–50% of *S. aureus* isolated in hospitals from infected patients [89]. In Japan, it was noted that high antibiotic consumption rates lead to increased MRSA burden over time [108]. In contrast, some countries such as the Netherlands and the Scandinavian countries have a low incidence of MRSA infections (around 1%) [109]. This is likely due to rigid infection control and surveillance policies, as well as restraint in antibiotic prescription in those countries.

At first, when cases of MRSA infection were identified in the community setting, an investigation usually exposed a history of recent hospitalization; close contact with a person who has been hospitalized; or previous antimicrobial-drug therapy. However, there were some notable exceptions. During 1980–1981, there was an outbreak of MRSA infections in Detroit among people with no history of hospitalization [110]. The majority of these patients were found to be injection drug users. The source of the Detroit outbreak was never identified, but it is assumed that frequent needle sharing was the mode of transmission in the community setting. The unexpected deaths of four Native American children with no known risk factors for acquiring MRSA in the late 1990s launched a new worry of community-associated MRSA (CA-MRSA) [111]. This more recent development in the spread of MRSA represents the migration of MRSA being completely healthcare associated (HA-MRSA) to now often finding CA-MRSA. These strains of CA-MRSA tend to be microbiologically distinct from HA-MRSA, typically more susceptible to commonly used antibiotics than are HA-MRSA. CA-MRSA is thought to have developed as an independent acquisition of *mecA* by a strain of methicillin-susceptible *S. aureus* (MSSA) [100]. A common clone of CA-MRSA belonging to pulsed-field gel electrophoresis (PFGE) type USA300 was originally widespread only in the USA; however, it has now become widespread throughout North America, Latin America, and the Caribbean [112–114]. CA-MRSA is discussed in detail in Chap. 3.

10.5.2 Penicillin-Resistant S. pneumoniae

S. pneumoniae is a major cause of illness and death worldwide, causing otitis media, acute sinusitis, community-acquired pneumonia, and meningitis [115]. Since the introduction of penicillin in the mid-1940s, the treatment of pneumococcal infections has primarily been with penicillin and other β-lactam antibiotics. Only 20 years

later, strains with decreased susceptibility to β-lactams were detected for the first time in 1967 [116]. PRSP were found periodically during the late 1970s but then rapidly emerged and disseminated soon after. In the USA, approximately 5% of pneumococcal isolates recovered during 1979 to 1987 were reported as testing with penicillin MICs of ≥1 μg/ml [117]. During the years 1993 to 1994, the percentage of nonsusceptible isolates was 14.1% but had increased to 25% by 1999 [118]. Antibiotic resistance in *S. pneumoniae* is now a global public health problem, although the incidence of PRSP differs by geographic region. 60–89% of *S. pneumoniae* are resistant to penicillin in parts of Latin America and Asia [27].

Penicillin resistance *in S. pneumoniae* is due to the expression of altered high-molecular-weight penicillin-binding proteins (PBPs) that have reduced affinity for binding and subsequent inhibition by β-lactam antibiotics [119]. It has been shown that alterations in the five high molecular-weight PBPs 1A, 2X, 2A, and 2B correlated with resistant patient isolates [25]. Acquisition of penicillin-binding protein gene segments from foreign donors, such as oral streptococci, thereby creating mosaic genes, is the primary mechanism for acquired resistance [120]. Following a recombination event in a single bacterium, drug-resistant progeny can proliferate with antibiotic pressure and subsequently can be transmitted both locally and globally by person-to-person spread [121]. The mechanisms of resistance in *S. pneumoniae* are discussed in detail in Chap. 2.

A good illustration of the rapid local clonal spread of antibiotic-resistant *S. pneumoniae* can be found by looking at the history of the development of resistance in Iceland. During a program to monitor of antibiotic resistance in *S. pneumoniae* in Iceland, no PRSP were isolated in years 1983 through most of 1988. However, in December of 1988, the first penicillin-resistant strain was isolated. The percentage of PRSP among *S. pneumoniae* then rose sharply 2.3% in 1989 to 17% by the first part of 1992 [122]. Approximately 70% of the isolates of PRSP were also resistant to multiple other antibiotics. Some of these isolates were characterized for relatedness, including serotype, PBP pattern, PFGE, and multilocus enzyme electrophoresis typing [123]. All of the isolates were found to be serotype 6B and had similar or identical patterns for each of the molecular markers examined. Interestingly, the PRSP isolated in Iceland were indistinguishable from a subgroup of serotype 6B PRSP that was frequently isolated in Spain. It was thought that the Spanish clone was imported to Iceland, as Spain was a popular vacation spot for Icelandic families with young children [124]. Continuing on with the study, it was noted that in 1995 PRSP comprised 24.2% of all pneumococci. There was then an interesting decline of the incidence of PRSP to 13.6% in 2001, followed by another rapid increase to 38.6% in 2010. The study found that at some point after 2001, 19F replaced type 6B in frequency, and by the end of the study, it accounted for 85.8% of all serogrouped PRSP [124]. The factors responsible for the spread of these clones of PRSP in Iceland remain unknown. However, it was noted that there was a high amount of trimethoprim-sulfamethoxazole and tetracycline usage in Iceland compared to other Nordic countries, which may have contributed to selecting the resistant clones. In addition, a high proportion of Icelandic children attend day care centers, which may have contributed to the rapid spread of PRSP in that country [27].

The incidence and spread of PRSP took a dramatic downturn following the introduction of the pneumococcal seven-valent conjugate vaccine (PCV7) in the year 2000 that included serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F, which are the most common serotypes found in invasive pneumococcal disease. As shown in Fig. 10.1, the incidence of PRSP among pediatric cases of invasive pneumococcal disease was 21.9 per 100,000 cases in 1999 [125]. Approximately 80% of PRSP isolated in the pre-PCV7 era in the USA were of serotypes commonly carried by healthy children (serotypes 6B, 9V, 14, 19F, and 23F), and serotype 19A was rare [118]. Following the introduction of the vaccine, the incidence dropped to 2.3 in just 10 years, followed by a further reduction to <1 PRSP in 2013. However, there was also a noted difference in the serotypes now responsible for β-lactam resistance in *S. pneumoniae*. Following the widespread implementation of the PCV7 vaccine, the highly resistant serotype 19A became the predominant serotype among PRSP. Fortunately, a 13 valent vaccine was introduced in 2010 that included serotype 19A; therefore, the further dissemination of PRSP was limited [126].

10.5.3 Vancomycin-Resistant Enterococcus *spp.*

Vancomycin-resistant *Enterococcus* (VRE) *faecium* was first encountered in clinical isolates in Europe in 1986, followed the next year by isolation of VRE *faecalis* in the USA [127, 128]. In Europe, the increased prevalence of VRE was primarily in the community setting, thought to arise from the use of a glycopeptide antibiotic avoparcin as a growth promoter in livestock, thus causing transmission from animal food products to humans [129]. In contrast, in the USA, the predominance of VRE was in the hospital setting a consequence of the increasing use of the glycopeptide antibiotic vancomycin due to the prevalence of MRSA in North America [130]. Subsequently, the USA experienced a rapid spread of VRE in hospitals in the 1990s, followed eventually by a worldwide spread of this resistance [131, 132].

The majority of VRE colonization occurs in the GI tract, but can also be found on the skin, and in the genitourinary tract [133]. GI colonization with VRE can persist for months to years and is often refractory to decolonization efforts [134]. Transmission of VRE within the hospital has been traced to the hands of healthcare workers [135]. In most patients, the VRE colonization does not result in infection. However, when a patient becomes immunosuppressed, the VRE can flourish, causing a clinical illness [133]. Additional risk factors for colonization and subsequent disease has also been linked to previous exposure to antibiotics [136].

E. faecalis is the most common cause of enterococcal infections. However, *E. faecium* is intrinsically more resistant to antibiotics with more than half of nosocomial isolates in the USA expressing resistance to ampicillin, vancomycin, and aminoglycosides [137]. Among Western countries, the prevalence of VRE is the highest in North America, with VRE comprising 35.5% of enterococcal hospital-associated infections. This ranks as the second most common cause of nosocomial infections in the USA [138]. In contrast, Canada has a much lower incidence of VRE, with

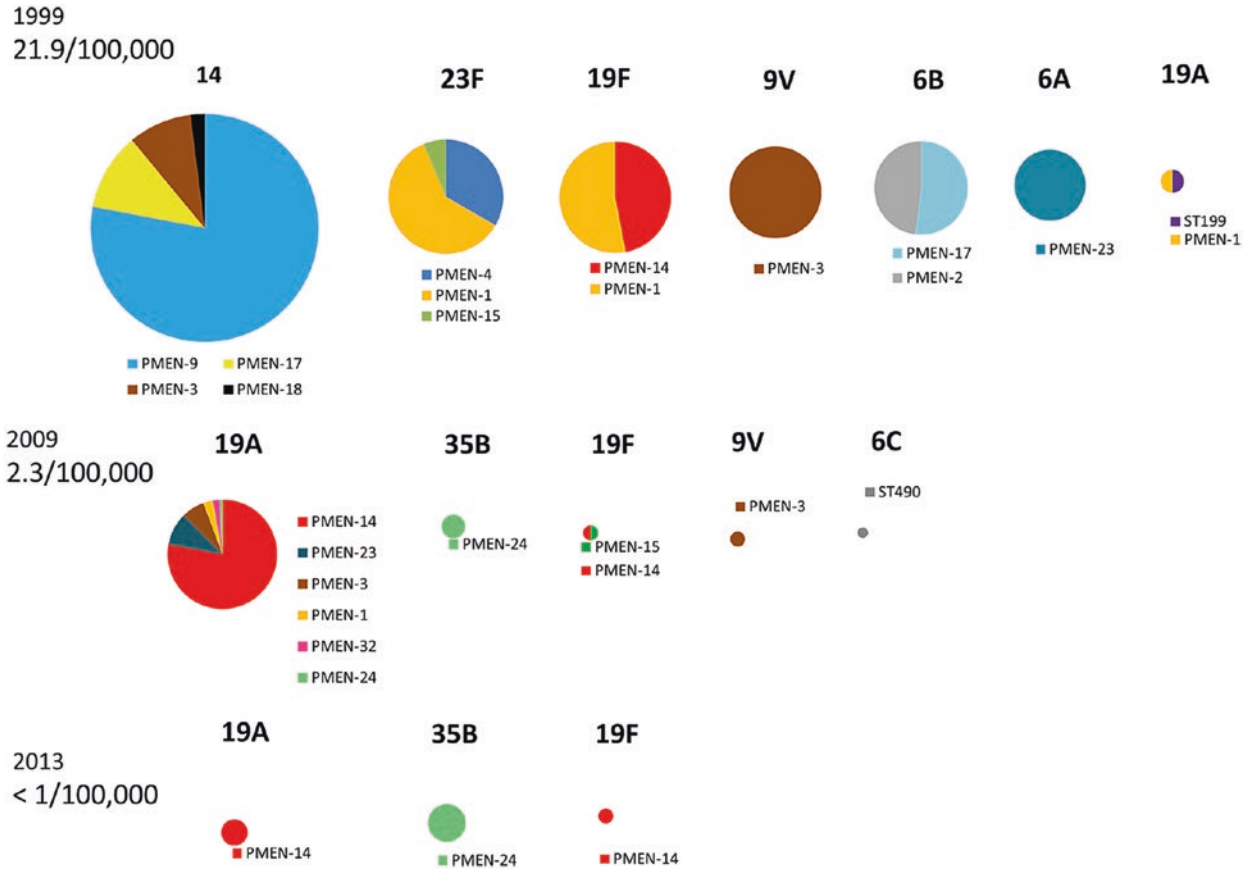

Fig. 10.1 Approximate numbers of cases per 100,000 individuals caused by penicillin resistance for each year (left). These data encompass all clonal complexes and serotypes associated with penicillin resistance (MIC of 2 μg/ml) in cases of pediatric invasive pneumococcal disease during 1999, 2009, and 2013. The circle diameters reflect relative incidence of disease [125]

only 6% of enterococci in Canada expressing resistance to vancomycin [139]. In Europe, VRE is less prevalent than in the USA but appears to be increasing in frequency. The European Antimicrobial Resistance Surveillance System (EARSS) reported a VRE *faecium* prevalence of 8.3% in 2015 [140]. The prevalence in the EU is variable depending on the country, with VRE ranging from less than 1% in France, Spain, and Sweden to greater than 20% in Greece, Portugal, and the UK, all the way up to 45% in Ireland.

10.5.4 KPC

The first incidence of the *Klebsiella pneumoniae* carbapenemase (KPC)-type β-lactamase was first identified in a 1996 isolate of *K. pneumoniae* from a single patient in North Carolina [141]. In the next decade, *K. pneumoniae* expressing KPC-2 gained a foothold in New York City [142]. Unfortunately, KPC-producing strains have spread very far beyond the Northeast USA and have spread worldwide [80]. During the mid-2000s, several outbreaks were detected in hospitals in Israel due to *K. pneumoniae* type ST258 that were nearly identical to the KPC-producing strains in New York, most likely due to the frequent travel back and forth of citizens of both countries [143]. In addition, KPC-type enzymes have been found among many different genera of *Enterobacteriaceae* and non-fermentative Gram-negative pathogens [144, 145]. In a recent survey of Gram-negative pathogens, KPC-producing isolates were found in 22 countries on four continents (Fig. 10.2). Although *K. pneumoniae* remained the most prevalent pathogen, KPC-type β-lactamases were also found in *Enterobacter aerogenes*, *Klebsiella oxytoca*, *Citrobacter freundii*, and several other genera [144]. Recent outbreaks of KPC-producing *K. pneumoniae* have been reported across the globe, including China, Korea, Poland, Portugal, and Switzerland [82, 84, 146–148]. A widely publicized outbreak of KPC-producing *K. pneumoniae* was documented in a clinical trial unit at the National Institutes of Health [149]. In this outbreak, 11 of 18 infected patients died as a result of their infections. The source of the outbreak was tracked to a single index case that left the unit 3 weeks prior to the next apparent case. The outbreak was subsequently halted using strict infection control practices; however, sporadic cases still occur [149]. In the last few years, the prevalence of KPC among in *K. pneumoniae* isolates in NYC has declined. This is most likely due to concerted effort to reduce the use of indwelling catheters and increased infection control [150].

KPC-2 and KPC-3 are the most prevalent isoenzymes among KPC-type β-lactamases. Bacterial pathogens expressing these enzymes are resistant to a variety of most β-lactam drugs including expanded-spectrum cephalosporins and carbapenems [151]. Some of the newer β-lactam/β-lactamase inhibitor combinations are active against strains expressing KPC [144, 152]. Bacterial pathogens expressing KPC significantly impact clinical management of patients with serious infections, in that they are often multi- or pan-resistant to many of the currently available

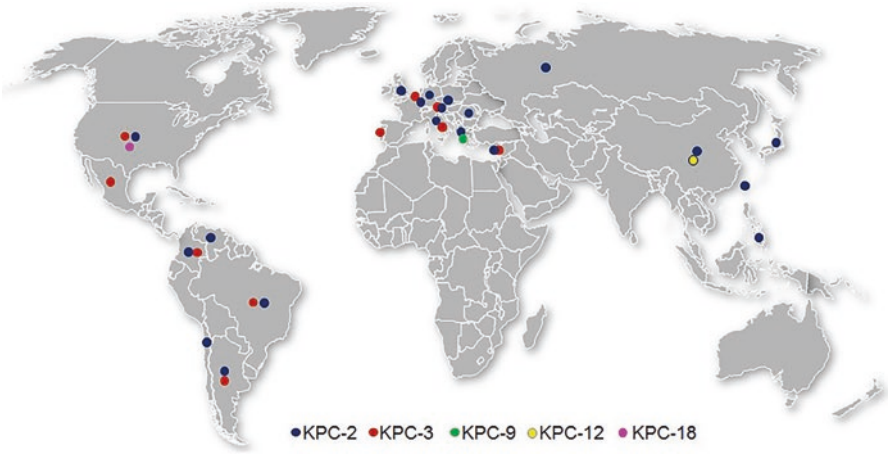

Fig. 10.2 Distribution of KPC-positive *Enterobacteriaceae* and *P. aeruginosa* collected in 2012 to 2014 from surveillance. (Figure from [144])

first-line therapeutic options [153, 154]. The successful spread of KPC has been primarily due to the spread of *K. pneumoniae* isolates belonging to the successful clonal complex ST258 [153, 154]. For isolates belonging to ST258, bla_{KPC} is most often found inside of the Tn*4401* transposon. Furthermore, the promiscuous plasmids harboring bla_{KPC} also commonly carry genes encoding resistance to aminoglycosides and additional β-lactamases, including ESBLs and metallo-β-lactamases [144, 145]. KPC has also been found in the same strain as metallo-β-lactamases. bla_{KPC} has also been identified the bacterial chromosome [155].

10.5.5 NDM

The New Delhi metallo-β-lactamase (NDM) was first described in an isolate of *K. pneumoniae* from a patient that had previously been admitted to two different hospitals in India during 2007 and then transferred to Sweden where the organism was identified and characterized [156]. The DNA sequence of NDM-1 shares very little identity with those of the other common MBLs found in *Enterobacteriaceae*, with only 32.4% amino acid identity share with VIM-1, its closest relative [157]. A 2010 study by Kumarasamy et al. showed that NDM-1 was endemic to all parts of India and Pakistan [158]. They also found that it had spread to the UK, mostly due to patients with a travel history to the Indian subcontinent (Fig. 10.3). In only 7 years since those initial few reports, NDM-1 has spread across the globe and has now been found in a multitude of countries. A recent surveillance study showed that NDM-type enzymes comprised 44.2% of all MBL-producing *Enterobacteriaceae* collected worldwide (Fig. 10.4) [159]. This study also showed that NDM has spread from *K. pneumoniae* and *E. coli* into multiple species of *Enterobacteriaceae*, *P.*

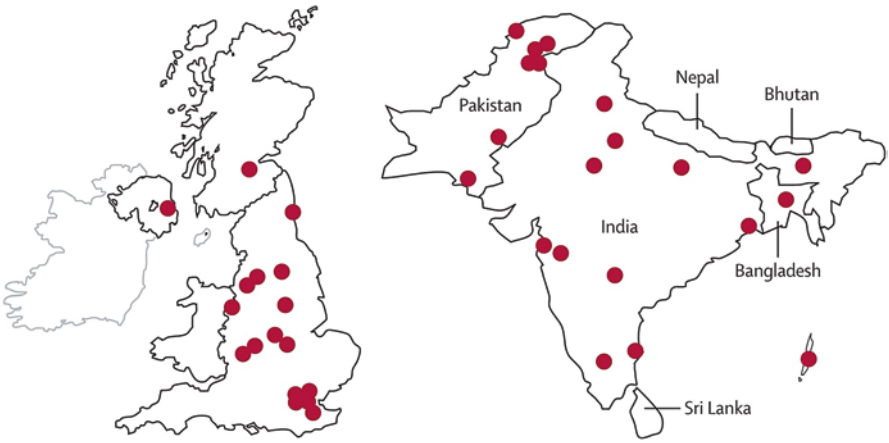

Fig. 10.3 Distribution of NDM-1 producing *Enterobacteriaceae* strains in Bangladesh, India, Pakistan, and the UK in 2010. (Figure from [158])

aeruginosa, and *A. baumannii*. In addition to the Indian subcontinent, NDM appears to now be endemic in the Balkan countries, Northern Africa, and on the Arabian peninsula, countries which may serve as a secondary reservoir [157]. Documented outbreaks caused by NDM-producing *Enterobacteriaceae* have recently been reported in a single hospital in Mexico, multiple centers within the Netherlands, and in a neonatal unit in China [160–162]. Unlike the case with KPC, high-risk clones and epidemic plasmids do not seem to play important roles in the global dissemination of NDMs [80].

10.5.6 Fluoroquinolone Resistance

Resistance to ciprofloxacin appeared quickly as single step mutations among many Gram-positive pathogens, following its widespread usage beginning in the 1990s. In Taiwan, 11% of *Streptococcus pyogenes* were found to be resistant to fluoroquinolones, and this was highly associated with the presence of the erythromycin resistance determinant *emm*12 [163]. However, susceptibility of *S. aureus* to levofloxacin remains high [164]. In contrast, fluoroquinolone resistance among *Enterobacteriaceae* is a growing concern. Current surveillance data indicates that worldwide levofloxacin resistance rates for *E. coli* have steadily increased. Reports from the National Healthcare Safety Network that monitors the prevalence fluoroquinolone resistance reports that among *E. coli* isolated from bloodstream isolates in the USA fluoroquinolone resistance increased from 30.8% in 2006–2007 to 41.1% in 2011 to 49.3% in 2014 (greater than 2% increase per year) [165]. In Europe, the prevalence appears to be stable with percent fluoroquinolone resistance in invasive *E. coli* isolates from blood or cerebrospinal fluid at

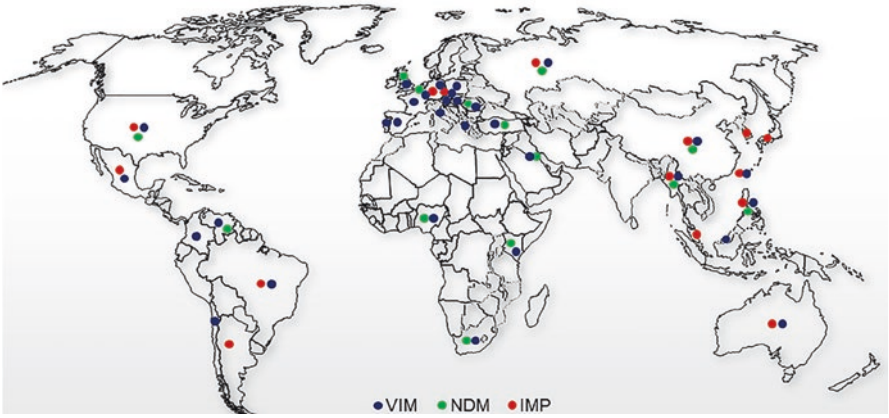

Fig. 10.4 Global distribution of metallo-β-lactamase-positive *Enterobacteriaceae* and *P. aeruginosa,* including NDM-type enzymes collected from 2012 to 2014 from surveillance. (Figure from [159])

approximately 22.8% during 2012–2015 [140]. However, there was a wide variation in the percent resistance among different European countries. The fluoroquinolone resistance for Europe ranged from 2.9% in Iceland up to 70% in Slovakia. A recent survey of 10,000 *E. coli* isolates from UTI in US hospitals showed an overall incidence of resistance to fluoroquinolones of 34.5% [166]. The epidemic CTX-M-15 expressing clone *E. coli* ST131 is often also resistant to fluoroquinolones, and this is thought to have played a significant role in the spread and maintenance of this strain [79]. The potential for the spread of fluoroquinolone resistance has increased with the development of plasmid-meditated fluoroquinolone resistance genes such as *qnr* [167]. In several longitudinal surveys, the incidence of *qnr* among *K. pneumoniae* in Taiwan increased from zero in 2000 to 7.6% in 2005 [168]. Similarly, among clinical isolates of *Enterobacter* spp. isolates from Israel, there were none positive for *qnr* during 1990–1993, but 6.8% were positive in 2005 [169].

10.5.7 Aminoglycoside Resistance

Aminoglycosides are used clinically to treat serious infections caused by Gram-negative pathogens, especially *P. aeruginosa*. However, as with most antibiotics that are widely used, resistance has become widespread. In the EU, the population-weighted mean percentage for aminoglycoside resistance in *E. coli* was 10.4% in 2015; however, there was a wide variation in the percentages of resistant isolates by country, with a low of 2.9% in Iceland to 24.2% in Slovakia in 2015 [140]. For this same population, the overall percent resistance among *K. pneumoniae* isolated in the EU was 22.5%. A survey of *Enterobacteriaceae*, *Acinetobacter* spp., and *P.*

aeruginosa showed that 91.9%, 79.6%, and 63.5%, respectively, of aminoglycoside-resistant isolates carried at least one aminoglycoside-modifying enzyme (AME) [170]. The most prevalent AMEs were *aac*(6′)-Ib (37.5%), *ant*(3″)-Ia (25.5%), and *aac*(3)-IIa (22.5%), with 13% of isolates carrying two or more AMEs. In another study from Spain, among 330 aminoglycoside-resistant isolates of *Enterobacteriaceae*, the predominant resistant determinant was *aph*(3″)-Ib (65.4% of isolates), which correlated to the streptomycin resistance in these strains [171]. AMEs are very often found in clinical isolates that produce other important resistance determinants, such as ESBLs and carbapenemases. A study showed that 98% of KPC-producing *K. pneumoniae* that were isolated from two hospitals in the US ($N = 50$) also expressed one or more AME [172]. Of these, 98% were positive for *aac*(6′)-Ib, which correlated with resistance to tobramycin. Aminoglycoside resistance due to the 16S rRNA methyltransferases (RMT) is much less widespread, and prevalence varies by region. A surveillance study conducted on isolates from 2007 to 2008 in Asia detected the presence of RMTs in 10.5% from India, 6.9% of isolates from China, 6.1% from Korea, 5.0% from Taiwan, and only 1.5% in Hong Kong [173]. A lower prevalence of RMTs (≤1.3%) was noted among isolates of *Enterobacteriaceae* from Europe [174]. RMTs are frequently found in the same isolates harboring the NDM-1 metallo-β-lactamase [175, 176].

10.5.8 Colistin Resistance

Until the recent years, the incidence of resistance to colistin was very low. However, because of the rise of carbapenem resistance among *Enterobacteriaceae*, there has been an increased usage of the drugs of polymyxin class, chiefly colistin [177]. Subsequently, reports of colistin resistance are being seen with increasing frequency. A recent study of colistin resistance among a global collection of clinical isolates of *Enterobacteriaceae* from 2012 to 2013 showed only 1.6% in the overall population; 12% in carbapenemase positive isolates were resistant [178]. Colistin susceptibility was higher among MBL-positive isolates (92.6%) than those positive for a KPC (87.9%) or OXA-48 (84.2%). In this study, approximately 2.4% of all *K. pneumoniae* isolates were colistin resistant compared to only 0.3% of *E. coli*. Interestingly, the prevalence of colistin resistance was relatively high among *Enterobacter* spp. but varied from 39% in *E. asburiae* to 0.4% in *E. aerogenes*. This study also noted regional differences in the prevalence of colistin resistance. For *K. pneumoniae*, the prevalence ranged from 5% in Greece, 4.7% in Italy, and 3.2% in Romania to 1.3% in North America [178]. Other studies have also shown *K. pneumoniae* resistance rates ranging from 10.5–20% in resistance hotspots such as Greece to 2.9% in Canada [179].

Previously, the spread of colistin resistance was limited to the expansion of resistant clones because the development of resistance depended upon mutations in the bacterial chromosome. However, the recent discovery of colistin resistance due to the plasmid borne *mcr-1* may have a very great impact in the coming years. The

mcr-1 gene was first described in a porcine isolate of *E. coli* in China [180]. Since that time, a multitude of reports from all over the globe have emerged. It has been found in patients with *Salmonella* spp. in China, *E. coli* in Belgium and Oman, and *K. pneumoniae* in South Africa [181–184]. The first detection of the *mcr-1* gene in *E. coli* from a patient in the USA was reported in 2016 [185]. In a survey of 390 colistin-resistant isolates collected in 2014–2015, none of the *K. pneumoniae* isolates harbored *mcr-1* (0 of 331 isolates), whereas 19 of 59 *E. coli* isolates had *mcr-1* [186]. A study of fecal carriage of mcr-1 in the Netherlands showed detection of the gene, although among very few patients (0.35%), but it was detected nonetheless [187]. There continues to be a high correlation of animal to human spread of *mcr-1*. This gene was found among patient and animal samples of *Salmonella* spp. in China [188]. Similarly, a recent study detected *mcr-1* in *E. coli* and *Salmonella* spp. isolates of animal origin in Europe, in every year from 2004 to 2014 [189]. It was also found among *E. coli* from a pig farm in Germany [190]. There have also been reports of bacteria containing *mcr-1* in sewage and wastewater in China, Germany, and Spain [191–193]. It is difficult to know if *mcr-1* is really spreading or if it is just an increased awareness of this resistance determinant which is causing its increased detection.

10.6 Epidemiology of Resistance in Special Populations

Most resistance among bacterial pathogens has been documented in hospitalized patients, many of which are in the ICU setting. Some infections are spread by contact with healthcare workers. However, studies have shown that drains, sinks, and faucets in ICU patient rooms were frequently colonized [194]. Although the hospital setting remains the most common location where resistant pathogens are encountered, resistant clones have become endemic in certain populations of people within the community. In addition, some outbreaks have occurred in some interesting places and groups.

10.6.1 Daycare Centers

Daycare centers that look after infants and preschool-aged children have been shown to harbor and spread resistant isolates. This is likely due to the fact that these children frequently share bodily secretions and receive multiple courses of antibiotics in their first few years of life. The high incidence of PRSP in Iceland (discussed in Sect. 10.5.2 above) was partially attributed to the large number of children that attend daycare in that country [27]. A study to document the increased carriage of PRSP by children in daycare centers was undertaken in Japan. Researchers sampled the nasopharyngeal passages of children from newborns to 6 years attending two daycare centers [195]. From 363 children cultured, they found that the overall

carriage incidence of *S. pneumoniae* was 3.3%. Of these, the percentages of penicillin nonsusceptible (PNSP) and erythromycin nonsusceptible *S. pneumoniae* strains were 36.73% and 71.3%, respectively. Interestingly, using PFGE, they found many different strain types among the isolates and attributed the genetic diversity of the resistant strains to the high turnover among the children in the daycare centers [195]. A longitudinal study of children in Guatemala revealed that from the years 2001 to 2006, the percentage of children colonized with PNSP rose from 1.5% to 33.3% [196].

Similarly, the prevalence of MRSA was studied in two daycare centers in the USA [102]. In one center, 2 of 61 (3%) children were colonized with MRSA, and 9 of 40 (24%) children were colonized at the second. Ten of 11 of the children were in classes for 2–3-year-old toddlers. In the second center, all of the MRSA belonged to one of two PFGE types. None of the daycare workers nor household members of the toddlers had any previous contact with a hospital, or healthcare worker, indicating that the daycare center itself was the reservoir for MRSA in that community [102]. A similar study was conducted in 500 children attending multiple daycare centers in Brazil [197]. They found that 48% of the children were colonized with *S. aureus* and 6.2% were colonized with MRSA. This study also looked at socioeconomic factors associated with resistance. They found that children attending daycare in low-income public housing sectors were 3.3 times more likely to be colonized with MRSA than children in other areas [197].

10.6.2 Long-Term Care Facilities

In the early 1990s, long-term care facilities (nursing homes), mostly comprised of elderly patients, became recognized as an important reservoir for antibiotic-resistant pathogens during one of the earliest outbreaks of ESBL producing (TEM-10) that occurred in Chicago [198]. The source of the outbreak was tracked to an index patient that had been admitted to the hospital from a long-term care facility. Subsequently, the authors conducted a study to determine the extent of prevalence of ESBL-producing strains among these patients [40]. With that goal, they identified 55 hospitalized patients who were colonized or infected with ESBL-producing *E. coli* or *K. pneumoniae*, during an 18-month period in 1990–1992. Of these 31 patients that were admitted from 8 different nursing homes were positive for TEM-10 expressing pathogens, all of which were resistant to ceftazidime, gentamycin, and tobramycin. As a case-control study, 24 nursing home patients colonized with resistant strains on hospital admission were compared with 16 patients admitted from nursing homes that were not colonized on hospital admission. A strong correlation ESBL carriage aligned with poor cognitive function, presence of a gastrostomy tube or decubitus ulcers, and prior treatment with antibiotics [40]. At the same time across the country, another outbreak of ESBL-producing *E. coli* (SHV-7) was described among elderly patients from nursing homes admitted to a New York hospital [199]. More recently, the prevalence of ESBL-producing *Enterobacteriaceae*

was studied among nursing home residents in Germany [200]. Using rectal swabs to survey, they found that 14.7% of the residents were colonized with ESBL-producing *E. coli*. All of the isolates expressed a variant of $bla_{\text{CTX-M,}}$ with the most common isolated being CTX-M-15 in 65.2% and CTX-M-27 in 21.7%. Moreover, 69.6% could be assigned by polymerase chain reaction (PCR)-typing to the epidemic clonal lineage *E. coli* ST131 [200].

Other studies have examined the prevalence of multiple types of resistant pathogens among residents of long-term care facilities. Trick et al. conducted a study to determine the frequency of and risk factors for colonization of nursing home residents by MRSA, vancomycin-resistant *Enterococcus* (VRE), and ESBL-producing *K. pneumoniae* or *E. coli* [201]. Of 117 residents that were sampled, 43% were culture positive in at least one antimicrobial-resistant pathogen as follows: MRSA 24%, VRE 3.5%, ESBL-producing *K. pneumoniae* 18%, and ESBL-producing *E. coli* 15%. At the time of the study, only three of the residents were under contact isolation precautions. Risk factors for colonization included a total dependence on healthcare workers for activities of daily living and prior therapy with antibiotics. Another study examined the molecular epidemiology and antimicrobial susceptibilities of *C. difficile* strains in long-term care facilities in Israel. Toxigenic *C. difficile* isolates were recovered 23.6% of the nursing home patients that were sampled. Many of these were of the predominant ribotype 027, which had increased MICs of vancomycin and metronidazole [47]. The authors recommend increased contact isolation and other infection control measures be implemented by the facilities [201].

10.6.3 Sports Teams

Outbreaks of MRSA usually have been associated with healthcare institutions. However, CA-MRSA is emerging as a cause of skin infections in the community, many of which have been documented in outbreaks among players of competitive sports, especially athletes who play contact sports [202]. These outbreaks have occurred in young, otherwise healthy high school and college students participating in wrestling, rugby, and American football [203, 204]. In the outbreaks among football players, risk factors have included skin trauma from turf burn and direct contact with lesions of other players [204]. The transmission of MRSA has also been documented through sharing equipment, towels, or personal items such as skin ointment, soap, or shaving razors [205, 206]. Among football players, MRSA infections were more likely to occur in linemen, a position that is more prone to receiving abrasions than some of the other players, and second in prevalence among cornerbacks and wide receivers that have frequent direct person-to-person contact with each other during scrimmage play [204, 207]. MRSA infections among wrestlers has been linked to contaminated wrestling mats, likely contaminated by infected lesions of the participants [203, 206]. Transmission has also been linked to whirlpools that are found in athletic department training facilities at most universities. A study found a strong correlation between the number of athletes using the whirlpool and the

presence of *S. aureus* in and around the whirlpools [206]. In a systematic review of the MRSA incidence, a statewide survey was conducted among high school athletes in Nebraska during the school years 2006–2007 and 2007–2008. Physician documented MRSA infections were reported among one or more athletes by 4.4% of the schools during the first year and 14.4% of the schools during the second. During 2007–2008, MRSA incidence per 10,000 wrestlers was 60.1 and 25.1 per 10,000 among football players [208].

Although less common, MRSA outbreaks have also been seen among athletes in noncontact sports. An outbreak of CA-MRSA was also reported among athletes participating in a fencing club in Colorado [205]. Five cases with a skin infection among the fencers plus one household contact were identified by culture. Because of the patients' low-risk history, patients with confirmed cases reported recurrent infections for which they made multiple healthcare visits before their wounds were cultured. Although the fencers did not share face masks or other equipment, they did share a sensor wire that is worn under clothing to record when they have been touched by an opponent's weapon. Following the implementation of routine cleaning of the sensor wires, no additional cases occurred [205].

A number of high profile college and professional football players have been infected with MRSA. Some of these have resulted in career-ending episodes, including amputation [209, 210]. In a very well-documented outbreak among the Saint Louis Rams, 5 of the 58 Rams players (9%) developed infections with MRSA during the 2003 football season [211]. All of the infections developed at turf-abrasion sites and was significantly associated with the lineman or linebacker position and a higher body mass index. Four of the five players had direct contact on the field during intra-team scrimmages (Fig. 10.5). None of the players were colonized with MRSA, but it was recovered from whirlpools and taping gel. Using PFGE, the MRSA from the Rams were determined to be of common CA-MRSA clone USA-300. An identical clone was found among some MRSA isolated from an opposing team that the Rams played shortly after the outbreak started, indicating the clone had been transferred from the Rams to the second team. In a review of the practices of the team, several things were noted that probably contributed to the spread of MRSA among the athletes: (i) hand hygiene products were not available to trainers that provided wound care to the players near the areas where wound care or physical therapy was provided; (ii) towels were frequently shared on the field during practice and games, with as many as three players using the same towel; (iii) players often did not shower before using communal whirlpools; and (iv) weight training and therapy equipment at the training facility was not routinely cleaned. The outbreak was contained after the implementation of infection control hygiene practices in the training facility (Fig. 10.5) [211].

Physicians should be aware of the potential for MRSA infections in sports participants when evaluating patients and making treatment decisions. As demonstrated by the cluster of MRSA infections among fencers, patients with recurrent MRSA infections might make multiple healthcare visits before a wound culture is obtained. Recurrence of infections might be avoided if physicians obtain cultures more routinely when athletes have infected wounds [205].

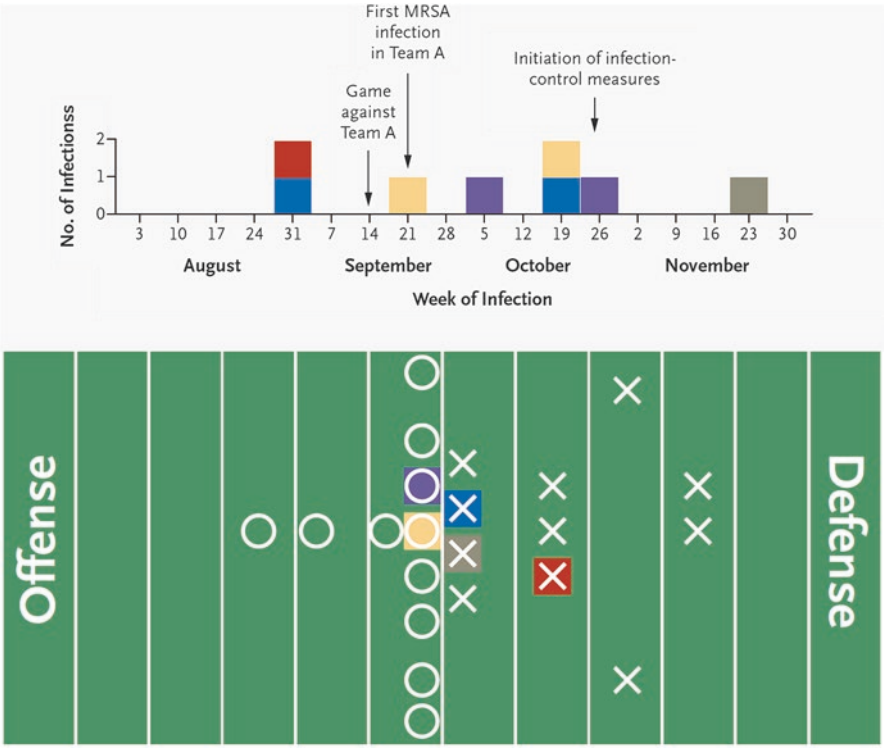

Fig. 10.5 Epidemic curve graph (top) and field position diagram (bottom) of cases of MRSA infection among St. Louis Rams professional football players in 2003. Each box on the epidemic curve graph and field diagram represents an MRSA infection; different colors designate different players; boxes of the same color thus represent recurrent infections. On the field diagram, X represents a defensive player position and O an offensive player position [211]

10.6.4 Resistance Among Travelers

With the ease of international travel in today's world, there has been an increase in documented transplantation of previously regional resistant strains into a naïve population of people. This mainly occurs when people return home from abroad, either infected or colonized with a resistant pathogen, and then transmit it to another individual. Recently, there have also been cases of resistant bacterial pathogens being imported to a destination country by refugees or those seeking asylum from their country of origin. A very well-documented example of the importation of a resistance mechanism was NDM-1 arriving in the UK in an individual who had recently returned from India (discussed above in Sect. 10.5.9) [158]. A study in Finland revealed that 46% of travelers returning from South Asia were colonized with ESBL-producing *Enterobacteriaceae* [212].

It has been shown that popular travel destinations can be a localized reservoir for resistant pathogens. In a systematic review of the literature from 2002–2017, Leangapichart et al. looked at reports of antibiotic resistance among pilgrims and

workers attending the Hajj in Mecca, Saudi Arabia [213]. MRSA carriage was reported in 20% of pilgrims and food handlers. Carbapenem resistance was detected in less than 10% of *E. coli* isolates, but up to 100% of *K. pneumoniae* and *A. baumannii* isolates. Colistin-resistant *Salmonella* spp., *E. coli*, and *K. pneumoniae*, including *mcr-1*-mediated colistin resistance, were detected among the pilgrims [213]. A cluster of genetically related, azithromycin-resistant *N. gonorrhoeae* was detected in Oahu, Hawaii, in a 1-month period in 2016. The majority of the isolates were also resistant to ceftriaxone, which eliminated both of the currently recommended therapies for treatment from consideration [214].

Antibiotic resistance has also been noted in cases of traveler's diarrhea, some of which caused larger outbreaks in the home country after return. Kim et al. studied an outbreak of intestinal illness caused by *Shigella sonnei* in a daycare center in South Korea. The outbreak strain was resistant to extended-spectrum cephalosporins (via CTX-M-15) and fluoroquinolones. The index case was a child who had recently returned from traveling to Vietnam [215]. Another study in Belgium examined antibiotic resistance patterns among isolates of *Campylobacter* spp. obtained from returned international travelers that were symptomatic for traveler's diarrhea and analyzed the data based on travel destination [216]. For the group as a whole, 60.9% of the isolates of *Campylobacter* spp. were resistant to ciprofloxacin, with the prevalence ranging from 50.8% in Africa to 75.0% in Asia. Resistance to erythromycin was 4.6%, with the highest incidence (15.2%) seen in isolates from individuals who had traveled to Southern Asia (six of seven patients returning from India) and 48.3% were resistant to tetracycline [216]. A recent study examined 453 cases of enteric fever in London caused by *Salmonella enterica* subspecies Typhi and Paratyphi. For patients with a history of travel, 88% of *S.* Paratyphi A isolates were resistant to ciprofloxacin. For isolate of *S.* Typhi, 80% were resistant to ciprofloxacin, 26% to ampicillin, and 27% to chloramphenicol [217].

Migration due to refugees and asylum seekers has been noted to be one of the risk factors for the spread of multidrug-resistant organisms, which can challenge local healthcare systems. Ravensbergen et al. analyzed cultures performed in Dutch hospital on asylum seekers during 2014–2015 and compared the results to those obtained from Dutch nationals. Of 118 asylum seekers with *S. aureus* in clinical cultures, almost 19% were MRSA positive compared to only 1.3% in the general public. In addition 20.3% were infected with *Enterobacteriaceae* that produced ESBLs [109]. Similarly, 20% *E. coli* isolated from refugees admitted to a German hospital were found to be ESBL producers [218].

10.7 Conclusions

The spread of resistance to antibacterial agents is dynamic and, at times, unpredictable. Although the major resistance determinants have been fully characterized and addressed with new therapies, new resistance genes continue to develop in response to new and changing therapies. The changeable nature of resistance due to the

movement of genes by plasmid, transposons, and integrons, plus the upregulation or downregulation factors such as of multidrug efflux and outer membrane permeability, contributes to the complexity of understanding resistance. Many global epidemics of resistance are the result of the spread of a few multidrug-resistant clones, but the exact reasons contributing to the success of particular lineages remain a mystery. Regardless of the cause of spread, we now have at our disposal a number of different molecular methods assisting in the tracking of resistant strains. Studies using these tools have aided in the identification of the source and elimination of several localized outbreaks of resistant pathogens. Resistance is a significant cause of excess morbidity, mortality, and cost to hospitalized patients, and with increasing frequency, previously healthy people in the community. In addition to the need for new and better antibacterial agents, continued monitoring of the epidemiology of infection-causing resistant pathogens paired with improved infection control will hopefully enable us to keep one step ahead of this threat to modern medicine.

Acknowledgment I thank Charles R. Dean for assistance with information gathering.

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Chapter 11
Transmissible Antibiotic Resistance

George A. Jacoby

11.1 Introduction

Transmissible antibiotic resistance was discovered by Japanese investigators in 1959 and publicized to the rest of the world in a review in English by Watanabe in 1963 [1]. Its discovery was motivated by a puzzling increase in resistance of clinical *Shigella* isolates in Japan following World War II with many strains co-resistant to chloramphenicol, streptomycin, sulfonamide, and tetracycline. All four resistances proved transmissible to *Escherichia coli* by conjugation and were carried on an element separate from the bacterial chromosome and able to replicate autonomously that came to be called a plasmid [2]. Plasmid-mediated penicillin resistance was inferred in *Staphylococcus aureus* in 1963 [3], and transmissible resistance to ampicillin was demonstrated in *E. coli* in 1965 [4]. Since then plasmid-mediated resistance to a particular antibiotic has followed its clinical use by from 2 to 58 years (Table 11.1) with the longer latencies generally associated with low use of the drug. For example, colistin was approved in 1958 but little used because of toxicity concerns until it became a drug of last resort in the 2000s and also widely used in animal husbandry before plasmid-mediated colistin resistance was reported in 2016 [9].

This chapter will address the biochemical and molecular mechanisms of resistance, the sources of resistance genes, and how they are acquired by transmissible elements. Resistance mediated by plasmids, insertion sequences, transposons, and related transmissible elements will be included along with a few considerations about what can be done to reduce or circumvent such spread.

The original version of this chapter was revised. The correction to this chapter is available at https://doi.org/10.1007/978-3-319-78538-7_25

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I. W. Fong et al. (eds.), *Antimicrobial Resistance in the 21st Century*, Emerging Infectious Diseases of the 21st Century, https://doi.org/10.1007/978-3-319-78538-7_11

Table 11.1 Timetable of antibiotic approval and appearance of transmissible resistance

Antibiotic	Approved for human use	Transmissible resistance reported	Organism	Reference
Sulfonamide	1936	1959	*Shigella* sp.	[1]
Penicillin G	1942	1963	*S. aureus*	[3]
Streptomycin	1944	1959	*Shigella* sp.	[1]
Bacitracin[a]	1945	2004	*E. faecalis*	[5]
Chlortetracycline	1948	1959	*Shigella* sp.	[1]
Oxytetracycline	1950			
Tetracycline	1955			
Chloramphenicol	1949	1959	*Shigella* sp.	[1]
Erythromycin	1952	1963	*S. aureus*	[6]
Nitrofurantoin	1953	1983	*E. coli*	[7]
Vancomycin	1955	1988	*E. faecium*	[8]
Colistin	1958	2016	*E. coli*	[9]
Metronidazole	1960	1989	*B. fragilis*	[10]
Ampicillin	1961	1965	*E. coli*	[4]
Trimethoprim	1961	1972	*E. coli*	[11]
			K. aerogenes	
Fusidic acid	1962	1966	*S. aureus*	[12]
Gentamicin	1964	1971	*K. pneumoniae*	[13]
Rifampin	1967	1998	*P. fluorescens*	[14]
Nalidixic acid	1967	1998	*K. pneumoniae*	[15]
Norfloxacin	1983			
Ciprofloxacin	1987			
Lincomycin	1967	Previously[b]		
Clindamycin	1970			
Fosfomycin	1973	1980	*S. marcescens*	[16]
Cefoxitin	1977	1989	*K. pneumoniae*	[17]
Cefotaxime	1980	1983	*K. pneumoniae*	[18]
Imipenem	1985	1991	*P. aeruginosa*	[19]
Mupirocin[a]	1985	1987	*S. aureus*	[20]
Quinupristin/ Dalfopristin	1999	Previously[b]		
		1977	*S. aureus*	[21]
Linezolid	2000	2006	*S. aureus*	[22]
Retapamulin[a]	2007	2006	*S. aureus*	[22]

[a]Topical

[b]The plasmid-mediated Erm 23S rRNA methyltransferase that confers resistance to lincomycin, clindamycin, and quinupristin as well as erythromycin was discovered before these non-macrolide antibiotics were approved for clinical use

11.2 Molecular Mechanisms of Resistance

Transmissible resistance utilizes the same three mechanisms involved in chromosomally mediated resistance: target alteration (including both protection and replacement), antibiotic modification, and drug exclusion (Table 11.2). For some antimicrobial agents all three mechanisms are utilized, with sometimes more than one determined by the same plasmid. Some resistances mechanisms are agent specific, other class specific, and a few affect susceptibility to several classes of antibiotics. A variety of antibiotic resistance gene databases are available on line and have been critically reviewed [23].

11.2.1 Aminoglycoside Resistance

More than a hundred plasmid-mediated enzymes are known that modify aminoglycosides by transferring acetyl, phospho, or adenyl groups to essential –OH or $–NH_2$ groups on the aminoglycoside core (Fig 11.1). Such modifications reduce the binding of the drug to its 16S rRNA target in the 30S ribosome. Two nomenclatures are in use. In one three letters identify the enzyme activity (AAC, APH, ANT) followed by the site of modification in parenthesis, a roman numeral for the resistance profile, and a lower case letter as an individual identifier [25, 26]. In the other system, the genes are labeled *aac*, *aph*, and *aad* followed by a letter or a number that identifies the site of modification and another number as a unique identifier [2]. Thus, *aac(6′)-Ib* and *aacA4* represent the same gene for an aminoglycoside *N*-acetyltransferase modifying position 6′ and giving a Ib pattern of resistance, a potentially confusing situation. A table of equivalent nomenclature is available [25].

The aminoglycoside acetyltransferase group is the largest. There are four subclasses named according to the position of the amino group that is modified. AAC(1) and AAC(3) target groups at positions 1 and 3 of the 2-deoxystreptamine ring, while AAC(2′) and AAC(6′) target groups found at the 2′ or 6′ position of the 2,6-dideoxy-2,6-diaminoglucose ring. The AAC(6′) group can be further subdivided into type I giving resistance to amikacin but not gentamicin and type II conferring resistance to gentamicin but not amikacin. Both AAC(6′) groups modify tobramycin and netilmicin and have many subgroups. AAC(6′) also exists as a bifunctional hybrid with APH(2″) in Gram-positive bacteria. The second largest is the phosphotransferase group, which targets 3′, 4′, and 2″ hydroxyls and provides resistance to kanamycin, to neomycin, and for some enzymes to additional aminoglycosides including amikacin. APH(3″) and APH(6) that modify streptomycin also belong to this group. The final group of nucleotidyltransferases attacks hydroxyls at the 2″, 3″, 4′, 6, and 9 positions. Clinically important members include ANT(2″) active on 4,6-disubstituted aminoglycosides with a deoxystreptamine ring including gentamicins, kanamycins, sisomicin, and tobramycin, ANT (3″) modifying the 3″ position of streptomycin and the 9-hydroxyl group of spectinomycin, and ANT(4′)

Table 11.2 Representative transmissible resistance genes

Antimicrobial agent	Modification	Target alteration	Efflux
Aminoglycoside	*aac(1)*, *aac(2′)*, *aac(3)*, *aac(6′)*, *aac(6′)-aph(2′)*, *ant(2′)*, *ant(3′)*, *ant (4′)*, *ant(6)*, *ant(9)*, *aph(2′)*, *aph(3′)*, *aph(3′)*, *aph(4′)*, *aph(6)*	*armA*, *npmA*, *rmtA*, *rmtB*, *rmtC*, *rmtD*, *rmtE*, *rmtF*, *rmtG*, *rmtH*	
Bacitracin			*bcrAB*
β-lactam	bla_{TEM}, bla_{SHV}, bla_{CMY}, $bla_{CTX\text{-}M}$, bla_{KPC}, bla_{NDM}, bla_{OXA}, bla_{PER}, bla_{VEB}, bla_{VIM}, *blaZ*, many others		
Colistin		*mcr*	
Fluoroquinolone	*aac(6′)-Ib-cr*	*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrE*, *qnrS*, *qnrVC*	*qepA*, *oqxAB*
Fosfomycin	*fosA*, *fosB*, *fosX*, *fosK*		
Fusidic acid	*catI*	*fusA*, *fusB*	
Glycopeptide		*vanA*, *vanB*, *vanD*, *vanE*, *vanG*, *vanL*, *vanM*, *vanN*	
Macrolide/ lincosamide/ streptogramin B (MLS_a group)	*ere*(A), *ere*(B), *vgb*(A), *vgb*(B), *lnu*(A), *lnu*(B), *lnu*(C), *lnu*(D), *lnu*(E), *lnu*(F), *lnu*(G), *vat*(A), *vat*(B), *vat*(C), *vat*(D), *vat*(E), *vat*(F), *vat*(G), *vat*(H), *mph*(A), *mph*(B), *mph*(C), *mph*(E), *mph*(F), *mph*(G)	*erm*(A), *erm*(B), *erm*(C), *erm*(F), *erm*(T), *erm*(Y), *cfr*(A), *cfr*(B) *cfr*(C)	*lsa*(B), *lsa*(E), *mef*(A), *msr*(A), *msr*(E), *vga*(A), *vga*(B), *vga*(C), *vga*(E), *optrA*
Mupirocin		*mupA mupB*	
Nitrofurantoin			*oqxAB*
Nitroimidazole	*nimA*, *nimB*, *nimC*, *nimD*, *nimE*, *nimF*, *nimG*, *nimH*, *nimI*, *nimJ*		
Oxazolidinone		*cfr*(A), *cfr*(B), *cfr*(C)	*optrA*
Phenicol	*catA*, *catB*	*cfr*(A), *cfr*(B), *cfr*(C)	*cmlA*, *cmlB1*, *cmr*, *cmx*, *fexA*, *fexB*, *floR*, *optrA*, *oqxAB*
Rifampin	*arr-2*, *arr-3*, *arr-4*, *arr-5*, *arr-7*, *arr-8*		
Sulfonamide		*sul1*, *sul2*, *sul3*	

(continued)

Table 11.2 (continued)

Antimicrobial agent	Modification	Target alteration	Efflux
Tetracycline	*tet*(X)	*tet*(M), *tet*(O), *tet*(Q), *tet*(S), *tet*(W), *tet*(36)	*tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), *tet*(J) *tet*(K), *tet*(L), *tet*(39), *tet*(42)
Trimethoprim		*dfrA*, *dfrB*, *dfrD*, *dfrG*, *dfrK*	*oqxAB*

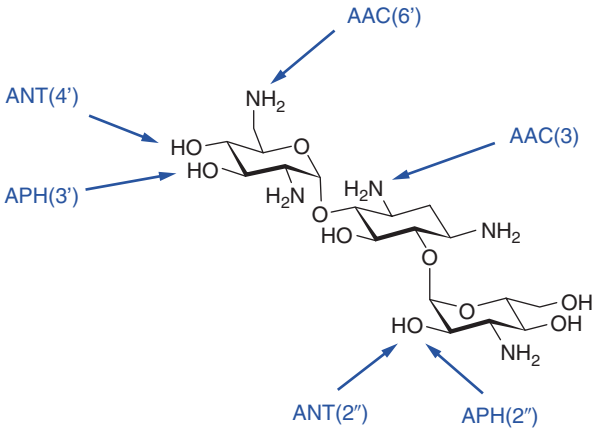

Fig. 11.1 Kanamycin B and sites of modification by representative aminoglycoside acetyltransferase (AAC), aminoglycoside nucleotidyltransferase (ANT), and aminoglycoside phosphotransferase (APH) enzymes. (Adapted from [24])

which targets kanamycin A, B, and C, gentamicin A, amikacin, tobramycin, and neomycin B and C [27]. ANT(6) adenylates the 6 position of streptomycin, while ANT(9) attacks the 9-hydroxyl of spectinomycin. The detailed substrate spectra and properties of these and other aminoglycoside-modifying enzymes have been cataloged in reviews [25, 26]. The resistance spectrum of clinically important aminoglycoside-modifying enzyme is shown in Table 4.6.

Target modification leading to high-level and broad aminoglycoside resistance due to S-adenosyl-L-methionine-dependent methylation of 16S rRNA was reported in 2003 [28]. Several gene families are involved, and either position 1405 or 1408 within the aminoacyl or A-site of 16 S rRNA can be modified. ArmA and RmtA, RmtB1, RmtB2, RmtC, RmtD, RmtD2, RmtE, RmtF, RmtG, and RmtH methylate guanine 1405 and confer high-level resistance to gentamicin, tobramycin, and amikacin but not to streptomycin, neomycin, or apramycin [29]. NpmA methylates adenine 1408 and provides resistance to all these aminoglycosides [30]. Initially these methyltransferases were rare, but they have subsequently been found on plasmids linked to common ESBLs and carbapenemases and are becoming more prevalent.

11.2.2 Bacitracin

Bacitracin acts by binding to and sequestering an undecaprenol pyrophosphate carrier in the cell membrane that transports peptidoglycan monomers for cell wall biosynthesis. During transport, undecaprenol monophosphate is phosphorylated to the pyrophosphate, which must be converted back to the monophosphate to allow further transport. Plasmid-mediated bacitracin resistance in *E. faecalis* is produced by an ATP-binding cassette (ABC) transporter encoded by the *bcrABD* operon under the control of *bcrR*, a membrane-bound bacitracin sensor that binds to the operon [31]. BcrA is an ATP-binding domain that together with the BcrB membrane-spanning domain makes up the homodimeric bacitracin transporter. BcrD is an undecaprenol kinase that converts undecaprenol to undecaprenol monophosphate and could thus counter the block in monophosphate synthesis by bacitracin but seems to play no role in resistance [5, 32]. *bcrAB* genes have been found in a variety of enterococcal species. Some strains lack *bcrR* but still express high-level (MIC >256 μg/ml) bacitracin resistance [32].

11.2.3 β-Lactam Resistance

The plasmid-mediated mechanism for β-lactam resistance is drug inactivation by β-lactamase enzymes. More than a thousand individual β-lactamases have been described and can be classified into classes A, B, C, and D by structure [33] or into 16 or more groups based on such functional properties as substrate spectrum and response to inhibitors [34, 35]. Class A, C, and D β-lactamases have serine at the active site and form acyl intermediates between the β-lactam and the serine hydroxyl in the course of hydrolyzing the β-lactam ring. Class B enzymes are metallo-β-lactamases with one or two Zn^{++} ions required to activate water for a direct attack on the β-lactam core.

The clinically important β-lactamases in Gram-positive organisms are found in *Staphylococcus* spp. About 90% of *S. aureus* make the class A (group 2a) BlaZ (also known as PC1) β-lactamase. Four immunotypes of the enzyme are known of which at least three can be distinguished by substrate profile [36] and by sequence [37]. The type A staphylococcal enzyme has also been found occasionally in *E. faecalis* on enterococcal-specific plasmids [38]. Unlike most plasmid-mediated β-lactamases, *blaZ* is inducible.

In Gram-negative bacteria, the commonest β-lactamase is TEM-1, the first one reported in 1965 [4]. Development of isoelectric focusing allowed β-lactamases to be differentiated further. TEM-2, which has similar biochemical properties, differs from TEM-1 by a single amino acid that changes the isoelectric point of the enzyme. Advances in DNA technology made sequencing a prime mode of characterization. Naturally occurring enzymes that vary from each other by from one amino acid to about 10% of their sequence constitute a β-lactamase family, many of which are

described in an online database [39]. TEM-1 and TEM-2 belong to class A or group 2b as does SHV-1, another common plasmid-determined β-lactamase, differing in sequence from TEM-1 by 36%. Group 2b enzymes hydrolyze penicillins such as ampicillin and early cephalosporins such as cephalothin or cefazolin and are inhibited by clavulanic acid, sulbactam, or tazobactam (Table 11.3).

Pharmaceutical chemists devised oxyimino-β-lactams such as cefotaxime, ceftazidime, ceftriaxone, and the monobactam aztreonam that are effective against TEM-1-, TEM-2-, or SHV-1-producing bacteria. Within a few years, transmissible resistance to oxyimino-β-lactams appeared and proved to be due to mutations in TEM-1, TEM-2, and SHV-1 that changed the charge and configuration of the active site allowing access to β-lactams with the oxyimino side chain. Many such extended-spectrum β-lactamases or ESBLs in the TEM (TEM-3, TEM-10, TEM-12, TEM-26, etc.) and SHV (SHV-2, SHV-4, SHV-5, etc.) families are known and belong to group 2be. Group 2be also includes a large family of CTX-M enzymes and smaller ones of PER and VEB β-lactamases.

Combinations of clavulanic acid, sulbactam, or tazobactam with β-lactamase susceptible drugs were also developed. Inhibitor-resistant TEM and SHV varieties followed (group 2br) such as TEM-30 or SHV-10 as well as a few enzymes that combined the extended-spectrum and inhibitor-resistant phenotypes (group 2ber), including TEM-50 and TEM-68. β-lactamases pay a price for broader spectrum or inhibitor resistance in terms of efficiency, so that compensatory promoter mutations increasing gene expression often accompany such mutations [40] as well as changes in the bacterial host to decrease β-lactam accumulation.

Carbenicillin and ticarcillin were developed to treat infections caused by *P. aeruginosa*. β-lactamases with high activity toward carbenicillin (group 2c) were found and, since they were at first thought to be pseudomonas-specific, were given names like PSE-1 (for *Pseudomonas*-specific enzyme) or, for carbenicillinase activity, CARB-3. A related variety able to hydrolyze cefepime as well as carbenicillin (group 2ce) has also evolved.

Cephamycins, such as cefoxitin and cefotetan, were also developed as agents effective against class A β-lactamase-producing bacteria and others. Class C (group 1) enzymes can hydrolyze cephamycins, but these enzymes were determined by chromosomal *ampC* genes and hence restricted to bacteria expressing them until plasmid-mediated class 3 enzymes (CMY-1, MIR-1, ACT-1, DHA-1, FOX-1, MOX-1, and more in each family) were discovered [41]. These β-lactamases could also hydrolyze oxyimino-β-lactams and were naturally resistant to the first generation of β-lactamase inhibitors. Group 1e enzymes have short deletions or amino acid substitutions in the R2 loop that enhance activity toward particular substrates, especially ceftazidime. With host porin and efflux pump changes to reduce drug accumulation, AmpC enzymes can even provide carbapenem resistance [42].

Carbapenems such as imipenem, meropenem, doripenem, and ertapenem came to be widely used against ESBL and AmpC β-lactamase-producing bacteria with the all too predictable appearance of carbapenemases belonging to class B (group 3a: VIM-1, IMP-1, NDM-1) and class A (group 2f: KPC-2, IMI-1) β-lactamases, which have spread worldwide [43] (see Chaps. 10 and 12 for further details). In the United

Table 11.3 Classification of transmissible β-lactamases

Group	Class	Distinctive substrates	Inhibited by			Examples
			A[a]	B[b]	C[c]	
1	C	Cephalosporins	No	Yes	No	ACC-1, ACT-1, DHA-1, FOX-1, CMY-1, MIR-1, MOX-1
1e	C	Cephalosporins	No	Yes	No	CMY-10, CMY-19, CMY-37
2a	A	Penicillins	Yes	Yes	No	BlaZ (PC1)
2b	A	Penicillins, early cephalosporins	Yes	Yes	No	TEM-1, TEM-2, SHV-1
2be	A	Extended-spectrum cephalosporins, monobactam	Yes	Yes	No	TEM-3, SHV-2, CTX-M-15, GES-1, PER-1, VEB-1
2br	A	Penicillins, early cephalosporins	No	Yes	No	TEM-30, SHV-10
2ber	A	Extended-spectrum cephalosporins, monobactam	No	Yes	No	TEM-50, TEM-68
2c	A	Carbenicillin	Yes	Yes	No	PSE-1, CARB-3
2ce	A	Carbenicillin, cefepime	Yes	Yes	No	RTG-4
2d	D	Oxacillin	Variable	Variable	No	OXA-1, OXA-10
2de	D	Extended-spectrum cephalosporins	Variable	Variable	No	OXA-11, OXA-15
2df	D	Carbapenems	Variable	Variable	No	OXA-23, OXA-48
2f	A	Carbapenems	No	Yes	No	KPC-2, IMI-1
3a	B	Carbapenems	No	No	Yes	NDM-1, VIM-1, IMP-1

Modified from [35]

[a]Clavulanic acid, sulbactam, or tazobactam

[b]Avibactam and related diazabicyclooctane derivatives

[c]EDTA

States, most carbapenem resistance in *Enterobacteriaceae* is due to KPC-type enzymes, which efficiently hydrolyze penicillins, oxyimino-, and other cephalosporins including cephamycins and aztreonam as well as carbapenems. bla_{KPC} is carried by transposon Tn*4401* allowing its spread to a variety of plasmid types many with other resistance genes. Worldwide, the most successful carbapenemase is NDM-1, which hydrolyzes all β-lactams except the monobactam aztreonam and for which no clinically useful inhibitor is yet available. In *A. baumannii*, NDM-1 is carried by composite transposon Tn*125* with two copies of IS*Aba125* and downstream the ble_{MBL} gene for resistance to the antitumor agent bleomycin [44]. In *Enterobacteriaceae* bla_{NDM-1} is located on plasmids belonging to several incompatibility groups linked to single or truncated copies of IS*Aba125* and ble_{MBL} (for further details of the epidemiology of NDM spread see Chaps. 10 and 12).

OXA β-lactamases (class D, group 2d) were originally distinguished by their ability to hydrolyze oxacillin or cloxacillin at a rate at least 50% of that for benzylpenicillin [34]. A few are susceptible to inhibition by clavulanic acid, sulbactam, and tazobactam but most are resistant. Response to avibactam is also variable. Class D β-lactamases are a large (more than 500) and diverse group, some members of which are ESBLs able to attack oxyimino-β-lactams (group 2de: OXA-11, OXA-15) or even act as carbapenemases (group 2df: OXA-23, OXA-48) [45]. 2df enzymes have been most frequently identified in *Acinetobacter* spp. but are becoming more common in *Enterobacteriaceae*. Spread of OXA-48 is mainly due to a specific plasmid in which the bla_{OXA-48} gene is part of composite transposon Tn*1999*.

β-lactams and β-lactamases have thus evolved together with the introduction of derivatives with increased enzymatic stability followed by the appearance of β-lactamases with broader spectrum able to hydrolyze the new agents [46]. The evolution is ongoing: TEM and SHV ESBLs have been largely replaced by ESBLs in the CTX-M family, especially CTX-M-15 and CTX-M-14 [47]. The CTX-M success story seems due not so much to superior properties of the enzyme as to capture of the genes by mobile genetic elements and their association with plasmids carrying other effective resistance determinants in highly successful sequence type strains [48, 49].

11.2.4 Colistin Resistance

Long after colistin (polymyxin E) was first used clinically but within a few years of its extensive use in animal husbandry, plasmid-mediated colistin resistance was reported in 2016 in an isolate from an intensive pig farm in China. The plasmid gene *mcr-1* encodes a membrane-bound phosphoethanolamine transferase, a zinc metalloprotein, that modifies lipid A decreasing the binding affinity of polymyxins and thus conferring a colistin MIC of 4–8 μg/ml [9]. Within a year of its discovery, *mcr-1* was found on a wide variety of plasmids in a diversity of *Enterobacteriaceae* in countries on five continents and in isolates from as early as the 1980s [50]. IS*Apl1* is often found upstream from the *mcr-1* gene [51]. *mcr-1* has been found in 10% of

NDM-1-producing *Enterobacteriaceae* in the United Kingdom and in KPC-producing pathogens in Italy and Greece so it is already compromising colistin use as a last resort antibiotic. Several alleles, including *mcr-1.2*, *mcr-1.6*, *mcr-2*, and *mcr-3*, have been reported [52].

11.2.5 Fluoroquinolone Resistance

Plasmid-mediated quinolone resistance was reported in 1998, 31 years after nalidixic acid began to be used clinically and 15 years after modern fluoroquinolones were approved for use [15]. The first transmissible quinolone resistance was discovered in a 1994 urinary isolate of *K. pneumoniae* from Alabama that could transfer low-level ciprofloxacin resistance on a multiresistance plasmid to a variety of Gram-negative bacteria. In *E. coli*, the plasmid caused an 8-fold decrease in susceptibility to nalidixic acid and a 30-fold decrease in susceptibility to all fluoroquinolones tested. When the responsible gene, named *qnr* and later *qnrA*, was cloned and sequenced facilitating its identification by PCR, *qnrA* was soon found at low frequency on plasmids in Gram-negative isolates around the world with the strain carrying the earliest known plasmid-mediated *qnr* collected in 1988. Further searches led to the discovery of plasmid-mediated *qnrS*, *qnrB*, *qnrC*, *qnrD*, and *qnrE* [53, 54]. The *qnrVC* gene from *Vibrio cholerae* can also be located on a plasmid or an integrated conjugative element. These new *qnr* genes generally differed by 35% or more from *qnrA* and each other. Allelic varieties that differ by 10% or less have been described in almost all families; currently, there are 8 *qnrA*, 85 *qnrB*, 1 *qnrC*, 2 *qnrD*, 1 *qnrE*, 9 *qnrS*, and 6 *qnrVC* alleles. *Qnr* genes are also present on the chromosome of a variety of Gram-negative and Gram-positive bacteria from both clinical and environmental sources. Their utility prior to the clinical use of quinolones is not known.

Qnr proteins are composed of tandemly repeating five amino acid units. Two Qnr molecules dimerize carboxyl terminus to carboxyl terminus and fold into a right-handed quadrilateral β helix with size, shape, and charge mimicking the B-form of DNA. Loops of 8 and 12 amino acids project from their otherwise rod-like structure. Deletion of the larger loop or even one amino acid in this loop compromises protective activity, suggesting that the loops are essential for proper positioning of Qnr on topoisomerase.

In vitro purified Qnr proteins protect DNA gyrase or topoisomerase IV from quinolone inhibition [55]. As the quinolone concentration is increased, more Qnr is needed for protection suggesting that they both compete for a site on the topoisomerase. In a gel-displacement assay or bacterial two-hybrid system [56], Qnr binds to both topoisomerase holoenzymes and their individual subunits. Subinhibitory concentrations of ciprofloxacin, however, reduce binding to GyrA but not to GyrB, suggesting that Qnr protects gyrase by blocking quinolone access to GyrA sites essential for its lethal action. The molecular details of this interaction are still being debated [57].

Many naturally occurring antibiotics and synthetic agents target DNA gyrase besides quinolones. Qnr protects against compounds with a somewhat quinolone-like structure, for example, 2-pyridone, quinazoline-2,4-dione, or spiropyrimidinetrione, so it is not strictly quinolone specific. Qnr, however, does not block agents acting on the GyrB subunit [58].

Expression of *qnrB*, *qnrD*, and *qnrE* is regulated by components of the bacterial SOS system in which DNA damage induced by quinolones activates RecA protease to cleave LexA protein which otherwise blocks expression by binding to specific DNA sequence upstream from these *qnr* genes. Expression of *qnrS*1 is also induced by quinolone and requires DNA sequence upstream from the gene, but *qnrS1* regulation is independent of the SOS system. Expression of *qnrA* in the aquatic organism *Shewanella algae*, on the other hand, is induced not by quinolone but by cold shock.

Qnr plasmids have been found around the world in a variety of *Enterobacteriaceae*, especially *E. coli*, *E. cloacae*, *K. pneumoniae*, and *S. enterica* but rarely in non-fermenting bacteria such as *P. aeruginosa* or *A. baumannii*. Plasmids carrying *qnr* genes vary in size and incompatibility specificity, indicating that the spread of multiple plasmids has been responsible for their dissemination and that plasmid acquisition has occurred multiple times. A mobile or transposable element is almost invariably associated with plasmid-mediated *qnr* genes, especially insertion sequences IS*CR1*, IS*Exp1*, and IS*26*. The complex is often inserted into a sul1-type integron. *qnrVC* is so far the only *qnr* gene located in a cassette with a linked *attC* site ready by itself for integron capture. Because of such linkage, *qnr* genes are often found on plasmids with genes for other resistance determinants such as ESBLs and carbapenemases. *Qnr* prevalence seems to be increasing and has reached as high as 39% in a sample of *E. cloacae* isolates at one hospital in China [59].

The second plasmid-mediated quinolone resistance mechanism to be discovered involves modification. AAC(6′)-Ib-cr is an acetyltransferase providing resistance to kanamycin, tobramycin, and amikacin that by two amino acid substitutions has broadened its spectrum to acetylate quinolones with an amino nitrogen on the piperazinyl ring, such as ciprofloxacin and norfloxacin [60]. Acetylation decreases the antibacterial potency raising the ciprofloxacin MIC eightfold. Both the substitutions Trp102Arg and Asp79Tyr are required for quinolone-acetylating activity. Seven nucleotide variants of *aac(6′)-Ib-cr* are known, one with a 26 amino acid insertion changing the position of the amino acid changes essential for anti-quinolone activity [61]. The *aac(6')-Ib-cr* gene has been found worldwide in a variety of *Enterobacteriaceae* (especially *E. coli*) and is often more common in surveys than *qnr* alleles. It is usually found in a cassette as part of a class 1 integron in a multiresistance plasmid, which may contain other plasmid-mediated quinolone resistance genes. Association with CTX-M-15 is particularly common.

The third mechanism of plasmid-mediated quinolone resistance involves efflux pumps. QepA is a plasmid-acquired efflux pump in the major facilitator family that decreases susceptibility to hydrophilic fluoroquinolones. *qepA* has often been found on plasmids together with aminoglycoside ribosomal methyltransferase *rmtB*. At least three *qepA* variants are known. OqxAB is an efflux pump in the

resistance-nodulation-division family that was originally recognized as responsible for resistance to olaquindox used for growth enhancement in pigs. OqxAB can efflux other antimicrobial agents including chloramphenicol, trimethoprim, and nitrofurantoin [62]. *oqxAB* has been found on plasmids in clinical isolates of *E. coli* and *K. pneumoniae* and on plasmids (especially IncHI2 plasmids) and the chromosome of *S. enteritis*, flanked in both locations by IS*26*-like elements.

By themselves none of the plasmid-mediated quinolone resistance determinants raises the MIC above the CLSI designated susceptibility breakpoint, but they raise mutant prevention concentration (MPC) [63] as well as the MIC and facilitate the selection of a variety of additional chromosomal mutations of higher MIC via activation of efflux pumps and altered membrane LPS [64].

11.2.6 Fosfomycin Resistance

Plasmid-mediated resistance to fosfomycin involves enzymes that open the oxirane ring of the antibiotic inactivating it [65]. FosA, FosB, and FosX are members of the same metalloenzyme superfamily but differ in their preferred cofactor. FosA utilizes glutathione, while FosB requires bacillithiol or L-cysteine and FosX uses water. Subtypes of each gene have been described: *fosA1–6* and *fosB1–6*.

fosA has been reported on plasmids in *Enterobacteriaceae*, *Pseudomonas* spp., and *Acinetobacter* spp. *fosB* has only been described in Gram-positive strains: on small plasmids in *S. aureus* and other staphylococcal species, on the chromosome in *B. anthracis*, and on transferable circular intermediates in *E. faecium* [66]. *fosX*^{CC} is transferrable between strains of *Campylobacter* but is located in a multidrug resistance genomic island rather than a plasmid [67]. Another allele, *fosK*, has been found in an integron in a highly fosfomycin-resistant isolate of *Acinetobacter soli* [68].

11.2.7 Fusidic Acid Resistance

Transmissible resistance to fusidic acid in staphylococci is produced by *fusA* and *fusB* genes that encode proteins that bind and protect elongation factor G (EF-G), the antibiotic target, from inhibition [69–71]. Related gene *fusC* and *fusD* are chromosomal, and *fusB* can also be found on the chromosome in a genomic island [72]. In countries where fusidic acid is used to treat *S. aureus* infections, resistance is slowly increasing [73], but it remains very low in US isolates [74].

In addition to the *fus* genes, some type A chloramphenicol acetyltransferases bind fusidic acid conferring resistance. For example, in fusidate-sensitive *E. coli* DB10, a cloned *cat$_I$* gene increased the chloramphenicol MIC from 2 to 50 μg/ml and the Na fusidate MIC from 5 to 80 μg/ml [75]. The two structurally unrelated antibiotics compete for binding to the enzyme at the same site [76].

11.2.8 Glycopeptide

The glycopeptide vancomycin, the lipoglycopeptide teicoplanin (approved for use in Europe), and the semisynthetic lipoglycopeptides telavancin, oritavancin, and dalbavancin bind to the D-alanyl-D-alanine (D-Ala-D-Ala) terminus of intermediates in peptidoglycan synthesis, inhibiting bacterial cell wall cross-linking. Glycopeptide resistance involves bypass or target modification by enzymes that substitute D-lactate (D-Lac) or D-serine (D-Ser) for the terminal D-alanine, thus reducing glycopeptide binding. These enzymes act together with others that eliminate the normal C-terminal D-alanine precursors [77]. Nine such gene clusters are currently associated with vancomycin resistance in enterococci [78] and are distinguished by the sequence of the structural gene for the resistance ligase: va*nA*, *vanB*, *vanD*, and *vanM* create a precursor ending in D-Ala-D-Lac with a 1000-fold decrease in vancomycin binding, while *vanC*, *vanE*, *vanG*, *vanL*, and *vanN* produce intermediates terminating in D-Ala-D-Ser with only a sevenfold binding decrement. The *vanC* cluster is intrinsic to *Enterococcus gallinarum*, *Enterococcus casseliflavus*, and *Enterococcus flavescens*; the rest are acquired with *vanA*, *vanB*, *vanD*, *vanM*, and *vanN* located either on plasmids or the chromosome and *vanG*, *vanE*, and *vanL* only chromosomal. The *vanA* and *vanD* clusters provide high and moderate levels of resistance, respectively, to both vancomycin and teicoplanin. *vanB* and the *van* clusters producing D-Ala-D-Ser confer only vancomycin resistance.

Figure 11.2 details the mechanism of VanA-type resistance. The *vanA* cluster is typically located in 11-kb transposon Tn*1546* or a related element on transferable or nontransferable plasmids or the host chromosome. It includes nine genes. Two genes are involved in transposition. VanS and VanR encode a two-component regulatory system that modulates transcription of the cluster. VanS is a membrane-bound kinase containing a histidine residue that is phosphorylated in the presence of glycopeptide. The phosphoryl group is transferred to an aspartate residue on VanR, which can then activate transcription from promotors P_H and P_R upstream from *vanH* and *vanR* [80]. The dehydrogenase VanH converts pyruvate to D-Lac, and the ligase VanA combines D-Lac and D-Ala to make the depsipeptide D-Ala-D-Lac, which is incorporated into peptidoglycan precursors in place of D-Ala-D-Ala. VanX is a D,D-dipeptidase that hydrolyzes the dipeptide D-Ala-D-Ala formed by the endogenous D-Ala-D-Ala:ligase thus reducing vancomycin susceptible targets. VanY, not essential for resistance, is a D,D-carboxypeptidase that cleaves the terminal D-Ala of any pentapeptide precursors synthesized from D-Ala-D-Ala that escaped VanX hydrolysis. VanZ contributes to teicoplanin resistance but its function is not known in detail.

The organization of the VanB cluster is similar to that of VanA but differs in its regulation. While VanA strains are inducible to high levels by either vancomycin or teicoplanin, VanB strains have variable levels of inducibility only by vancomycin. The VanB operon is found in Tn*1547* and other transposons and includes genes encoding a dehydrogenase, a ligase, and a dipeptidase with 67–76% identity to the corresponding VanA enzymes and a two-component $vanR_BS_B$ set of regulatory genes

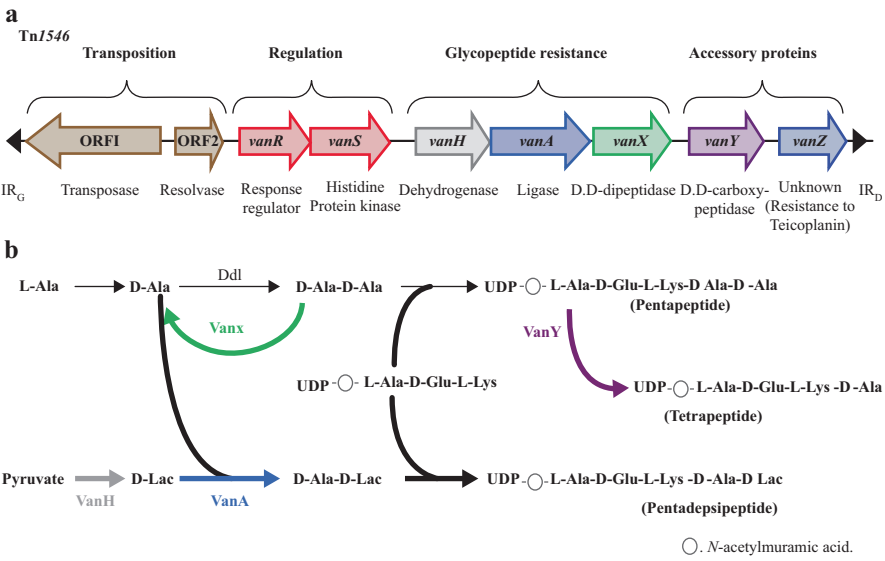

Fig. 11.2 VanA-type glycopeptide resistance. a: Organization of the *vanA* operon in Tn*1546*. Open arrows represent coding sequences and indicate the direction of transcription. IR_L and IR_R, inverted repeat left and right, respectively. b: Synthesis of peptidoglycan precursors in a VanA-type-resistant strain after induction with glycopeptide; Ddl, D-Ala:D-Ala ligase [79]

only distantly (34% and 24%) related to those of VanA. A *vanW* gene of unknown function is included but no *vanZ*.

The VanC cluster produces peptidoglycan precursors ending in D-Ala-D-Ser and is chromosomal and not transferable. VanT is a racemase converting L-Ser to D-Ser which is joined to D-Ala by VanC ligase and incorporated into peptidoglycan precursors in place of D-Ala-D-Ala. The two enzymes of VanA or VanB that eliminate D-Ala-D-Ala targets are combined in VanC in a single $VanXY_C$ D,D-peptidase. Expression of *vanC*, $vanXY_C$, and *vanT* is again controlled by a two-component regulatory system $VanR_C$ and $VanS_C$. The VanE cluster has a similar organization and 41–60% identity to VanC [81].

VanA and VanB are the most frequent causes of vancomycin resistance in *Enterococcus* spp. and have occasionally been detected in coryneform bacteria and other streptococci. VanA has also appeared in *S. aureus* causing high-level resistance but fortunately only rarely.

In a sample of enterococci causing bloodstream infections between 2001 and 2014 from the United States, the frequency of vancomycin resistance in *E. faecalis* was 6.5% in 2008 declining to 1.9% in 2014 but reached 80.7% in *E. faecium* in 2010 and 68.4% in 2014. In Europe, the corresponding vancomycin resistance frequencies were 2.6% in *E. faecalis* and peaked at 27.3% in *E. faecium* in 2012. All three lipoglycopeptides are active against VanB enterococci, while oritavancin and telavancin have activity as well against VanA strains but of unproven clinical significance [82].

11.3 Macrolide/Lincosamide/Streptogramin B (MLS_B Group)

Macrolides in clinical use include erythromycin and clarithromycin with 14-membered lactone rings, azithromycin with a 15-membered ring, and josamycin, spiramycin, and tylosin with 16-membered rings. Lincosamides include lincomycin and clindamycin, and for veterinary use pirlimycin. The streptogramin B group includes pristinamycin, virginiamycin, and quinupristin, which is combined with dalfopristin, a streptogramin A agent, in Synercid®. This group of antibiotics is considered together because they share overlapping binding sites on the bacterial 50S ribosomal subunit and often have resistance mechanisms in common. Inactivating enzymes, target-modifying rRNA methylases, and efflux pumps are all involved. Coresistance with pleuromutilins (retapamulin, valnemulin, and tiamulin) and phenicols may also be seen. Resistance to this group of antibiotics has recently been reviewed [83].

Inactivation of these antimicrobials involves esterases, hydrolases, nucleotidyltransferases, acetyltransferases, and phosphotransferases that are more or less specific for structurally related antibiotics. *ere*(A) and *ere*(B) coding for erythromycin esterases are found in transposons and integrons on plasmids in Gram-negative and only occasionally in Gram-positive bacteria. Both inactivate drugs through hydrolysis of the macrolactone ring. Ere(A) is specific for erythromycin, while Ere(B) inactivates azithromycin as well. Neither attacks the ketolide telithromycin or the lincosamides [84]. *vgb*(A) and *vgb*(B) (virginiamycin B lyase) encode enzymes catalyzing the linearization of cyclic streptogramin B-type antibiotics (such as quinupristin) via a cleavage that requires a divalent metal ion [85]. Several varieties have been found in staphylococci and enterococci. The *lnu* family encodes lincosamide-specific nucleotidyltransferase on plasmids and transposons in staphylococci, streptococci, enterococci, anaerobes, *Salmonella* spp., and *E. coli*. Lnu(A) modifies a hydroxyl group of clindamycin and lincomycin at positions 3 and 4, respectively, while Lnu(B) attacks a hydroxyl at position 3 in both clindamycin and lincomycin, although only resistance to lincomycin is observed with both enzymes in Gram-positive organisms [86]. Nucleotidyltransferases Lnu(C), Lnu(D), Lnu(E), Lnu(F), and Lnu(G) differing in size and origin have also been described. The *vat* family encodes acetyltransferases on plasmids in *Staphylococcus* and *Enterococcus* spp. that inactivate streptogramin A-type antibiotics (such as dalfopristin). Vat(A), Vat(B), Vat(C), Vat(D), Vat(E), Vat(F), Vat(G), and Vat(H) are currently known. *mph* genes encode macrolide-2′-phosphotransferases that preferentially inactivate 14- and 15-membered macrolides [*mph*(A)] or 14- and 16-membered ones [*mph*(B)] [87]. *mph*(A) and *mph*(B) have been found in Gram-negative organisms, while *mph*(C) has been described in staphylococci, corynebacteria, and *Stenotrophomonas maltophilia*. Plasmid-mediated *mph*(E), *mph*(F), and *mph*(G) have also been reported.

The MLS_B antibiotics bind to 23S rRNA of the 50S ribosomal subunit at the peptidyltransferase center blocking protein synthesis. More than 40 *erm* methylase

genes have been distinguished based on >20% difference in sequence. Many are determined by plasmids, transposons, or integrative and conjugative elements [88]. Erm proteins add one or two methyl groups to adenine 2058 in domain V of 23S rRNA preventing MLS_B antibiotic attachment. Resistance is produced to 14-, 15-, and 16-membered macrolides, ketolides, lincosamides, and streptogramin B antibiotics. *erm*(A), *erm*(B), and *erm*(C) are typically found in staphylococci. *erm*(B) and a subclass of *erm*(A) [*erm*(TR)] are widespread in enterococci and streptococci. *erm(*F) has been found in anaerobes and *H. influenzae*. *erm*(A) is part of transposon Tn*554* or its close relative Tn*6133*, while *erm*(B) is part of transposons Tn*917* and Tn*551*. *erm*(C) is often located on small plasmids in staphylococci and *erm*(T) on larger ones. *erm*(33) is the result of in vivo recombination between *erm*(A) and *erm*(C). *erm* expression may be inducible or constitutive. Erythromycin and other 14- and 15-membered macrolides tend to be good inducers via a mechanism that involves ribosome stalling while translating an upstream leader peptide with consequent changes in the structure of *erm* mRNA that allows it to be translated. Ketolides and lincosamides are usually not inducers but may become so by deletions, insertions, and point mutations in this attenuator system [87, 89, 90]. Hence a staphylococcal strain with *erm*(A) or *erm*(C) may appear erythromycin resistant but clindamycin susceptible, but if exposed to clindamycin, it can mutate to resistance to both agents [91].

A different methyl transferase is encoded by the *cfr* gene and confers resistance to lincosamides, streptogramins A, phenicols, oxazolidinones, and pleuromutilins and decreased susceptibility to such 16-membered macrolides as josamycin and spiramycin. It adds a methyl group from S-adenosyl-L-methionine to the C8 position of adenine 2503 at the peptidyltransferase center in domain V of 23 rRNA by a two-step mechanism involving intermediate methylation of a Cys residue on the enzyme [92]. The *cfr* gene has been found worldwide in *Staphylococcus* spp., *Enterococcus* spp., other Gram-positive organisms, and *P. vulgaris* and *E. coli* on plasmids or together with insertion sequences. Cfr(B) with 74.9% amino acid identity to Cfr(A) has been described in *E. faecium* [93] and Cfr(C) with 55.1% identity in *Campylobacter* spp. [94].

Plasmid-mediated efflux genes are also involved in MLS_B resistance. Msr(A) in the ABC transporter family confers resistance to 14- and 15-membered macrolides, and streptogramin B antibiotics and low-level resistance to ketolides, while Mef(A) in the major facilitator superfamily provides resistance to most 14- and 15-membered macrolides but not 16-membered macrolides, lincosamides, or streptogramin B [87]. Msr(A) has mainly been found in *Staphylococcus* spp. but also in *Streptococci*, *Corynebacterium*, and *Pseudomonas* [95]. Mef(A) has been detected in streptococci including pneumococci and Group A and D organisms and also in Gram-negative bacteria. Plasmid- or transposon-borne *vga*(A), *vga*(A_V), *vga*($A)_{LC}$, *vga*(B), *vga*(C), *vga*(E), and *vga*($E)_V$ encode ABC transporters that export streptogramin A antibiotics, while *vga*(A), *vga*(C), and *vga*(E) export lincosamides and pleuromutilins as well. *lsa*(B) found on a plasmid in *S. sciuri* encodes an ABC transporter active on clindamycin but probably not streptogramins [96]. *lsa*(E) on the other hand confers resistance to lincosamides, streptogramins A, and pleuromutilins and

has been found in *S. aureus* and several species of *Enterococcus* [97]. OptrA in the ABC transporter family confers resistance to oxazolidinones and phenicols and has been found in *E. faecium*, *E. faecalis*, *Staphylococcus sciuri*, and *Streptococcus suis* [98, 99]. Insertion sequence IS*1216E* has been implicated in the spread of *optrA* among enterococcal plasmids and to the streptococcal chromosome [100, 101].

11.3.1 Mupirocin

High-level resistance to the topical anti-staphylococcal agent mupirocin involves *mup* genes determining mupirocin-resistant isoleucyl-tRNA synthetases [102]. *mupA* (also known as *ileS2*) is determined by readily transmissible plasmids, while the 65.5% identical *mupB* (*ileS3*) gene is located on a nonconjugative plasmid in the single strain studied [103]. In a recent investigation of 358 *S. aureus* isolates cultured from children attending a Dermatology Clinic in New York City, 96 of 112 mupirocin-resistant isolates had high-level resistance typical of the plasmid-determined mechanism [104].

11.3.2 Nitrofuran

Nitrofurantoin resistance transferable from clinical *E. coli* to laboratory strains of *E. coli* was reported in 1983. The nitrofurantoin MIC rose from 5 to 50–70 μg/ml. Plasmids were not demonstrated physically, and the mechanism of resistance was not established, but resistance was cured by rifampin treatment and transmissible by conjugation. More than 30 years later, plasmids discovered for resistance to olaquindox and later fluoroquinolone were found also to confer nitrofurantoin resistance of the same degree via the resistance-nodulation-division family efflux pump OqxAB [62].

11.3.3 Nitroimidazole

Plasmid and chromosomally located *nim* genes (A through J) [105, 106] encode nitroimidazole reductases that convert 5-nitroimidazole to 5-aminoimidazole thus blocking formation of toxic nitroso derivatives that are essential for bactericidal activity by metronidazole and tinidazole [107]. The *nim* genes are usually transcribed from promotors located within different insertion elements: IS*1168* for *nimA-nimB*, IS*1169* for *nimD*, and IS*1170* for *nimC*. *nim* plasmids characterized in *Bacteroides* spp. have been nonconjugative (7.2 to 10–kb in size) but mobilizable by larger plasmids or transferable by electroporation [10, 108]. Metronidazole resistance in the *B. fragilis* group has been quite rare in the United States [109].

11.3.4 Oxazolidinone

Linezolid targets the peptidyltransferase center of the bacterial 50S ribosomal subunit and, like other drugs with the same target, is blocked by the Cfr 23S rRNA methyltransferase with an increase in the MIC of *S. aureus* from 0.5 to 8–16 μg/ml [22]. The plasmid-mediated ABC transporter OptrA also exports linezolid with typical MIC increases in *S. aureus* or enterococci of 2 to 8–16 μg/ml. Susceptibility of tedizolid is affected to a lesser extent by OptrA [98] and not at all by Cfr [110].

11.3.5 Phenicol

Chloramphenicol resistance is most often due to chloramphenicol acetyltransferase (CAT), which transfers an acetyl group from acetyl-CoA to the C3 position of the antibiotic. The acetyl groups then shifts to the C1 position making chloramphenicol available for diacetylation. The fluorine group in florfenicol (licensed for use only in animals) occupies the C3 position making florfenicol resistant to inactivation by CAT. There are two main types of transmissible CAT enzymes with many subgroups [111]. They are found in plasmids, transposons, integrons, and integrative and conjugative elements in both Gram-negative and Gram-positive pathogens. Some *cat* genes on plasmids in *S. aureus* are induced by chloramphenicol via upstream translation attenuators but most are expressed constitutively. Both CAT types have a trimeric structure composed of three identical monomers.

The *cfr* gene was first recognized by its production of combined chloramphenicol and florfenicol resistance in *S. sciuri* [112] and later appreciated to provide resistance to lincosamides, streptogramins A, oxazolidinones, pleuromutilins, and 16-membered macrolides as well as via methylation of 23S rRNA at the peptidyltransferase center.

In addition to drug and target modification, a number of plasmid and transposon-mediated phenicol exporters have been described. They belong to the major facilitator superfamily and include chloramphenicol-specific *cmr*, *cmx*, *cmlA*, and *cmlB1* genes and *floR* and $floR_V$ genes that export florfenicol as well [113]. *cmlA* and *cmlB1* are expressed inducibly via translational attenuation. In Gram-positive organisms, *fexA* and *fexB* encode chloramphenicol/florfenicol exporters that are plasmid-mediated. Both phenicols are also exported by the OptrA pump that also transports oxazolidinones [98], and at least chloramphenicol is effluxed by the multidrug OqxAB pump [114].

11.3.6 Rifamycin

In 1998 transfer of rifampin resistance on a multiresistance plasmid from *P. fluorescens* to *E. coli* or *P. putida* was reported. Accumulation of rifampin was blocked by the plasmid and relieved by the energy uncoupler potassium cyanide, suggesting that an efflux pump was involved [14]. The gene was not named or sequenced nor have subsequent reports elaborated on the evidence for its nature.

The next year, a gene was found in a class 1 integron in *P. aeruginosa* related to the rifampin ADP-ribosylating transferase responsible for rifampin resistance in *Mycobacterium smegmatis* [115]. It was named *arr-2* and has been subsequently found in integrons on plasmids in *K. pneumoniae*, *E. coli*, and species of *Enterobacter* and *Acinetobacter*. Additional alleles *arr-3*, *arr-4*, *arr-5*, *arr-7*, and *arr-8* have been reported in integrons, some associated with carbapenemases KPC-2 or NDM-1 [116, 117].

11.3.7 Sulfonamide

Plasmid-mediated sulfonamide resistance adopts the simple solution of providing a resistant dihydropteroate synthase to substitute for this usually sulfonamide-sensitive enzyme in the pathway to folic acid. Sulfonamides are structural analogs of p-aminobenzoic acid with which they compete in the synthesis of dihydropteroic acid. The resistant enzymes efficiently distinguish between its normal substrate dihydropteroic acid and the inhibitor. There are three *sul* genes encoding this resistance mechanism on plasmids in Gram-negative organisms: *sul1* usually found with other resistances in a Tn*21*-type integron, *sul2* found on small plasmids in the IncQ or pBP1 families or larger conjugative plasmids of several Inc groups, and, least common, *sul3* located in a composite transposon [118].

11.3.8 Tetracycline

More than 60 genes conferring resistance to tetracycline are known, most associated with mobile elements that allow for gene exchange. Most common are genes encoding energy-dependent efflux proteins. Others code for ribosomal protection proteins or inactivating enzymes. Further details can be found in the web site maintained by M. Roberts [88].

tet(X) encoding a NADP-dependent monooxygenase that requires oxygen to degrade tetracycline was originally discovered as part of a conjugative transposon in *Bacteroides* sp. where, lacking oxygen, it does not confer tetracycline resistance on its host. Subsequently, it has been found in *E. cloacae*, *K. pneumoniae*, and other

Enterobacteriaceae [119] and deserves attention since it inactivates all tetracyclines including tigecycline, a derivative designed to overcome resistance.

Ribosomal protection involves proteins that displace tetracycline from its ribosomal binding site allowing protein synthesis to proceed [120]. Further details of the mechanism can be found in Sect. 4.2.4.2. Resistance is conferred to tetracycline, doxycycline, and minocycline but not to tigecycline. The ribosomal protection proteins have sequence similarity to ribosomal elongation factors EF-G and EF-Tu and like them are GTPases. The *tet*(M), *tet*(O), *tet*(Q), *tet*(S), *tet*(W), and *tet*(36) ribosomal protection genes have been found in both Gram-negative and Gram-positive organisms. *Tet*(M), *tet*(Q), and *tet*(W*)* are usually associated with conjugative transposons, while *tet*(O) and *tet*(S*)* have been found on conjugative and nonconjugative plasmids. A subgroup of ribosomal protection genes are mosaics, made up of segments of two or three different known *tet* genes [121].

The *tet* efflux genes belong to the major facilitator superfamily and encode membrane-associated proteins that exchange a proton for the tetracycline cation thus reducing the intracellular concentration of the antibiotic. Most export tetracycline and doxycycline but not minocycline or tigecycline. Tet(B), however, exports minocycline as well. *tet*(A) and presumably most other efflux genes are regulated by a divergently transcribed repressor gene that produces a protein that binds to the *tet* operator. Tetracycline complexed with Mg^{++} binds to the repressor spreading its binding domains apart so that they no longer interact with the operator thus allowing transcription to take place [122]. Widely distributed *tet* efflux genes include *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), and *tet*(J) found in Gram-negative bacteria and *tet*(K), *tet*(L), *tet*(39), and *tet*(42) found in both Gram-negative and Gram-positive organisms. Some may be found integrated into the host chromosome as well as on plasmids.

Overexpression of plasmid-mediated *tet*(M) ribosomal protection protein and *tet*(L) encoded efflux has been associated with tigecycline resistance in *E. faecium* [123].

11.3.9 Trimethoprim

Like that for sulfonamide, the strategy for plasmid-mediated trimethoprim resistance is a resistant substitute for the trimethoprim target, dihydrofolate reductase. A genetically diverse set of more than 30 *dfr* genes are known, mostly located in Gram-negative organisms in integron cassettes or associated with IS*CR* elements. The *dfrA* genes encode dimeric dihydrofolate reductases and include at least 26 alleles with *dfrA1* and *dfrA17* the most common [124]. *dfrB* genes encode smaller trimeric enzymes of seven varieties [125, 126]. In addition, several *dfr* genes conferring high-level trimethoprim resistance are known in Gram-positive organisms: *dfrA* located on transposon Tn*4003* in *S. aureus* and other *Staphylococcus* spp., *dfrD* found on small plasmids in staphylococci and *Listeria monocytogenes* [127],

dfrG which seems to be the most common variety in *S. aureus* [128], and *dfrK* found on small plasmids in species of *Staphylococcus* [129].

The multidrug OqxAB pump effluxes trimethoprim [114].

11.4 Source of Resistance Genes

Resistance genes existed long before the antibiotic era and have been found, for example, in ancient permafrost, at the bottom of isolated caves, and in the gut microbiome of a pre-Columbian mummy [130]. An origin in the organisms that produce antibiotics with hence a need to be protected from their action is plausible [131, 132]. Alternatively, organisms living in the same environment as antibiotic producers, often soil, also need resistance genes to be able to compete. In other cases, a housekeeping gene playing no apparent role in antibiotic production or defense could have been adapted to this new use [133]. Sophisticated metagenomic studies have found sequences in DNA from soil, oceans, and human feces 100% identical to genes for resistance to aminoglycosides, β-lactams, glycopeptides, phenicols, tetracycline, and other agents supporting an environmental source for exchange of resistance genes, although the direction of this transfer is not always obvious [134, 135].

Some examples of potential sources are listed in Table 11.4. *Streptomyces griseus*, producer of streptomycin, makes phosphotransferases modifying the same-OH groups as APH enzymes in pathogens. *Streptomyces fradiae*, producer of neomycin, has acetyltransferases with the same specificity as the plasmid-mediated AACs. *Micromonospora purpurea*, producer of gentamicin, has methylases that modify 16S rRNA like ArmA and Rmt enzymes. *Streptoalloteichus tenebrarius*, producer of tobramycin, has an rRNA methylase similar to the acquired NpmA methylase. *Bacillus licheniformis*, producer of bacitracin, protects itself with a BcrABC transporter similar to that determined by plasmids in *E. faecalis*. *Streptococcus* (*Saccharopolyspora*) *erythreus*, producer of erythromycin, has 23S rRNA methylases similar to acquired Erm methylases. *Amycolatopsis orientalis* and *Streptomyces toyocaensis*, glycopeptide producers, have Van-like systems for self-protection. *Pseudomonas fluorescens*, producer of mupirocin, has a resistant isoleucyl-tRNA synthetase like the acquired Mup enzyme. *Streptomyces rimosus*, an oxytetracycline producer, has ribosome protecting Tet(M) and Tet(O)-like proteins. In each case, although the mechanisms are the same, the amino acid identity between producer and plasmid-mediated resistance protein is too low to accommodate a direct transfer. Both could have a common ancestor, but convergent evolution has not been ruled out.

Candidates with much closer sequence identity have been found for other resistance genes. AAC(6′)-Ih, so far found only on plasmids in *Acinetobacter* spp., and the more broadly distributed APH(3′)-VI are 99–100% identical to chromosomal enzymes from particular species of *Acinetobacter*. QnrA, QnrC, and QnrS have 97–99% identical analogues in aquatic bacteria such as *Shewanella* and *Vibrio* spp.,

Table 11.4 Suggested sources of transmissible resistance genes

Antibiotic	Resistance gene	Representative source	Identity %[a]	Reference
Aminoglycoside	*aph(3″)*	*Streptomyces griseus*	50	[132]
	aph(6)	*Streptomyces griseus*	34	[132]
	aph(3′)-VI	*Acinetobacter guillouiae*	99	[136]
	aac(3)-IIa	*Streptomyces fradiae*	37	[132]
	aac(6′)-Ih	*Acinetobacter gyllenbergii*	100	[137]
	armA	*Micromonospora purpurea*	27	
	rmt	*Micromonospora purpurea*	33	
	npmA	*Streptoalloteichus tenebrarius*	28	
Bacitracin	*bcrA*	*Bacillus licheniformis*	52	[5]
β-lactam	bla_{SHV}	*Klebsiella pneumoniae*	100	[138]
	bla_{CTX-M}	*Kluyvera* spp.	99	[139]
	bla_{OXA-48}	*Shewanella oneidensis*	92	[140]
	bla_{OXA-51}	*Acinetobacter baumannii*	97	[141]
	bla_{ACT-1}	*Enterobacter asburiae*	98	[142]
	bla_{CMY-1}	*Aeromonas* sp.	95	[143]
	bla_{CMY-2}	*Citrobacter freundii*	96	[144]
	bla_{MIR-1}	*Enterobacter cloacae*	99	[145]
	bla_{MOX-1}	*Aeromonas hydrophila*	94	[146]
	bla_{FOX-1}	*Aeromonas caviae*	99	[147]
	bla_{DHA-1}	*Morganella morganii*	99	[148]
	bla_{ACC-1}	*Hafnia alvei*	99	[149]
	bla_{KPC}	*Chromobacterium piscinae*	76	[150]
	bla_{NDM-1}	*Erythrobacter litoralis*	55	[151]
Chloramphenicol	*catA*	*Streptomyces albus*	36	[152]
	cfr(A)	*Bacillus amyloliquefaciens*	74	[153]
Colistin	*mcr-1*	*Moraxella porci*	63	[154]
Fluoroquinolone	*qnrA1*	*Shewanella algae*	99	[155]
	qnrB	*Citrobacter freundii* complex	99	[156]
	qnrC	*Vibrio parahaemolyticus*	97	[157]
	qnrE	*Enterobacter* spp.	96	[158]
	qnrS1	*Vibrio parahaemolyticus*	97	[157]
	oqxAB	*Klebsiella pneumoniae*	100	[159]
	qepA	*Pseudorhodoferax* sp.	84	
Fosfomycin	*fosA3*	*Klebsiella pneumoniae*	80	[160]
	fosA6	*Klebsiella pneumoniae*	99	[161]
	fosC2	*Achromobacter xylosoxidans*	28	[160]
Erythromycin	*ermA*	*Streptococcus erythreus*	21	[162]
	ermB	*Streptococcus erythreus*	24	[162]
	ermC	*Streptococcus erythreus*	24	[162]
Glycopeptide	*vanA*	*Amycolatopsis orientalis*	62	[163]
Mupirocin	*mup*	*Pseudomonas fluorescens*	35	[164]

(continued)

Table 11.4 (continued)

Antibiotic	Resistance gene	Representative source	Identity %[a]	Reference
Rifampin	*arr*	*Mycobacterium* sp.	63	
Tetracycline	*tet(M)*	*Streptomyces rimosus*	33	[132]
	tet(O)	*Streptomyces rimosus*	36	[120]

[a]Calculated from data available in GenBank May 2017

while QnrB originates from *Citrobacter* and QnrE from *Enterobacter* spp. The QepA efflux pump is related to ones in the order *Burkholderiales* such as species of *Pseudorhodoferax*, while the OqxAB pump has close relatives in *K. pneumoniae*, an organism that is also the likely source of *fosA* genes. Plasmid-mediated *fosA3*, *fosA5*, and *fosA6* are surrounded by truncated genes that also delimit the *fosA* gene on the chromosome of strains of *K. pneumoniae*.

Many β-lactamases also have a clear pedigree. The origin of TEM-1 is not known, but $bla_{\text{SHV-1}}$ is a chromosomal as well as a plasmid gene in *K. pneumoniae* and has been mobilized onto plasmids at least twice [165]. Close homologues of $bla_{\text{CTX-M}}$ genes can be found on the chromosome of rarely pathogenic *Kluyvera* species with $bla_{\text{CTX-M}}$ groups 1 and 2 related to genes of *K. ascorbata*; $bla_{\text{CTX-M}}$ groups 8, 9, and 25 related to genes of *K. georgiana*; and $bla_{\text{CTX-M-37}}$ related to genes of *K. cryocrescens* [48]. Several plasmid-mediated OXA-type carbapenemases are close enough in sequence to chromosomal genes in *Acinetobacter* or *Shewanella* spp. to make them likely progenitors. Plasmid-mediated AmpC-type β-lactamases have close homologues in chromosomally determined enzymes of various species. Enzymes in *Chromobacterium* spp. are as much as 76% identical in amino acid sequence to KPC β-lactamase. NDM β-lactamase appears to be a chimera formed, probably in *A. baumannii* [166], between the aminoglycoside resistance gene *aphA6* and a metallo-β-lactamase such as ElBla2 from *Erythrobacter litoralis* [151].

Aminoglycoside nucleotidyltransferases are missing among aminoglycoside producers. Several ANTs, however, share structural similarity and catalytic mechanism with housekeeping enzymes such as DNA polymerase β, which has a similar relationship with lincosamide nucleotidyltransferases LnuA and LinB [167]. Chloramphenicol acetyl transferase has been found in species of *Streptomyces*, such as *S. albus*, but not in *Streptomyces venezuelae*, the organism known to produce it [152]. The *cfr* methyltransferase gene has homologues in *Bacillus* spp. *Moraxella* spp. contain chromosomal *mcr*-like genes and also IS*Apl1* that is often associated with them [154]. Species of *Aeromonas* have also been suggested for the origin of *mcr*-like genes [52]. *Mycobacterium* sp. has a rifampin ribosyltransferase 63% identical to the plasmid Arr-2 enzyme. The origin of acquired *sul* and *dfr* genes is not known.

11.5 Plasmids and Other Mobile Genetic Elements

Plasmids vary in size from a few to more than 500-kb. Core plasmid functions include systems for maintenance (replication, stability, and copy control), for partitioning between daughter cells at the time of bacterial division, and for mobility (mobilizability and conjugal transfer). Small plasmids usually exist in multiple copies within the cell, while replication of larger ones is limited to a few copies. Plasmids in Gram-negative organisms smaller than about 25-kb lack space for the genetic machinery involved in mating pair formation but may be mobilized by a conjugative helper plasmid. In Gram-positive organisms, many plasmids rely on chemical signals mediated by oligopeptides for mating pair formation [168]. A third group of plasmids, found across the size spectrum, is neither conjugative nor mobilizable and is thought to rely on transformation or transduction for transfer [169].

Several plasmid classification schemes have been developed but can be compromised by plasmid plasticity and recombination [170]. Historically, plasmids were classified into Inc or incompatibility groups based on whether two plasmids were unable to coexist stably in the same bacterial host, a property based on replication specificity and copy control. Inc grouping is now tested with specific primers by PCR-based replicon typing. In *Enterobacteriaceae* as of 2014, PCR-based replicon typing could identify 24 distinct plasmid replicons with IncFII, IncA/C, IncL/M, and IncI1 being the most common groups among typed resistance plasmids [171, 172]. Since the system was based on established Inc groups, it relates directly to the older classifications. In *Acinetobacter baumannii*, plasmids have been subdivided into 19 GR types based on replicon sequences [173], and in *Enterococcus* and *Staphylococcus* spp., more than 25 rep families have been defined [174, 175]. Alternatively, MOB classification is based on variations in relaxase, an enzyme in the plasmid mobilization system that nicks DNA at a specific site to produce a single-stranded substrate for transfer. Use of degenerate primers recognizing the conserved N-terminal portion of the relaxase gene allows five MOB types to be distinguished for plasmids of *γ-Proteobacteria* [176]. Advantages of the MOB scheme include broad applicability to plasmids of *Acinetobacter* and *Pseudomonas* spp. as well as *Enterobacteriaceae*, and inclusion as well of integrative conjugative or mobilizable elements (ICE and IME). A disadvantage is the limited resolution inherent in the current number of MOB types. For some plasmid groups, a multilocus sequence typing (pMLST) system based on 2–6 core plasmid genes is available for subtyping [172, 177]. With neither replicon nor MOB typing, however, can all plasmids be classified at present, so further evolution of plasmid taxonomy can be anticipated.

Plasmids vary in host range, due mainly to specificity of replication rather than requirements of the conjugative system itself. In liquid culture, little if any mating occurs between Gram-negative and Gram-positive bacteria or between strict anaerobes and facultative organisms. Plasmids found in *Enterobacteriaceae* are usually transferable within that family, but only those belonging to a few Inc groups are transferable to *P. aeruginosa*, which has its own set of plasmids transferable to other *Pseudomonas* spp. [178]. Similarly, among plasmids in Gram-positive organisms,

some are species specific, while others have a broad host range and can be found in both *Enterococcus* and *Staphylococcus* spp. [174].

Plasmids carry accessory functions besides antimicrobial resistance such as metabolic pathways, colonization and virulence factors, sex factor activity, bacteriocin production and resistance, restriction/modification systems, biocide resistance, and heavy metal ion resistance. Hughes and Datta examined over 400 enterobacterial isolates collected in the "pre-antibiotic era" between 1917 and 1954 and found that 24% contained conjugative plasmids, many in the same Inc groups as contemporary resistance plasmids, but none carried antibiotic resistance genes [179, 180]. How then did naked plasmids and other mobile genetic elements acquire the genes for resistance?

Figure 11.3 shows tools that bacteria have used to capture genes and incorporate them into plasmids [181]. An insertion sequence (IS) is a 700 to 2500-bp DNA segment usually bounded by short, identical, sometimes imperfect inverted repeats (IR_L and IR_R) and containing one or two transposase (*tnp*) genes that code for enzymes that recognize the IRs and catalyze movement to another DNA site where integration generates direct repeats of 2 to14-bp depending on the IS [182]. As originally defined, classical IS did not carry resistance genes but may locate to provide an active promoter to activate an adjacent gene. Two copies of the same IS or related ones can, however, surround a resistance gene creating a composite transposon that can now move as a unit to another plasmid or chromosomal location. In particular, 820-bp IS*26* is very common in multiresistance regions of plasmids and is a frequent flanking element in composite transposons.

A few IS are unusual in that a single copy of the element can capture and move an adjacent resistance gene. For example, 1656-bp IS*Ecp1* is bounded by 14-bp IRs but on moving can utilize IR_L and a new IR_R distal to an adjacent gene which consequently becomes part of the mobile unit. IS*Ecp1* has been implicated in the mobilization of $bla_{CTX\text{-}M}$, *qnr*, *rmt*, and other resistance genes [183]. IS*CR* elements differ in moving by rolling circle replication and can incorporate larger segments of DNA than IS*Ecp1*. They are bounded not by IRs but by a downstream origin (*ori*IS) and an upstream terminus (*ter*IS) and do not create DR. Failure to recognize *ter*IS allows replication to continue into an adjacent gene which is thus mobilized. More than 20 IS*CR* elements have been distinguished based on the sequence of their transposases. IS*CR* have been involved in the mobilization of virtually every class of antibiotic resistance genes in Gram-negative organisms [184] .

An integron is an even more sophisticated system for capturing resistance genes packaged in cassettes. A cassette contains the gene, often preceded by a ribosome binding site but usually not a promoter, and an *attC* recombination site. The integron is made up of an *intI* gene encoding an integrase of the tyrosine recombinase family, an *attI* recombination site, and a Pc promoter. The integrase catalyzes site-specific recombination between the *attI* and *attC* sites capturing or releasing gene cassettes which can be lined up in tandem, all under the control of the Pc promoter. One hundred or so different cassettes are known carrying antibiotic resistance genes and three main groups of integrons, classified on the basis of *intI* sequences. Class 1 integrons are the most common [185, 186].

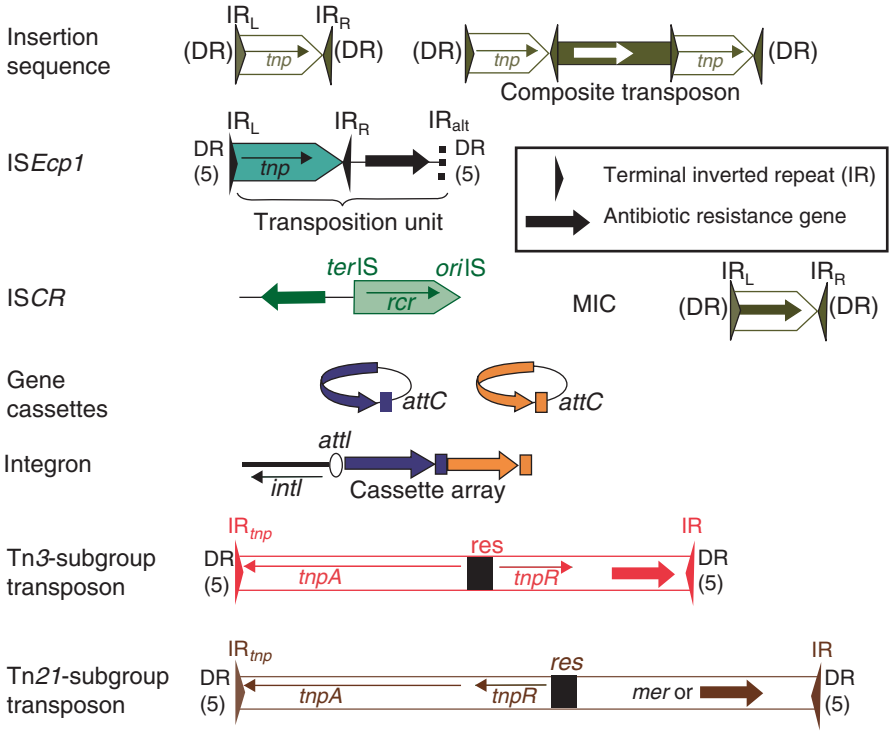

Fig. 11.3 Mobile elements involved in the capture and mobilization of antibiotic resistance genes in Gram-negative bacteria. DR, direct repeat; *tnp*, *tni*, transposition functions; IR_L and IR_R, left and right inverted repeats; IR_{alt}, alternative IR; *rcr*, rolling circle replicase; *ori*IS, origin of ISCR elements; *ter*IS, terminus of IS*CR* elements; *attC*, cassette recombination site; *attI*, integron recombination site; *res*, resolvase site. Elements that create DR are indicated and the DR length given, except for IS, where the DR length varies for different elements. Tn*21*-subfamily transposons may carry resistance genes as part of class 1 integrons inserted in or near the *res* site. MIC mobile insertion cassette. (Adapted from [181])

The first moveable units on plasmids to be described were complex or unit transposons, such as 4957-bp Tn*3* encoding TEM-1 β-lactamase. Members of the Tn*3* family are bigger than IS and include a transposase gene (*tnpA*), a resolvase gene (*tnpR*), and a resolution (*res*) site as well as one or more resistance genes all bounded by 38-bp IR. Movement is replicative and involves formation of a cointegrate intermediate consisting of two copies of the transposon linking donor and recipient molecules. Tn*21* (19,671-bp) and Tn*21*-like transposons contain the same *tnpA*, *tnpR*, and *res* genes in different orientations and often include *mer* genes for resis-

tance to Hg^{++}. Integrons are often found within transposons and IS*CR* elements within integrons.

Another transposable element termed *mic* for mobile insertion cassette is composed of a resistance gene bracketed by IR but lacking an integrase/transposase, which must be supplied in trans for the unit to move [187].

Integrative and conjugative elements (ICE) (also known as conjugative transposons) encode a phage-like integrase (*int*) that catalyzes recombination between an *attP* site on the unit and the host chromosome. The chromosomal integration site is typically specific for a particular ICE family. They are bounded by IRs, and most ICE also encode an excisionase (*xis*) that removes the ICE from the chromosome as a circular molecule. Transfer of the circular form to a new host requires plasmid-like genes that control DNA transfer and genes that form a mating pair between donor and recipient. Lack of the latter function produces an integrative mobilizable element (IME) that requires the missing functions in trans for transfer by conjugation. Both ICE and IME can contain transposons, ISs, and integrons. ICE and IME thus share many of the functions of conjugative and nonconjugative plasmids except for their preference for a chromosomal location. Surveys of prokaryotic genomes indicate that ICE are more common than plasmids and mobilizable elements outnumber self-conjugative ones [188]. They occur in Gram-positive, Gram-negative, and strictly anaerobic organisms.

Genomic islands are gene clusters, some very large, fixed in the chromosome with features that suggest a foreign origin. In one strain of *A. baumannii*, an 86-kb resistance island containing a variety of ISs, transposons, integrases, and 45 resistance genes has been identified and obviously allows for rapid development of panresistance [189]. Genomic islands are also important in the evolution of multiresistance in *P. aeruginosa* [190].

Phage particles carrying resistant genes (bla_{TEM}, $bla_{CTX\text{-}M}$, *qnrA*, *qnrS*, *armA*) have been identified in wastewater or the human gut and constitute another class of mobile elements [191, 192].

These elements can interact in various and complex ways. For example, a plasmid in *K. pneumoniae* carrying genes for both carbapenem, aminoglycoside, and quinolone resistance was found to contain a complex transposon incorporating $bla_{KPC\text{-}3}$ inserted into a Tn*3*-family complex transposon with aminoglycoside resistance genes and $bla_{TEM\text{-}1}$ that also contained *qnrB19* mobilized by IS*Ecp1* [193]. Because these mobile elements are built in modules, they can exchange, rearrange, insert, delete, and recombine to generate remarkable diversity [194]. They have also been doing this for a long time. Plasmid NR1 (also known as R100), one of the original transmissible elements discovered in Japan in the 1950s, already contained both integrons and transposons [195].

11.6 Overcoming Transmissible Antibiotic Resistance

Despite detailed investigation, no clinically useful direct attack on plasmid replication, stability, or mobility has been discovered. The most successful application of knowledge about resistance has been the development of antibiotics that escape resistance mechanisms and of inhibitors that restore effectiveness to agents that would otherwise be inactivated. Table 11.5 lists examples of successful antibiotic modification or discovery. β-lactam antibiotics provide many examples. The 7-α-methoxy group of the cephamycins cefoxitin and cefotetan allow activity against many class A β-lactamase-producing bacteria as well as enhanced activity against anaerobes. The oxyimino group of cefotaxime, ceftazidime, ceftriaxone, cefepime, and aztreonam gave these antibiotics an even broader activity against class A β-lactamases, and the carbapenems imipenem, meropenem, doripenem, ertapenem, and others have the broadest activity spectrum of all. Success was met with counterattack in the form of plasmid-mediated AmpC enzymes active against cephamycins, extended-spectrum β-lactamases active against oxyimino-β-lactams, and carbapenemases of classes A and D as well as class B [196].

Other successful antibiotic modifications include amikacin, a semisynthetic derivative of kanamycin, with a 2-hydroxy-4 aminobutyric acid side chain that makes it less susceptible to many aminoglycoside-modifying enzymes, and florfenicol, a fluorinated derivative of thiamphenicol, that is insensitive to CAT enzymes and some chloramphenicol efflux pumps. The oxazolidinone tedizolid is more potent than linezolid particularly against strains with the Cfr methyltransferase because of facilitated binding to methylated 23 S rRNA [197], and the minocycline derivative tigecycline is active against most organisms with transmissible tetracycline resistance, although it can be overcome by a combination of *tet*(L) and *tet*(M) [123]. See also Sect. 4.2.4.3 for other mechanisms of emerging tigecycline resistance. Finally, the semisynthetic lipoglycopeptides telavancin, oritavancin, and telavancin are active against vancomycin-resistant enterococci containing the VanB gene cluster, and oritavancin and telavancin are also active against VanA strains [82] with the caution that resistance may emerge if used as monotherapy [198].

Clavulanic acid, sulbactam, and tazobactam are β-lactamase inhibiting β-lactams that have been combined with otherwise enzyme-susceptible agents (amoxicillin, ampicillin, ticarcillin, piperacillin) to expand their spectrum of action. Problems with their use are that many β-lactamases are intrinsically resistant to inhibition (Table 11.3) and that initially sensitive enzymes can develop inhibitor resistance by mutation, as happened first for TEM and SHV-type β-lactamases and recently with a CTX-M variety [199]. A new group of diazabicyclooctane compounds (avibactam, relebactam, zidebactam, and others) with a broader spectrum of inhibition is currently undergoing evaluation (see Sect. 4.2.2.3 for further details). Several have direct antibacterial activity and attack organisms producing metallo-carbapenemases

Table 11.5 Antibiotics with improved resistance properties

Parent antibiotic	Improved derivative	Escapes resistance from
Benzylpenicillin Ampicillin Cephalothin and other 1st generation β-lactams	Cefoxitin, cefotetan	Many class A β-lactamases
	Cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam	Most class A β-lactamases
	Imipenem, meropenem, doripenem	Most class A, C, and D β-lactamases
Chloramphenicol	Florfenicol	CAT, some chloramphenicol efflux pumps
Kanamycin	Amikacin	Many aminoglycoside-modifying enzymes
Linezolid	Tedizolid	Cfr methyltransferase
Tetracycline	Tigecycline	Tet efflux and ribosomal protection agents
Vancomycin	Telavancin, oritavancin, telavancin	VanB,? VanA

as well as acting as β-lactamase inhibitors [200]. The ceftazidime-avibactam combination has been approved for clinical use, and already inhibitor-resistant KPC-3 mutations have been reported in patients treated for *K. pneumoniae* infections producing the carbapenemase [201].

Much can also be done to reduce the selective pressure for developing and maintaining resistance. More than half of the antibiotics produced commercially are used for other than human medicine. For example, streptomycin was sprayed for years on apple and pear trees to prevent a destructive bacterial disease known as fire blight until the responsible organism *Erwinia amylovora* became streptomycin resistant, and its use had to be abandoned. Other nonhuman uses that contribute to resistance development include antibiotic use for livestock growth promotion; use for pest control; use for therapy of household pets; use for treatment of cows, pigs, chickens, fish, and other animals produced for food; use as biocide in toiletries, skin care creams, and cleaning products; and use in research and industry [202]. For example, the glycopeptide avoparcin was used as an animal growth promoter with selection of glycopeptide-resistant enterococci that were shown to be similar to those from human infections [203] leading to a ban of avoparcin use in Europe. Attention needs to be paid also to agents other than antibiotics that can select for resistance, such as use of olaquindox and carbadox as feed additives for pigs that led to the spread of the plasmid-mediated OqxAB multidrug efflux pump.

More than 40 years ago, studies in hospitals showed that more than half the antibiotics used clinically were not needed, were given inappropriately, or were dosed incorrectly [204]. Recent studies indicate little if any improvement, but up-to-date guidelines for antibiotic stewardship in human medicine are available [205], and their implementation is now a requirement for hospital and nursing care center approval by the Joint Commission that accredits healthcare organizations in the United States.

Major Points

Our adversaries turn out to be cleverer than we thought with an abundant reservoir of resistance genes and a toolkit of efficient genetic devices to mobilize, incorporate, and share them. Resistance is increasing, and one by one agents that we thought could still be counted on have become less reliable. Knowledge of resistance mechanisms has allowed the development of antibiotics and combinations effective for a time against resistant pathogens, but bacteria will continue to evolve resistance. Speedier diagnostic tests will facilitate choice of effective agents, but new antibiotics and new ideas to combat resistance are urgently needed.

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Chapter 12
Antibiotics and Resistance in the Environment

Marilyn C. Roberts

12.1 Introduction

The discovery and use of antibiotics was one of the greatest public health achievements of the twentieth century. Antibiotics have saved millions of human and animal lives, reduced agricultural losses, and contributed to increased food production. These agents have extended the lives of people with genetic conditions and have become indispensible in modern medicine. The majority of antibiotics currently in use were originally produced by living microbes that were then modified by man. Antibiotics either inhibit growth of other microbes or kill them by interacting with specific microbial targets. Most of the targets are unique to microbes, which has led to the agents being safe enough to use with eukaryotic organisms.

In the mid-twentieth century, antibiotics became the foundation for treating bacterial infections in both humans and animals. Antibiotic-resistant bacteria [ARB] and antibiotic resistance genes [ARGs] were recognized within a year after penicillin was first used in humans, and soon after it was seen with agricultural use [1, 2]. ARB infections now contribute to thousands of deaths each year plus increased morbidity and medical cost. Currently, it is estimated that ~10 million deaths due to antibiotic-resistant infections occur each year; this number is expected to rise in coming years [3]. In essence, antibiotic resistance has changed treatable infections into untreatable diseases, thereby moving us closer to the "post-antibiotic era."

Multidrug-resistant pathogens were first identified in the 1950s [4]. ARB were initially limited to hospital settings and few outbreaks occurred; ARB were not seen as a major concern for general community medicine. Today it is known that antibiotic use in humans and agriculture results in increased antibiotic resistance in

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I. W. Fong et al. (eds.), *Antimicrobial Resistance in the 21st Century*, Emerging Infectious Diseases of the 21st Century, https://doi.org/10.1007/978-3-319-78538-7_12

all types of bacteria, ranging from pathogenic to environmental species. A major paradigm shift occurred in the 1970s, with the identification of ampicillin-resistant *Haemophilus influenzae* and penicillin-resistant *Neisseria gonorrhoeae*, both community-acquired pathogens. Resistance to the preferred therapy led to changes in the recommended therapies for disease arising from these pathogens. The need for monitoring ARB and ARGs and periodic changes of first-line therapies has become an ongoing issue for many different pathogens. Resistance has also lead to a new industry of diagnostics in which new methods and techniques continue to be developed for rapid identification of resistance in clinically important bacteria.

In the past, surveillance of the environment locally, nationally, and internationally has not been a priority, but that has changed [5], as we are beginning to examine the issue and assess the impacts of ARB/ARGs on human and animal health, agricultural and food production, aquaculture, human and animal waste management, and the impact and contamination of the environment globally [6–11].

Antibiotic uses and abuses are directly responsible for the increases in the level of ARB and ARGs isolated in agricultural as well as aquacultural settings, the food chain, man, and built and natural environments [12–14]. Much has been said about the uses of antibiotics as growth promoters in Europe and the USA as being a major source of antibiotic resistance. In June 2015, the US Food and Drug Administration published a final rule known as the veterinary feed directive (https://www.gpo.gov/fdsys/pkg/FR-2015-06-03/pdf/2015-13393.pdf), which limits the use of antibiotics as feed additives for growth promotion. The rule became effective on October 1, 2015, and may have widespread impact on use and prescribing of medically important antibiotics in food animals, both in the years leading up to implementation and after implementation (Jan 2017).

In the early years of antibiotic usage, there were new antibiotics available to replace the older antibiotics as bacteria became resistant. Thus, when one antibiotic failed to work, another was available to take its place. Today there are very few new antibiotics in development to replace the less effective, older antibiotics [3]. The current lack of new and novel antibiotics coming into the market, along with the high cost of newer antibiotics, has led researchers to anticipate a time when there will be no useable antibiotic for many common bacterial diseases. Thus, animals, plants, and people will die of infections that were once easily treated with antibiotics but are now resistant to all available therapies [11]. The factors that contribute to emergence and dissemination of bacterial resistance are complex and require attention in both industrialized and developing countries [12, 13].

Concerns over the spread of antibiotic resistance have stimulated several groups to assess the impact of ARB/ARGs on human and animal health, agricultural and food production, and agricultural and human waste management [15, 16]. One of the primary outcomes of emerging reports is a call for increased surveillance of ARB and ARGs in agricultural and environmental settings, with a particular interest in identifying transmission routes of ARB and ARGs throughout the world [11, 15]. Keys to the success of current and future surveillance efforts are strategies to determine which types of resistant genes to monitor and how to support the surveillance effort, especially for environmental settings and in developing and resource-poor

countries. This is a major task given that in the USA there is no national surveillance program for the most common pathogens across most states. Instead, the Centers for Disease Control and Prevention (CDC) has used surveillance systems that focus on nine representative states [17]. The European Union does a more comprehensive job of covering their member states (http://ecdc.europa.eu/en/healthtopics/antimicrobial-resistance-and-consumption/antimicrobial_resistance/EARS-Net/Pages/EARS-Net.aspx); other parts of the world have varying success with human surveillance systems [17]. The problem is difficult, because ARGs are not randomly distributed among bacterial species. Data suggests that a clear link exists between bacterial taxonomy and specific types of ARG [18, 19]. This phenomenon has been particularly well documented with tetracycline resistance genes [20–22].

The environmental dissemination of ARGs and the development of ARGs are thought to be primarily due to horizontal gene transfer. The most common way bacteria exchange ARGs is by conjugation, which allows rapid transfer of ARGs between species and genera within and between ecosystems [21]. However, our knowledge is limited in regard to how the environment contributes to transmission between the environment, wildlife, domesticated animals, plants, and humans. It is critical when examining specific antibiotic resistance genes to know whether a given gene is normally associated with a mobile element and whether that element has a narrow or broad host range. Clearly a mobile element with a broad host range will allow for wider transmission across multiple genera than a narrow host range element [23, 24]. It is important to identify the specific ARGs associated with specific bacterial species and/or genera within the environment. Durso et al. [18] suggested that the same antibiotic resistance gene might have different risks for environmental transmission that depends on the specific bacterial taxa within which it is found. For example, if the bacteria are widely distributed among a variety of environments, the ARGs associated with them are more likely to spread widely. If, on the other hand, the bacteria have a limited environmental range, the ARGs will tend to remain associated with them specifically. If they have a limited host range, they may also not be widely distributed. It is equally important to know how ARGs and ARB are distributed among human and animal populations and how these ecosystems interact with various environments. Moreover, we need to know how microbial distribution differs by region, nation, and worldwide [25]. Other issues include the fact that most environmental studies look at a selected group of ARGs by qPCR, which determines the presence or absence of particular genes [26], or they use microbiome studies that usually do not look specifically for selected ARGs [25]. Thus, environmental studies should include bacterial culturing, in addition to molecular studies, to fully understand the distribution within the bacterial ecosystem of various environments. The more comprehensive analysis is especially important because many of the new ARGs are coming from the environment rather than from either human or animal sources, which makes it difficult to know the bacterial source of a given ARG (http://faculty.washington.edu/marilynr/).

Organized environmental surveillance of ARGs/ARB will hopefully allow identification of major gaps in our understanding of the forces that act on selection and transmission of bacterial resistance. This effort in turn may lead scientists in direc-

tions that could either slow or stop the march to a time when common infections and minor injuries kill, as they did prior to the introduction and widespread use of antibiotics (this phenomenon is well illustrated by the recent spread of NDM-1 β-lactamase carrying bacteria [27]). It is clear that a global "One Health" approach is needed in which animal and human usage and environmental contamination are considered together, along with an understanding of how ARGs and ARB move between the ecosystems.

12.2 Antibiotics Used for Conditions Due to Non-bacterial [Noninfectious] Conditions

Some antibiotics have non-bacterial effects on humans and animals and have been used to treat non-bacterial conditions, especially skin diseases. A review of the non-antibiotic properties of minocycline by Garrido-Mesa et al. [28] is a useful guide to other properties that this antibiotic has and the non-bacterial conditions for which minocycline is used as treatment. A 2013 paper [29] reported that minocycline improves symptoms of fragile X syndrome when given to children and adolescents. Another study explored the use of tetracyclines with cancer targets through a randomized phase II trial [30]. In a third case, the macrolide azithromycin stimulated immune and epithelial cell modulation of transcription factors AP-1 and NFκB with subsequent delayed inhibitory effects on cell function and may cause lysosomal accumulation of the macrolide with disruption of protein and lipid transport through the Golgi apparatus and effects the surface receptor expression, including macrophage phenotype changes and autophagy [31]. In addition, azithromycin inhibits quorum sensing and biofilm formation by *Pseudomonas aeruginosa*, even though the drug does not inhibit growth. Moreover, azithromycin, given prophylactically, can reduce the incidence of ventilation-associated pneumonia [31]. It is important to note that the use of antibiotics for non-bacterial conditions increases the exposure of individuals' microbiomes to the selective pressures underlying the emergence of bacterial resistance (M. Roberts unpublished results). It also increases the potential for environmental contamination by the antibiotic, its residues, and ultimately selection of ARGs and ARB resistant to these antibiotics.

12.3 The One Health Approach

The One Health approach contrasts with the traditional practice of human and animal medicine, which have been studied and practiced in isolation rather than as part of an ecosystem. The environmental contribution to global health has not been generally considered, or if studied, rarely, until recently in connection with the health of man and/or animals. The world microbial ecosystem includes the microbiomes associated with each domain of life and the direct and indirect mixing of

the different microbiomes that, in some cases, may lead to disease. The human-animal interface is ancient, but it has expanded with the development of farming animals and fish. It is a continuum of contacts and interactions that allow for barrier breaches of pathogens to occur and an increased driver of infections. This is illustrated by the estimation that ~75% of emerging infectious diseases in humans over the last 20 years have been zoonotic, i.e., the pathogen spread from animals or insects to people. In some cases, the pathogen becomes established and then spreads within human populations. However, more commonly, there are recurrent events of transmission from an animal/insect reservoir to humans, with limited human-to-human transmission. An example of this situation is observed with the Zika virus [32, 33]. Other examples include many foodborne bacterial infections, such as those caused by *E. coli* O157:H7 and enterotoxigenic *E. coli* O114:H4. *E. coli* O114:H4 caused a huge outbreak in 2011, which, besides causing death and infections, created tension among EU members involving boycotts of vegetables within the EU [34]. Dealing with emerging and reemerging infections that cross species barriers not only impacts humans but also impacts livestock, pets, wildlife, crops, and aquaculture. These pathogens can contaminate the environment, and in worse cases, they may impact food resources and food security. The importance of global ecological changes due to human impact on the environment and technological changes in society, along with important changes in how food is produced, processed, and transported, combines to increase the potential risk of disease transmission [32]. With environmental contamination as a major by-product of these endeavors, changing the downward spiral of increasing global contamination can only be addressed by improved communication, cooperation, and collaboration across disciplines and the realization that there are multiple ways contamination can enter the food chain.

How a particular antibiotic can influence where and how antibiotic resistance and ARB develop and spread from one domain to all three has been illustrated in the literature. One good example is the development of vancomycin-resistant *Enterococcus faecium* (VRE) in North America, the EU, and the rest of the world. In Europe and other parts of the world, a vancomycin-related drug avoparcin was used as a growth promoter in livestock. Over time, VRE developed in chickens and swine to where it could be readily detected in processed meat [35]. Transmission of VRE genes, or the intact bacterium, from animals to humans occurs in the EU setting. Once VRE was established in livestock populations, farmers and those slaughtering the VRE+ animals acquired VRE in their intestinal tracts. VRE ultimately was isolated from hospitalized persons [36]. In contrast, avoparcin was never used as a food additive in the USA. Early studies suggested that VRE was not found in chickens in the USA, and there was little evidence to suggest transmission of VRE in healthy adults prior to 2000 [35]. In contrast to the EU, which did not use vancomycin heavily in the hospital setting, vancomycin was used extensively in US hospital. The result was the emergence of VRE as a major nosocomial pathogen within US hospitals [38]. This was due, in part, to the persistence of viable VRE on contaminated surfaces within the hospital for weeks and even months. Rooms housing patients colonized or infected with VRE were difficult to clean.

Consequently, these rooms served as reservoirs for transmission of VRE to new patients [37]. More recently, VRE has been cultured from the general community environment in the USA, as illustrated by VRE recovered from wild crows and recreational beach sand and water in North America [39–41]. Currently, if a patient enters a hospital and exhibits a VRE infection within 48 h of entrance, the infection is considered community acquired rather than nosocomial. The occurrence of outpatient VRE depends on geographic location, occupation of the people, differences between urban or rural settings, and/or recent attendance at a medical/dental outpatient clinic or office. VRE in the USA spread from hospitalized humans to the community and environment, while in the EU and other parts of the world, VRE developed in livestock receiving avoparcin and then spread to the farm workers, local communities, and ultimately hospitalized patients.

12.4 The Environment

Most studies on ARGs, over the last 70 years, have focused on clinically important bacteria found in humans and animals. It is estimated that there are ~5×10^{30} bacteria on earth, with only a small subset adapted to live either in or on humans and animals. More striking is the estimate that <1% of the total number of bacteria in the world have been cultured [42]. The natural world is rich in chemicals made by living organisms and human activity – antibiotics are not the only compounds that have influenced the evolution of microbiomes [43].

As stated above, knowing which type of bacterium carries a particular ARG can be critical in designing studies of the environment. For example, many in the field use *E. coli* as a model system for ARG carriage. Yet many ARGs found in *E. coli* are unique to Gram-negative facultative aerobes and not found in anaerobes, other Gram-negative bacteria, or Gram-positive species [22]. Thus, when *E. coli* is the model, most acquired ARGs are associated with plasmids that independently replicate and tend to have a host range limited to Gram-negative bacteria. In contrast, many ARGs in Gram-positive and anaerobic species are on mobile elements that are normally found in the chromosome; thus, they have a different host range dynamics that can be much broader than classical Gram-negative plasmids. Therefore, by looking for both classical Gram-negative and Gram-positive ARGs, researchers can select ARGs that are likely to be most important for a particular ecosystem from a large set that confers resistance to the same class of antibiotic. This is especially important when molecular methods of detection are used, because only a limited number of genes can be assayed, and if the rare genes are chosen, it will bias the results leading to an unrealistic picture in that ecosystem.

Environmental studies are moving away from culturing bacteria, instead of determining which ARGs are present by using either PCR or qPCR. These molecular assays have now been used for direct detection of ARGs in food [44], animal feeding facilities, and agricultural soils amended with manure [45] and as indicators for water quality changes [46]. In these studies, known ARGs were used without

determining their likely distribution in the particular sample source, which can lead to biased results. For example, if only ARGs present in Gram-negative bacteria are used for screening, then no information will be obtained regarding the Gram-positive and anaerobic component of the sample source. Such studies have a very limited ability to identify novel resistance genes.

To overcome the shortcomings of nucleotide sequence-dependent methods, soil bacteria were screened for the ability to degrade or inactivate antibiotics. In one study, strains were randomly isolated from 11 diverse rural and urban soils, and they were then tested for the ability to utilize 18 different antibiotics as sole sources of carbon and nitrogen [47]. Many of the bacteria were *Burkholderia* spp. and *Pseudomonas* spp., which are naturally part of the soil microbiome and only rarely cause disease. These bacteria could grow on antibiotic-supplemented media and were resistant to multiple antibiotics at clinically relevant concentrations, suggesting the presence of an unappreciated reservoir of antibiotic resistance genes in these soils [47]. This work led to development of the functional metagenomic approach (described below) in which the antibiotic resistome of an environment is examined. This has led to identification of novel resistance genes in addition to know ARGs [22].

Functional genomic studies have also been used to study a variety of microbial environments [48]. This assay determines whether the cloned DNA can be expressed and confer resistance when transferred into a host *E. coli*. When the cloned DNA allows the host bacteria to grow in the presence of antibiotic-supplemented media, the resistance-conferring DNA fragments can be sequenced and compared to known ARGs. The Antibiotic Resistance Genes Database now lists ~20,000 potential resistance genes([49]; http://ardb.cbcb.umd.edu/), while the Comprehensive Antibiotic Resistance Database [CARD] also has a large number of resistance genes that can be used to screen sequences [50]. A variety of new potential ARGs have been identified using this method ([51]; http://faculty.washington.edu/marilynr/). One issue with these databases is they rely on GenBank information, which can be confusing and inaccurate because the system allows authors to name their own genes rather than going through a system. They also make it difficult to change names. Thus many of the ARGs in GenBank do not have the correct nomenclature for specific ARGs. Consulting other sources such as http://faculty.washington.edu/marilynr/ and http://www.lahey.org/studies/ should be used to get the correct names for tetracycline, for macrolide-lincosamide-streptogramin genes, and for β-lactamase genes, respectively. Recent reviews are also good sources for the current nomenclature [22].

The term antibiotic "resistome" is defined as the collection of all genes that can either directly or indirectly contribute antibiotic resistance to its bacterial host [52]. Research groups have been examining the microbial resistome of natural and clinical environments [51, 53–55]. Studies have looked for ARGs in samples linked to human activity, such as food production [56, 57], polluted waterways, and wastewater treatment plants [26]. Resistome studies suggest that environmental bacteria may be antibiotic resistant by virtue of both previously characterized, known genes and unknown resistance genes, mutations, and resistance genes on

mobile elements [55, 56]. A number of new ARGs have been identified from these studies; in most cases, the bacterial host is unknown (http://faculty.washington.edu/marilynr/). One example of a ARG identified in a molecular study is the *tet*(43), which encodes an efflux protein. It was isolated from metagenomic analysis of soil taken from an apple orchard that had been repeatedly treated with streptomycin [56]. It is unknown what type of bacterium actually carries *tet*(43), and little is known about the distribution of this gene. Similarly, the nine genes [*tet*(47)-*tet*(55)], identified more recently, code for enzymes that inactivate tetracycline. These were identified, cloned, and sequenced from soil samples where functional metagenomic analysis was done [58]. Again the bacterial sources of each of the genes are not known. This later work increased the number of characterized enzymes that inactivate tetracycline from 3 to 13 (http://faculty.washington.edu/marilynr/), clearly showing that a variety of new ARGS may be present in environments.

Many bacteria, including environmental bacteria, encode β-lactamases, which hydrolyze and inactivate β-lactam antibiotics (http://www.lahey.org/studies/). They are the most widely distributed of all ARGs [3]. One example is *ampC*, which was originally an inducible chromosomal cephalosporinase found in a variety of Enterobacteriaceae. This gene has been found in opportunistic pathogens belonging to normal intestinal floral of humans and animals, in bacterial species that normally live in either soil or water, and in both pathogenic and nonpathogenic bacteria [59, 60]. It has been proposed that *ampC* originated in environmental bacteria. The first AmpC-positive clinical strains were *E. coli* isolated in the 1940s, just as the first antibiotics were being developed and used. In a host background that has porin deficiencies, *ampC*, when expressed, confers carbapenem resistance due to increased production of the AmpC β-lactamase. This increased production of the AmpC β-lactamase is usually due to mutations that up-regulate expression of the enzyme. Today the chromosomal AmpC β-lactamases are associated with plasmids, which was first noticed in the 1980s. These mobile plasmids often tend to be large, and they carry multiple ARGs. Plasmid-mediated AmpC β-lactamases have greatly expanded the host range of this group of enzymes that are now found in epidemic human pathogens such as *E. coli* ST131. This *E. coli* strain has been isolated from fresh vegetables, food-producing animals, fish farms, pets, and water environments [61, 62].

Many ARGs are associated with soil antibiotic producers such as *Streptomyces*. Some of these natural ARGs have the same mode of action as those found in clinically resistant bacteria [3]. In the past, it was assumed that most environmental bacteria were poorly adapted for life in humans and animals. However this idea is changing, as progress in medical science allows severely immunocompromised patients to live in the community where they can be infected with environmental organisms. Other susceptible persons include those who have foreign objects permanently implanted in their bodies and persons with various types of occupational exposure [63]. Moreover, the distinction between environmental and non-environmental bacteria has become difficult, because the mixing of the two sources of bacteria has become increasingly common as human and agricultural contamination of the environment has become widespread. Indeed, very few

ecosystems around the world have not been touched by the activities of human civilization – whether it is in polar regions or the Amazon jungle [64, 65]. The continual mixing of environmental and non-environmental bacteria provides opportunities for horizontal genetic exchange of ARGs between man, animal, and environmental bacteria.

Antibiotics, antibiotic residues, ARBs, and ARGs move by water and wind [66], wastewater treatment discharges [26, 67], biosolids, and manure applications [68], isolated from recreational beaches [40]. They are also moved along with the transportation of goods and people around the world [69, 70]. One result of this movement has been the global spread of specific strains, such as *Clostridium difficile* NAP1/027/BI [71]. *C. difficile* spores are robust, and they can survive in hospital dust for extended time periods. *C. difficile* was originally classified as a nosocomial pathogen. Today *C. difficile* is known to be a foodborne and community pathogen. Similarly, 25 years ago, *Acinetobacter baumannii* was a rarely identified human pathogen. At that time, *Acinetobacter* spp. were primarily found in the environment. They are well adapted to grow at a wide range of temperature and pH values, can use a variety of carbon and energy sources, and persist in both moist and dry locations for extended time periods. Today multidrug-resistant *A. baumannii* is considered an opportunistic pathogen that has become a major concern for military trauma patients and causes infections that are very difficult to treat due to limited therapeutic options [72].

12.5 Agriculture

Antibiotics are used for both human and agricultural activities for prevention and treatment of infections. They are also used as food additives and growth promoters in food production in the USA. However this widespread use is changing. In June 2015, the US Food and Drug Administration published a final rule, known as the veterinary feed directive (VFD), that extends the use of veterinary feed directives to an increased number of medically important antimicrobials used in food animal production [73]. The rule became effective on October 1, 2015, and may have impacted use and prescribing of medically important antibiotics in food animals in years prior to implementation because evidence supporting this idea is derived from the experience of the EU. On July 1, 1989, an EU-wide ban on the use of four growth-promoting antibiotics, spiramycin, tylosin, bacitracin zinc, and virginiamycin, came into effect. The result of this ban was a dramatic drop in the sales of antimicrobial growth-promoting agents. In 2006, the remaining antibiotics used as growth promoters (monensin, avilamycin, salinomycin, and flavomycin) came under an EU-wide ban. It is projected that a further dramatic decrease in sales of growth promoters will occur [74]. Therefore, it is hoped that the US FDA ruling will reduce overall uses of antibiotics used annually in livestock raised in the USA.

Antibiotics can be found in domestic animal manure, which may be transferred when manure is applied to fields or stored in lagoons. In the USA, manure, regardless of whether the host animals are treated with antibiotics or not, is considered an organic product. Domestic animal manure can be placed on crops that will be grown organically. In addition, "organic farms" are usually on land that was originally farmed conventionally. Therefore, antibiotics, antibiotic residues, ARGs, and ARB are normally present in the farm environment. The ARB can colonize the "organic" livestock, while the ARGs may be incorporated into the livestock microbiome. As a result, both organic and conventionally grown meats may have ARB [75].

Antibiotics are sprayed onto crops which contaminates the surrounding soil, sediment, and groundwater. This practice exerts selective pressure on the associated microbiomes and increases the prevalence of resistance in bacterial pathogens of fruit orchards. Antibiotics may also be incorporated into food given to farm animals and fish, which will, in turn, contaminate the surrounding area and ultimately enter the water system.

Antibiotics from human therapeutic use, especially from hospital effluents, are a continual source of pollution and are considered part of the "emerging contaminants" in municipal waste (concentrations of tetracycline vary from ng/L to μg/L). At these levels, antibiotics may select for tetracycline-resistant environmental bacteria which, once present, may persist in the environment for long times. The environment may become a reservoir for tetracycline resistance genes and for other antibiotic resistance genes, since co-selection with other ARGs is common. Antibiotic-resistant bacteria and residues have been identified in tap water, urban water supplies, milk, meat, vegetables, and processed and unprocessed foods [76]. All of these sources contaminate both built and natural environments, either directly or indirectly, and provide selective pressure on the resident environmental bacteria to become antibiotic resistant [3]. In some cases, transfer of specific antibiotic resistance genes is increased with exposure to low levels of antibiotics [77].

12.6 Human Influences on Environments

Human activity may directly influence the development of ARBs in built-up environments. For example, several studies have recovered antibiotic-resistant *E. coli* and *S. aureus* from air in homes that are enriched relative to samples from outside of the home, even though the latter have higher bacterial levels. However, there was variability both in study design and results [66]. Potentially, ARB may contaminate the environment either directly, as occurs when manure is applied to enrich agricultural fields, or indirectly due to sewage contamination of receiving waters where the final effluent is deposited such as a river, lake, or ocean. The first description of the *tet*(M) gene in *Bacillus* spp. and of Tc^r*Bacillus cereus* strains carrying the *tet*(M) gene, on a Tn*916* mobile element, was found in animal manure and in fields where the manure was spread. These results suggested that the presence of *tet*(M)-carrying *B. cereus* in fields was a direct result of manure application to the soil.

Whether *tet*(M)-carrying *B. cereus* will act as a donor and transfer the *tet*(M) gene to either related *B. anthracis* or *B. thuringiensis* is unknown. However, some toxin-encoding plasmids are shared among these three species [68].

An example of human wastes increasing ARB was illustrated by a 1980's study that observed three groups of wild baboons in Kenya. Two of the groups lived in their natural habitat with either limited or no human contact; these groups had low levels of antibiotic-resistant Gram-negative enteric bacteria. The third group lived close to a tourist lodge that provided opportunities for daily contact with unprocessed human refuse. From these animals, high levels of antibiotic-resistant Gram-negative enteric bacteria were recovered with >90% tetracycline resistant [Tc^r]. These results suggested that contact with human refuse greatly increased the carriage of Tc^r bacteria in these wild primates [78]. Unfortunately, the surrounding environmental bacteria were not sampled. One could speculate that the level of environmental ARB was likely higher around the human refuse site than in areas where the two other baboon groups lived in a more natural setting. Other studies have recovered antibiotic-resistant *E. coli* from arctic and subarctic seals [79], wild boars [80], and wild rabbits [81]. More recently, bacteria carrying extended-spectrum beta-lactamases (ESBLs) have been isolated from water birds in remote locations [82]. Birds and wild animals can also be found feeding either in or around wastewater treatment ponds, waste landfill sites, and septic tank discharges. Birds have the potential for long-distance dissemination of ARB and ARGs to remote environments. Such transmission sources may explain why ARB and ARGs can be found in environments having little anthropogenic activity, such as the remote arctic [66].

In many studies it has been assumed that ARG flowed from humans and animals to the environment. But in other cases, the use of antibiotics for food production has created antibiotic-resistant bacteria in animals and farm environment that has spread to man. One classic example of animal-to-human spread is the use of avoparcin in farm animals in the EU [83]. Vancomycin-resistant enterococci [VRE] develop on these farms, contaminating the farm ecosystem, including animal, environmental, and human microbiomes. The VRE strains were passed to farm workers and families living on the farm. In other cases, the plasmids carrying the *vanA/vanB* genes were transmitted from animal to human enterococci [40]. In contrast, VRE development in hospital settings in North America has occurred because vancomycin was commonly used in hospitalized individuals but not in the general community population. More recently, VRE strains have spread to the environment in the USA where they are now isolated in a variety of settings, from recreational beaches to birds to farms [39, 40, 84].

12.7 Aquaculture

As the taste for seafood and shellfish increases, the use of aquaculture around the world, especially in Asia, has increased. Integrated aquaculture is a traditional practice used by small-scale farmers in Asia. The fish are raised in ponds along

with livestock. The livestock manure is used to feed the fish. This system allows for mixing of ARGs and ARB, as well as for creating recombinant influenza viruses [85]. Other parts of the world are less likely to practice integrated aquaculture. Varying sizes of fish farms, both of the fresh water and marine type, grow many types of fish for global export. Tilapia (*Oreochromis niloticus*) is among the most cultured and internationally traded food fish, with an estimated 1.45 million tons produced in China in 2013 [85].

ARGs are enriched in sediments below fish farms in Finland, even though selective pressure from antibiotics was low. A new study, which looked at 364 PCR primer sets for detecting ARGs, mobile genetic elements, and 16S rRNA genes, detected 28 genes in fish feces and fish farm sediments. The ARGs included aminoglycoside (*aadA1*, *aadA2*), chloramphenicol (*catA1*), macrolide (*mef*(A), *msr*(A)), sulfonamide (*sul1*), trimethoprim (*dfrA1)*, and tetracycline ribosomal protection genes [*tet*(32), *tet*(M), *tet*(O), *tet*(W)]. The same ARGs were found in fish feces, suggesting that fish contribute to the ARG enrichment of the farm sediments even though no antibiotic treatment of the fish in the farms was performed. Individual farms had their own unique resistome compositions [86]. The Baltic Sea has no tide, and water circulation is slow; thus, ARGs in the sediment underneath the fish pens and up to 200 m from the fish farms were expected to reflect activity in the farm. Muziasari et al. [86] concluded that their findings provide indirect evidence for the hypothesis that selected ARGs are introduced into the sediment underneath fish farms in the Northern Baltic Sea by farmed fish. The antibiotic concentrations in the sediments were ~1–100 ng/g of sediment.

Tetracyclines have been used extensively in aquaculture, and Tc^r bacteria have been characterized from numerous sources, including fish pathogens and environmental bacteria associated with finfish aquaculture from around the world [87–91]. Tc^r bacteria can be found in fish feed, in the sediment under the fish pens, as well as in the water entering and leaving fresh water ponds [92]. Some of the greatest diversity in Tc^r genes has been identified in the aquaculture environment. In one of our studies, ~40% of the Tc^r bacteria isolated from Chilean salmon fish farms carried previously unidentified Tc^r genes, suggesting that the diversity in the types of *tet* resistance genes is higher than routinely found in collections from either man or other food animals [57]. Some of these bacteria were later found to carry *tet*(39), while other genes are still unknown [93]. It is common to find previously characterized *tet* genes in new bacterial genera. Many of these *tet* genes were not readily transferred under laboratory conditions, thereby raising the question of how some of the genes are transferred to bacteria across the world and from very different environments [57]. The diversity of type and number of Tc^r bacteria found in the aquaculture setting suggests that this may be one environment where there is rapid evolution of Tc^r bacteria and a hotspot for ARG transmission.

12.8 Wastewater Treatment Plants (WWTP)

Municipal wastewater is a mixture of everything that is flushed down a toilet or washed down a drain. This can include commercial, industrial, hospital, and residential waste, in addition to stormwater. The latter is especially important when excessive rain leads to floods. Flooding is expected to become more common, as the climate continues to change. Contamination of the sewer system by stormwater may also occur when storm and sanitary sewers are combined. Previously, municipal wastewater and biosolids were considered waste products that required disposal. However, as drought conditions continue, there has been a paradigm shift. Municipalities are increasingly considering the final wastewater and biosludge as resources to be utilized, rather than as waste products to be disposed of [94, 95]. This change is occurring throughout the world, although it is not a new idea ([96]; https://woods.stanford.edu/news-events/event/wastewater-resource-focus-bay). WWTP do not specifically have a goal of reducing the level of ARGs and ARBs in their final waste products.

Relatively little is known about the risk to farmers, exposed community members, and WWTP workers to the pathogens, ARGs, and ARB present in WWTP products. In most cases, a link between the presence of WWTP products and human health has not been established. However, one study looking at the reuse of wastewater found higher levels of intestinal parasitic infections among Uganda farmers than in other persons [97]. Fenollare et al. [98] found that sewage workers were more likely to be colonized with *Tropheryma whipplei*, the causal agent of Whipple's disease, than nonexposed people. Few other studies have looked at occupational risk of WWTP products.

Human pathogens, including shiga toxin-producing *E. coli* and enteric viruses, typically die off within a 3-month period in WWTP products, while *Clostridium* spp. can persist for years as dormant endospores [99]. Spores include those from *C. perfringens* and *C. difficile*, with the majority of work focused on *C. perfringens* [100]. Several examples of the human opportunistic/pathogens associated with WWTP effluents and biosolids are discussed below.

Wastewater treatment plants and their by-products [biosludge and effluents] have been considered potential reservoirs, amplifiers, and transmitters of ARGs and ARB in a variety of settings [95, 101, 102]. This is of concern because biosludge is an important by-product of the WWTP process and is now considered an economically important resource. Biosludge has been used for a variety of agricultural purposes, including growing food for public consumption; effluent has been used to recharge aquifers, for water landscaping and agriculture, and as a contributor to drinking water [94]. These uses suggest that ARGs/ARB found in biosludge and effluent may be transferred to food products, including shellfish. They can also contaminate waterways, lakes, rivers, recreational waters, and oceans worldwide. Some studies have speculated that the wastewater treatment process may increase the proportion

of ARB in outlets [102]. Hotspots of ARGs and ARB may be at WWTP outflows where wastewater effluents are discharged into bodies of water. Thus, WWTP effluent may contribute to the dissemination of specific ARGs in the natural environment [102, 103]. Similarly, other studies have shown that use of reclaimed water is a reservoir for ARGs which increase in the soils after repeated irrigation with reclaimed water. This has potential implications for human health [104].

Residual ARB/ARGs in the final effluent are normally deposited into bodies of water where they can then be taken up by fresh water and marine wildlife and ultimately cycle back to humans, land animals, and/or marine life [105]. Preliminary data supports this hypothesis. High levels of ARGs were detected where WWTP and CSO outflows discharged into Puget Sound WA USA (Dr. L. Rhodes personnel communications). This release may be one reason why the southern resident killer whales carry Gram-negative and Gram-positive resistant and multiresistant bacteria in their respiratory tracts, as determined by cultures from exhaled breath samples [106]. Similarly, antibiotic-resistant enterococci have been isolated from feces of sea turtles, seabirds, and marine mammals from the southern coast of Brazil [105]. We conclude that the major waterways are sources and reservoirs of ARGs and ARB worldwide.

Conventional wastewater treatment does reduce the total number of fecal bacteria, but it does not necessarily reduce the fraction of ARGs/ARB present. Over 30 years ago, Walter and Vennes [107] showed that between 0.35% and 5% of the coliforms from a domestic sewage system were resistant to ≥1 antibiotics, with ~75% of the multiple resistant strains capable of resistance gene transfer. Other studies have isolated and characterized multidrug-resistant fecal coliforms and/or enterococci from municipal water from multiple geographical areas [108, 109]. To complicate the issue, wastewater effluent is now being used for urban landscaping and to replenish urban aquifers. Thus what is in the effluent can make its way into the drinking water ([110]; http://www.ocwd.com/what-we-do/water-reuse/).

The wastewater treatment process, besides increasing the abundance of ARGs and the diversity of ARBs, may also provide selective pressure to increase the diversity of antibiotic-resistant phenotypes and transmission of ARGs to new bacterial species. These final WWTP products can ultimately contaminate a variety of ecosystems, with particular impact on health through aquaculture, agriculture, the human workers in these industries, and persons who consume these products [104]. Occupational exposure risk to human and animal health is just now being recognized [110]. ARGs and ARB have been found throughout the wastewater treatment process, from raw influent, primary and secondary effluent, aeration tanks, activated sludge, and residual biosolids [111, 112]. The biosolids represent the majority of the biomass and thus the highest concentration of the ARGs and ARB from the treatment process. This material is now widely used to enrich both urban and agricultural environments. This can lead to environmental contamination of soil and water and, most importantly, the potential to contaminate food consumed by the general public [101]. This potential contamination needs to be considered when

trying to determine where the bacteria causing an outbreak were introduced into the food product of interest. Moreover, knowing which specific ARG(s) are found in which bacterial species and/or genera in WWTP products is critical when selecting specific ARGs for regional, national, and international surveillance studies. It is likely that there are common microbes in most WWTP systems (*E. coli* and enterococci), but they may differ in the carriage of ARGs. Thus, unlike isolating bacteria, which can also lead to biases, determining which ARGs are carried by specific bacteria is key to the success of future surveillance efforts using molecular methods. The use of whole genome sequencing of WWTP products with emphasis on a large number of different ARGs would be extremely useful in determining which suite of ARGs should be examined when screening various components of the WWTP. This needs to be done in different types of WWTP systems in both rural and urban setting and both economically advantaged and disadvantaged nations.

Few studies have been conducted concerning metagenome analysis of plasmids [113] or the microbiome of human sewage [114]. More research needs to be done to determine whether there are variations by geographical location, seasons, and other factors. Thus most studies in the literature that screen for specific ARGs and/or resistant plasmids are inherently biased, because of the very large number of different ARGs that are known. This bias should be taken into account when reviewing the literature, including studies cited below.

A variety of studies have looked for specific ARGs in influent wastewater, after primary settling, treated effluent, activated sludge, and treated biosolids. Most of these studies select a small subset of the known antibiotic resistance genes characterized by conferring resistance to a particular antibiotic class. For example, one study looked at 10 different *tet* genes out of 59 that are known ([22]; http://faculty.washington.edu/marilynr/). The genes included Gram-negative-specific efflux genes *tet*(A), *tet*(E), and *tet*(G) and ribosomal protection genes *tet*(M), *tet*(O), *tet*(Q), and *tet*(S) that are found in both Gram-negative and Gram-positive bacteria [115] from the 18 samples over a 12-month period. The Gram-negative efflux *tet*(A) and *tet*(C) genes were identified from all samples (n = 18). The other Gram-negative efflux genes were isolated from 9–16 of the samples. The least common Gram-negative efflux gene, *tet*(D), was identified in 9 of the 18 samples. The results are not surprising, given the distribution of the different *tet* genes (http://faculty.washington.edu/marilynr/). It is interesting that most common efflux gene, *tet*(L), which is isolated in similar numbers of Gram-negative ($n = 19$) and Gram-positive ($n = 22$) bacteria, was not examined ([22]; http://faculty.washington.edu/marilynr/). This is a common issue with many of the environmental sample studies published. The authors selected tetracycline resistance genes to survey based on what previous studies have used rather than base the work on abundance or on those most widely distributed ARGs among different genera in the system they are studying. This approach provides a significant bias to many of the environmental studies, including those on WWTP products [101, 116].

12.9 Selective Examples of ARGs Found in Environmental Bacteria

Bacteria carrying Tc^r are widely distributed throughout the world. They have been isolated from deep, subsurface trenches; in wastewater, surface water, and groundwater, sediments, and soils; and in pristine environments untouched by human civilization, such as penguins in Antarctica and seals in the Arctic [42, 56, 65, 79]. Seventeen (39%) of the 43 known *tet* genes including 12 (44%) of the efflux, 3 (25%) of the ribosomal protection, and 2 (66%) of the enzymatic *tet* genes are uniquely ascribed to environmental bacteria. Whether this is an accurate representation, with some *tet* genes being truly "unique" to environmental bacteria, or whether these genes have not been used in surveillance studies of either animal or human bacteria is unclear. As of 2017, there are 59 *tet* genes with many of the new genes not having been identified in specific bacteria (http://faculty.washington.edu/marilynr/).

Five different resistance genes from *Streptomyces*, designated *otr*(A), *otr*(B), *otr*(C), *tcr3*, and *tet*, have been identified in the chromosome of antibiotic-producing strains. Today the *otr*(A) and *otr*(B) are now found in classical *Bacillus* and *Mycobacterium* species that were primarily environmental bacteria but recently have caused animal and human disease. It is possible that over time other environmental "*tet* genes" will move into bacteria of clinical importance and become associated with animals and man. For example, *Clostridium* spp. are found in the environment, but they are also associated with the intestinal tract of humans and animals. The *tetA*(P) and *tetB*(P) genes appear to be unique to *Clostridium* spp. Other environmental genes included are the *tet*(V) gene that has been found in *Mycobacterium smegmatis*, which is thought to be an environmental bacterium; the *tet*(30) gene in *Agrobacterium*; the *tet*(33) that has been found in environmental *Arthrobacter* and *Corynebacterium* spp.; the *tet*(35) gene in *Vibrio* and *Stenotrophomonas* spp., which can cause human disease; and the *tet*(41) gene in *Serratia* spp. which rarely causes human disease. The *tet*(42) gene found in *Bacillus*, *Microbacterium*, *Micrococcus*, *Paenibacillus*, and *Pseudomonas* spp. was isolated from a deep-sea trench. The *tet*(34) gene was first described in *Vibrio* spp. and more recently identified in *Pseudomonas* spp. and *Serratia* spp.(http://faculty.washington.edu/marilynr/). To determine if these genes are truly environmental will require new surveillance studies in human and animal bacteria to determine if some of genes currently assigned as "uniquely environmental" are really only associated with bacteria isolated in the environment.

Among the 97 genes that confer resistance to one or more macrolide, lincosamide, and streptogramin (MLS) antibiotics, there are a number of resistance genes that are exclusively identified in the *Streptomyces* spp. including rRNA methylase genes [*erm*(H), *erm*(I), *erm*(N), *erm*(O), *erm*(S), *erm*(U), *erm*(Z), *erm*(30), *erm*(31), and *erm*(32)], ATP-binding transporters [*car*(A), *ole*(C), *srm*(B), *tlr*(C)], and a major facilitator [*lmr*(A)] gene. Other rRNA methylases are found innately in vari-

ous environmental *Mycobacterium* spp., [*erm*(37) to *erm*(41)], while environmental bacteria carry a variety of the known MLS resistance genes (http://faculty.washington.edu/marilynr/). Other than genes associated with *Streptomyces* spp. and *Mycobacterium* spp., there are relatively few genes exclusively associated with environmental bacteria. Why a difference occurs in the distribution between *tet* and MLS genes in environmental bacteria is not clear.

β-Lactamases are enzymes that provide resistance to β-lactam antibiotics such as penicillins, cephamycins, and carbapenems (ertapenem). These β-lactamase enzymes have random mutations that modify the spectrum of resistance to varying classes of this antibiotic group. There are hundreds of these modified β-lactamase genes. β-Lactamase genes are ancient and have been identified in remote and isolated environments, suggesting that β-lactamases occur in nature [66]. Another class of β-lactamases, the CTX-M genes, which hydrolyze expanded-spectrum cephalosporins, originated in environmental *Kluyvera* spp. Bacteria with CTX-M genes were first identified in 1989. Today these genes can be found across the world [3]. The *qnr* genes originated in waterborne *Aeromonas*, *Shewanella*, and *Vibrio* spp. [52]. Data from a 30,000-year-old permafrost sample showed that the sample carried genes conferring resistance to a variety of different classes of antibiotics [β-lactams, tetracycline, and glycopeptides]; thus, resistance existed in the environment before antibiotics were used by man.

12.10 Conclusions

The environmental microbiome, which is difficult to define, remains largely unexplored. However, a few studies suggest the wide distribution of ARB and ARGs in the environment. For example, antibiotic-resistant marine bacteria have been isolated 522 km offshore and at depths of 8200 m [117]. The degree of pollution in the environment correlates with the prevalence of resistance, suggesting that over time even the more "pristine" environments will become increasingly contaminated with ARGs and ARB. This phenomenon will ultimately increase resistance among opportunistic and pathogenic bacterial species having human and animal importance. Increased selection pressure for antibiotic resistance in environmental microorganisms is likely to continue, since human activities will likely continue to pollute the environment. Natural forces, such as wind and movement of water, will continue to contaminate areas of relatively uninhabited environments.

The One Health concept is a worldwide strategy for expanding interdisciplinary collaborations and communications in all aspects of health care for humans, animals, and the environment. The aim is for inclusive collaborations dedicated to improving the lives of all species through the integration of human medicine, veterinary medicine, and environmental science. This concept recognizes that using compartmentalized (silo) mentality to approach the three disciplines is not adequate, since the distinction of environment from non-environment, especially at the bacterial level, has become increasingly difficult. It is clear that the introduction of

a new ARG into a human, animal, agricultural, or environmental microbial ecosystem often leads to cross-transmission and dissemination of ARGs and ARB within and between the ecosystems [3].

The data summarized in this chapter indicate that the environment is an important reservoir for ARGs and ARB; it needs to be considered in future studies. There is a large diversity of resistance genes in the environment, and many of these genes have yet to be identified or characterized. Horizontal gene transfer within the microbial world knows few boundaries, and our ability to experimentally mimic what occurs in nature has significant limitations. Indeed, the role that the natural environment plays in the evolution, maintenance, and transmission of ARB and ARGs is just now being examined. However, it is generally agreed that human anthropogenic changes are impacting natural ecosystems that will ultimately impact human and animal health.

It is clear that ARB and ARGs are spread among animals, the environment, and humans and from one geographic location to another throughout the world. The environment is an important reservoir for these resistance genes, with WWTP products being an important component as reservoirs, potential amplifiers, and/or transmitters of ARB and ARGs in the environment. These contaminants not only degrade the local environment but ultimately influence the health of humans and animals associated with that environmental landscape. The environment has provided an increasing number of novel ARGs that have not been found in bacteria traditionally associated with animals or humans (http://faculty.washington.edu/marilynr/). It is unclear whether these "new genes" will impact the treatment of animal and human infections in the future, but NDM-1 and CTX-M genes have been associated with bacterial pathogens. Evidence also exists that WWTP plays a role in the evolution of multidrug-resistant opportunistic and pathogenic bacteria. WWTP is thought to be a hotspot for the contamination of environments including receiving waters of effluent and of soil and agricultural lands where biosolids are utilized. This is very important, as WWTP biosolids and final effluents are considered to be resources that should be used for agricultural purposes and, in some communities, as water resources. Thus it is plausible that there is a human health risk associated with WWTP products; however, data backing this hypothesis is currently very limited. Reducing the levels of ARGs/ARB in WWTP by-products before they are recycled is an important component in the multipronged approach to reduce the global spread and distribution of ARGs. Advanced wastewater treatments using ozone, UV, ultrafiltration, chlorination, dry-air beds, and membrane bioreactor processes are effective in reducing the number of bacteria. These processes may be useful in reducing the level of ARB/ARGs in effluents and biosolids before they are utilized by communities, thereby reducing the risk to humans (113). Unfortunately, recent studies report that UV/H_2O_2 disinfection processes do not eliminate the possible spread of antimicrobial resistance in the receiving environment [118]. Moreover, cost-effectiveness is an important consideration with advanced wastewater treatment options. To comprehensively assess AMR-related impacts on risks to human health, we need to gain a better understanding of the role that biosolids and effluents play as amplifiers, reservoirs, and transmitters of these bacteria and genes.

It is important that members of human communities understand that they contribute to the contamination of their environment – practices such as discarding food and food waste products inappropriately may have downstream consequences. Thus education of the general community, from young children through adults, is an important mission that many scientist in the field neglect – it is potentially the most cost-effective use of resources.

Major Points

Limited work on various environmental ecosystems limits our understanding the relationships between environmental bacteria and the stressors that lead to selections and retention of ARGS/ARB in only one system. Preliminary data indicates that certain places such as WWTP and the receiving waters of this material along with the biosolids produced in the WWTP are hotspots for the exchange of ARGs among the bacterial microbiome. How to deal with these products to reduce the number and diversity of ARGs and ARB is not clear. Using a One Health approach, it is clear that ARGs and ARB can flow from humans and/or animals into the environment and environmental bacteria, and genes can flow back into human and/or animal bacteria. Looking at the complete picture will provide better information for specific ARGs and ARB and with this knowledge perhaps ways of reducing overall transmission from one sector to the other. This requires resources and science at all levels to stabilize and hopefully reduce the human-generated impact on the environment including contamination as well as changes in climates which can disturb the natural web of life as well as increase food insecurity to millions.

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Chapter 13
Phenotypic Tolerance and Bacterial Persistence

Carl Nathan

13.1 Introduction

Antibiotics are among the most important achievements of biomedical science. However, they are also among the most endangered. Not only are antibiotics susceptible to rapid emergence of heritable resistance, but their action is resisted by a much less well understood set of processes collectively termed "phenotypic tolerance" that gives rise to "persisters." Persisters are the members of a population of an antibiotic-susceptible strain of bacteria that survive exposure to the antibiotic at concentrations that kill the vast majority of the population when tested under the conditions used to define the antibiotic's minimum inhibitory concentration (MIC) and that when expanded in number and retested give rise to a population whose MIC is unchanged. The major theme of this chapter is that mechanistically distinct forms of phenotypic tolerance present different challenges for the development of effective therapeutic approaches.

The chapter begins by describing what is at stake with the rise of antimicrobial resistance (AMR) and then contrasts heritable AMR with its nonheritable form, phenotypic tolerance. With tuberculosis (TB) as a case in point, I review the contribution of host immunity to phenotypic tolerance. This sets the stage for contrasting two major classes of phenotypic tolerance. Turning to the history of how phenotypic tolerance was recognized, we will see that evidence for two major classes was evident from the outset, although the distinction was not perceived at the time. Finally,

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This chapter reproduces, adapts, and updates portions of a wider-ranging essay, "Fundamental Immunodeficiency and Its Correction" by C. Nathan, published in the *Journal of Experimental Medicine* 214: 2175-2191, 2017. Passages from that publication are reproduced here with permission of The Rockefeller University Press.

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I. W. Fong et al. (eds.), *Antimicrobial Resistance in the 21st Century*, Emerging Infectious Diseases of the 21st Century, https://doi.org/10.1007/978-3-319-78538-7_13

I discuss what is known about the mechanisms for each class and approaches to overcome them.

13.2 Unique Features of Antimicrobial Agents among Medicines

Over the past six generations, humans have found or invented several thousand medicines. Among them, the antimicrobial agents, discovered over the past four generations, are unique in two aspects. First, until recently, antimicrobial agents were the only medicines that cured large numbers of the sick, and they remain the only medicines that do so routinely. Within the last two generations, some antineoplastic regimens have been curative, including some that are immunity-based, and corticosteroids sometimes cure temporal arteritis. Second, antimicrobial agents are the only medicines whose use hastens their loss of usefulness for people who have not yet taken them.

The first claim hinges on using "cure" in the true sense. Administration of an appropriately chosen antimicrobial agent has the routine capacity to restore an individual to the state of wellness that prevailed before the onset of an illness that would not otherwise have resolved, that would not otherwise have resolved as quickly, or whose unaided resolution would not restore the individual to their prior state of wellness. In contrast, when the administration of most other medicines stops, the individual returns to the state of illness that invited intervention, unless the illness had resolved spontaneously or from a change in contributory factors, such as diet. Some other medicines help prevent the onset of illness rather than treating it.

The definition of "cure" given above is admittedly idealized. Clinical cure can be ambiguous. "Cure" does not return the patient to the previous state of health if tissue damage already caused by the pathogen or the host's reaction to it is irreparable, as is often the case in successfully treated TB. Finally, cure achieved with broad spectrum antimicrobial agents often comes at the cost of a long-lasting perturbation of the microbiota, and in that sense an important component of the host's overall makeup has not returned to its preexistent state. Nonetheless, within the bounds of these ambiguities and qualifications, antimicrobial agents stand out among medicines for their ability to cure large numbers of people routinely.

However, the ability of antimicrobial agents to cure the majority of patients for whom such drugs are appropriately prescribed is handicapped by the second unique feature of this class of medicines: their use eventually selects for resistance. The resistant pathogens are eventually shared among hosts, or the determinants of resistance are eventually shared among pathogens. Thus we are all likely to need antimicrobial agents, yet the more a given agent is used, the nearer it comes to being useless.

In sum, antimicrobial agents are at once among the most important and least permanent achievements of medicine.

13.3 Rising Stakes: The Growing Reach and Recognition of Antimicrobial Resistance (AMR)

Beginning with the use of penicillin in civilian populations in the mid-1940s, physicians, scientists, and much of the public quickly came to regard antimicrobial agents as both indispensable and invincible [1]. Beginning just 20 years later, taking antimicrobial agents for granted put us on a path to losing them.

Over the past few decades, a declining rate of success in discovering new antimicrobial agents discouraged much of the pharmaceutical industry from continuing the search [2]. Meanwhile, levels of AMR continue to rise. These respectively falling and rising curves have crossed in recent years for one pathogen after another; antimicrobial agents are now lacking to treat a significant proportion of formerly curable infections caused by nearly a dozen different bacterial species. As the remaining agents become less often useful, elective surgery and cancer chemotherapy may become prohibitively risky, trauma care ineffective, premature babies nonviable, and incidental wounds potentially lethal.

To imagine what it might be like to return to a pre-antibiotic era, consider the reaction to the introduction of penicillin to public use after World War II. Alexander Fleming "was showered with gifts of carnations… people whose lives had been saved by penicillin … now knelt before him to kiss his hands" [3]. In 1964, the city of Madrid installed statues of Fleming and of a bullfighter saluting him outside the municipal bullring, because antibiotics had so greatly reduced the lethality of matadors' wounds.

One of the first postwar impacts of penicillin was the cure of gonorrhea with a single injection. Yet *Neisseria gonorrhoeae* is one of the bacterial pathogens some of whose clinical isolates are now resistant to most antibiotics. Others include *Enterococcus faecium*; *Staphylococcus aureus*; *Klebsiella pneumonia*; *Acinetobacter baumannii*; *Pseudomonas aeruginosa*; *Enterobacter* species; some *Salmonella*, including invasive, non-typhoidal strains; some *Shigella*; and *Mycobacterium tuberculosis*. Leaving out the single most prevalent instance of AMR—drug-resistant tuberculosis— it is estimated that drug-resistant bacterial pathogens now kill some 700,000 people a year, and if present trends continue, the toll will rise to 10 million deaths per year by 2050 [4]. Authorities seem reluctant to factor drug-resistant tuberculosis into this tally, perhaps fearing that its unfamiliarity to the citizenry of economically advanced countries might blunt their concern. Nearly 500,000 people a year develop drug-resistant tuberculosis; as matters now stand, over 50% of them will die from it.

After decades of advocacy by scientists and physicians, beginning with Fleming himself in his Nobel Prize acceptance speech in 1945, acknowledgment of the gravity of AMR has finally come from leaders in business and government, as voiced by the World Health Organization, the World Economic Forum, the G20, and the G7. In 2015, President Obama issued a National Action Plan for Combating Antibiotic-Resistant Bacteria [5]. In May 2016 a panel commissioned by the British government issued cogent recommendations for coordinated global action [6]. In July

2016, NIH, the Department of Defense's Biomedical Advanced Research and Development Agency, the Wellcome Trust, the California Life Sciences Institute, the Massachusetts Biotechnology Council, and the AMR Centre in the United Kingdom announced that Kevin Outterson, a Boston University law professor interested in incentives to overcome AMR, will oversee the award of $350 million in grants via a consortium called the Combating Antibiotic-Resistant Bacteria Biopharmaceutical Accelerator (CARB-X) [7, 8]. In September 2016, NIH announced a $20 million Antimicrobial Resistance Diagnostic Challenge, and the government of China announced a national initiative to counter antimicrobial misuse and to find new antimicrobials (http://scim.ag/Chinaresistance). Also in September 2016, the United Nations General Assembly declared AMR to be a risk to global health security, placing it alongside HIV/AIDS, noncommunicable diseases, and Ebola virus as only the fourth global health issue prioritized for discussion and action in the history of the General Assembly. The UN's 193 member nations agreed to develop an action plan [9].

13.4 AMR as a Scientific Challenge; Tuberculosis as a Case in Point

There is now a cross-sector consensus that preserving antibiotics as a mainstay of human medicine will require overcoming obstacles of four kinds—scientific, regulatory, economic, and political [1, 10–12]. Among the several scientific challenges confronting the development of new antimicrobial agents [13], one stands out as most needful of fresh thinking: the nature of AMR itself.

The discussion that follows deals only with bacterial infections and antibacterial agents, now generally called "antibiotics" without regard to whether they are of microbial origin, as the term was originally used. This focus is for purposes of illustration; it is not meant to discount the urgency of developing antimicrobial agents for viral, fungal, protist, and helminthic infections.

M. tuberculosis (Mtb) serves, for further focus, for the following reasons [14]. That these four points are all true reveals serious shortcomings in existing approaches to antibiotic development and use: (i) Mtb is now the single leading cause of death from infectious disease, (ii) despite causing a curable infection, (iii) one that is now becoming progressively incurable because of AMR. (Among potentially lethal bacterial pathogens displaying AMR, Mtb is estimated to account for the highest number of cases, even though the vast majority of cases of drug-resistant tuberculosis go undiagnosed, given that drug sensitivity testing is lacking in many endemic areas. The fate of people whose tuberculosis displayed extensive AMR was recently monitored: 5% were cured, 73% died, and 10% failed all efforts to treat them and were discharged into the community in a contagious state [15, 16].) (iv) Even in its drug-sensitive form, tuberculosis takes longer to cure than almost any other bacterial infection.

That an immunologic perspective might help derives from four additional points: (i) Mtb has no known naturally transmitting host but humans. (ii) As noted earlier, for its transmission, Mtb needs a live human whose immune response is vigorous enough to liquefy infected lung and erode into an airway. (This dependency probably accounts for the striking finding that the nucleotide sequences most highly conserved among 1226 clinical isolates of Mtb were those encoding human T cell epitopes, that is, the specific oligopeptides within a given protein that bind to antigen receptors on T lymphocytes [16].) (iii) Untreated, the active disease has a fatality rate of 50% or more. (iv) Nonetheless, after an estimated 70,000 years of parasitism, neither species—Mtb nor humans—has eliminated the other.

From these considerations we can reach four conclusions: Mtb has evolved the ability to incite, titrate [17], survive, and exploit the human immune response.

To the degree that we understand the host-pathogen relationship in tuberculosis, we should be able to apply strategies for drug development that accommodate or even capitalize on those relationships rather than ignoring them and paying the price for unappreciated antagonism.

13.5 Heritable AMR

The best understood form of AMR is heritable. There are bacterial genes that encode resistance to antibiotics that were not invented or deployed at the time that the bacteria acquired the genes [18], and it is usually possible to isolate bacteria that have become heritably resistant to any new antibiotic as soon as there is enough of the antibiotic on hand to conduct a selection [19]. Apparent exceptions [20–22] are likely to involve compounds with multiple targets or no specific target. Only a few such agents are sufficiently selective to be clinically useful. In general, the issue with heritable AMR is not whether but when the deployment of a given antibiotic will select for the emergence of heritable resistance in clinical settings.

While correct use of antibiotics will usually lead in time to heritable AMR, other forms of use hasten its emergence: misuse, overuse, and underuse.

Misuse is exemplified by feeding over half of the United States' antibiotic tonnage to healthy food animals and plants to accelerate their growth; the proportion is thought to be higher in China [23]. Another form of misuse is the routine failure to account for individual variation in drug levels attained with standard dosing, although it is possible to conduct therapeutic drug monitoring on finger-prick blood spots [24]. Without dose adjustment, peak rifampin levels in the blood vary by nearly two orders of magnitude in people treated for tuberculosis [25], with some 40–70% being undertreated [26]. Undertreatment fosters the emergence of resistance.

Overuse results from lack of rapid, point-of-care diagnostics. An estimated 30% of antibiotic prescriptions in the United States are written for the wrong indication, typically a viral infection [27]. Overuse is also fostered in settings where the

prescribers are the purveyors or the consumers, that is, where doctors sell the drugs or patients purchase them without recourse to doctors.

Underuse is a problem when the drugs are diluted by inexpert manufacture or fraudulent intent or when patients discontinue them prematurely because they feel better, feel worse, or cannot afford to buy more of them.

Mechanisms of heritable AMR are still being discovered. They include mutation or posttranslational modification of the target so that it continues to support the viability of the organism but no longer binds the antibiotic, increased expression of the target so that it titrates the antibiotic, expression of a pathway that compensates for the impairment caused by the antibiotic, inactivation of the antibiotic inside the bacterium [28] or by a secreted bacterial product [29], decreased activation of a prodrug form of the antibiotic, and decreased uptake or increased export of the antibiotic.

Discovery of mechanisms of AMR has profoundly impacted both basic science and clinical care. In basic science, studies of heritable AMR played a prominent role in introducing the concept that small chemical compounds can have specific macromolecular targets in biological systems and can serve as tools to identify the targets' functions [30]. Clinically, mechanistic understanding of heritable AMR allowed the design of combination chemotherapy with agents that thwart resistance. For example, the World Health Organization's list of essential medicines includes the combination of amoxicillin, which is a β-lactam, with clavulanate, an inhibitor of some bacterial β-lactamases. Moreover, mechanistic understanding of heritable AMR allows combination chemotherapy with agents to which bacteria manifest resistance by different mechanisms. Combination chemotherapy was introduced to the practice of medicine in the 1950s with the discovery that there was no other way to avoid routine emergence of resistance in the treatment of TB [31]. The practice was later adopted for the treatment of cancer and HIV/AIDS.

13.6 Antagonism Between Immunity and Antimicrobial Agents

To set the stage for a discussion of phenotypic tolerance as a major form of AMR, it helps to acknowledge the seemingly paradoxical negative impact of host immunity on the action of anti-infectives that were developed without taking immunity into account.

Because a primary function of the immune system is to protect the host from infection and the purpose of administering antimicrobial agents is the same, then immunity and antimicrobial chemotherapy can be expected to exert additive or synergistic effects, and no special effort should be necessary to take advantage of their common actions.

Indeed, it is sometimes difficult to cure an infection with antibiotics in someone whose encoded immune system is dysfunctional. For example, most patients with

nontuberculous mycobacterial infections who are discovered to have autoantibodies that neutralize interferon gamma (IFNγ) fail to clear the pathogen in response to treatment with antimicrobial agents [32]. One of the genes induced by IFNγ is inducible nitric oxide synthase (iNOS) [33]. Tuberculosis can be cured in most mice with isoniazid and pyrazinamide [34], but apparent cure is quickly followed by relapse if the mice are deficient in iNOS [14]. Such observations indicate that antimicrobial agents not only synergize with host immunity but can depend on host immunity to effect clinical cure.

At the same time, immune mechanisms often act at cross-purposes with antimicrobial agents. When antibiotics are selected for their activity against replicating bacteria, as is almost always the case, they usually work best, or only, against replicating bacteria. When immunity serves to halt the replication of some infecting bacteria but fails to kill all of them, as is often the case at the time that an infection manifests as clinically apparent disease, then immunity can antagonize antibiotic action. Such antagonism has been demonstrated in axenic culture [35], in cultured macrophages [36], in rabbits [37], and in mice [38].

In fact, some of the foregoing examples underscore that the same antibiotic and the same element of host immunity can work both for and against each other in the same disease. As noted, apparent clinical cure of tuberculosis in mice with isoniazid and pyrazinamide was sustained in the majority of wild type mice [34] but was rapidly followed by relapse in all mice that lacked iNOS [14]. Yet the action of isoniazid in Mtb-infected mice was partially impaired by iNOS [38] because products of iNOS block replication of Mtb and, in vitro at least, isoniazid only kills Mtb when the bacteria are replicating. There may be diverse mechanisms for such antagonisms. For example, reactive nitrogen species (RNS) target cytochromes involved in electron transport; the reduction in energy generation can block uptake of aminoglycoside antibiotics [39]. Bacteria themselves can generate RNS that induce their own antioxidant defenses, covalently modify antibiotics, and confer resistance [40]. Host-derived RNS may do the same.

Like generation of RNS, generation of reactive oxygen species (ROS) is a major element of host immunity against infection. Genetic deficiency in the primary ROS-generating enzyme of phagocytes, NADPH oxidase 2 (NOX2), predisposes to life-threatening bacterial and fungal infections [41], including by *Staphylococcus aureus*. Yet the autotoxicity of NOX2-derived ROS for host myeloid cells can impair the ability of antibiotics to cure *S. aureus* pneumonia [42].

When immunity adversely impacts the action of antimicrobial agents, it creates a form of AMR. The more we understand about the mutual antagonism between antimicrobial chemotherapy and partially effective host immunity, the more opportunity we have to identify drug targets in the bacterial pathogen whose inhibition may convert a non-curative response to chemotherapy into a cure [11, 43].

13.7 Nonheritable AMR: Phenotypic Tolerance and Its Subtypes

In contrast to the situation with heritable AMR, we have very limited understanding of nonheritable AMR, also called "phenotypic tolerance," a term introduced by Tuomanen [37]. Phenotypic tolerance can be defined as conditional drug resistance that is not attributable to changes in the nucleic acid sequence of the pathogen's genome. Phenotypic tolerance gives rise to bacterial persistence: survival of bacteria during treatment of a host with a drug to which the same strain of pathogen is susceptible under standard laboratory conditions at concentrations achieved in the host. Phenotypic tolerance predisposes to emergence of mutants with heritable resistance [44].

The first two studies of phenotypic tolerance hold such important lessons for today that they deserve detailed discussion. The purification of penicillin was reported in 1942 [45]. That same year, Gladys Hobby and her colleagues reported that at 37 °C, about 1 streptococcus remained viable after 48 hours of exposure to penicillin for every 10^6 present in the control culture at the end of that period. The authors did not comment on that but drew attention to the survival of nearly all the penicillin-treated streptococci if the exposure took place at 4 °C, conditions in which there was no increase in bacterial number in the untreated control culture. The authors concluded, "It is apparent that penicillin is capable of destroying bacteria only if multiplication takes place" [46].

In 1944, Joseph Bigger repeated and extended the experiments using staphylococci [47]. He introduced the term "persisters" to stress the observation that about 1 in 10^6 staphylococci survived the treatment of logarithmically replicating cultures at body temperature. He inferred that persisters to penicillin must be "cocci … which happen to be, when exposed to it, in a phase in which they are insusceptible to its action," because "If persisters had an abnormally high resistance, either natural [that is, heritable and existing prior to the experiment] or acquired [that is, heritable but acquired during the experiment], it is probable that their descendants would also possess abnormally high resistance. The descendants of a number of persisters which had survived contact with 1 unit per c.cm. penicillin for 3–5 days were found to be killed by 1/8 unit per c.cm. within 46 hours and to have no greater tendency than normal forms to produce persisters" [47].

Bigger went on to confirm the observation of Hobby et al. [46] that cooling the bacteria elevated the frequency of persisters to nearly 100%, that is, by 6 orders of magnitude. He demonstrated the same effect by acidifying the medium or lowering its tonicity. He concluded that "persisters are cocci which survive contact with penicillin because they are in dormant, non-dividing phase" [47].

In fact, within 2 years of the report of penicillin's publication, the two groups mentioned above, working on two continents with two different pathogens, had each observed two different classes of phenotypic tolerance, but without distinguishing them. It took another 70 years before the distinction was made, driven by the recognition that the two classes have different implications for drug discovery [11].

Class I phenotypic tolerance can be viewed as a form of bacterial bet-hedging manifest by a minority of a population in conditions permissive for growth. The upper limit of the size of the minority population that can display class I phenotypic tolerance is set by the precision of the assay used to determine the minimum inhibitory concentration (MIC) of the antibiotic. If the MIC is defined as the concentration that inhibits growth by 90%, then 10% of the population could be phenotypically tolerant without changing the population's MIC. Typically, in a wild type population, the frequency of class I phenotypic tolerance is about 1 in 10^6. Certain mutations can increase the frequency of class I phenotypic tolerance by orders of magnitude without changing the MIC and without conferring heritable AMR. The phenotypically tolerant minority may be non-replicating at the time, as Hobby et al. [46] and Bigger [47] inferred and others then assumed and asserted, or it may be replicating, as documented in later studies. The key feature is that a population of class I persisters, once expanded in the absence of the antibiotic, succumbs in the same proportion to the same concentration of antibiotic as did the population from which the persisters were recovered.

Heritable AMR can emerge more readily after antibiotics select for a mutation that increases the frequency of class I phenotypically tolerant bacteria in the population. Such mutations can arise in diverse genes, including those encoding antitoxins or enzymes that catalyze metabolic processes [44]. Mutations that augment class I phenotypic tolerance increase the proportion of bacteria that survive one exposure to antibiotic, providing a larger population in which mutants may arise that confer heritable resistance to a subsequent exposure [44].

In contrast, class II phenotypic tolerance is a bacterial response to exogenous stress, including non-sterilizing immunity. It is imposed by conditions that impair growth and pertains to all of the bacteria whose growth is impaired, which may be most or all of the bacterial population in a given site at the time that chemotherapy is administered. Conditions that impair growth can be imposed by the host environment, host immune chemistries, or exposure to sublethal levels of other antibiotics (Table 13.1). The stresses that lead to class II phenotypic tolerance can foster the emergence of heritable AMR by increasing the frequency of mutation [49, 50].

A particularly challenging form of class II phenotypic tolerance is displayed by bacteria whose non-replicative state is not reversed by plating them on a rich medium rendered semisolid with agar. That is, they are not colony-forming units, yet their viability is demonstrable by some other means, such as growth after limiting dilution in liquid culture or injection into an experimental host. Over 80 bacterial species have been shown to have the property of becoming what Rita Colwell and colleagues originally called "viable but non-culturable" [51]. Strikingly, in two studies to date, most of the Mtb in the sputum of most treatment-naïve patients with tuberculosis were unable to replicate as CFU and were detected instead by limiting dilution [52–54]. Similarly, limiting dilution rather than plating on agar was necessary to detect 90–99% of the Mtb remaining in vitro after sequential starvation and exposure to a rifamycin in an in vitro model of "differentially detectable" Mtb [55].

Table 13.1 Classes of phenotypic tolerance and their therapeutic implications

	Class I	Class II
Growth state of bacterial population	Most cells replicating	Most cells not replicating
Persistence phenotype	Small minority; different cells tolerate different antibiotics	Large majority; same cells tolerate many antibiotics
Inducers of persistence	Unknown; stochastic	Acidification, ROS, RNS, hypoxia, deprivation of C, N, P or Fe; sublethal exposure to antibiotics
Speculative mechanisms	Epigenetic, transcriptional, translational, or posttranslational expression or suppression of any process for which genetic change can produce heritable resistance	Decreased uptake, increased export, or increased catabolism of drug; metabolic stress leading to oxidative stress and adaptation; increase in proteostasis pathways; preferential transcription and translation; alternate respiratory pathways and electron acceptors
Therapeutic implications	Combine different drugs that each reach the sites of infection	Include new kinds of drugs active on non-replicating cells that reach the sites of infection

Based on Nathan [11] and modified from Nathan and Barry [48]

Not all the anti-infectives that kill Mtb in some non-replicating states kill Mtb in other non-replicating states. For example, rifampin generated rather than killed the differentially detectable Mtb described above, while thioridazine did not generate such cells but did kill them [55]. These antimicrobial agents serve as chemical probes to teach us that class II phenotypic tolerance encompasses a spectrum of states—at our present state of knowledge, at least two. Class IIa phenotypic tolerance is characteristic of bacteria that stop replicating in response to a given set of stresses but form CFU when those stresses are relieved. Class IIb phenotypic tolerance is a feature of bacteria that stop replicating in response to different stresses and remain viable when those stresses are removed, but do not form CFU [55]. This complicates the task of finding anti-infectives that can kill bacteria displaying phenotypic tolerance.

To the extent that individual bacteria in an otherwise antibiotic-susceptible population manifest class I phenotypic tolerance to two different antibiotics by different mechanisms, then the cells that are phenotypically tolerant to the first antibiotic are likely to be susceptible to the second. In such a case, to kill the whole population, it should suffice to combine antibiotics in such a way that no one bacterium is phenotypically tolerant to all of them, provided that each of the drugs in the combination reaches the bacteria in adequate concentrations at the same time. (In vitro, class I phenotypically tolerant Mtb could be killed by forcing them to produce extra ROS in the presence of rifampin or isoniazid by supplying them with small thiols [56].) In contrast, if all the bacteria in a population are phenotypically tolerant to several different antibiotics, then each individual bacterium must be tolerant to each of

them, and combinations of those antibiotics are unlikely to be effective. Instead, it will be necessary to discover antibiotics that can kill non-replicating bacteria.

The foregoing theses constitute a practical imperative for distinguishing classes of phenotypic tolerance (Table 13.1). Other classifications of nonheritable AMR are also useful, for example, to frame mechanistic questions [57]. A caveat of all classifications based on in vitro observations is that the relationship is complex and variable between the MIC measured in low-protein, host cell-free media over short periods of time and the dosing regimens of antibiotics required for clinical cure [58, 59].

13.8 Mechanisms of Class I Phenotypic Tolerance

Class I phenotypic tolerance can theoretically arise by any mechanism that confers heritable AMR, from epigenetic regulation to posttranslational modification, as long as the mechanism does not depend on a change in the pathogen's coding sequence. As noted earlier, the size of the tolerant subpopulation may be affected by a change in coding sequence, as long as the tolerant subpopulation remains such a minority that the overall population does not manifest an increase in the antibiotic's MIC.

Much of the research in this field has wrestled with a descriptive question, whether class I phenotypic tolerance is as tightly linked with non-replication as Hobby et al. [46] and Bigger [47] inferred. In short, the answer is "no."

The first study to use time-lapse photomicroscopy of bacteria in microfluidic chambers to study phenotypic tolerance at the single cell level [60] revealed that in an otherwise replicating population of *E. coli*, most of the few cells that survived ampicillin were non-replicating at the time of exposure to the drug. However, some of the other surviving *E. coli* had been replicating. This study was rendered feasible by using *E. coli* with compound mutations in *hipA* that raised the frequency of class I phenotypically tolerant *E. coli* by several orders of magnitude without changing the MIC of the overall population.

Nine years later, a study of similar design reached a different conclusion while studying the action of isoniazid on Mtb [61]. Isoniazid is a prodrug whose activation depends on the Mtb catalase-peroxidase KatG. The investigators showed that stochastic extinction of KatG expression conferred resistance to isoniazid. Growth rate had nothing to do with it [61].

The same year, Orman and Brynildsen showed that *E. coli* persisters to ampicillin and fluoroquinolones are enriched among the non-replicating subpopulation, but not confined to it nor highly prevalent in it [62]. Natural clinical and veterinary isolates of *E. coli* each showed the same MICs to a given antibiotic, yet each showed different levels of persistence to different sets of antibiotics [63]. This suggested that different individual cells were phenotypically tolerant to different antibiotics, meaning that non-replication of a given cell could not be a universal explanation for phenotypic tolerance.

The same conclusion was reached in studies of persisters among antibiotic-stressed *E. coli* during diauxic transition. The frequency with which *E. coli* persisted in the face of exposure to ampicillin increased from about 1 in 10^4 to about 1 in 2.5×10^3 during the transition from replication in glucose to utilization of fumarate [64]. Results were similar with ofloxacin. However, co-treatment with ampicillin and ofloxacin reduced the frequency of persisters in diauxie by about tenfold, suggesting that about 90% of them were phenotypically tolerant to one or the other of the antibiotics, but not both [64]. In this instance as well, non-replication could not serve as a universal explanation for phenotypic tolerance to all antibiotics tested.

Working with Mtb, Javid and co-workers discovered a growth-rate independent form of class I phenotypic tolerance to rifampin and defined its molecular mechanism [65]. Individual Mtb cells mistranslate different proportions of individual copies of rifampin's target, RNA polymerase subunit B (RpoB). The basis of mistranslation is the propensity of Mtb's glutaminyl-tRNA synthetase to charge tRNA not only with glutamine but also with glutamate and of Mtb's asparaginyl-tRNA synthetase to charge tRNA not only with asparagine but also with aspartate. The errors are corrected by a glutamine amidotransferase, but not perfectly. If a given cell's collection of RpoB molecules includes enough copies in which Asn170 has been replaced with Asp, the cell can survive a dose of rifampin that kills genetically identical siblings. Heritable mutations in the gene encoding a subunit of the amidotransferase increased the frequency of class I phenotypically tolerant Mtb in a population but, as with *hipA* mutations in *E. coli* discussed above, did not allow the persisters, when grown up without antibiotic, to display a higher MIC than the population from which they were recovered [65].

Some view class I phenotypic tolerance as an outcome of noise: random variation arising from imperfect execution or synchronization of various processes. In contrast, others argue that the high value of class I phenotypic tolerance for survival of a replicating population in the face of emergent stress, together with its susceptibility to genetic regulation, make a case for the existence of specific, evolved mechanisms. Both views are likely to be correct, depending on the setting.

Our understanding of class I phenotypic tolerance in diverse bacterial species would be greatly enriched if we could study the phenomena not only in mono-species planktonic cultures in optimal growth media during exposure to clinically relevant antibiotic concentrations but in natural, multi-species environments with their complex chemical language of cooperation and competition.

13.9 Mechanisms of Class II Phenotypic Tolerance

One of the most important challenges for antibiotic research is to understand mechanisms of class II phenotypic tolerance, a state for which incompletely effective immunity and sublethal antibiotic therapy bear much of the responsibility.

We have a long way to go. We do not know if a given bacterial species that enters a non-replicating state in response to different host conditions manifests class II

phenotypic tolerance to the same antibiotic by different mechanisms nor whether a given bacterial species that enters a non-replicating state in response to the same host condition manifests class II phenotypic tolerance to different antibiotics by different mechanisms.

Following the reasoning that Bigger advanced three quarters of a century ago [47], some scientists today argue that non-replicating bacteria are phenotypically tolerant to inhibitors of biosynthetic processes because they are "dormant," where dormancy is inferred from the cells' survival of exposure to inhibitors of biosynthetic processes. For example, it was recently stated that "Tolerance is a property of dormant, nongrowing bacterial cells in which antibiotic targets are inactive, allowing bacteria to survive." [66].

Such reasoning is circular. Although class II phenotypic tolerance is associated with non-replication by definition, non-replication does not constitute a mechanistic explanation of class II phenotypic tolerance. In fact, non-replication offers bacteria no blanket reprieve from the need for biosynthetic processes, such as generation of energy to maintain membrane potential. Generation of energy requires the action of enzymes. Stresses associated with imposition of non-replication cause damage to macromolecules. Some such damage is reparable; most repair requires energy. Some damage is irreparable. Replacement of irreparably damaged molecules requires synthesis, which again requires energy, and usually requires transcription as well. Indeed, non-replicating Mtb maintains its membrane potential [67–69] and a large, altered transcriptome [70, 71].

In short, non-replication is a state associated with class II phenotypic tolerance but not a mechanism accounting for it. Only recently have underlying mechanisms begun to come into focus. Non-replicating states can lead to reduced antibiotic uptake [72] or reduced retention [73] and perhaps to altered drug catabolism. Stress can lead to upregulation of antioxidant pathways, as seen, for example, in a proteomic analysis of *M. smegmatis* exposed to sublethal concentrations of rifampin [74]. To the extent that antibiotic action is augmented by generation of reactive oxygen species secondary to disordered metabolism [75], the increase in antioxidant defenses may contribute to phenotypic tolerance [76], as may the increased expression of proteostasis pathways for macromolecular preservation and repair. Non-replicating bacteria may switch to alternate respiratory pathways and use alternate electron acceptors. During non-replication, an essential process may occur so slowly that its corruption by the antibiotic only leads to death after the period of observation. Condition-dependent changes in gene essentiality may lead to prioritization of the transcription and translation of newly essential genes in the face of partial inhibition of overall transcription or translation.

It is a separate question how stresses suppress replication. Some stresses limit the supply of exogenous precursors for an increase in biomass. Many stresses activate the stringent response, leading to inactivation of antitoxins in toxin-antitoxin modules, of which Mtb has over 80 [77]. The activated toxins can cleave specific tRNAs, mRNAs, or ribosomal RNAs; phosphorylate and inhibit specific tRNA synthetases; interfere with DNA gyrase; ADP-ribosylate DNA [78]; and reduce the proton motive force [77]. The stringent response in some organisms includes induction of

hibernation factor and ribosome modulation factor, proteins that bind ribosomes and inhibit translation [79]. It is clear how these actions could suppress replication, but as noted above, suppression of replication does not suffice as a general explanation of phenotypic tolerance.

13.10 Is It Possible to Find New Antibiotics that Can Kill Bacteria Displaying Class II Phenotypic Tolerance to Existing Antibiotics?

Tuberculosis illustrates the importance of answering this question. A central hypothesis is that class II phenotypic tolerance to existing TB drugs is a major contributor to the failure of these drugs to reduce the time it takes to cure TB to less than 6 months for over 86% of individuals with drug-sensitive disease. If most of the Mtb at a given site in the host are non-replicating because of conditions they encounter at that site, such as hypoxia, nutritional restriction, acidity, or reactive species of oxygen or nitrogen, and, in association with those conditions, are phenotypically tolerant to every antibiotic that reaches the site, then chemotherapy that combines those drugs is not likely to be effective.

The following considerations illustrate one way that immunologic thinking can suggest new targets for unconventional antibiotics against Mtb to complement the action of conventional antibiotics.

Mechanisms of Mtb's resistance to host immunity can be understood in terms of successive lines of resistance. First, Mtb can suppress host immunity (e.g., [80]). Failing that or in addition, Mtb can detoxify host effector molecules (e.g., [68, 81–84]). Next, the pathogen can adapt to effector molecules whose production it failed to block and whose level it failed to reduce (e.g., [85]). If macromolecules are nonetheless damaged, the bacteria can repair them (e.g., [86]). If repair is inadequate, the bacteria can degrade damaged macromolecules to avoid their toxic gain of function (e.g., [87, 88]). Some macromolecules are too damaged to be repaired, such as irreversibly oxidized proteins that cannot be unfolded for degradation by chambered proteases. These can be sequestered [89]. If all else fails, some bacteria can survive long periods without replicating, awaiting the return of conditions in which replication can be sustained. In many cases, enzymes have been identified that mediate these microbial defenses and compounds have been identified that inhibit these enzymes [81, 88, 90–92]. Where human homologs exist, it has been possible to identify Mtb-selective inhibitors that spare the corresponding human enyzmes [81, 88, 90–92].

Almost all antibiotics that were selected on the basis of their ability to kill replicating bacteria are much less effective, or ineffective, against the same organisms when they are non-replicating. While rifampin, fluoroquinolones, and bedaquiline are active against non-replicating Mtb, much of that effect in short-term in vitro assays appears to be an artifact of carry-over of antibiotic from the non-replicating

stage of the assay to the stage of the assay where recovery is assessed under conditions that support replication [93]. Rifampin has genuine bactericidal action on non-replicating Mtb in vitro but at far higher concentrations than needed to kill replicating Mtb, and even then, the maximum extent of killing in vitro is far less [93]. This is not meant to disparage the proven clinical utility of these drugs but rather to suggest that they do not represent an ideal solution to the problem of class II phenotypic tolerance.

Fortunately, compounds can be found that extensively kill bacteria in a state that confers class II phenotypic tolerance to conventional antibiotics. An early example was a thioxothiazolidine that killed Mtb only when the Mtb was non-replicating, without regard to diverse conditions tested that imposed non-replication [81]. Another target-based screen led to two chemically distinct classes of Mtb-selective proteasome inhibitors [88, 92] that killed Mtb that was rendered non-replicating by nitrosative stress [88, 92] or starvation [94]. A whole-cell screen designed to identify compounds that kill non-replicating Mtb identified oxyphenbutazone [35] and other compounds [95]. Subsequently, over 100 compounds have been reported to kill non-replicating Mtb selectively, including novel cephalosporins [96]. However, in only a few cases did the investigators exclude the possibility that carry-over of compound into the replicative phase of the assay may have led to a false impression of activity in the preceding, non-replicative phase of the assay [30].

Why are some compounds only able to kill non-replicating bacteria, sparing the same cells when they replicate? Barring compound modification under one of the two sets of assay conditions, and assuming equivalent uptake under both, the question becomes why some targets are nonessential under conditions that support replication but essential under conditions that do not. For example, at least four sets of Mtb enzymes involved in central carbon metabolism—hydroxyoxoadipate synthase, dihydrolipoamide acyltransferase, lipoamide dehydrogenase, and the isocitrate lyases—are dispensable for survival under nonstressed conditions but become essential for Mtb to withstand oxidative or nitrosative stresses that impose non-replication [68, 76, 81, 84]. This invites the speculation that some pathways that would afford redundancy in a critical function targeted by the antibiotic are inactivated under non-replicative conditions, or a singular essential pathway incompletely inhibited by the antibiotic is further inhibited by the non-replicative conditions.

Even more encouraging are antibiotics that can kill bacteria extensively not only when they are replicating but also when they are not replicating and are phenotypically tolerant to other antibiotics. With respect to tuberculosis, this has been reported with 8-hydroxyquinolines [97, 98] and nitazoxanide, an antibiotic approved for other indications [20]. In vitro, the nitroimidazole PA-824 (Pretomanid) kills both replicating and non-replicating Mtb to comparable extents and at comparable concentrations [30, 99]. Under non-replicating conditions, the mechanism involves generation of reactive nitrogen species [99], a striking example of a synthetic antibiotic mimicking host immunity [100].

Major Points

- Phenotypic tolerance prevents an antimicrobial agent from eradicating a pathogen population; it likely accounts for relapse and contributes to the emergence of heritable resistance.
- Type I phenotypic tolerance occurs when a minority (subpopulation) survives antibiotic treatment in conditions permissive for growth of the majority population and individual tolerant bacterial cells are each tolerant to a different antibiotic.
- Type II phenotypic tolerance is a bacterial response to exogenous stress that impairs growth and pertains to all of the bacteria whose growth is impaired and individual bacterial cells are each tolerant to multiple antibiotics.
- Host cell immunity can foster phenotypic tolerance and thereby work at cross-purposes with antimicrobials.
- Better mechanistic understanding of the different classes of phenotypic tolerance will help improve antimicrobial chemotherapy and help reduce the emergence of heritable antimicrobial resistance.

Acknowledgments I am grateful to K. Burns-Huang, B. Gold, K. Rhee, K. Saito, and T. Warrier (Weill Cornell Medicine) for critical comments. I am thankful for the support of the Tri-Institutional TB Research Unit (NIH U19 AI111143) and the Milstein Program in Chemical Biology and Translational Medicine. The Department of Microbiology and Immunology is supported by the William Randolph Hearst Charitable Trust.

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Chapter 14
Staphylococcus aureus Adaptation During Infection

Bo Shopsin and Richard Copin

14.1 Introduction

Bacterial survival critically depends on the ability to swiftly respond to environmental change. To efficiently monitor the surrounding environment, microbial genomes encode numerous, highly diverse proteins, such as two-component signaling systems, that sense particular extracellular stimuli. In response to diverse cues, including nutrients, light, gases, and host and synthetic antimicrobial stress, systems transmit signals to the intracellular environment and thereby elicit a response.

Molecular dissection of these signaling networks has increased our understanding of communication processes and provides a platform for therapeutic intervention. When organisms are forced into environments far beyond their normal situation and when their mechanisms for responding to the new environment are overwhelmed, an alternative path to adaptive evolution may occur through selection of heritable genetic changes that "capture" the phenotype produced by a stimulus. The emergence of antibiotic resistance in pathogenic microorganisms provides an excellent example of such evolution, one that has profound consequences for human health.

Antimicrobial resistance is based on selection for organisms that have an enhanced ability to grow in the presence of a host or synthetic antimicrobial. The evolution of drug resistance can be attributed to multiple factors that include: (1) an increased frequency of intrinsically resistant variants, (2) the acquisition of mobile

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I. W. Fong et al. (eds.), *Antimicrobial Resistance in the 21st Century*, Emerging Infectious Diseases of the 21st Century, https://doi.org/10.1007/978-3-319-78538-7_14

resistance determinants, and (3) de novo accumulation of resistance mutations. The evolutionary dynamics depend on the biology and population size of the microbe in question, the drug, and the opportunity for genetic exchange of resistance determinants. The observation that the emergence of drug resistance outpaces the development of new antimicrobial agents underscores the crucial importance of understanding the evolutionary mechanisms that lead to the development of resistance.

Antimicrobial resistance has traditionally been approached from a mechanistic perspective focused on identifying the cellular determinants that prevent a drug from entering a cell, remove a drug from the cell, inactivate a drug, or prevent a drug from inhibiting the normal activity of its target. Selection may occur at key regulatory loci, or it can be focused downstream at the effectors of the phenotype. None of these mechanisms acts alone. Moreover, the phenotypic effects of mutations that confer resistance depend on the genetic background of the strain and changes in the genome that occur during clinical infection. This complexity is illustrated by the observation that the development of resistance is often accompanied by a fitness cost or deleterious effect on pathogen growth in the absence of the drug. Fitness costs are often mitigated by the accumulation of compensatory mutations that enhance the fitness of the resistant genotype in the absence of the drug. Fitness in the presence of a drug is a complex trait affected by multiple loci, the bacterial species involved, the ecological niche, and the host.

Using *Staphylococcus aureus* as an example pathogen, the present chapter focuses on the mechanisms that potentiate the evolution of drug resistance, with an emphasis on the central role of mutations in "off-target" genes having pleiotropic effects. Substantial support exists for the role of metabolic changes that fuel the accumulation of reactive oxygen species (ROS; superoxide, peroxide, and hydroxyl radicals) in the live-or-die decision made by bacteria [1–4]. Thus, emphasis is placed on how these principles apply to the lethal (bactericidal) cellular responses to a variety of antimicrobials during bacterial growth. In addition, mechanisms by which alterations in cellular states can influence the emergence of drug resistance, including the effects of tolerant cells, will be highlighted. We also discuss how bacterial adaptations that are potentially beneficial within hosts and hospitals can be used to track the evolution of hospital clones using whole-genome sequencing, underscoring the need for rapid containment. Finally, the possibility of harnessing evolution for therapeutic benefits through cellular perturbations will be explored.

14.2 The Accesory Gene Regulator (*agr*) Paradox

14.2.1 agr *and Clinical* S. aureus *Infections*

S. aureus is responsible for a large variety of diseases in both community and hospital settings [5]. Despite advances in care, *S. aureus* infections remain associated with considerable morbidity and mortality. In addition, treatment of methicillin-resistant

S. aureus strains (MRSA), an increasing cause of healthcare-associated infections, is complicated by the emergence of intermediate and fully vancomycin-resistant strains [6–8]. MRSA surgical site infections are particularly devastating when hardware is implanted in the patient (e.g., prosthetic joint, pacemaker, and vascular graft infections). As human populations age, more invasive procedures are being performed. Consequently, the incidence of implant infection by MRSA is certain to increase.

While the outcome of an *S. aureus* encounter is usually asymptomatic colonization, the propensity of *S. aureus* strains to produce invasive infection defines a capacity to resist host innate immune clearance mechanisms. Infection is likely transformative for the bacterium, since it must overcome host, and possibly synthetic antimicrobials, to live within as well as upon the host. Thus, hope for developing new ways to control *S. aureus* rests in part on understanding how the bacterium adapts to the new, in-host environment.

In a general sense, mutations and natural selection are expected to shape the evolutionary dynamics of *S. aureus* within an individual host; however, the type, frequency, and interaction of these events are largely unknown. Work on adaptation to the host has focused on gene regulation; in contrast, population genetics has addressed the genetic basis of evolution, with little overlap between the two disciplines. Recent work has combined molecular typing of field isolates with in vitro experiments that examine how *S. aureus* evolves within hosts. These studies have identified within-host variation in the *agr* locus, a quorum-sensing, global regulator of virulence in *S. aureus* [9] (Fig. 14.1).

agr mutants are attenuated for virulence in animal models of infection [14], and the majority of clinical isolates have a functional *agr* locus. However, *agr*-defective strains are a common clinical occurrence, particularly in persistent infections in which biofilms are thought to play a role, such as device-related infection and endocarditis [15–20]. In vivo selection for *agr*-defective strains was suggested by studies of sequential isolates recovered from the blood of patients during antimicrobial treatment [18, 21, 22], as well as in animal infection models [23]. The existing data led to the paradoxical conclusion that survival of *S. aureus* in the bloodstream may be *enhanced* by the inability of *S. aureus* to produce numerous virulence factors, including cytotoxic leukocidins. Moreover, among patients with MRSA bacteremia, the development of an *agr*-defective phenotype serves as a predictor of persistence of the organism and of a higher incidence of infectious endocarditis [24] and mortality [25]. Thus, the clinical consequences of *agr* activity are not obvious – depending on the patient – they could even make efforts to use *agr* and virulence as targets for new antimicrobials ill advised [26].

14.2.2 Epidemiology of agr *Dysfunction*

The temporal and spatial variation in environmental conditions that opportunistic microbes are exposed to within an individual host and during transmission between hosts likely promote adaptation to a lifestyle that accomodates rapidly changing

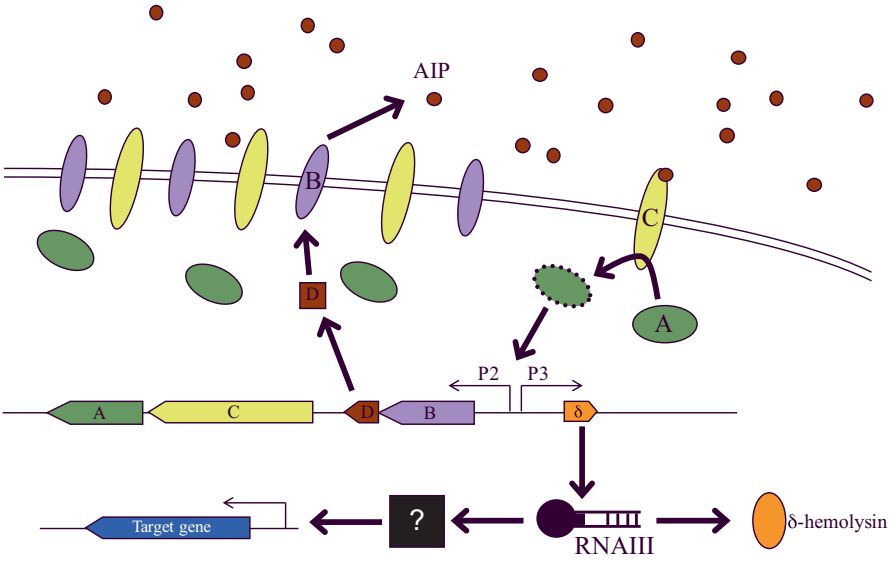

Fig. 14.1 The *agr* quorum-sensing system. (A) The *agr* locus consists of two divergent transcription units driven by promoters P2 and P3. The P2 operon encodes the signaling module, which contains four genes – *agr*B, D, C, and A – each of which is required for transcriptional activation of the *agr* regulon (reviewed in [9]). AgrC is the receptor-histidine kinase, and AgrA is the response regulator. AgrD is the autoinducing, secreted peptide that is derived from a propeptide processed by AgrB. The P3 transcript is a regulatory RNA (*RNAIII*) that also encodes the structural gene for hemolysin. Regulation of target genes by *agr* occurs through two pathways: (1) an *RNAIII*-dependent regulation of virulence genes and (2) an *RNAIII*-independent, AgrA-mediated regulation of metabolic genes and small cytolytic toxins known as phenol-soluble modulins (modulins). The regulatory connection between these processes links virulence to metabolism. A*gr* has a dual, time-dependent regulatory role (in vitro) that is characterized by (1) increased post-exponential production of toxins and exoenzymes (e.g., α-hemolysin) that facilitate dissemination of bacteria via tissue invasion; (2) decreased production of cell surface proteins that facilitate adherence and attachment (e.g., fibronectin-binding proteins); and (3) decreased production of factors that promote the evasion of host defense (e.g., protein A). Thus, the *agr* locus coordinates a switch from an adherent state to an invasive state dependent on bacterial population density. This important duality has been exploited by the use of *agr* quorum-sensing inhibitors for the prevention and treatment of experimental *S. aureus* infections, including catheter and vascular prosthetic graft infection [10–13]

environments. Thus, a better understanding of how *S. aureus* redirects and fine-tunes its gene expression in response to the challenges of colonization, transmission, and infection is central to understanding the causal pathway between commensalism and serious, complicated disease.

The observation that a significant fraction (~20% overall and ~70% in patients with persistent bacteremia [18, 24]) of clinical isolates of *S. aureus* from infections have genotypic *agr* defects provides a way to delineate the epidemiology-host-microbe relationship of this system, and therefore virulence, in disease. Nasal carriage is an important prerequisite for *S. aureus* infection, indicating the importance

of examining the role of *agr* in colonization. Screening assays to detect *agr* functionality among isolates from healthy subjects indicate that although *agr* dysfunction is not an absolute barrier to colonization and transmission, carriage of *agr*-defective strains is strongly associated with hospitalization rather than with healthy patients [27]. Collectively, these observations suggested that: (1) *agr*-defective mutants are fit for transmission (they are not a "dead-end" state), and (2) the hospital environment is a reservoir of attenuated, *agr*-defective variants. Presumably, disruption of protective barrier functions by disease and clinical intervention (e.g., intravenous catheter use) permits *S. aureus* lacking full virulence to cause infection. Analysis of paired *S. aureus* clones from blood infection and nasal carriage sites in individual hospitalized patients presenting with bacteremia indicate that recovery of an *agr*-defective mutant from blood is usually predicted by the *agr* status of carriage isolates [28]. Thus, fieldwork supports the idea that the transition from commensalism to opportunism in *S. aureus* does not require full virulence in hospitalized patients.

The strong association of *agr* dysfunction with the hospital environment and infection suggests an unappreciated role for *agr*: colonization by *S. aureus* is responsible for maintaining *agr* function. Indeed, although fully *agr*-defective mutant isolates colonize and transmit, they do not persist indefinitely in natural populations of hospital-associated MRSA [29]. This suggests that, in the case of *agr* mutation, attenuation of virulence is the product of short-sighted evolution within hosts – although attenuation of *agr*-mediated virulence may help *S. aureus* adapt to host tissues in the short term, it appears to put *S. aureus* at a disadvantage in the long term.

The combination of ubiquity and relatively short lifespan suggests that the occurrence of *agr*-defective mutants results from frequent within-host selection in situations such as persistent bacteremia [18, 21, 22, 28]. However, an experimental system demonstrating transmission following invasive bacteremia was lacking, and thus implications of within-host adaptation for between-host transmission – and therefore for hospital epidemiology – were unknown. While a disease-promoting *agr* mutation that occurs during the course of bacteremia could confer a transient advantage to the bacterium, such an adaption would be a dead end for the bacterium in the absence of transmission. Recently, *S. aureus* was found to disseminate to the gastrointestinal tract of mice via the gall bladder following intravenous injection, and the bacterium readily transmits to cohoused naive mice [30]. These findings established an animal model to investigate gastrointestinal dissemination of *S. aureus* and the role of adaptive mutations in genes such as *agr*. The work suggests that selective processes taking place over the course of blood infection can go beyond a single host. Both intestinal dissemination and transmission were linked to the production of virulence factors based on gene deletion studies of two-component virulence regulatory systems, including *agr*. Thus, the animal data are consistent with data from hospital isolates that indicate that *agr* inactivation can attenuate colonization-transmission but is selected during bacteremia.

14.3 Pleiotropic Point Mutations in *agr* Illustrate a General Mechanism of Adaptive Evolution During Infection

Adaptive evolution to a new niche, such as a novel host or host environment, involves either fluctuating or completely novel conditions, and generally requires a rapid shift in the expression of genes [31, 32]. Mutations in transcriptional regulators produce a rapid pleiotropic, phenotypic effect on the expression of multiple genes, and the mutations correlate with adaptive radiation [33]. Indeed, experimental [34–36] and observational [37–40] work suggests that global regulators constitute a "one-step" mechanism of adaptation that drive adaptive leaps made by microbes.

Global regulators and two-component signaling systems are highly abundant in the *S. aureus* genome ([41], see also Chap. 15). They form a complex regulatory network that modulates phenotypic plasticity and the expression of virulence genes, cell division, and stress responses in response to environmental change [42]. Quorum sensing can be considered to lie at the top of the transcriptional regulatory network hierarchy, not just in *S. aureus* but also in other pathogens. Consequently, mutations that affect quorum-sensing loci during adaptation to novel environments are likely to be a general phenomenon. Indeed, the *lasR* quorum-sensing system, which is involved in the repression of biosynthesis of virulence factors and biofilm in *Pseudomonas aeruginosa*, is a hot spot for mutations in isolates from chronically infected cystic fibrosis patients [40, 43–46]. Accordingly, the present chapter focuses on the role of quorum-sensing mutations as a prototype adaptive mutation. Moreover, *agr* dysfunction and virulence attenuation are similar to the phenotype of strains that have mutations or dysfunction in other regulators during infection. For example, *S. aureus* mutationally adapts the global regulator *rsp* and virulence factor expression in the course of infection [38]. Thus, multiple genetic mechanisms, as well as the genetic background of the strain, control the induction of host-adatpted states, indicating that the interplay between factors and the associated selective loss of any one regulator are complex.

14.4 Evolution of *agr*-Defective Mutants

14.4.1 Host-Pathogen Interactions

Several explanations can potentially account for the selection of *agr*-defective strains and their association with persistent infections. For example, endocarditis vegetations may be regarded as biofilms in which the organisms are protected from attack by phagocytes (and antibiotics). It has been demonstrated that *agr*-defective mutants are enriched in biofilms [20, 47]. Organisms at the surface of a biofilm express *agr* and those in the underlying layers have *agr* repressed [48]. Thus, *agr*-defective strains could provide adhesins to stabilize the vegetations, and the

agr-positive strains could adhere to the *agr*-defective variants while producing their toxic exoproteins. Additionally, inactivation of *agr* upregulates fibronectin-binding proteins, which play an important role in the ability of *S. aureus* to colonize, persist within, and damage cardiovascular tissue [49]. *agr* inactivation also increases resistance to endogenous thrombin-induced microbicidal proteins [50], key mediators of host defense that are secreted by platelets at sites of cardiovascular damage and infection.

It is also possible that *agr* mutation promotes *S. aureus* survival inside host cells, as opposed to within-host tissues. Although considered an extracellular pathogen, *S. aureus* clearly thrives inside host phagocytic, epithelial, and endothelial cells ([51–56]; reviewed in [57]). The importance of this intracellular lifestyle is highlighted by the recent finding that ablation of intracellular *S. aureus* improves outcome from experimental infection [54, 55]. While the large majority of work on MRSA-phagocyte interactions, including work from our laboratories, has been performed with neutrophils, recent findings indicate that disseminated *S. aureus* infection is tied to survival inside macrophages [52–55]. For some cell types, *agr*-defective mutants exhibit prolonged intracellular residence due to attenuated cytotoxicity and delay in initiation of host cell death [58, 59]. But the *agr*-defective phenotype is not noted in isolates from primary skin and soft tissue infection (e.g., [60]), suggesting that such attenuated toxicity and intracellular survival may be particularly important in infections in which persistence is an issue. Persistence is a particular problem with *S. aureus* endocarditis, while it is not such an issue with acute infection of skin and soft tissue. Thus, a better understanding of the interaction between-host cells, *agr*-mutant, and wild-type strains will generate the knowledge needed to confront the growing problem of complicated disease and poor outcomes.

Other investigators showed that the use of antibiotics, such as fluoroquinolones or beta-lactams, is a risk factor for loss of *agr* functionality in vitro and during treatment of infection in the hospital [61, 62]. Furthermore, *agr*-defective strains are associated with the development of vancomycin tolerance in vitro and during treatment of patients with bacteremia in vivo, perhaps owing to defects in autolysis and consequent changes in cell wall structure that mitigate fitness costs associated with the evolution of vancomycin resistance (reviewed in [17]). Indeed, all known vancomycin intermediate-resistant *S. aureus* (VISA) strains are *agr*-defective. Moreover, recent work demonstrates that *vraR*, a member of the two-component *vraRS* regulatory system that is upregulated in vancomycin-resistant strains, suppresses transcription of *agr* [63]. Thus, vancomycin resistance appears to be an example of how a fitness trait that is initially dependent on attenuation of *agr* can evolve transcriptional independence.

Enhanced fitness when *agr* function is compromised can enrich underlying polymorphisms in the locus such that a threshold is passed and the phenotype is expressed in all members of the population. The effect of antimicrobials on selection for *agr*-defective strains is discussed further in sections below in a different context – diminished antimicrobial lethality (tolerance) rather than inhibition of growth (resistance). We conclude this section by noting that *agr* dysfunction offers potential advantages

to *S. aureus* not just during infection but also more generally by promoting protection against antimicrobials. Indeed, loss of *agr* expression has been described as a potential "win-win" situation for a nosocomial pathogen [64].

14.4.2 Fitness and Protection from Antimicrobials in agr*-Defective Mutants*

The widespread increase in *S. aureus* antibiotic resistance has dramatically narrowed treatment choices, especially with the appearance of resistance to key antimicrobials such as vancomycin and daptomycin. Resistance often emerges in vivo during persistent infection, and a number of studies have investigated the genomic basis for this phenomenon. Many mutations, such as those involved in target modification, are thought to lead directly to antimicrobial resistance. Other mutations accumulate under combined antibiotic and host selective pressures, leading not only to antimicrobial resistance but also to altered host-pathogen interactions that favor persistent infection. For example, host thrombin-induced platelet microbicidal proteins (tPMP) are one of the first-line innate defense mechanisms against *S. aureus* infection, and a link has been demonstrated among *agr mutations* associated with reduced vancomycin and daptomycin susceptibility, persistence, and reduced tPMP susceptibility [24, 50, 65].

As discussed above, inactivation of *agr* also correlates with antibiotic use in patients, suggesting that *agr* functionality is subject to a tradeoff – *agr* activation promotes survival in host niches favoring acute virulence but represents a liability to the bacterium during growth stress, especially antimicrobial treatment. The frequent occurrence of *agr* mutants in serial passage of laboratory cultures in the absence of antimicrobials supports the hypothesis that *agr* activity is metabolically costly [66], as does the observation that the locus is itself highly expressed and that *agr* activates the expression of many more genes than it inhibits [67]. Futhermore, previous reports indicate a growth advantage for Δ*agr* mutants in the presence of subinhibitory concentrations of several antibiotics [42]. Fitness gains for Δ*agr* mutants were associated with inactivation of RNAIII, indicating that the growth advantage is *agrA* independent. *agr* mutation may also mitigate fitness defects of resistance mutations. For example, deformylase inhibitor-resistant *S. aureus* strains partly regain fitness through mutation of *agr* while still retaining high-level resistance [68].

Superoxide, a metabolic product, may play a central role in the live-or-die decision made by bacteria when challenged with lethal antimicrobials, such as ciprofloxacin, a gyrase-mediated DNA-damaging agent [1–4]. Activation of antioxidant/oxidative stress-protective responses in bacteria would therefore be expected to promote antimicrobial tolerance (loss of lethal activity but retention of bacteriostatic activity). From a clinical point of view, tolerance presents a major challenge: in contrast to the specificity of resistance, tolerance confers a survival advantage against a broad spectrum of drugs and stresses. Additionally, it is likely that tolerance provides

a reservoir for relapse and the evolution to antibiotic resistance. Thus, understanding tolerance is critical for addressing the decreasing efficacy of antibiotics. We note that, although definitions have been debated, tolerance is related to but distinct from another important contributor to pathogen survival – the phenomenon of persistence. Persisters are considered to be slow growing, metabolically dormant cells that exhibit tolerance and are less likely than growing cells to exhibit ROS-mediated killing by antimicrobials (for additional discussion of tolerance, see Chap. 13).

One oxidative stress-protective mechanism that might promote antimicrobial tolerance in *S. aureus* involves mutation of *agr*, which has a built-in oxidation-sensing mechanism through an intramolecular disulfide switch possessed by the DNA-binding domain of the response regulator AgrA [69]. Oxidation of AgrA decreases DNA-binding activity, which results in derepression of the *bsaA* gene, which encodes the antioxidant glutathione peroxidase. As a result, *agr*-defective mutants are less susceptible to oxidative stress.

The frequent occurrence of in vivo-selected *agr*-defective mutants during persistent infection highlights a possible link between oxidative stress and antibiotic tolerance in this organism. The mechanism underlying *agr* dysfunction among strains derived from serial passage in vitro and from clinical isolates is almost always traced to inactivating mutations in *agrC and agrA,* the sensor component and response regulator, respectively, of the *agr* system (Fig. 14.2). The intuition explaining this observation is that selection for *agr*-defective strains occurs in mixtures containing *agr*-positive parental strains. Accordingly, inactivation of *agrD* or *agrB* does not silence *agr* owing to the production of autoinducing peptide in trans by the *agr*-positive strain. However, this scenario does not explain why RNAIII, the effector of the *agr* response, is not targeted by selection for loss of *agr* function. We hypothesize that an *agrA*-dependent, *bsaA*-mediated antioxidant phenotype provides protection against antibiotic-dependent oxidative damage, thereby resolving the dilemma.

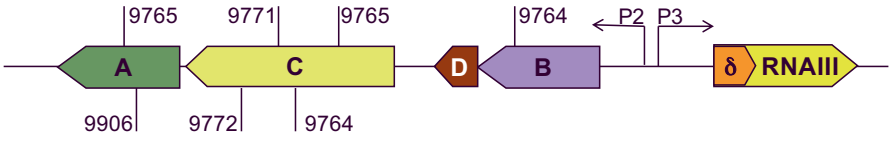

Fig. 14.2 Localization of inactivating mutations in *agr*. The mechanism underlying *agr* dysfunction can usually be traced to mutations in *agr* that inactivate the locus [18, 22, 27, 29, 66, 70, 71]. Representative mutations identified by DNA sequencing of the *agr* operon in different clinical isolates that were negative for δ-hemolysin. (Adapted from [18]). The numbers on the figure refer to the location of *agr* mutations for different isolates. The isolates were derived from patients with various clinical infections (the strain number is an arbitrary designation). Some strains had more than one mutation in *agr*, but complementation with the relevant gene on a plasmid showed that only one mutation per strain resulted in *agr* inactivation. Notably, *agr* defects in *S. aureus* and *Staphylococcus epidermidis* usually result from quorum-sensing deficiency (due to a mutation in *agrA* or *agrC*) rather than from quorum-signaling deficiency (due to a mutation in *agrB* or *agrD*). Presumably, selection for *agr*-defective strains occurs in mixtures with *agr*-positive parental strains. Thus, inactivation of *agrD* or *agrB* cannot silence *agr* owing to the production of AIP in trans by the *agr*-positive strain

To test our *agrA-bsaA* hypothesis, we used a range of both drug concentration and treatment time to probe effects of *agr* status on the response of *S. aureus* to lethal stress [72]. We note that, to study *agr*-stressor effects, it is important to distinguish phenotypes related to growth from those specific to survival. For example, treatment with an antimicrobial leads to damage that is specific to the test agent. This primary damage halts growth, which is measured as the minimal inhibitory concentration (MIC). MIC reflects drug uptake, efflux, and target affinity; high MIC values are associated with antimicrobial resistance. Some forms of primary damage also kill cells, with much of the lethal process arising from a self-destructive bacterial response to the primary damage (reviewed in [2, 3]; also Chap. 20). To focus experimental measurements on the lethal response, lethal drug concentrations were normalized to MIC. It is also important to recognize that lethal stress may be transient. For example, with *S. aureus* ROS can accelerate killing without increasing the extent of killing [73]. Consequently, overnight killing assays, such as those commonly used to measure minimal bactericidal concentration (MBC), may be not provide much information [73].

Using highly lethal antimicrobials as probes for studying bacterial responses to lethal stress, we found that wild-type *agr* stimulates the lethal action of several stressors, including gentamicin and ciprofloxacin; thus, defective mutants will tend to persist under stressful conditions rather than being killed by stressors that may include synthetic antimicrobials and host defenses such as neutrophil-generated ROS. Disruption of the RNAIII-dependent pathway had no effect on the stress responses to lethal stress. In contrast, disruption of the *agrA*-dependent pathway had effects, but they varied from one stressor to another. For example, with ciprofloxacin-mediated killing, *agr* facilitated the accumulation of toxic ROS, which is known to be involved in quinolone-mediated killing of *Escherichia coli* and *S. aureus*. *agr* action appears to be exerted by downregulating *bsaA*; thus, an *agr* defect allows expression of a protective protein.

Within our sample of stressors, daptomycin was unusual in exhibiting greater lethality with the *agr*-deficient mutant. Test conditions are important, as indicated by consideration of previous work in which the opposite result was obtained with nongrowing *S. aureus* in deep stationary phase, long after induction of *agr* and expression of *agr* transcripts [74]. Daptomycin, a calcium-dependent molecule that acts as a cationic antimicrobial peptide, releases membrane phospholipids that bind to and inactivate the antibiotic. Although both wild-type and Δ*agr* strains released phospholipid in response to daptomycin, *agrA*-triggered secretion of phenol-soluble modulin (PSM) cytotoxins prevents antibiotic inactivation by wild-type cells. In previous experiments, killing assays were performed using overnight cultures, long after induction of *agr* and expression of its transcripts. Thus, the results reflect *agr*-mediated exoprotein secretion rather than a cellular response to stress-mediated killing. Our experiments were performed in late exponential phase when PSM levels may be lower and less protective [75]. The complex relationship between daptomycin lethality, *agr* status, and bacterial physiological state illustrates the importance of understanding *agr* biology before applying novel therapies that target *agr* [26].

Collectively, the data support the hypothesis that inactivation of *agrA* can result in degradation of both antimicrobials themselves and the lethal response to antimicrobial-mediated stress. Given the data indicating that fitness gains are associated with inactivation of RNAIII (mentioned above, [42]), we conclude that two distinct subsets of *agr* antimicrobial fitness exist: an RNAIII-independent one that impacts antimicrobial *lethality* and an RNAIII-dependent form that controls antimicrobial-associated fitness for *growth* (Fig. 14.3).

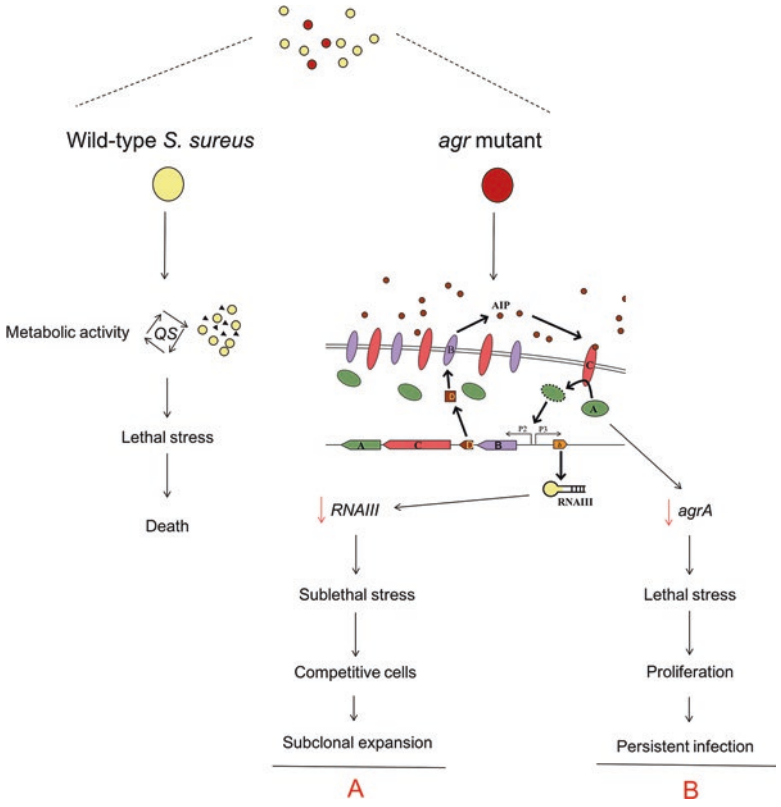

Fig. 14.3 Overview of *agr* mutation and its consequences in complex host environments. Effect of *agr* deficiency on sublethal and lethal action. (**A**) Sublethal stress. By switching to the inactive form and modulating expression of appropriate factors, *S. aureus* cells gain enhanced replicative fitness (RNAIII pathway). In this model, RNAIII deficiency enhances energy resources. Decreased protein synthesis and ATP could be involved in this coupling; however, as with many factors in the *agrA* pathway, the physiological relevance, as well as the mechanism by which these factors act, is poorly understood. (**B**) Lethal stress. *agr* deficiency modulates survival against lethal stress (*agrA* pathway). *agrA*-mediated effects are stress dependent, for example, derepression of the antioxidant *bsaA* results in protection against ciprofloxacin, whereas upregulation of unknown factors control survival in trans against gentamicin (not shown). For both RNAIII and *agrA* pathways, enhanced survivability is achieved at the cost of virulence, which may or may not be compensated for by the presence of coinfecting *agr*-positive strains

14.4.3 Social Cheating

Given the metabolic burden associated with *agr* function, the question arises as to whether it is advantageous for *S. aureus* populations to consist purely of signaling-proficient cells or whether there might be situations in which "cheaters" would be favored. Social cheaters reap the benefit of public goods while contributing less than average to the cost [76, 77]. In *S. aureus*, many *agr*-regulated products are released into the extracellular environment and benefit not only the producing cell but also its neighbors. Mutants that do not respond to *agr* autoinducing peptide signals do not incur the cost of producing these "public goods," but they may gain the benefit of production of goods shared by neighbors. Cheating theory therefore predicts that *agr*-defective cells should be at a disadvantage to their wild-type counterparts when grown in monoculture. In support of this hypothesis, it is well known that *agr*-defective cells are rapidly eliminated and are less virulent in animal models of acute infection [14, 23, 78, 79]. Indeed, it was unequivocally demonstrated that blocking of *agr* attenuates staphylococcal virulence and that the administration of an *agr*-positive supernatant along with *agr*-defective organisms protects the bacteria in an abscess model of infection [14]. This suggested that the spread of *agr*-defective strains within populations in vivo is due in part to the exploitation of shared products produced by their wild-type neighbors. Thus, social cheating may be relevant during infection owing to the differential impact of host defenses on bacterial survival.

14.4.4 **agr** *and Mutability*

Evolution by natural selection involves two main steps: the generation of heritable variations (e.g., mutations) and the differential proliferation of the variants in the environment. Hypermutability may function to create a diverse bacterial population, increasing the likelihood of environmentally adapted variants. Thus, enhanced mutability may provide the substrate for selection of attenuated *agr*-mediated virulence in *S. aureus* infection. However, recent work by Plata et al. suggests that heritable elevations of mutation frequency are not likely the cause of *agr* diversification: *agr*-defective mutants, and their parent strains showed similar mutation frequencies in the range of what is commonly found in the species [80]. Nonetheless, the authors reported that generation of heterogeneous resistance to oxacillin is enhanced by *agr* mutation and antimicrobial-related stress [80], giving rise to the related ideas that (1) *agr* suppresses genome plasticity and (2) *agr* dysfunction can result in bursts of mutations. In this scenario, *agr* dysfunction potentially serves as a "driver" mutation that promotes accumulation of additional genetic alterations and rapid evolution in the complex environmental milieu of invasive infection. *agr* inactivation and genetic instability in turn may result in subclones that display emergent properties,

including antimicrobial resistance, that pose challenges for therapeutic intervention. *agr*-mediated effects may be superimposed on those of antimicrobials themseleves, which may induce mutagenic "SOS" responses that contribute to the emergence of resistance (e.g., see [81]).

14.4.5 Implications of agr*-Mediated Antimicrobial Protection*

The observations described above provide an entry point for additional screening to identify other bacterial regulators having activities that can be self-protective, depending on the type and level of lethal stress. Elucidating the basis of such effects can be clinically significant when they inform efforts to personalize management of antimicrobials through pathogen strain-specific characteristics. For example, the use of anti-*agr* agents or therapeutic vaccines [26] may be counter-productive for applications in which the absence of *agr* reduces lethal activity of an antimicrobial. Likewise, identification of adaptations that erode the lethal activities of antimicrobials might inform the development of novel strategies to selectively bolster antimicrobial effectiveness [82–84].

14.5 Role of Virulence in Acute Infection

14.5.1 Virulence and Outcome in Nosocomial Pneumonia

In persistent infections, such as complicated bacteremia, low virulence, and enhanced adherence to prosthetic or host material might be expected to lie on in the causal pathway leading to persistent infection and poor patient outcome. In contrast, *agr*-mediated cytotoxic activity is integral to increased *S. aureus* virulence in most models of acute infection, and agents that block *agr* and quorum-sensing exhibit antiinfective properties [14]. Recently, virulence phenotypes were characterized among *S. aureus* isolates obtained at the time of diagnosis from a large, prospective clinical trial that compared the efficacy of linezolid and vancomycin for treatment of nosocomial pneumonia due to MRSA [85]. The analyses took into account host-related factors (virulence) and organism-related factors (antimicrobial resistance). Virulence was measured by screening for functionality of *agr*. Since *agr* functionality alone does not imply efficient expression of virulence, an additional, direct measure of virulence factor production was sought. The leukocytotoxins, which are *agr*-regulated pore-forming toxins (bicomponent leukocidins and alpha-hemolysin), and membrane-damaging cytolytic peptides, which are found in virtually all staphylococcal isolates, are attractive candidates to be wide-ranging virulence factors whose presence can effectively distinguish patient outcomes. Accordingly, relative leukocytotoxic activity (in which individual leukotoxic

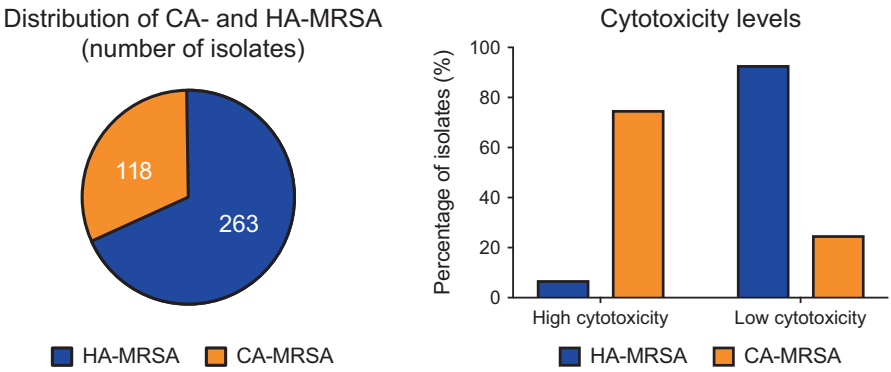

Fig. 14.4 Neutrophil cytotoxity values for CA-MRSA (orange) and HA-MRSA (blue) strains. In our recent survey of more than 380 MRSA isolates from patients enrolled in a randomized controlled trial of nosocomial pneumonia [85, 86], CA-MRSA lineages were found to be almost uniformly highly cytotoxic to human neutrophils (82% vs 11%; $p < 0.0001$). In contrast, HA-MRSA lineages were weakly cytotoxic, often below levels expected to correlate with mortality in animals. Furthermore, many strains (22/381 [6%] HA-MRSA) produced no detectable cytotoxic activity. Left, overall, 69% (263/381) of isolates were assigned to CA- or HA-MRSA subsets based on SCC*mec* type. More than 75% of CA-MRSA demonstrated ≥90% killing of neutrophils within 2 h. In contrast, ≥90% of HA-MRSA strains demonstrated cytotoxicity levels below 90%

activities are inseparable) were measured under normalized conditions [85], as discussed below.

Cytotoxicity levels varied remarkably within each MRSA clonal complex and even within groups of *agr* functional strains. Thus, *agr* functionality does not imply full cytotoxic virulence. Community-acquired MRSA (CA-MRSA), defined by the presence of the community-associated staphylococcal cassette chromosome *mec* type IV genes that confer methicillin-resistance, was almost uniformly highly cytotoxic (Fig. 14.4). In contrast, most hospital-associated MRSA (HA-MRSA) strains were weakly cytotoxic, often below levels expected to correlate with mortality in animals. Indeed, several strains of HA-MRSA produced no detectable cytotoxic activity. The low cytotoxicity phenotype was stable and heritable, indicating a genetic perturbation.

HA-MRSA strains were the predominant isolate from patients with nosocomial pneumonia. Moreover, the crude mortality rates for adequately treated patients with MRSA due to strains of *low* cytotoxic activity were fourfold *higher* than for patients infected with strains of high virulence, even after multivariate logistic regression analysis with careful adjustment for bacterial, clinical, and host factors. Thus, the low cytotoxic activity associated HA-MRSA strains is a predictor of mortality in MRSA-mediated nosocomial pneumonia. Moreover, isolates having low cytotoxicity, which were derived largely from healthcare-associated clones, were recovered more often from patients who were older and frailer. Collectively, these data suggested that the discrepancies in clinical outcome and survival of low-virulence strains stem from confounding factors

related to differences in populations of patients infected with highly cytotoxic and weakly cytotoxic MRSA. In this scenario, cytotoxic activity is a proxy for a subtle factor that makes hospitalized patients more susceptible to infection with hospital-associated bacteria of low virulence. That factor, referred to as "host quality" [85], appears to be a multidimensional syndrome of decreased reserve and resistance to stressors leading to increased vulnerability to adverse outcomes.

The results provide a framework with which to further explore the relationship between strain variation in MRSA and clinical outcomes. The issue is that, depending on the type of infection, patients who are infected with low-virulence MRSA are inherently different from those infected with virulent MRSA and, in turn, that both the natural history of disease and treatment effects between some drugs may be different in these patients. Future outcome studies should be designed so that the host quality factor is assessed and evenly balanced between study groups.

14.5.2 Hypothetical Model of Adaptation of Low-Virulence MRSA in the Hospital Environment

Attempts to draw direct relationships between in vitro and animal model-based virulence attributes, strain success, and patient outcome appear to have been too simplistic. Substantial evidence supports the idea that adaptation to the host and hospital environment, plus poor clinical outcome in invasive infection, are associated with low – not high – virulence [24, 25, 28, 38, 87–90]. Likewise, adaptation is often accompanied by decreased susceptibility to killing by host factors and synthetic antimicrobials. These observations enable formulation of a tentative model for the events occurring during hospital adaptation as follows: after hospitalized patients are infected with "wild-type" bacteria, host and antimicrobial factors promote diversification and the emergence of low-virulence variants. By evolving to a host and potentially antimicrobial-tolerant form, the bacterium gains enhanced ability to persist in human tissues despite treatment. This persistence, however, requires a downregulation of virulence (and possibly metabolic) functions. In this way, modulation of hospital adaptation is achieved at the cost of virulence, which may or may not be compensated for by the presence of coinfecting wild-type strains. This duality of virulence and persistence may explain why such variants are preferentially isolated from patients in the hospital compared with isolates from colonizing sites in healthy community subjects. Supporting this hypothesis, *agr* mutations having moderate or weak functional defects, such as the Gly55 amino acid change in *agrC,* are associated with the successful and deadly hospital- and bacteremia-associated clonal complex 30 lineage [88–90]. Apparently, the Goldilocks metaphor applies; when high- and low-virulence states are balanced, infections are maintained, and bacterial proliferation is not restricted, resulting in interhost spread and poor clinical outcomes (Fig. 14.5).

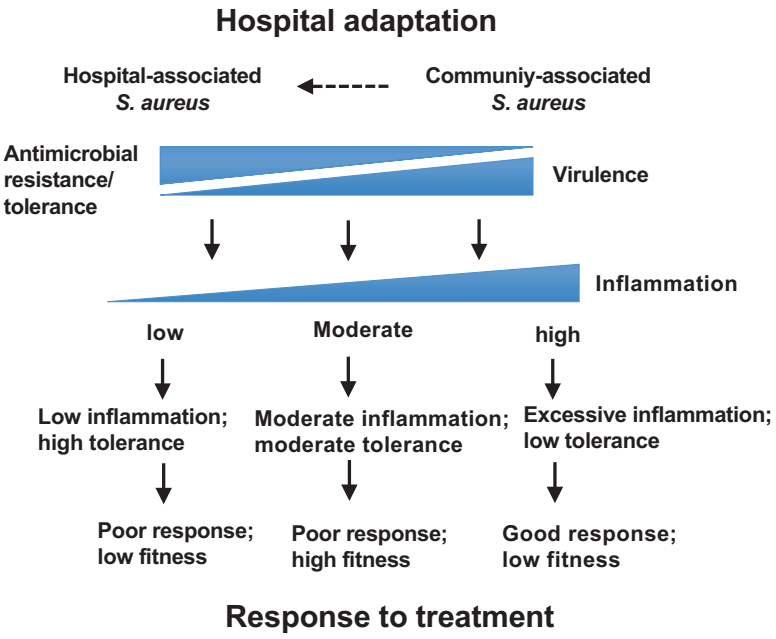

Fig. 14.5 Variations in resistance and virulence, represented by the continuum symbol, influence the balance between stimulation of proinflammatory host responses and antimicrobial-tolerant states. High levels of virulence but low antimicrobial resistance results in immune and antimicrobial clearance among hospitalized patients with bacteremia. The same characteristics result in exuberant bacterial growth and increased inflammation among hosts in the community [91–94]. In the community, lack of prompt antimicrobial treatment leads to severe inflammation, tissue necrosis, and poor outcomes. Although lower levels of virulence result in decreased inflammation during infection, high tolerance to antimicrobials and lack of effective immune responses may still result in high bacterial burden and poor outcomes among hospitalized patients

14.6 Molecular Basis of Adaptation of CA-MRSA to Low Virulence States in the Hospital Environment

14.6.1 Genetic Mechanisms Other than agr *Mutation in the Evolution of Attenuated Cytotoxicity*

We now know that bacterial isolates from invasive infection often differ phenotypically from naturally occurring strains that colonize subjects in the community, exhibiting high frequencies of decreased cytotoxicity and reduced *agr* activity. Moreover, they are associated with worse outcomes [24, 25, 38, 70, 87]. Colonizing isolates represent the source for disease [28], and isolates in hospital-associated settings are the reservoir for low-virulence phenotypes [27]. A better understanding of low-virulence strains is critical not only to evaluate therapeutic efficacy of vaccine and monoclonal antibody strategies under

development for prophylaxis and therapy but also for the important first step of target identification. To date, efforts to define the polymorphism and expression heterogeneity of antigens associated with targets of protection have been mostly limited to individual *agr*-regulated toxin antigens, such as *hla*, that are expressed poorly, if at all, among HA-MRSA lineages that are implicated in complicated infections, such as bacteremia [24, 89, 90]. However, it is possible that adaptive variants result from changes that do not alter *agr* sequences or expression. To test for non-*agr* specific selection processes, additional mutations can be sought using genome sequencing to define the complete set of changes that accompany the transition to host and hospital-adapted phenotypes.

Recent studies have used genome sequencing to provide evidence for rapid pathogen genome diversification, some of which could potentially affect the course of disease (reviewed in [95]). Much of the variation described has been measured between bacterial isolates of a single patient during infection, either at a single time point or longitudinally. Although longitudinal studies in single patients have been highly informative (e.g., [21]), they often focus on resistance mutations during antibiotic exposure; effects of selection are superimposed upon mutational patterns generated by antimicrobial damage and repair processes. In contrast, the overlap between the within-host and the between-host levels that defines adaptation to the hospital environment results in heterogeneity of selective pressures that derive from from functional trade-offs, wherein mutations in genes such as *agr* may promote survival in certain host niches in the short term but represent a liability to clones over the longer term. A consequence of the differential populational stability of mutations is that pathogens evolve toward fitness on a global scale. Thus, to understand adaptation to a complex environment, such as the hospital, we must examine evolution in populations as well as in single patients.

Divergence of HA-MRSA strains from community isolates occurred decades ago, complicating the identification of specific genetic changes associated with the transition from community to hospital. CA-MRSA strains, such as USA300, the predominant CA-MRSA clone in the United States, are only distantly related to hospital-associated MRSA; until recently, they were not linked to the healthcare environment [96, 97]. To limit confounding effects of genetic variability, genetic background can be controlled by leveraging the recent introduction and dissemination of CA-MRSA strain USA300 into hospitals. The limited number of functional mutations associated with this lineage helps identify specific variants that are associated with the evolution to low-virulence phenotypes in hospitals. Using such an approach, recent genomic studies demonstrate that a number of MRSA genes, including but not limited to *agr*, are targets for mutation during hospital adaptation [70, 98]. Among the non-*agr* genes are *sucD, clpC*, *tsK*, and *rpsA* [70].

Identification of genetic changes that potentially drive or are associated with emergence of host- and hosptial-adapted strains highlight the need to rapidly reduce bacterial burden and restrict the emergence of adapted mutants, as well as vigorous surveillance and early public health intervention to limit further adaptation. Additionally, the mutations that accumulate during adaptation pro-

vide vital biological information crucial for the interpretation of outbreak sequence evolution. Unique epidemiology and pathogens make the course of nosocomial outbreaks unpredictable, complicating efforts to plan for the next outbreak. Nonetheless, a better understanding of infection traits involved in the epidemiology (e.g., virulence, metabolism, resistance) will likely facilitate integration of genomic and epidemiological analysis, allowing more effective targeting of intervention strategies.

14.6.2 Potential Role of Mobile Genetic Element Diversity During Hospital Adaptation

Although genome sequencing has improved our understanding of the mutations and genetic polymorphism associated with *S. aureus* strains isolated from patients during invasive and hospital-associated infections [99–101], the contribution of mobile genetic elements (MGE) remains under-investigated. MGE discovery and identification are important goals for genome analysis because almost all *S. aureus* strains harbor one or more MGE with potentially syndrome-specific and tissue-specific functions. Staphylococcal MGEs include plasmids, transposons, integrons, genomic islands, *S. aureus* pathogenicity islands (SaPIs), integrative conjugative elements, staphylococcal chromosome cassettes, and phages [102–104]. MGEs are abundant, representing 15–20% of the *S. aureus* genome. Staphylococcal MGEs are thus an important source of variation in *S. aureus* strains and remain, for the most part, functionally uncharacterized. Together, phages and plasmids are the main source of MGE diversity among *S. aureus* strains [105].

Phages are bacterial viruses that can integrate into the *S. aureus* chromosome. As the primary vehicles for horizontal transfer of MGEs between strains, phages are major drivers of staphylococcal genome evolution [106]. All *S. aureus* genomes sequenced to date contain at least one phage, and many strains carry up to four [107]. In addition to numerous hypothetical proteins, phages carry antibiotic resistance genes and virulence factors, such as the Panton-Valentine leukocidin (PVL) toxin [108, 109]. Phages also mobilize other MGEs, such as plasmids and staphylococcal pathogenicity islands (SaPIs) [103]. SaPIs, which integrate into specific attachment sites in the chromosome, employ phage machinery for replication and dissemination [102]. Thus, phages impact both the virulence and adaptability of *S. aureus*.

Genomic comparisons between successful *S. aureus* clones and distantly related strains have provided insights into MGE contribution to *S. aureus* adaptation [110–112]. For example, genomic studies have pinpointed the dramatic impact of MGE on the success of MRSA clones, such as USA300, the predominant CA-MRSA clone in the United States [113]. Notably, they highlighted the importance of the association between phage-derived PVL with purulent skin and soft tissue infection and with necrotizing pneumonia and other syndrome-specific MGE, such as the arginine catabolic mobile element [111, 112]. Other

studies have extensively characterized the contribution of MGE to *S. aureus* antibiotic resistance [108, 114].

Recent data suggest that genomic rearrangement through MGE exchange can contribute to the success during within-host adaptation of *S. aureus* infection [115]. An example is given by the regulation of β-toxin expression through bacteriophage φSa3 excision. β-toxin is a sphingomyelinase, encoded by virtually all *S. aureus* strains, that exhibits human cell cytotoxicity [116]. Despite the presence of the gene, the majority of human *S. aureus* isolates are reported not to express β-toxin due to integration of the bacteriophage φSa3 into the β-toxin structural gene *hlb* [117]. φSa3 encodes accessory virulence genes reported to be involved in immune evasion, including superantigens, staphylokinase, chemotaxis inhibitory protein, and staphylococcal complement inhibitor [117]. Given the human specificity of these gene products and the high incidence of φSa3 among human isolates, it has been suggested that the bacteriophage-encoded virulence factors provide a greater advantage to *S. aureus* for human colonization and initial survival than β-toxin production [115]. Recent studies, however, demonstrated that φSa3 inactivation of β-toxin is reversible [115, 118]. Indeed, *S. aureus* strains from infective endocarditis patients showed evidence of phage excision and β-toxin restoration compared to isolates from nares of healthy individuals or to laboratory strains. Collectively, these observations suggest that during invasive infection, host pressure favors MGE rearrangements with consequent variation in the regulation of genes, possibly benefiting *S. aureus* survival and disease progression in vivo.

Identification and discovery of novel MGEs involved in adaptation is complicated by a multitude of repeat regions and mosaic elements that cannot be assembled using traditional "short-read" sequencing instruments. However, de novo sequence assembly using a combination of short-read and long-read third-generation sequencing can provide a comprehensive characterization of MGEs and repeat regions [119, 120]. Although "hybrid" long- and short-read sequencing is currently costlier than short-read sequencing alone, the cost is offset by much greater accuracy in MGE analysis. Moreover, increases in long-read sequencing accuracy, throughput, and reduced multiplex sequencing costs will likely eliminate the need for hybrid analysis in the near future.

Inconsistent annotation and nomenclature in public databases is a complicating factor. For example, ~40% of *S. aureus* genes have inconsistent annotation across *S. aureus* lineages due to the lack of an adequate framework for ensuring standardized genome analysis methods and curation of *S. aureus* meta-data [105]. This percentage reaches 90% when applied to phages and plasmids, which represent the main source of MGE diversity among *S. aureus* public sequences [105]. As whole-genome sequencing tools improve and the number of sequenced genomes multiply, the need for curated databases will be crucial for rapid sequence analysis across strains. Elimination of the time-constraining process of creating tools for analyzing strain-specific orthology promises to produce a significant resource for researchers seeking a greater understanding of *S. aureus* and other pathogens.

14.6.3 Potential Role of Metabolic Changes During Adaptation to the Hospital

Even modest genetic changes in a bacterial pathogen can have a dramatic impact on virulence factor expression and the host-pathogen interaction [121–123]. Although the presence or absence of a particular gene or polymorphism can be determined, we do not yet have tools to readily extract information about gene expression in an organism from the aggregate of its sequence data alone. Thus, given that multiple mutations and MGEs are involved in the process of host and hospital adaptation, it is not obvious that an understanding of any one mutation or change will be sufficient to define what constitutes a hospital-adapted strain.

The research challenge posed by these observations is to reduce the heterogeneity of hospital adaptation so that we can identify a proxy for hospital adaptation that can explain epidemiological patterns and clinical outcomes. To augment mutation discovery, we propose a novel approach focused on the phenotypic effects of mutation: analysis of gene expression, proteomic, and metabolic pathways using information-rich datasets derived from low-virulence MRSA populations. Once identified, pathways can be directly assessed for phenotypic and genetic variation in individual strains.

The metabolic underpinnings of bacterial virulence, host defense, fitness, and antimicrobial lethality underscore metabolic capacity as an attractive candidate to determine the ability of MRSA to adapt to the hospital environment. The frequent occurrence of dysfunctional tricarboxylic acid (TCA) *cycle* activity in clinical isolates of staphylococci supports the idea that genetic adaptation can redirect metabolism [124], as does the observation that downregulation of TCA cycle activity is common among small colony-variant phenotypes that are associated with chronic infections and antimicrobial resistance [125–127].

These observations give rise to the hypothesis that MRSA use mutation to fine-tune their metabolism to sustain fitness in response to selective pressures in the hospital environment. As CA-MRSA become HA-MRSA, metabolic heterogeneity ensues. Our working model is shown in Fig. 14.6.

14.7 Concluding Remarks

We expect the cancer-biology style systems biology approach to enable characterization of the genotypic and phenotypic pathways of low-virulence MRSA populations that are evolving heterogeneity. The knowledge base generated can be used to describe the range of features of hospital-associated, low-virulence MRSA that are associated with complicated infection. Identification of adatpive phenotypes would open new avenues of further study to identify how mutations may create new vulnerabilities that can be used for treatment. This hope arises by analogy with the situation in typical cancers in which mutational loss of DNA repair checkpoints favors clonal expansion but also confers sensitivity to DNA-damaging agents.

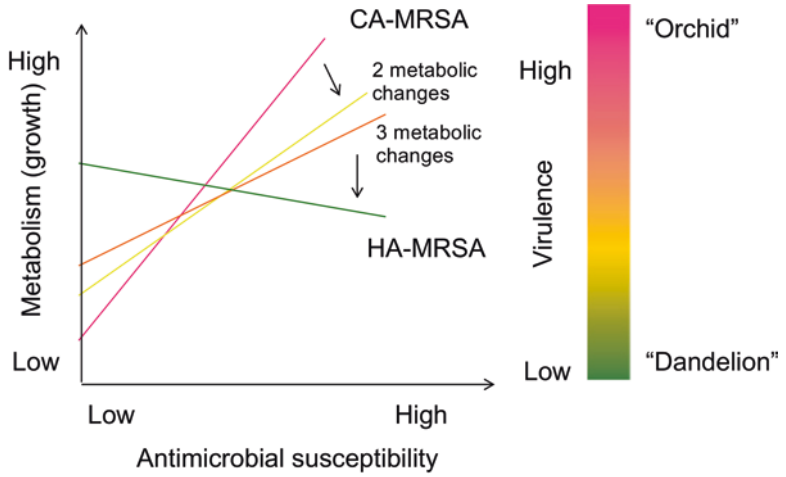

Fig. 14.6 Differential susceptibility hypothesis of nosocomial adaptation. Current work focuses on resistant and susceptible *S. aureus*; we hypothesize that *S. aureus* displays a spectrum of metabolic states that causes strains to differ in the degree to which they tolerate host and antimicrobial stress. Hospital-adaptation is accompanied by distinct stress tolerance states that are associated with distinct metabolic states, and these metabolic states can be identified as signatures that define specificity for different pathways to metabolize substrates, as well as differences in virulence phenotypes and functions. Differential susceptibility of an organism to stress is sometimes called the orchid hypothesis. In the figure, the boundary between community and hospital-adapted states is not distinct; however, CA-MRSA are like delicate orchids; and HA-MRSA are like dandelions. The former are highly virulent, and if left untreated causing more severe disease, they quickly wither if exposed to antimicrobial stress. The latter prove resilient to the effects of antimicrobials, but at the same time, they are not particularly virulent. CA-MRSA represents a "high" metabolic state, although high-metabolic states may be favored during certain hospital environments, which can account for inter-strain variability. In this scenario, the Goldilocks metaphor is applicable; extreme states impair fitness and are considered either deleterious or "short-sighted." Metabolism refers to growth rate and metabolic "complexity"

Efforts to understand the pathogenesis of infections often assumes that for a given pathogen, organisms respond equally to management attempts and that interventions, such as antibiotics, should therefore equally help all patients with a given infection. During adaptation to the hospital, a variety of strong selective pressures are exerted on the pathogen. The resulting genetic changes that occur might influence clinical outcomes and affect diagnosis and epidemiology. Identification of hospital and host-adaptive distinctions could be used with future point-of-care diagnostics to screen for characteristics that define patterns of MRSA epidemiology and clinical outcomes. Adaptations that erode the activities of bactericidal antimicrobials might also inform the potential use of novel strategies to provide an adjunctive to selectively bolster the effectiveness of antimicrobials [82–84, 128–130].

Finally, we note that dentification and characterization of infection-associated phenotypes is also significant from a basic research perspective: identification of

changes that have a profound impact on the *S. aureus* transcriptome and on virulence has important implications for the study of gene regulation in the pathogenicity of CA-MRSA infection. For example, differences in pathology observed in infections caused by mutants constructed in the laboratory may be obscured or compounded by a failure to undergo mutation of *agr* to one form or another: only careful comparison of strains going into and coming out of a host can identify the extent to which this is a problem. Thus, field work is essential. By unraveling the evolutionary steps occurring during infection and characterizing the genetic basis of such changes, we expect to provide a framework for interpreting the phenotype of newly emergent clones.

Major Points

- Functional compromise of pleiotropic regulators, which control many genes in complex genetic networks, provides a broad framework in which alterations in cellular circuitry promote the emergence of new traits.
- Mutation of *agr* is an explicit example of a mechanism by which hospital adaptation can alter the relationship between virulence, infectivity, and host and synthetic antimicrobial susceptibility.
- Mechanisms of adaptation-dependent changes in antimicrobial susceptibility include direct and indirect effects on fitness and antimicrobial tolerance.
- Mutations in genes such as *agr* may be adaptive for survival in the infected host, but they are counter-adaptive outside infected host tissues or in situtations that involve high attack rates, such as outbreaks.
- Hospital adaptations are likely to derive from a range of heritable changes in virulence attenuation, metabolic activity, and antimicrobial susceptibility, all of which are linked to the oxidative stress network that participates in host and synthetic antimicrobial-mediated killing.
- Identification of polymorphisms and targets of selection that affect the cellular response of MRSA to antimicrobial drugs may increase our understanding of drug tolerance mechanisms; such identification will also provide an initial step toward predictive tailoring of drug treatments to individuals to maximize therapeutic benefit.

Acknowledgments We thank Karl Drlica for helpful discussions and critical comments. Our work was supported by NIH grants AI103268 and N272201400019C.

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Chapter 15
Bacterial Signal Transduction Systems in Antimicrobial Resistance

Andrew T. Ulijasz, Sarah C. Feid, and David G. Glanville

15.1 Introduction

Bacteria must continually sample and assess their environment to survive. Once pathogens are inside a host, they must detect important external stimuli, including nutrient availability, oxygen levels, osmolarity, temperature, and even light quality [1]. Pathogens must also be able to respond to assaults from other competing microbes within the host, as well as antibacterial activity from the host itself. Indeed, bacteria produce an entourage of antimicrobial compounds to assure that a particular niche is maintained in the presence of other bacterial competitors or, in the case of the host, factors that are produced with the sole intent of clearing the invasive microbe. It is important that the microbial pathogen responds to such environmental signals only when necessary. This phenomenon is often referred to as inducible responses. Bacteria (and most life as we know it) use inducible systems to respond to a present threat rather than to anticipate one, as the response itself is usually entergetically costly (*e.g.*, transcription and translation require ATP, *etc*.). Consequently, such energy, if its use is not required, is better redirected to another task or stored for a later time. From these considerations it is thus reasonable to conclude that antimicrobial resistance and/or tolerance can have a severe fitness cost, and thus they must be tightly regulated to ensure the long-term survival of an invading microbe.

In order to properly respond to their changing environment in the most efficient manner, bacteria have evolved intricate detection mechanisms called signal transduction systems. These systems are based largely on the transfer or "relay" of a phosphate, as a posttranslational modification, from one protein moiety to another

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I. W. Fong et al. (eds.), *Antimicrobial Resistance in the 21st Century*, Emerging Infectious Diseases of the 21st Century, https://doi.org/10.1007/978-3-319-78538-7_15

[2], although many other posttranslational modifications are now surfacing as equally important signaling components (*e.g.*, acetylation [3] and oxidation [4]). Signal transduction systems sense the threatening antimicrobial and subsequently elicit a tailored relay to counter the drug's deleterious effects [5–7]. In addition, signal transduction systems and other proteins that may not have explicitly evolved for the sole purpose of conveying antimicrobial resistance (AMR) can incur amino acid changes that provide an evolutionary advantage over wild-type cells in avoiding the effects of antibiotic treatment [8]. These resistance sensory and response mechanisms were recognized shortly after the first antibiotic, penicillin, was discovered in 1929. However, it appears that AMR predates the widespread use of antimicrobials in modern times, suggesting that these molecular mechanisms have been repurposed through evolutionary pressures in today's antibiotic era [9]. The result is that signal transduction systems contribute to one of the most important medical problems of our time: antimicrobial ineffectiveness [10].

In the most simplistic bacterial signaling system, one protein directly binds the antimicrobial and elicits a transcriptional response. These systems, commonly referred to as "one-component" systems, are the most common among the bacterial signaling family and often take the form of a single transcription factor [11]. However, more complex signaling cascades are usually observed with antibiotic resistance mechanisms. Those involving two or more components, the most prevalent family, are aptly referred to as two-component signaling (TCS) systems [5]. TCS systems usually consist of a membrane-bound sensory histidine kinase that, upon stimulation by a specific environmental que (e.g., the direct or indirect sensing of an antimicrobial), dimerizes and autophosphorylates at a conserved histidine residue (Fig. 15.1). This action leads to a phosphoryl relay from the modified histidine residue to a conserved aspartate residue on a transcription factor called a response regulator [2, 5]. Although exceptions to this canonical mechanism exist [12], in general the response regulator then changes conformation to allow its DNA-binding domain to access specific *cis* elements within bacterial promoters, which ultimately leads to transcriptional initiation and an ensuing targeted response to the threatening antimicrobial. TCS systems usually respond to a specific stimulus, and they phosphorylate only one target: their cognate response regulator (Fig. 15.1). Both the histidine kinase and its cognate regulator target are most often found as co-transcribed operons in bacterial genomes; however exceptions to this rule exist [12]. The number of TCS systems present in bacteria can range from one to over a hundred [5].

In recent years, additional bacterial signaling systems have been discovered that parallel eukaryotic signal transduction systems. These include the so-called bacterial eukaryotic-like serine/threonine kinases (eSTKs) [2, 6], phosphatases (eSTPs), and tyrosine kinases [13, 14] (Fig. 15.1). In the case of eSTKs, which are most relevant to our discussion in this chapter, the domain architecture usually consists of an N-terminal intracellular kinase domain, which strongly resembles the eukaryotic versions, followed by a single transmembrane segment and an extracellular/periplasmic sensory domain that is unique to the microbial systems. Interestingly, atomic structure comparisons indicate that eSTK kinase domains have a structural

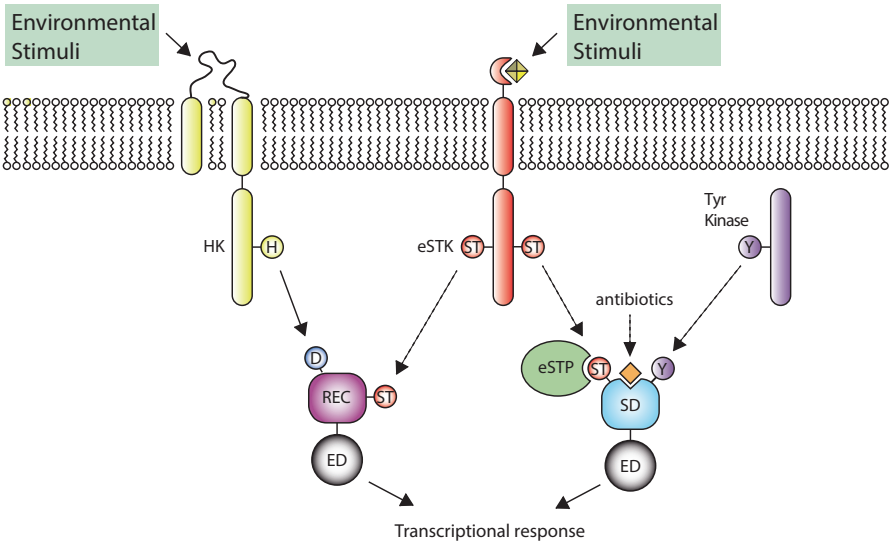

Fig. 15.1 Bacterial signaling basics. Classical two-component signaling (TCS) involves an initial sensory input, usually detected from outside the bacterial cell by a membrane-bound histidine kinase (HK). This signal results in autophosphorylation of the HK on a conserved histidine (H) residue, whose phosphate group is then transferred to a conserved aspartate residue (D) on a receiver (REC) domain. This posttranslational modification (PTM) then results in conserved structural rearrangements that ultimately activate the effector domain (ED) of the receiver, which can take many forms including DNA binding (most common). Eukaryotic-like serine-threonine kinases (eSTKs), their cognate phosphatases (eSTPs), and tyrosine (Tyr) kinases can also phospho-regulate TCS systems or one-component systems, the latter which have been shown to directly bind antibiotics in many cases through a sensory domain (SD). The end result is the sensory input initiates a transcriptional response to the external threat

fold that is almost identical to their eukaryotic homologs [6, 15], suggesting that the mechanism by which they act is evolutionarily conserved between kingdoms. Especially prominent, and relevant to our discussion, are the so-called PASTA (penicillin-binding protein and serine/threonine kinase associated) eSTKs found in Gram-positive pathogens. PASTA eSTKs contain repeat PASTA domains that are suspected to directly interact with the peptidoglycan and/or free peptidoglycan fragments. They serve as crucial regulatory players in bacterial cell division, cellular stress responses and infection [6, 15]. In general, Gram-positive bacteria have only one eSTK/eSTP pair, or a single tyrosine (Tyr) kinase, although some species have many more. For example, *Mycobacterium tuberculosis* possesses 11 eSTKs (but only a single eSTP [6]). Another difference from the two-component systems is the pleiotropic nature of the targets for eSTKs and Tyr kinases, where many substrates have been identified, including transcription factors ([6, 14, 15]; Fig. 15.1). Surprisingly, these eukaryotic-like signaling proteins in bacteria also directly regulate TCS proteins [16]. In addition to the multitude of other signaling proteins involved in bacterial AMR sensory systems, this observation suggests a complexity

in bacterial signaling that has been underappreciated. Understanding such complexities should lend insight into how bacteria use their signal transduction systems to avoid treatment effectiveness and host immunity assaults. Thus, research in this area has the potential to generate novel therapeutic modalities.

This chapter focuses on these bacterial signaling systems and how they play a pivotal role in AMR. Examples of specific signaling systems that lead to resistance will first be discussed, followed by signaling systems that respond to host cell antimicrobial peptides (AMPs), systems involved in biofilm regulation, and finally mechanisms of bacterial "persistence." It should be noted that these topics are not mutually exclusive, and therefore they overlap. For example, bacterial persisters are a large component of biofilms and greatly contribute to the tolerance of biofilms to antimicrobial treatment [17].

15.2 Examples of Signaling and Antibiotic Resistance

15.2.1 Signaling Mechanisms of Vancomycin Resistance

One of the first examples of inducible signal transduction-regulated AMR emerged from vancomycin-resistant enterococci isolates that were obtained in 1988 in Europe, 34 years after this antibiotic's introduction [18]. Vancomycin-resistant and vancomycin-tolerant isolates were subsequently found in the USA and, unfortunately, have now spread throughout the world to include diverse microbial species [18]. Vancomycin is a "drug of last resort" and is on the World Health Organization's list of essential medicines, as it is used to treat Gram-positive infections that are unresponsive to other treatments, in particular methicillin-resistant *Staphylococcus aureus* (MRSA).

Vancomycin was isolated from the soil-dwelling *Actinomycete*: *Amycolatopsis orientalis* [18]. The drug targets the bacterial cell wall, binding with high affinity to the D-Ala-D-Ala portion of the pentapeptide component, effectively preventing transglycosylation to the nascent peptidoglycan chain. This binding results in a subsequent blocking of transpeptidation cross-linking [19]. Resistance to vancomycin is due to replacement of the D-Ala-D-Ala vancomycin target with D-Ala-D-Lac or D-Ala-D-Ser, both of which exhibit an approximately 1000-fold lower affinity for the antibiotic. Several different variations of aminoglycoside resistance are conferred by this same mechanism, depending on the precise microbe and genes involved (VanA-, VanB-, VanD-, VanE-, and VanG-type resistance).

15.2.1.1 *Enterococcus* sp.

The classic example of inducible vancomycin resistance is observed with the enterococci (*e.g.*, *E. faecium* and *E. faecalis*), where it was originally observed in the late 1980s [18]. Among the enterococci, VanA-type strains display high levels of

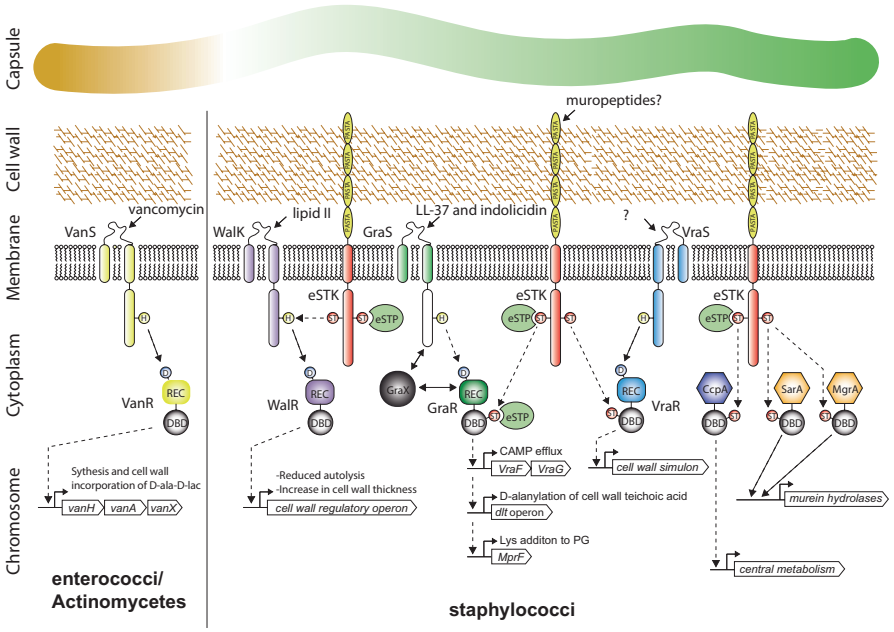

Fig. 15.2 Vancomycin resistance and tolerance signaling mechanisms in the enterococci and *S. aureus*. In the enterococci, a single two-component signaling (TCS) system conveys vancomycin resistance through the vanHAX operon. This histidine kinase VanS has been shown to likely bind vancomycin directly to initiate a D-Ala-D-lac modification of the cell wall pentapeptide. In *S. aureus* there are at least three TCS systems responsible for the VISA phenotype, WalKR, GraSR, and VraSR, all of which are controlled through the *S. aureus* serine-threonine kinase Stk1 (an eSTK). The serine-threonine phosphatase (eSTP) acts on Stk1 and its substrates for counter-regulation. The histidine kinase WalK responds through contact with lipid II, and GraS binds and responds to the human CAMP LL-37. The VraS ligand remains unknown. The ligand for Stk1 is still under debate, but is suspected to be cell wall muropeptides. Dotted lines represent direct regulation by phosphorylation; dashed lines represent regulatory interactions with GraX. ST, serine-threonine phosphorylation; D, aspartate phosphorylation; H, histidine phosphorylation; REC, receiver domain; DBD, DNA-binding domain; PASA, penicillin-binding and serine-threonine kinase associated domain

inducible resistance to both vancomycin and teicoplanin aminoglycosides, whereas VanB-type strains are only resistant to vancomycin [19]. In the more common VanA-type strains, the resistance operon generally contains three core genes required for peptidoglycan modification and resistance, namely, *vanH*, *vanA,* and *vanX*. The VanH protein is a dehydrogenase that reduces cellular pyruvate to D-Lac, VanA is a ligase that catalyzes the formation of the new D-Ala-D-Lac dipeptide that is incorporated into the cell wall, and VanX is a dipeptidase that assists in eliminating the wild-type D-Ala-D-Ala dipeptide pool by cleaving the D-Ala-D-Ala peptide bond. Accessory proteins VanY and VanZ participate in the hydrolysis of the terminal pentapeptide D-Ala residue. VanZ confers resistance to teicoplanin by an as-of-yet unknown mechanism ([19]; Fig. 15.2).

Adjacent to the *vanHAXYZ* operon is the TCS pair VanRS, which controls the synthesis of *vanHAXYZ* and therefore renders vancomycin resistance inducible [19]. Upon addition of vancomycin, the histidine kinase VanS phosphorylates the response regulator VanR. VanR then binds the *vanHAXYZ* promoter, inducing the operon, thereby conferring resistance to the drug [20]. Deletion of VanS results in a constitutively active phenotype [19]. To date, the precise mechanism for how vancomycin interacts with the Gram-positive versions of VanS to induce resistance has remained enigmatic. However, in recent years progress has been made with the study of the VanS histidine kinase-driven system from *Actinomycetes*, the bacteria that naturally produce vancomycin and therefore require their own inducible resistance mechanism when manufacturing the antibiotic. Wright and colleagues were the first to show that the *Actinomycete* version of VanS directly binds vancomycin [21]. The antibiotic binds within the first 41 N-terminal residues of the protein, which comprise the first transmembrane region plus a predicted short extracellular sensory peptide containing the sequence DQGW. Vancomycin is predicted to interact with this peptide based on biochemical data [21] (Fig. 15.3). These data hold true for *Actinomycete* versions of VanS, which directly bind vancomycin and confer intrinsic resistance (*i.e.*, resistance to vancomycin by these native producers [21]). On the other hand, the mechanism by which the enterococci, staphylococci, and Gram-positive pathogens in general sense the presence of vancomycin through the VanS histidine kinase receptor has yet to be determined. The primary amino acid sequences of the *Actinomycete* VanS are divergent enough from the enterococcal and staphylococcal versions to suggest a possible alternative mechanism (Fig. 15.3) that might include more indirect signaling mechanisms, such as interactions with cell envelope components. However, a recent study indicates that vancomycin and teicoplanin might bind directly to the receptor at aromatic residues, similar to what was observed with *Actinomycetes* [22]. Indeed, an alignment of VanS receptors shows that some aromatic residues, such as the tryptophan residue proposed to directly interact with vancomycin [21], are conserved among VanS versions (Fig. 15.3). An interesting topic of future research would be to resolve exactly how VanS from pathogenic Gram-positive bacteria sense the presence of vancomycin.

15.2.1.2 *Staphylococcus aureus*

Although it is clear how vancomycin resistance in both the *Actinomycetes* and pathogenic enterococci rely on the specific *vanRS/vanHAX* operons, the signaling cascade and ensuing mechanism of resistance in *S. aureus* and other staphylococci appear more diverse and are therefore far less clear [23]. In rare cases, the *Enterococcus* VanA genes have been found within vancomycin-resistant *S. aureus* strains. These *S. aureus* strains, which have presumably acquired the enterococcal resistance by horizontal transfer, produce the high levels of resistance observed in vancomycin-resistant *Enterococcus* strains; they are collectively referred to as high-level vancomycin-resistant *Staphylococcus aureus*, or VRSA strains. A more common form of *S. aureus* vancomycin resistance seen in the clinic results from acquired

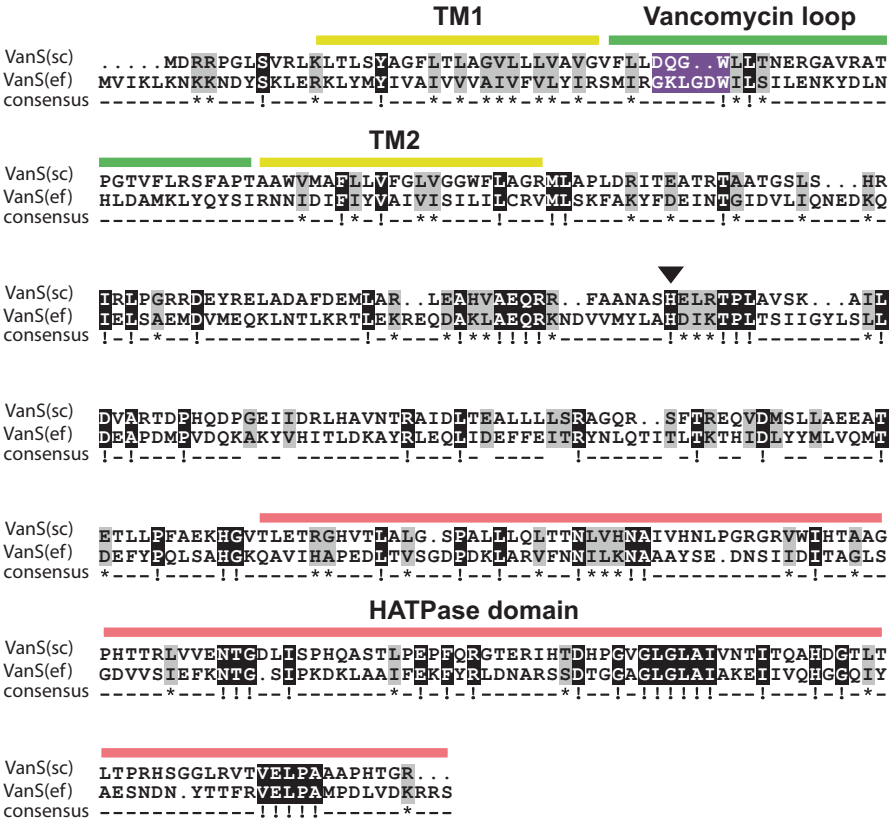

Fig. 15.3 Alignment of VanS from *Streptomyces coelicolor* (VanS(sc)) and VanS from *Enterococcus faecium* (VanS(ef)). Yellow bars indicate transmembrane (TM) regions, the green bar the vancomycin sensory loop, and the red bar the histidine kinase ATPase domain. The arrow indicates the phosphorylated histidine. The purple highlighted area indicates the residues (DQGW) proposed to bind vancomycin directly in VanS(sc). Black-colored residues are identical and gray similar. For consensus,! is identical residues and * similar

mutations that lead to in an intermediate resistance phenotype. These strains are commonly referred to as vancomycin-intermediate *Staphylococcus aureus*, or VISA [8, 23] (see Chap. 9 for additional discussion of VISA). The VISA mechanism appears to involve decreased cell wall cross-linking and, importantly, a thicker cell wall that is thought to act as a mechanical barrier to vancomycin uptake by acting as a "vancomycin sponge" (a phenomenon sometimes referred to as the "cell wall clogging effect"). Genomic comparisons between VRSA and VISA strains indicate that many genes are involved in producing VISA traits, although only a handful of specific contributions have been experimentally verified (see Refs [8] and [23], and references therein). Not surprisingly, many of these mutations occur in signal transduction proteins that control the intermediate-resistance phenotype.

Signal transduction system genes that produce *S. aureus* VISA phenotypes include three TCS pairs (VraSR, GraSR, WalKR) plus the *S. aureus* eSTK/eSTP signaling pair [6, 8] (Fig. 15.2). Although complex, it is now becoming more clear how these signaling proteins might act to reduce susceptibility. The VraS (histidine kinase) and VraR (response regulator) pair regulate a cell wall synthesis stimulon in *S. aureus*. Mutations within the VraSR TCS pair, found in a clinical isolate, correlate with an overactive or constitutively active cell wall synthesis phenotype [23]. However, VraSR mutations alone do not always confer VISA – only after additional mutations arise in the GraSR TCS pair is a VISA phenotype likely to emerge [24].

The GraSR system is responsible for host defense cationic antimicrobial peptide (CAMP) resistance [25]. Upon stimulation by direct binding of host CAMP LL-37, the GraS histidine kinase phosphorylates the GraR response regulator transcription factor. The phosphorylated GraR then initiates transcription of GraX and an ABC transporter called VraFG. Together, these five proteins change the net surface charge of the microbe, which results in repulsion of the CAMP [25]. Alone, mutations in the GraSR system cause only a marginal effect on susceptibility to the antibiotic [23, 24] (Fig. 15.2).

A third TCS system that contributes to the VISA phenotype is the WalKR TCS cognate pair (also referred to as YycGF). The WalK histidine kinase and WalR response regulator are rare among the TCS family of signaling proteins as they are essential for bacterial viability. Interestingly, their essential nature is conserved in many other Gram-positive pathogens, as is their important function in controlling the regulatory connection between cell wall biosynthesis and cell division (for review see Ref. [26] and references therein). Mutations resulting in downregulation of the WalKR function result in a marked decrease in vancomycin effectiveness, whereas mutations resulting in WalKR upregulation result in cells becoming more susceptible to treatment [8, 23]. Unlike the VraSR and GraSR systems, it appears that WalKR mutations can autonomously control vancomycin susceptibility in *S. aureus* [23].

Mutations within the *S. aureus* eSTK (also referred to as PknB or Stk1) and eSTP (also called Stp1) pair also contribute to the VISA phenotype (Fig. 15.2). Indeed, VraR is a substrate for the *S. aureus* eSTK, such that phosphorylation negatively impacts DNA binding and thus VraR activity [27]. Furthermore, eSTK phosphorylates several other transcription factors associated with vancomycin susceptibility, including catabolite control protein A (CcpA) and the murine hydrolase regulators SarA and MgrA [28] (Fig. 15.2). Each of these transcription factors has been associated with a loss of susceptibility to vancomycin [6]. Interestingly, the *S. aureus* eSTK (Stk1) directly regulates SarA and MgrA by phosphorylating a cysteine residue on each. This observation represents the first example of a cysteine posttranslational modification having a clear regulatory role [28]. Finally, deletion of the *S. aureus* eSTP (Stp1), which would result in a constitutive eSTK phosphorylation of the GraR, VraR, CcpA, and SarA transcription factors, produces the thickened cell wall phenotype associated with vancomycin and teicoplanin intermediate resistance [29]. Although not yet demonstrated in *S. aureus*, it has recently been shown that an eSTK directly phosphorylates the WalK histidine kinase to regulate antibiotic sus-

ceptibility [16]. This novel discovery demonstrates that eSTKs and TCS can work together to co-regulate essential cellular processes and antibiotic susceptibility, and opens new opportunities and strategies for bacterial signaling perturbation to facilitate novel antibiotic treatments.

15.2.1.3 *Streptococcus pneumoniae*

S. pneumoniae is the most common cause of community-acquired bacterial pneumonia and a major cause of many other serious infections, such as meningitis and otitis media [30]. Despite an available vaccine, pneumococcal infections continue to be a problem, as the immunization covers only a small subset of strains (see Chap. 2). After introduction of a pneumococcal vaccine, an epidemiology shift occurs away from the vaccine serotypes. This "vaccine escape" phenomenon, combined with an increase in antibiotic-resistant strains, has placed *S. pneumoniae,* along with *Enterococcus* and *Staphylococcus* species, on the list of the Center for Disease Control's (CDC) "biggest threats" to public health due to AMR [10]. Although vancomycin resistance in *S. pneumoniae* has yet to be described, tolerant clinical isolates have been reported.

S. pneumoniae possesses 13 TCS pairs and 1 orphan response regulator [31] (*i.e.*, a TCS response regulator that lacks a known cognate kinase). The VncRS TCS system was initially reported to be responsible for vancomycin tolerance in *S. pneumoniae* [32]. However, with the exception of the ABC transporter (Vex123) that was described as the VncRS target-regulated gene in the original report [33], these findings were later proven to be artifactual [34]. More recently it was found by another group that the CiaHR TCS pair helps confer the vancomycin tolerance phenotype, although CiaHR is better known for controlling beta-lactam resistance in this pathogen (see below) [35]. Surprisingly, only a single mutation within the CiaH histidine kinase gene (within Ser198) is enough to convey tolerance, but only in the presence of the pneumococcal polysaccharide capsule and in the absence of the pneumococcal autolysin LytA [36]. Another recent report implicates a PadR family transcription factor called PtrR and its vancomycin-inducible control over a four-gene operon (*ptvABC*) in vancomycin tolerance. The *ptvABC* operon reportedly encodes membrane-associated proteins whose precise function remains enigmatic [37]. The mechanism with which these seemingly unrelated transporters, signaling proteins, and the capsule components act to confer loss of susceptibility to vancomycin in *S. pneumoniae* remains to be deciphered.

15.2.2 Signaling Mechanisms of Beta-Lactam Resistance

Beta-lactams, which are among the most widely prescribed antibiotics, were the first to be discovered (in the 1920s by Alexander Fleming at St. Mary's Hospital, now part of Imperial College London) [38]. Members of this antibiotic class target

the aptly named penicillin-binding proteins (PBPs), enzymes that are responsible for catalyzing the later steps in the assembly of the bacterial cell wall. When applied, the beta-lactams can cause malformation of the cell wall, cell lysis, and death. Due to the early introduction and overuse of beta-lactams, especially with Gram-positive pathogens, resistance is now widespread. As with many cell envelope-acting antibiotics, beta-lactam susceptibility is controlled through bacterial signaling proteins.

15.2.2.1 *Streptococcus pneumoniae*

A classic example of a signal transduction system controlling beta-lactam resistance is found with the *S. pneumoniae* CiaRH TCS pair, which has also been implicated in competence (DNA uptake) regulation and cell lysis [35]. This TCS system monitors cell envelope integrity; however, the mechanism by which this occurs, including the exact inducer/molecule sensed by the CiaH kinase, remains unknown. What is understood is that CiaRH responds to a diverse array of beta-lactams, including advanced generation derivatives, such as cefotaxime and the Gram-negative-acting piperacillin. Using these antibiotics, Hackenbeck and colleagues have isolated resistant laboratory mutants [39]. Additionally, the genomes of several clinical isolates have been sequenced and the influence of the mutations on beta-lactam resistance and CiaRH promoter-regulated activity assessed (11 mutations in total [39]). Data from these studies demonstrated that the mutations generally result in an increase in CiaRH activity and ensuing regulatory gene expression, which ultimately leads to a loss of beta-lactam susceptibility. A more in-depth survey of 3085 Thai and 616 US pneumococcal isolates as independent datasets demonstrated a high mutation frequency within the *ciaH* kinase allele, resulting in its hyperactivation and ensuing beta-lactam resistance phenotype [40]. Combined with previous data, these findings indicate that signal transduction genes can mutate extensively during treatment, presumably to rapidly accommodate new environmental conditions and avoid the effects of antibiotics.

15.2.2.2 *Staphylococcus aureus*

S. aureus presents one of the most serious problems with respect to beta-lactam-resistant hospital infections [10]. After the introduction of penicillin and the ensuing development of resistance provided by penicillinase, a semisynthetic penicillinase-resistant beta-lactam called methicillin was introduced in 1959. Within a year resistant strains emerged, and MRSA was born as a problematic nosocomial pathogen [41]. Although methicillin is no longer in clinical use, MRSA strains of *S. aureus* continue to become resistant to new beta-lactams. Resistance is conferred by introduction of an alternative PBP target (called PBP2a), which lowers the affinity of the antibiotic. PBP2a is encoded by the *mecA* gene, which is in a mobile genetic element that is referred to as *SCCmec*. MecA is under control of an inducible signaling system, as depicted in Fig. 15.4.

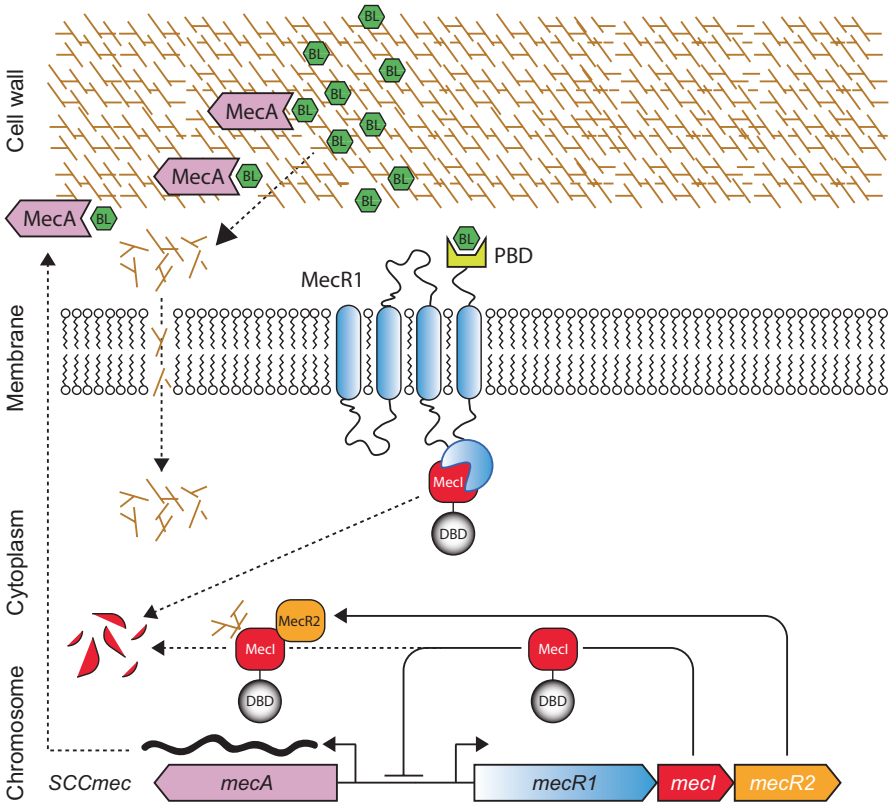

Fig. 15.4 MecA (PBP2a)-mediated regulation in *S. aureus*. MecI is a transcription factor that binds to the promoter of *mecA* (PBP2a) and represses its transcription until it is degraded via MecR1-mediated proteolysis. MecR1 contains a penicillin-binding domain (PBD) that senses the beta-lactam (BL, green octagons). The binding of cell wall fragments (specifically g-D-Glu-L-Lys) and MecR2 to MecI destabilizes its binding to DNA and enhances MecR1-mediated degradation. Once *mecA* repression is remediated, MecA is produced and binds the beta-lactam to lessen drug susceptibility

In contrast to the inducible resistance pathways discussed above, beta-lactam resistance in MRSA is induced via a proteolytic signaling system encoded within *SCCmec*. This resistance mechanism parallels those associated with the "bla" systems found in wild-type *S. aureus* and *Bacillus licheniformis*. In MRSA, the signaling is carried out by an integral membrane receptor, MecR1, and its intracellular relay target, the winged helix-turn-helix transcription factor MecI. MecR1 senses the presence of beta-lactams though its extracellular penicillin-binding domain (PBD). Intriguingly, upon activation though drug binding, MecR1 acts as a Zn-dependent metalloendopeptidase zymogen, autocatalytically cleaving its intracellular signaling domain, which then, directly or indirectly, binds/inactivates by cleavage of the MecI transcription factor. This action then results in the induction of MecA and resistance (Fig. 15.4). Interestingly, cell wall fragments, specifi-

cally γ-D-Glu-L-Lys, bind the C-terminal domain of the MecI repressor and act as a cofactor that mediates MecI cleavage and inactivation by MecR1 (Fig. 15.4). Once produced, the *mecA* (PBP2a) gene product interacts with beta-lactams. Other reports show that MecA is more complicated, as it is apparently also controlled allosterically through binding the D-Ala-D-Ala terminus of the pentapeptide stem, likely generated by the inhibition of cell wall synthesis by beta-lactams. The allosteric site requires occupation to then unveil the active binding pocket for the beta-lactam [42, 43].

15.2.2.3 *Enterococcus* sp.

The Gram-positive opportunistic nosocomial pathogen *Enterococcus faecalis* is a major cause of urinary tract infections and endocarditis, and it has now been widely associated with inflammatory bowel disease [44] and more recently with colon cancer [45]. *E. faecalis* and other enterococci are unfortunately naturally resistant to the cephalosporin beta-lactam antibiotics and many other antimicrobials including bile, a natural human-produced antimicrobial [46]. In 2007 it was determined that resistance is largely conferred by the sole eSTK in this organism [46].

Kristich et al. found that the *E. faecalis* eSTK (now renamed IreK) is responsible for controlling both antimicrobial resistance and intestinal persistence in this pathogen. An IreK mutant exhibits increased susceptibility to cephalosporins, as well as to bile; the mutation rendered *E. faecalis* unable to colonize the mouse intestine [46]. Although such a pronounced effect as seen with cephalosporins was not observed, resistance through IreK was also extended to other cell envelope-acting antibiotics, including ampicillin, bacitracin, and vancomycin. These data suggest a broad-based sensory mechanism. This broad specificity is likely due to unlinked peptidoglycan fragments that result from antibiotic-induced cell wall disruption [47]. Later work by the Kristich lab showed that IreK modulates its antimicrobial activity through phosphoryl regulation of a small protein substrate, IreB, and the IreK cognate eSTP, IreP [48]. Interestingly, identification of IreB in many other Gram-positive organisms having low GC content in their DNA suggests that this signaling system may be conserved outside of enterococci species [48].

Another two-component system, CroRS, was also found to be required for intrinsic beta-lactam resistance in *E. faecalis* [49]. A deletion of the CroRS system presents a dramatic phenotype, with a 4000-fold reduction in the MIC for the new, third-generation cephalosporin ceftriaxone. This MIC reduction is facilitated by the induction of PBP5 [47]. As with the IreK signaling system, resistance to structurally unrelated cell wall-targeting antibiotics bacitracin and vancomycin was also observed in the CroRS mutant. An expanded study determined that another TCS system, CisRS, could compensate for the loss of CroRS signaling. Interestingly, this system also compensates for the absence of the VanG-type resistance system in *E. faecalis*, suggesting that CisRS might act to compensate as a "surrogate" TCS cell wall stress response system [50]. The molecules that bind and activate the CroRS

and CisRS systems and the molecular mechanism underlying antibiotic resistance remain to be deciphered. Based on the differing targets and structures of the antibiotics that CroRS/CisRS respond to, it appears that the mechanism may involve general sensing of cell envelope stresses.

15.2.2.4 Gram-Negative Pathogens

Although beta-lactam resistance is widespread in Gram-negative bacteria, unlike Gram-positive microbes, the focus has generally been on the mechanism of the beta-lactam-degrading enzymes (beta-lactamases) that are responsible for resistance, rather than on the sensory systems involved. Nevertheless, like many antibiotic resistance mechanisms, beta-lactam resistance in Gram-negative bacteria is also controlled by signal transduction systems. In this section we discuss two of these signaling cascades.

In the *Enterobacteriaceae* family, the beta-lactam resistance determinant AmpC (a beta-lactamase [51]) is induced by the AmpG-AmpR (AmpRG) TCS pair. As a consequence of a beta-lactam being present, anhydrous *N*-acetylglucosamine-N-acetylmuramic acid (GlcNAc-MurNAc) oligopeptides from peptidoglycan accumulate in the periplasm. These oligopeptides are then transported into the cytoplasm by the inner membrane-associated AmpG transporter. Once in the cytoplasm, the GlcNAc moiety is removed by the glycoside hydrolase NagZ, liberating anhydro-MurNAc. Anhydro-MurNAc then binds the LysR-type transcription factor AmpR, resulting in a conformational change that enables promoter binding and activation of AmpC transcription (Ref. [52] and references therein). As production of AmpC taxes the overall energy requirements of the cell, another protein, the *N*-acetylmuramoyl-L-alanine amidase (AmpD), mitigates AmpC synthesis by cleaving the muropeptides and reducing accumulation of the inducing anhydro-MurNAc molecule.

Variations of the AmpG-AmpR-AmpC resistance theme are also found within other Gram-negative bacteria. For example, *E. coli* and *Shigella* species, as well as *Acinetobacter baumannii,* lack AmpR, which results in a low level of constitutive AmpC production. Introduction of a heterologous AmpR regulator into these AmpR-deficient species results in a reinstatement of the inducible system. In another example of AmpC operon divergence, the problematic cystic fibrosis pathogen *P. aeruginosa* genome harbors three redundant copies of AmpD, which results in a hyper suppression of AmpC transcription/translation. As successive AmpD copies are mutated during, for example, chronic infection with *P. aeruginosa,* beta-lactam susceptibility drops in a stepwise fashion [52].

In addition to the AmpG-AmpR system, TCS systems have also been implicated in AmpC induction by the BlrAB signaling cascade. The BlrAB histidine kinase response regulator phosphoryl relay has been studied in *Aeromonas* species, which are facultative anaerobes that cause a variety of human diseases (Ref. [52] and references therein). In *Aeromonas*, overexpression of the BlrB histidine kinase results in a marked increase in AmpC, presumably due to enhanced phosphorylation of the

BlrA response regulator. Standard bioinformatics analysis reveals that the closest homolog to BlrAB is the CreBC TCS system. Interestingly, the *Aeromonas* CreBC TCS, when introduced into in a heterologous host (*E. coli*), regulates the *E. coli* beta-lactamases. This regulation occurs through the *E. coli* CreC response regulator binding a conserved CreC-binding motif (TTCACnnnnnnTTCAC), which activates gene expression. A recent report confirmed this same binding motif for CreC in *Aeromonas*, and it importantly demonstrated that the CreBC system is specifically responsive to inhibition of PBP4 by beta-lactams [53]. The authors hypothesize that the BlrAB and CreBC TCS systems respond to peptidoglycan recycling and therefore levels of AmpC-inducing muropeptides that control beta-lactam susceptibility [53]. Future work will need to elucidate the precise mechanisms underlying the BlrAB and CreBC systems and determine whether their roles are ubiquitous among Gram-negative pathogens.

15.2.3 Signaling Mechanisms of Polymyxin Resistance in Gram-Negative Bacteria

Polymyxins are antimicrobials classified as cyclic peptides that contain a hydrophobic tail; the two classic examples are polymyxin B and colistin [54]. They are predominantly used against Gram-negative infections, but sometimes they are administered in combination with other antibiotics to treat Gram-positive infections. Because this antibiotic class was originally found to be both neuro- and nephrotoxic, their use dwindled in the wake of newer, less harmful choices (*e.g.*, beta-lactams and aminoglycosides). A further complication for their clinical use is that polymyxins are not absorbed in the gastrointestinal tract, and they must therefore be administered intravenously, by inhalation or by application topically for skin infections, for example. However, the recent rise of untreatable infections due to multidrug resistance has led to reintroduction of the polymyxins as a "new" drug of last resort.

Unlike the beta-lactams and aminoglycosides, the polymyxins target the bacterial cell membrane. There they bind to the lipopolysaccharide (LPS) through their cyclic peptide moiety and then disrupt both inner and outer membranes of the Gram-negative cell envelope. This mechanism of action is facilitated by the hydrophobic "tail" of the polymyxins, which is suggestive of detergent-like qualities [55].

Unfortunately, with the increase in polymyxin use has come the emergence of resistance. In general, a common theme among the polymyxins is that they work by ultimately changing the charge, or electrostatic repulsion properties, of the LPS such that the initial (and required for efficacy) cyclic peptide binding is blocked. This is accomplished by substituting moieties, such as 4-amino-4-deoxy-L-arabinose (L-Ara-4N), phosphoethanolamine (pEtN), or galactosamine enzymatically into Lipid A or the LPS core (Fig. 15.5). In some cases, the LPS is simply lost [54].

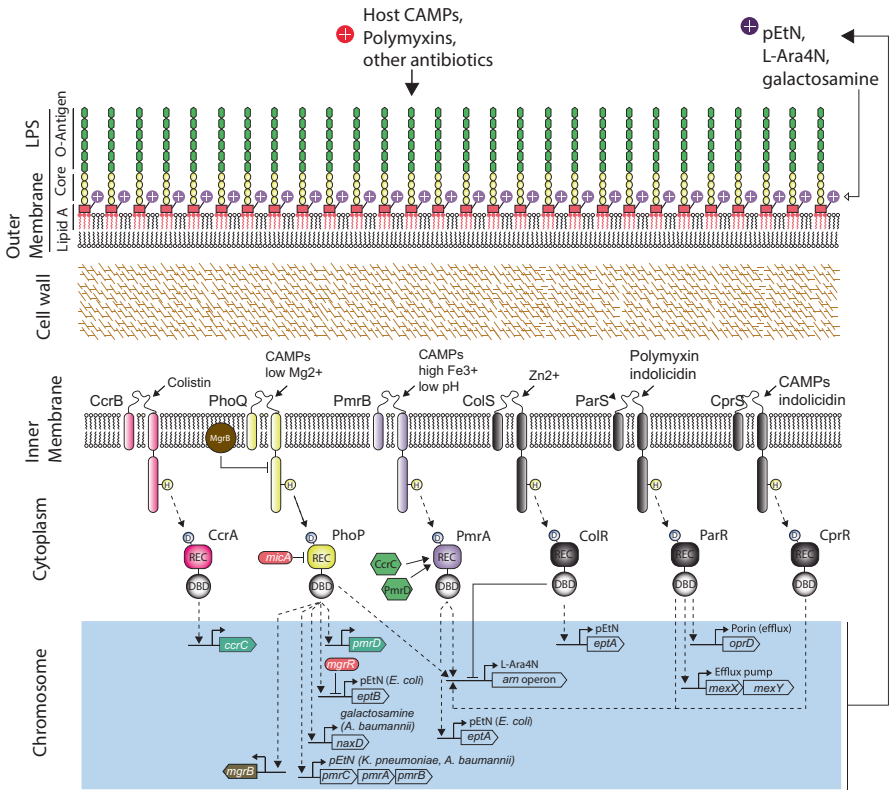

Fig. 15.5 The complex signaling pathways of Gram-negative polymyxin and CAMP resistance. TCS systems shown in gray (ColR, ParR, and CprR) are found in *P. aeruginosa*. The CcrABC system and MgrB are found in *K. pneumoniae*. Small regulatory RNAs *micA* and *mgrR* are found in *E. coli*. Sensory inputs vary depending on the pathogen. Here we show some common histidine kinase stimulants (*e.g.*, Mg^{2+}, Fe^{3+}) among the Gram-negative pathogens. For a complete list, refer to the text and references therein. Phosphoethanolamine (pEtN), galactosamine, and 4-amino-4-deoxy-L-arabinose (L-Ara-4N) molecules are all added to Lipid A of the LPS to modify the outer membrane charge. *A. baumannii* lacks a L-Ara-4N-modifying system and instead has galactosamine and pEtN enzymes. The mexXY/oprD multidrug resistance efflux system is found in *P. aeruginosa*

Since resistance costs valuable energy, signal transduction networks have been adapted by bacteria to control the antibiotic response on an as-needed basis.

15.2.3.1 *Enterobacteriaceae*

With the *Enterobacteriaceae* species, *Salmonella* species (*Salmonella*), *E. coli*, and *Klebsiella pneumoniae,* polymyxin resistance is controlled via two main TCS systems: PhoPQ and PmrAB (for comprehensive reviews see Ref. [54] and Ref. [56]). Induction of these systems has been investigated extensively, especially in

Salmonella, the pathogen species in which they were originally discovered [56–58]. Although much is still not understood about the complexities of how polymyxin resistance arises, resistance appears to be induced nonspecifically by the presence of cationic compounds through the action of TCS networks. Inducing agents include polymyxins, low magnesium (Mg^{2+}) concentrations, high ferric iron (Fe^{3+}) concentrations, and acidic pH [54, 56]. In some cases metals such as aluminum and zinc induce the polymyxin signaling cascade. Specifically, magnesium or cationic drugs stimulate phosphorylation of the histidine kinase PhoQ by direct interaction with an acidic patch within its sensory domain. PhoQ then dimerizes and phosphorylates the intracellular response regulator PhoP, which then modulates transcriptional activity [59]. In *K. pneumoniae*, PhoP directly activates the *arn* operon to initiate a chemical modification that changes the net charge of LPS. In *E. coli* and *Salmonella* species, signaling is accomplished through an intermediary relay protein PmrD, which signals between the PhoPQ and a second TCS cognate pair, PmrAB [58]. The PhoPQ/PmrAB systems have common activators and are also able to respond to their own individualized signals. Both signaling systems respond to cationic peptides (*i.e.*, polymyxins) and low pH; however, they differ in that the PhoQ histidine kinase responds to low magnesium, while the PmrB histidine kinase responds to high ferric iron (Fig. 15.5). Interestingly, in *E. coli* PmrB responds to other metals, such as zinc and aluminum [54].

Downstream signaling can differ among *Enterobacteriaceae* species. However, it is important to note that independent of species, the end result of PhoPQ/PmrAB induction is the same phenotypic change: the enzymatic restructuring of the LPS as a charge switch through upregulation of 4-amino-4-deoxy-L-arabinose (L-Ara-4N), phosphoethanolamine (pEtN), or galactosamine additions. Nevertheless, there are slight variations among *Enterobacteriaceae* that are exploited to achieve this common goal. For example, in *K. pneumoniae*, aside from activating LPS-modifying operons, PhoP also activates synthesis of a membrane protein called MgrB, which inhibits the PhoP kinase to complete a negative feedback regulatory loop (Fig. 15.5). To further complicate matters, in *K. pneumoniae* a third TCS, CrrAB, can respond to polymyxins by activating the transcription of an intermediary signaling protein, CrrC, which then activates the PmrB response regulator to induce the PmrC LPS-modifying enzyme (Fig. 15.5). *E. coli* PhoPQ/PmrAB signaling differs from *K. pneumoniae* regulation by adding another layer of regulatory complexity in which two small regulatory RNAs, *mgrR* and *micA*, are involved in inhibition of the phosphoethanolamine LPS-modifying operon and PhoP, respectively (Fig. 15.5).

The genomes of many polymyxin-resistant clinical isolates have been sequenced, thereby verifying that mutations within the *Enterobacteriaceae* PhoPQ/PmrAB TCS relays and associated signaling systems are sufficient to result in polymyxin resistance. In particular, MgrB has been the subject of several reports, as a mutation within this gene is sufficient to result in colistin resistance by strongly activating the PhoPQ signaling system [60]. These single mutations within the *K. pneumoniae mgrB* gene have been identified in clinical isolates from globally sampled patients, indicating that this form of resistance is common and possibly arises from independent mutagenic events. In fact, one study reported that over 40% of colistin-resistant isolates, collected from several countries worldwide, had an *mgrB* mutation [60]. In

addition to MgrB, independent mutations within the PmrB kinase also produce resistance to colistin. This observation was noted in a study of globally collected and sequenced *K. pneumoniae* colistin-resistant genomes in which a single conserved amino acid change in the PmrB regulator (threonine-157 to proline) resulted in (up to) a 170-fold induction in the PmrC LPS-modifying enzyme [61].

15.2.3.2 *Pseudomonas aeruginosa* and *Acinetobacter baumannii*

As in *Enterobacteriaceae*, *P. aeruginosa* activates its polymyxin resistance system through upregulating an operon that adds the L-Ara-4N moiety to LPS (Fig. 15.5). However, in this pathogen the signaling that controls this process is complex, with at least five TCS networks being involved in resistance, including PhoPQ and PmrAB (Fig. 15.5). In addition to the presence of polymyxins, in *P. aeruginosa* PhoPQ and PmrAB can both respond to low magnesium and calcium levels. A third TCS system, ColRS, responds to zinc as it activates an operon that adds phosphoethanolamine groups to the LPS. Lastly, two other systems, ParRS and CprRS, respond to a variety of polymyxins and cationic peptides (Fig. 15.5; see section below on cationic antimicrobial peptides).

Clinical isolates of *Pseudomonas* that exhibit resistance to polymyxin have mutations within the histidine kinases PmrB (*i.e.*, similar to *Enterobacteriaceae*), PhoQ, ParS, or the response regulator ParR that are associated with MICs that range from 2 mg/L up to 512 mg/L. Higher MICs can be reached when additional mutations accumulate in genes encoding CprS, CprR, ColS, or ColR TCS proteins in a PhoQ-negative genetic background [62]. These findings suggest that, in many cases, resistance is a result of the accumulation of mutations within several alleles, rather than a single mutation in one allele. Interestingly, the ParRS TCS pair also positively controls the important MexXY multidrug transporter operon (Fig. 15.5). Thus clinical isolates with mutations in ParRS not only lead to LPS modification but also result in low-to-moderate loss of susceptibility to a broad spectrum of antimicrobials, including polymyxins, aminoglycosides, fluoroquinolones, and beta-lactams, contributing to the broad-spectrum resistance/tolerance *P. aeruginosa* is known for. In contrast, *A. baumannii* possesses only a PmrAB TCS system that when activated by polymyxins can alter the LPS with pEtN, or alternatively with galactosamine, through the action of the NaxD enzyme [54] (Fig. 15.5). Why some bacterial species have evolved more or less complex signaling networks to lower susceptibility to antimicrobials is an open question.

15.2.4 *Daptomycin Resistance in* Staphylococcus aureus

Daptomycin (DAP) belongs to the same family of antimicrobials as the polymyxins; however, unlike polymyxins, DAP is a cyclic lipopeptide that specifically targets Gram-positive bacteria. Since it received approval from the US Food and Drug Administration for the treatment of soft tissue infections (2003) and *S. aureus*

bacteremia (2006), it has been reserved for the most serious infections caused by methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *E. faecium* (VRE) [63]. The mechanism by which DAP exerts its bactericidal activity is not fully understood; however, it is thought to bind and insert itself into the Gram-positive bacterial membrane via a process that is enhanced by the presence of calcium and is dependent upon interaction with the anionic membrane phospholipid phosphatidylglycerol (PG) [64–67]. How this results in cell death is still unclear, but it may involve the formation of oligomeric DAP pore-like structures that results in ion leakage and disruption of membrane potential [66]. Another possibility is the recently proposed lipid extracting effect in which the accumulation of DAP in the cell membrane leads to the aggregation and subsequent release of lipid from the cell membrane [67], both of which would affect permeabilization, metabolism, and cell division.

Due to its distinct mechanism of action, resistance to DAP is rare. Nevertheless, treatment failure is a serious concern [68, 69]. Some of the mechanisms that underlie DAP resistance (DAP-R) involve enzymes participating in phospholipid metabolism and membrane homeostasis (Fig. 15.2) [70]. Regarding signal transduction genes, the previously mentioned VraSR-regulated cell wall synthesis stimulon and the essential TCS, WalKR, have been shown to contribute to the DAP-R phenotype in clinical and laboratory isolates [71, 72] (*see section above regarding Vancomycin resistance/Staphylococcus aureus*). VraSR is involved in the regulation of cell wall biosynthesis via transcription of a number of genes including *pbp2* (penicillin-binding protein 2) [73] and is upregulated in the presence of DAP. Differential gene expression analysis between DAP-R and DAP-susceptible (DAP-S) clinical isolates reveals an upregulation of the VraSR TCS system. In addition, deletion of VraSR from a DAP-R isolate conferred a DAP-S phenotype, pointing to the importance of these signaling systems in producing daptomycin AMR. In *S. aureus*, the WalKR TCS system is involved in the control of peptidoglycan biosynthesis and can influence peptidoglycan turnover, cross-linking, and chain length by sensing membrane fluidity, likely through lipid II (Fig. 15.2) [74, 75]. Due to the similarities between a DAP-R and a WalKR-deficient phenotype that includes thickened cell walls, increased membrane fluidity, and resistance to membrane disruption, it has been proposed that mutations in WalKR may lead to the downregulation of cell wall homeostasis, which leads to an increase in bacterial survival in the presence of DAP [70].

The success of *S. aureus* as a pathogen can be attributed largely to its ability to produce a wide range of virulence factors and accessory genes, many of which are under the control of the Agr quorum-sensing system, a classical TCS module (see also Chap. 14). Importantly, dysfunction of this system has recently been implicated in a transient defense mechanism that protects against daptomycin activity [76]. Quorum sensing is a form of intercellular communication that enables bacteria to initiate density-dependent changes in gene expression, allowing populations of bacteria to restrict the expression of genes whose resulting phenotypes are most beneficial at high cell densities. Examples are biofilm production, bioluminescence, and

virulence factor secretion (for review see Ref. [77]). Quorum sensing involves the production and secretion of small molecules that are sensed by neighboring cells. Once the concentration of these small molecules, termed "autoinducers," exceeds a certain threshold, population-wide responses are initiated. Broadly speaking, *S. aureus* uses the Agr system to coordinate the upregulation of exotoxin expression and downregulation of surface proteins, such as adhesion molecules, at high cell densities [78].

The Agr quorum-sensing system is encoded by a four-gene operon (*agrBDCA)* and a regulatory RNA, RNAIII, which are expressed from two divergent promoters, P2 and P3, respectively (Fig. 15.6). AgrA and AgrC comprise a classical TCS system. AgrD is the activating ligand (or autoinducer) which is N- and C-terminally processed in the bacterial cell and then secreted via AgrB into the extracellular milieu to produce the final autoinducing peptide (AIP) (Fig. 15.6). AIP is sensed by the transmembrane histidine kinase AgrC, which induces phosphorylation of the cytoplasmic HPK domain upon AIP binding. This phosphate is transferred to AgrA, the response regulator TCS transcription factor, thereby activating AgrA and transcription of the two *agr* promoters, P2 and P3. The Agr system is an example of an autoactivating system; *agr* autoactivation leads to an exponential increase in expression of the two *agr* promoters. At high cell densities, RNAIII, whose transcription is under the control of the P3 promoter, is a regulatory molecule that is responsible for the differential expression of many genes. These large changes in gene expression inflict a significant metabolic burden upon the cell and have been hypothesized to partly explain the selective enrichment of Agr-defective mutants among seriously ill, hospitalized patients [78] (see also Chap. 14).

The development of Agr-defective mutants during invasive infection and the hypothesis that these mutations may incur a survival advantage in the presence of antibiotics led the Edwards lab to investigate the role of the Agr system in daptomycin susceptibility [76]. Somewhat counterintuitively, they discovered that the loss of AgrA or AgrC, the TCS module, allowed for survival of *S. aureus* in the presence of daptomycin. Investigation into the mechanism by which this occurred revealed that Agr-defective mutants can survive antibiotic exposure by actively releasing membrane phospholipids that bind to and inactivate daptomycin. This process also occurs in wild-type bacteria; however, a set of Agr-regulated genes that are expressed in wild-type bacteria mitigate this observed inactivating effect. The genes encode molecules called phenol-soluble modulins (PSMs), small cytolytic toxins that promote binding of daptomycin to the bacteria by sequestering the shed membrane phospholipid, likely via their surfactant (or detergent-like) properties [76]. Pader et al. also found that the enhanced survival of Agr-defective mutants in the presence of daptomycin could be mitigated by the addition of the β-lactam antibiotic oxacillin, which reduced the rate of lipid release from the bacterial membrane and therefore the inactivation of daptomycin. This result suggests addition of oxacillin as an immediate clinical remedy for daptomycin-tolerant *S. aureus*. This mechanism has been extended to include the enterococci and streptococci in a recent report [79].

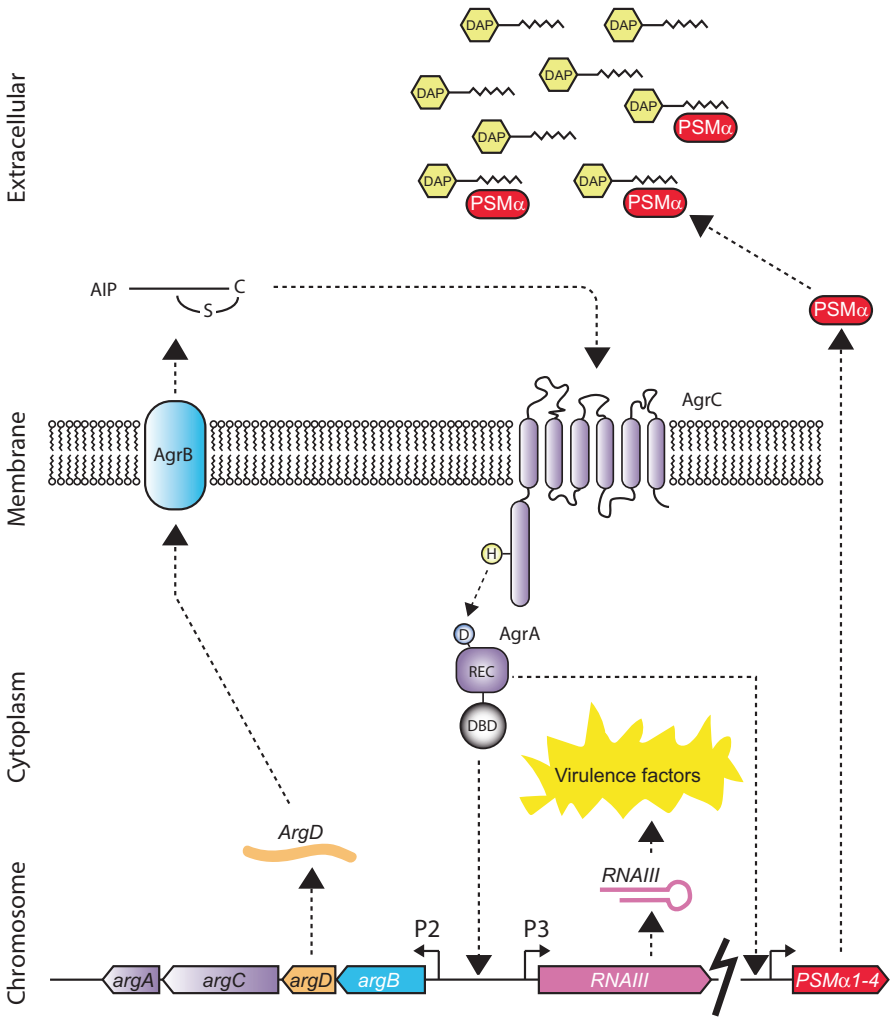

Fig. 15.6 The Agr signaling pathway and daptomycin resistance. AgrC and AgrA comprise a TCS pair that senses the AIP quorum-sensing autoinducer peptide (AIP) signal. AIP is made from ArgD peptide, which is processed and secreted by ArgB. The ArgCA TCS system also regulates (alpha 1–4 type) phenol-soluble modulin (PSM) production, which are excreted and bind daptomycin (DAP) to inactivate it. *agrBDCA* is transcribed from the P2 promoter, and the virulence regulatory *RNAIII* is transcribed from the P3 promoter. Both are controlled by AgrA

15.2.5 The CpxR TCS System and Fosfomycin Resistance

The drug fosfomycin, discovered in the late 1960s, has lately reemerged as a second-line treatment for urinary tract infections due to the increasing prevalence of resistance to commonly prescribed antibiotics, such as trimethoprim-sulfamethoxazole and ciprofloxacin [80]. Fosfomycin is primarily taken up by the glycerol-3-phosphate transport system (GlpT) but may also be internalized by the hexose phosphate uptake transport system (UhpT) when glucose-6-phosphate is present. Once inside the bacterium, fosfomycin inactivates cytosolic *N*-acetylglucosamine enolpyruvyl transferase (MurA). This impairs bacterial cell wall formation by inhibiting the first step in peptidoglycan synthesis, the formation of *N*-acetylmuramic acid from *N*-acetylglucosamine and phosphoenolpyruvate [81–83]. Since its clinical launch, several mechanisms for fosfomycin resistance have emerged, including inducible modulation of the GlpT and UhpT transporters by the Cpx TCS system [84]. Although this section will focus on the Cpx system as it is understood in *E. coli* and as it relates to fosfomycin, the Cpx system has also been implicated in resistance to other antibiotics in *E. coli*, such as aminoglycosides and beta-lactams, as well as forms of resistance in *Salmonella* and *P. aeruginosa* [85–87].

The Cpx system is comprised by the genes *cpxA* and *cpxR*, which encode the inner membrane sensor histidine kinase CpxA and the response regulator transcription factor CpxR [88]. CpxA responds to membrane stressors such as unfolded or misfolded proteins, as well as to changes in pH. In the absence of stressors, CpxA functions as a phosphatase, keeping CpxR in its unphosphorylated, inactive form. The activation of CpxA is modulated by the periplasmic protein CpxP, which interacts with the sensing domain of CpxA; CpxP is displaced by misfolded proteins [89, 90]. Free CpxA is activated by additional signals that are currently not well understood. In typical TCS fashion, once activated, CpxA autophosphorylates and then transfers the phosphate to CpxR [89, 91]. P-CpxR then acts as a transcription factor for a variety of genes, including the *cpx* regulon, *degP*, *glpT,* and *uhpT* (Fig. 15.7) [84, 88–91].

Interestingly, the Cpx response is shut off by feedback inhibition modulated by the amount of unfolded protein present in the bacterial cell. P-CpxR increases the transcription levels of *cpxP*, and CpxP subsequently inhibits the CpxA kinase, while also carrying out its function in binding unfolded proteins. Increases in P-CpxR also increase transcription and translation of the periplasmic protease DegP [88], whose function is to relieve membrane stress by degrading misfolded or unfolded proteins in the periplasmic space. DegP recognizes unfolded proteins bound to CpxP, and the CpxP complex is then degraded by DegP. Importantly, the digestion of CpxP does not occur in the absence of unfolded proteins. This careful balance suggests a mechanism whereby unfolded proteins displace CpxP from CpxA, allowing for autophosphorylation and the subsequent phosphorylation of CpxR, which, in turn, leads to increased levels of both CpxP and DegP through transcriptional activation (Fig. 15.7). While unfolded protein remains in the periplasmic space, CpxP will bind to it and target it for degradation by DegP. As unfolded protein levels fall, more

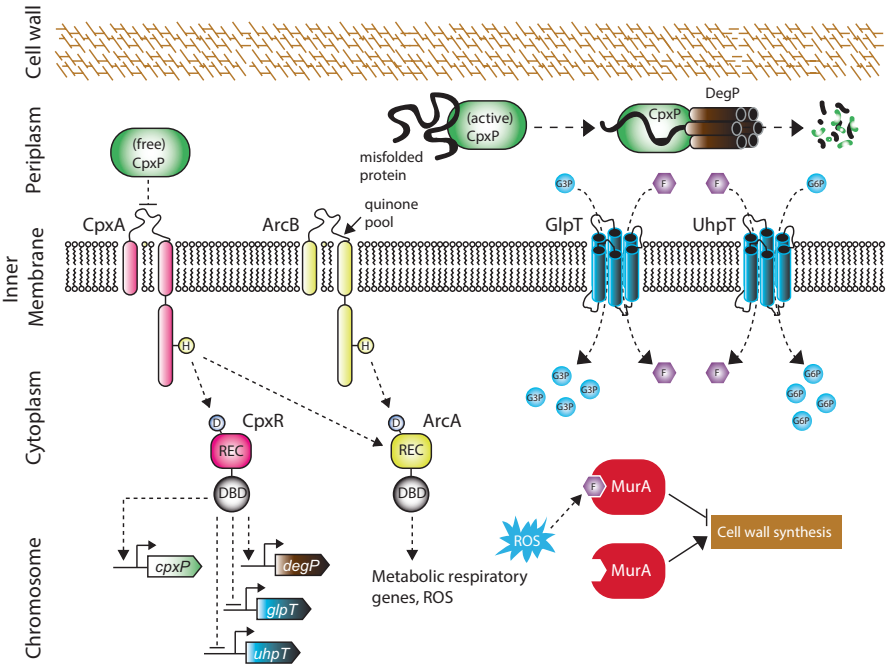

Fig. 15.7 Fosfomycin resistance and the Cpx regulatory system. Fosfomycin (F) acts on the early stages of cell wall synthesis by binding MurA and inhibiting its function. The drug enters the cell via GlpT and UhpT transporters that normally transport glycerol-3-phosphate (G3P) and glycerol-6-phosphate (G6P) sugars, respectively. The CpxAR TCS system represses *glpT* and *uhpT* transcription and activates *degP* transcription. Free CpxP inhibits CpxA activity. CpxP functions to bind unfolded protein and deliver it to the DegP protease in the periplasm. The TCS system ArcAB senses cellular quinone pools and, in turn, regulates aerobic metabolism and resultant reactive oxygen species (ROS) that is proposed to induce fosfomycin antibiotic lethality

CpxP is free to bind CxpA; this change in the CpxP/CpxA stoichiometry shifts activity from kinase to phosphatase function in CpxA, increasing the levels of CpxR relative to P-CpxR (*i.e.*, phosphorylated and active CpxR (Fig. 15.7)). The altering of the CpxR phosphorylation equilibrium ultimately results in a decrease in the transcription of membrane stress-response genes [92].

The Cpx system promotes fosfomycin resistance through P-CpxR by negatively regulating the transcription of *glpT* and *uhpT*. This limits fosfomycin entry into the cell via the GlpT and UhpT transporters (current data suggests that the GlpT transporter is the more physiologically relevant [84]). It is worth noting that the role of the Cpx system in mediating a response to antibiotics is still under debate. Some work suggests that the Cpx system activation is involved in antibiotic-mediated accumulation of reactive oxygen species (ROS), which then can lead to cell death [93]. This conclusion was drawn by observing that a null *cpxA* strain of *E. coli* reduced the lethality of several antibiotic classes and accumulated less ROS. Other studies show that mutations turning CpxA into a constitutively active kinase lacking

phosphatase activity confer a decrease in susceptibility to some antibiotics, while leaving the bacteria susceptible to others. These results indicate that some antibiotics might work through the CpxAR TCS system. Kohanski et al. [93] hypothesize that the ArcBA TCS may be a signaling pair that CpxAR communicates with to facilitate intracellular ROS accumulation and thus antibiotic-induced ROS-mediated killing (Fig. 15.7) [93]. Careful distinction between blocking growth and killing cells is required to assess the contribution of Cpx to ROS [94] (see Chap. 20).

The Cpx system as a means of decreasing fosfomycin susceptibility is of interest because other responses to fosfomycin challenge involve permanently altering the activity of important carbon transporters, such as MurA. This enzyme, which is necessary for bacterial cell wall production [95], is thought to be associated with a high biological cost to the cell and thus may explain why fosfomycin resistance rates in clinical practice remain consistently low [95, 96]. However rare, the Cpx mechanism of resistance presents an inducible means of decreasing GlpT activity and the potential for these mechanisms to contribute to decreased drug susceptibility, which is an important consideration in the development of new antibiotics.

15.3 Resistance to Host Antimicrobial Peptides (AMPs)

Although we usually focus on bacteria when contemplating the natural producers of antimicrobials, these compounds are produced by virtually every life form on earth, including humans. Human-produced antibiotics are mainly protein-based and are commonly referred to as antimicrobial peptides (AMPs; they have also been referred to as host defense peptides or HDPs; for review see Ref. [97]). AMPs are critical components of the host immune response to infections; however, recently they have also been implicated in many immunological roles, including modulation of pro- and anti-inflammatory responses, chemoattraction, enhancement of extracellular and intracellular bacterial killing, cellular differentiation and activation of the innate and adaptive immune compartments, wound healing, modulation of autophagy, apoptosis, and pyroptosis [98]. The mammalian versions of AMPs generally fall into two major categories: (i) defensins, which are structurally defined by the presence of a beta-sheet component, and (ii) cathelicidins, which are more heterogeneous and are characterized by a conserved and a highly variable cathelicidin peptide domain. AMPs are effective against microbial invaders, as they are generally positively charged (cationic), which enables them to more specifically target bacterial cell surfaces, as the bacterial cell envelope is naturally more negatively charged than the cell surface of eukaryotic cells. This phenomenon has earned AMPs the extended name of cationic AMPs (or CAMPs).

CAMPs are a major host defense against bacterial pathogens; they are especially exploited by innate immune cells, such as neutrophils, macrophages, epithelial cells, and specialized secretory cells (*e.g.*, Paneth cells that are important for gut microbiota composition [99]). In phagocytes (*e.g.*, neutrophils and macrophages), CAMPs are found within the granules where they aid in phagosomal killing once

bacteria are engulfed. However, CAMPs can also be released into the medium after phagosomal digestion to kill pathogens that have a more extracellular lifestyle, such as streptococci, staphylococci, and *Pseudomonas* species. In the case of epithelial-type cells, CAMPs can be secreted and act as an intrinsic barrier to pathogens entering organs or tissues where they are unwelcome. These activities place CAMPs at the forefront of maintaining the normal gut flora and general microbiome composition of the host [100].

In order to successfully colonize and infect their host, pathogenic bacteria have evolved a multitude of CAMP-resistance mechanisms. Among these mechanisms are (1) repulsion via recharging their cell envelope, (2) sequestration, (3) export via transporters, and (4) direct enzymatic-driven breakdown of the peptides [101]. Since these collective processes require energy, bacteria must have ways to detect and respond quickly to host CAMP assaults without compromising precious energy needed for colonization and pathogenicity. In this section we describe several specific examples of how bacterial pathogens sense and respond to CAMPs.

15.3.1 Salmonellae

Bacterial CAMP-resistance mechanisms often use the same general signaling and resistance determinants that they use for bacteria-produced antimicrobials. Probably the most well-studied CAMP-resistance signaling cascade is PhoPQ TCS pair [102]. Aside from being an integral signaling component of polymyxin resistance (see the above section on polymyxins), after engulfment of the bacteria by the host, PhoPQ is required for sensing the acidic shift in pH and an accompanying drop in manganese concentrations within the phagosome (see Fig. 15.5). This detection is crucial to the *Salmonella* species life cycle, since they are intracellular pathogens and must therefore escape and/or subvert innate immunity to remain viable and replicate within host cells, including innate immune cells such as macrophages. Since phagocytes produce CAMPs to lyse the engulfed *Salmonella*, once PhoPQ is activated it initiates expression of several genes that modify the cell envelope charge and repel CAMPs. To accomplish this, PhoPQ controls a large regulon containing more than twenty genes, which together change several chemical moieties within lipopolysaccharides, glycerophospholipids, and outer membrane proteins that alter the net charge of the cell envelope from negative to positive (Fig. 15.5). As described in the previous section on polymyxins, these changes are also influenced by the PhoPQ induction of a second TCS, PmrAB. For a more extensive description of the chemical basis for PhoPQ-induced CAMP resistance see Ref. [102] and references therein.

The collective action of PhoPQ/PmrAB induction results in CAMP resistance and a concomitant decrease in innate immune recognition that ensures the survival of *Salmonella* within macrophages and the host environment. One interesting aspect of the many outer membrane modifications is that PhoPQ/PmrAB may make specific changes that depend on the particular infected host tissue and environment. For

example, using a murine model of infection, it was found that PhoPQ/PmrAB-mediated induction of the specific aminoarabinose (L-Ara-4N) modification of Lipid A is required for full colonization of the lumen of the small intestine (Fig. 15.5) [101]. This modification is likely due to PhoPQ sensing host CAMPs found within the lumen and then activating PmrAB and downstream resistance regulons that code for enzymes necessary to resist CAMP assaults.

The association of PhoPQ signaling and CAMP resistance has been extended to several additional Gram-negative pathogens, including *Legionella pneumophilia*, *Bordetella* species, *Francisella* species, *Yersinia pestis,* and *P. aeruginosa* [103]. In the case of *P. aeruginosa*, chemically modified LPS was recovered from bacteria cultured from the cystic fibrosis lung, suggesting that PhoPQ/PmrAB signaling is important for this pathogen's ability to cause cystic fibrosis chronic infection [104]. Collectively, these findings suggest that the Gram-negative cell envelope is not static but rather chemically dynamic, since modifications occur through PhoPQ/PmrAB signaling to accommodate different host cell environments and threats in an energy-efficient manner.

15.3.2 Streptococci

Gram-positive bacteria are also subject to CAMPs from phagocytes and other host cells. Differing from Gram-negative microbes, Gram-positive bacteria lack an outer membrane and instead possess an expanded cell wall for fortification, often surrounded by a protective polysaccharide capsule (see Fig. 15.2). These layers collectively act as barriers to phagocytosis and unwanted host molecules. Taking these differences into consideration, it is not surprising that Gram-positive bacteria have evolved systems of CAMP resistance that may differ considerably from those of Gram-negatives, in both signaling and mechanistic outcomes. For example, streptococci (*e.g.*, *S. pneumoniae*, *S. agalactiae,* and *S. pyogenes*) and staphylococci species often rely on an L-lysinylation-protective strategy facilitated by MprF (for multiple peptide resistance factor). MprF conveys its phenotype by adding positively charged L-lysine groups to the membrane lipid phosphatidylglycerol, thereby enabling repulsion of host CAMPs [105–108]. Other strategies include the D-alanylation of cell wall teichoic acid by the *dlt* operon, a mechanism conserved among several streptococcal and other Gram-positive species. This chemical modification results in increased density and surface charge of the Gram-positive cell wall, which then acts to absorb and repel CAMPs. D-alanylation generated by the Dlt proteins also confers resistance to host-produced acid, lysozyme, neutrophil-produced neutrophil extracellular traps (NETs), and antimicrobial peptides known as bacteriocins [106]. Such broad resistance capabilities suggest that the *dlt* operon and other antimicrobial systems act as versatile signaling and host-responsive mechanisms, rather than specifically targeting a single antibiotic. This reoccurring theme is emphasized below.

The streptococci and staphylococci often possess a polysaccharide capsule that creates a natural barrier to CAMPs and therefore provides intrinsic resistance [106, 107]. Also present in the Gram-positive arsenal to counterbalance the host CAMP threat is a litany of: (i) efflux pumps dedicated to the export of host CAMPs, (ii) proteases that inactive CAMPs, and (iii) other proteins that sequester CAMPs within the extracellular milieu [106, 107]. Due to the environment that Gram-positive pathogens, such as the streptococci, encounter within the host, they must have a means to detect CAMP threats and quickly respond. This is accomplished by many signaling systems.

Unlike the PhoQ histidine kinase found in Gram-negative bacteria, no bona fide receptor for CAMPs has been discovered in the streptococci. However, several signaling systems that respond to these peptides have been identified with both laboratory-generated and clinical mutant collections. In *S. agalactiae*, *S. pyogenes*, and many related pathogens, the CovRS TCS system has been one of the most studied signaling systems [109]; it controls many virulence factors and, relevant to this section, a moderate level of CAMP resistance. Paralleling the Gram-negative PhoPQ TCS pair, the *S. pyogenes* CovRS signaling system activates genes in response to host CAMP LL-37, a major mammalian CAMP that is also responsible for repression of genes in response to magnesium. Collectively, the broad response network controlled by CovRS regulates induction of virulence factors during infection, including production of the capsule (Ref. [106] and references therein). Although the mechanism is largely unknown, CovRS CAMP resistance could result from some of the virulence factors it controls, which include the SpeB protease that directly cleaves the LL-37 CAMP. SpeB also regulates the *S. pyogenes* hyaluronic acid capsule locus (*has*). However, mutations that arise during infection within *covRS, speB,* and the *has* locus do not result in drastic reduction in susceptibility to CAMPs. Instead, it appears that multiple, cumulative mutations are necessary for a pronounced effect. An interesting regulatory twist is that in both *S. agalactiae* and *S. pyogenes* CovR function is also regulated through phosphorylation by the sole eSTK and eSTP cognate pair found in these pathogens [6, 110]. eSTKs and eSTPs (bacterial serine-threonine kinases and phosphatases) have been implicated in antibiotic resistance in many Gram-positive pathogens [29, 46, 111]. Whether eSTK/eSTP and CovRS integrate their sensory properties to confer resistance to antibiotics requires further investigation.

In addition to CovRS, other signaling mechanisms have been discovered that regulate streptococcal resistance to CAMPs. For example, *S. agalactiae* upregulates several CAMP-resistance factors in response to subinhibitory CAMP concentrations (specifically LL-37), the response to which is carried out by two TCS systems: CiaRS (discussed above) and LiaRS [112]. Although CiaRS/LiaRS homologs are found in many other streptococcal species, at present it is not known whether these systems directly bind CAMPs to facilitate resistance/tolerance. Based on their broad range of inducing signals, the CiaRS/LiaRS sensory mechanisms are likely to be indirect, functionally paralleling sensory attributes of other two-component responsive systems (*e.g.*, PhoPQ).

In another example of TCS regulation and responsiveness to the presence of CAMPs, *S. agalactiae* and other streptococci utilize LisRS (also referred to as LisRK). Originally discovered in *L. monocytogenes* for its role in nicin and cephalosporin resistance [113], the LisRS TCS pair appears to be present in many other Gram-positive pathogens and is seemingly functionally conserved. Similar to other TCS-induced CAMP resistance/tolerance strategies, LisRS controlled responses function to chemically alter the bacterial cell envelope to avoid a broad range of antimicrobials, including vancomycin, bacitracin, and polymyxins. As above, a common theme is the alteration of cell wall chemical bonds to repel the CAMP positive charge and their binding to the cell envelope. Interestingly, LisRS is also postulated to control the expression of antibiotic targets, such as PBPs. In doing so, the bacterial cell is able to withstand higher concentrations of certain antibiotics by downregulating their PBPs, resulting in less targets for the drugs to act upon (Ref. [112] and references therein).

As detailed above, the *S. pneumoniae* CiaRS system has been implicated in penicillin resistance [39]. CiaRS has also been implicated in conveying responsive protection to ROS produced by phagocytes, including hypochlorite and hydrogen peroxide, and also regulates several proteases that could be involved in the degradation of CAMPs. In contrast, another transcription factor called CrgR, found in *S. pyogenes*, likely controls a more specific CAMP-resistance/tolerance response. Evidence comes from an interesting study by Nizet et al. where they found that CrgR confers a competitive advantage to *S. pyogenes* only when infecting the skin of wild-type mice but not of CAMP knockout mice [114]. Additional work is required to understand the regulatory profile and mechanism of CrgR, but it is clearly important in avoiding CAMPs generated by the vertebrate skin during infection.

15.3.3 Staphylococci

The main CAMP sensory system in *S. aureus* and related species is the GraRS/VraFG signaling cascade, which modifies the overall charge of the cell envelope (described above with reference to its contribution to vancomycin resistance [25]; Fig. 15.2). Thus, this signaling cascade is a general, nonspecific means to facilitate resistance based on electrostatic repulsion of host-produced CAMPs. However, the resistance conveyed by GraRS/VraFG can differ greatly depending on the staphylococcus species. For example, GraRS/VraFG recognizes and responds to host LL-37 and indolicidin in both *S. aureus* and *S. epidermidis*, but only *S. epidermidis* GraRS/VraFG responds to human beta-defensin-3, which protects against skin infections. *S. epidermidis*, as its name suggests, is part of the normal flora of human skin and causes opportunistic skin infections. The species-specific recognition is facilitated by a short extracellular loop within the GraS histidine kinase sensory domain [115]. Induction of the GraRS signaling cascade (histidine kinase phosphorylation of the response regulator; Fig. 15.1) then initiates expression of the *dlt* operon, which is

responsible for enzymatic incorporation of D-alanine into the cell wall teichoic acid. Simultaneously, GraRS upregulates MprF, which adds a lysine moiety onto phosphatidylglycerol (Fig. 15.2). These modifications collectively alter the overall cell wall and membrane charge of the bacterial envelope, thereby electrostatically repelling host CAMPs. In addition, GraRS activates the VraFG TCS system, which has been proposed to govern the efflux of CAMPs from the bacterial cell [107]. The requirement of the GraRS and VraFG signaling systems for CAMP resistance therefore explains experimental evidence showing that these TCS pairs are required to survive neutrophil attacks and are critical to the success of staphylococcal infections [107, 116].

In addition to the all-important and very ubiquitous GraRS/VraFG systems, the staphylococci also use other signaling cascades to counteract CAMP activity. The global regulators Agr (see section above on daptomycin resistance) and SarA, as well as the TCS system SaeRS, have been implicated in CAMP resistance through activation of the controlled expression and release of CAMP-degrading proteases, such as the exoprotease SepA (Ref. [116]; for review see Ref. [107]). Finally, a report from 2013 describes another TCS pair, LytSR, which confers staphylococcal CAMP resistance. Interestingly, the authors proposed that LytSR senses subtle changes in membrane potential, alerting the bacterial cell of early exposure to host CAMPs that perturb the normal electrical gradient [117]. An appropriate response can then be elicited in time. These studies collectively suggest that *S. aureus* and other staphylococci have evolved complex regulatory networks to survive host onslaughts from innate immune cells, and specifically from CAMPs.

15.4 Biofilms and Antimicrobial Resistance

Bacteria are capable of growing either planktonically as free autonomous entities, or as a largely sessile, immobile community commonly referred to as a biofilm [118]. The latter state has been the subject of considerable research in recent years, and it is now well understood that once a biofilm is established within host tissues, bacteria become far less susceptible to host immunity and antimicrobial treatments (as much as a thousand times less susceptible [118]). As bacterial infections usually have a large biofilm component, knowing how to better treat biofilm infections, which include catheters, surgical implants, and chronic infections such as the cystic fibrosis lung, remain a consistent and unresolved clinical problem [119].

The mechanism responsible for reduced susceptibility of biofilms to antibiotic treatments is under debate. One hypothesis is that there is simply more of a physical and/or mechanical barrier to the drugs. This idea depicts the surface of the biofilm as being exposed to lethal doses, and as one moves deeper into the biofilm substrata, antibiotics are diluted to concentrations where they are less effective. However, there is now compelling evidence that the situation is more complex. For example, ciprofloxacin, a clinically relevant fluoroquinolone antibiotic, fully penetrates bio-

films rapidly, as is the case for several other antimicrobial classes such as the polyketide tetracycline (see Ref. [120] and references therein).

One commonality to all biofilms is the upregulation of genes producing a protective glycocalyx or capsular sugary matrix once the bacteria are attached to a host surface. This physiological change upon surface attachment is often accompanied by the initiation of a number of AMR resistance mechanisms, including enzymatically driven antibiotic degradation (*e.g.*, by beta-lactamases) and the production of multidrug resistance efflux pumps. Indeed, both are hypothesized to contribute significantly to the staying power of the bacterial biofilm [119, 120].

Biofilms also contain a large DNA component, aptly named "extracellular DNA" or eDNA, which was found to act as a protectant against innate immunity and some antibiotics by adsorption [121]. A large fraction of the bacteria within the biofilm community are also metabolically less active, especially within the deeper layers of the substratum. This phenotype has now been associated with bacterial stress responses that include the ppGpp-driven stringent response, or in other cases the SOS response. Both are involved in controlling bacterial cell death and longevity. This slowing of metabolism is a hallmark of the so-called "persister" cell phenotype, or the subset of cells that "persist" in the wake of antimicrobial or other environmental stress [17, 120]. Data now show that persister cells comprise a considerable proportion of the biofilm and thus might contribute significantly to the longstanding question of why biofilms render antibiotics less effective (for an expanded description on persisting microbes see the "Persisters" section below).

The transition from a planktonic state to initial adherence to gradual, stepwise increments toward a mature and resistant biofilm is a highly complex process that requires bacterial cells to undergo dramatic physiological, metabolic, and phenotypic changes [118]. This process is reversibly controlled through a variety of signaling mechanisms that include quorum-sensing systems (*i.e.*, cell-cell communication) and signal transduction cascades. In many cases, biofilms are induced by subinhibitory concentrations of the antibiotic itself, strongly suggesting that signal transduction systems are involved at sensing and responding to the threat and play an integral role in tolerance and resistance [122]. Here we will discuss specific examples from both Gram-positive and Gram-negative bacteria concerning how pathogens undergo biofilm formation and, importantly, the relevant signaling proteins involved in biofilm-mediated resistance and tolerance to antibiotics.

15.4.1 Pseudomonas aeruginosa

P. aeruginosa is arguably the most well-studied microorganism with respect to biofilm formation and its contribution to pathogenicity. This problematic pathogen has a relatively large genome (5.5–6.8 million base pairs depending on the strain) and an arsenal of virulence factors that the cell controls through multiple and diverse signaling cascades. Although its natural habitat is soil and water, its versatility enables it to colonize and infect a range of animal and plant tissues. In humans, *P.*

aeruginosa is problematic with implanted and indwelling devices, as well as with skin wounds and many internal organ infections, including infections of the urinary tract (most common) and kidneys. This microbe is most infamous for infecting the lungs of patients with chronic obstructive pulmonary disorder (COPD) or cystic fibrosis. For the latter, *P. aeruginosa* is the major cause of death [120]. One of the reasons for its disproportionate morbidity and mortality rates is that *P. aeruginosa* is naturally resistant to several classes of antibiotics, which is largely associated with its ability to form biofilms in the chronically infected cystic fibrosis lung. To regulate biofilm formation, many signaling proteins are involved, some key examples of which we describe in this section. We focus on cell signaling and AMR rather than on the in-depth fundamentals of biofilm formation and clinical consequences. For a comprehensive review on biofilms and how they are regulated in *P. aeruginosa* and related microbes, see Ref. [118].

As previously mentioned, *P. aeruginosa* possesses an arsenal of multidrug resistance (MDR) pumps and antibiotic-degrading enzymes to combat both host onslaughts and therapeutic treatments [120]. As with most resistance mechanisms, these actions are energetically costly, and they must therefore be coordinated with environmental ques so they are used only when explicitly required (*e.g.*, in the presence of an antimicrobial). The so-called antimicrobial "resistome" used by the *P. aeruginosa* pathogen was recently shown to be controlled by a biofilm-specific transcription factor called BrlR (for biofilm resistance locus regulator [123, 124]). BrlR accomplishes this by upregulating the well-known multidrug resistance transporter *mexAB-oprM* and *mexEF-oprN* operons. Interestingly, BrlR does not appear to respond to specific antibiotics directly, as seen in canonical MerR-type transcription factors, but rather to the cell-cell (or quorum-sensing) messenger cyclic-di-GMP (c-di-GMP; for review see Ref. [118] and references therein). This finding revealed the important connection between regulation of antibiotic-induced biofilms and quorum sensing, the latter of which had already been associated with the general transition from a planktonic to sessile state. c-di-GMP directs key phenotypic changes in *P. aeruginosa* biofilm formation that include the production of the protective extracellular matrix, a hallmark of the established biofilm. This matrix contributes loss of susceptibility due to its electrostatic repulsion and absorption properties [118]. Another report by Sauer and colleagues connects the activity of the histidine kinase SagS with control of the levels of cellular c-di-GMP and therefore BrlR responsiveness and biofilm-associated antimicrobial resistance. A *sagS* deletion mutant was found to have lower c-di-GMP levels, and therefore was more susceptible to antibiotic treatment [125].

BrlR has also been shown to confer tolerance to host CAMPs, as well as to the polymyxin colistin and the aminoglycoside tobramycin, the latter of which is typically used to treat Gram-negative infections, especially in cystic fibrosis patients. Sauer and colleagues also demonstrated that BrlR regulates the PhoPQ and PmrAB TCS networks of *P. aeruginosa*, which change the cell envelope charge conferring tolerance to CAMPs and polymyxins (Fig. 15.5). When expressed, BrlR represses PhoPQ/PmrAB network (described in detail in the Polymyxin section) and therefore its signaling. An interesting finding from these studies revealed that

many *P. aeruginosa* clinical isolates from cystic fibrosis patients' sputum contained mutations within BrlR, suggesting that in chronic infections BrlR might be inactivated to enhance tolerance to host-produced CAMPs [126]. Indeed, recent clinically relevant cystic fibrosis models developed in swine have shown that *P. aeruginosa* and other bacteria are better able to colonize the diseased cystic fibrosis lung due to the drastic change in lung pH. It was hypothesized that the pH change alters the charge of host-produced CAMPs, making the lung far less effective at clearing microbes from the usually more or less "sterile lung" environment [127]. On the other hand, when present in its wild-type form, BrlR is responsible for activation of biofilms and, as a result, tolerance to the aminoglycoside tobramycin. Collectively, these studies suggest that tobramycin is more effective with isolates that have lost BrlR function in the more chronic, later stages of the cystic fibrosis disease and, conversely, might be less effective if given early on.

Almost a decade before the discovery of BrlR the association of a signaling pathway connecting aminoglycoside resistance and biofilm formation was discovered. Miller and colleagues described the aminoglycoside response regulator Arr as being responsible for induction of biofilm formation at subinhibitory concentrations of aminoglycosides, such as tobramycin, in both *P. aeruginosa* and *E. coli* [128]. Arr contains a c-di-GMP phosphodiesterase (or EAL) domain that directly regulates biofilm formation and tobramycin resistance. This regulation is accomplished by responding to subinhibitory levels of antibiotic via an as-of-yet unknown mechanism and, in turn, regulating c-di-GMP levels through breakdown by the Arr EAL domain [128].

Other regulators that affect the levels of c-di-GMP have also been implicated in antibiotic resistance, such as the PvrR response regulator, originally described as controlling *P. aeruginosa* phenotypic variance and antibiotic resistance. With a *pvrR* mutant, the aminoglycoside kanamycin induced small-colony variants, which are hyper-adhesive and better resist antimicrobials [129]. Similar to Arr, PvrR contains an EAL domain and is therefore involved in responding to and controlling cellular c-di-GMP levels. Results from these studies collectively point to bacterial cell-cell communication systems responding to often subinhibitory concentrations of antibiotics and then changing the cell phenotype to enable resistance/tolerance. In this way the bacteria can communicate the threat to their community and respond in a timely fashion. The concept of subinhibitory levels of antibiotic resulting in biofilm formation has now been demonstrated with many diverse pathogens, including Gram-positive bacteria such as *S. aureus*. The general response to antimicrobial treatment and induction of the protective biofilm state is a result of the ability of signal transduction systems to detect and quickly eliminate the threat. For a comprehensive review on antimicrobial induction of biofilms see Ref. [122].

As mentioned in the introduction of this section, biofilms also contain an extensive amount of eDNA. These web-like structures are either secreted by dedicated export systems in the bacteria or, more indirectly, can be a consequence of cell lysis [121]. Once outside the cell, eDNA can act as neutrophil extracellular traps (or NETs) to enable immune system evasion. Interestingly, within the context of the *Pseudomonas* biofilm, eDNA was recently shown to play a role in signaling CAMP

and other AMR mechanisms [121]. Due to the highly anionic nature of eDNA, it was shown to act as an absorber of cationic metal ions, such as magnesium. As a consequence, eDNA depletes extracellular magnesium and activates the PhoPQ/PmrAB TCS, which chemically modifies the *P. aeruginosa* polysaccharide to repel host CAMPs (see Fig. 15.5). The same effect holds true with other Gram-negative bacteria, such as *Salmonella* species [121]. A surprising discovery was that addition of eDNA to planktonic cultures of *P. aeruginosa* cells induced the expression of a three-gene cluster adjacent to PmrAB that is responsible for the production and export to the outer membrane of the cationic molecule spermidine. It was hypothesized that spermidine then acts as a positively charged surrogate to magnesium, repelling CAMPs and positively charged antibiotics, including polymyxins and aminoglycosides (*e.g.*, tobramycin and gentamicin) [121].

15.4.2 Staphylococci

Gram-positive bacteria are naturally more susceptible to many cell envelope-acting antimicrobials, as they lack the outer membrane possessed by Gram-negatives. Therefore, to aid in antibiotic resistance and immune avoidance, it would seem especially advantageous for them to form a protective, structured biofilm community. Although it is well established that Gram-positives also form biofilms, in comparison with their Gram-negative counterparts, Gram-positive biofilm formation and mechanisms are currently less clear and understudied. One difference is that many Gram-positive biofilm phenotypes tend to be strain-specific rather than a species-wide attribute, as in the case of *S. pyogenes* and *S. aureus*, for example [130]. Although many species of Gram-positive bacteria form biofilm structures (*e.g.*, oral pathogen dental plaques and *S. pneumoniae* colonization of the nasopharynx [131]), in this section we focus mainly on the staphylococci, as their biofilm formation and associated signaling have been studied more extensively than with other Gram-positive microbes.

S. aureus is an animal and human bacterial pathogen capable of causing a wide-range of infections and known for its formation of biofilms aiding in its success at inflicting substantial morbidity and mortality in the USA and abroad. *S. aureus* infections that are assisted by the biofilm matrix include infective endocarditis and implant-associated disease. In addition, the skin-dwelling opportunistic pathogen, *S. epidermidis*, contributes to many of these same difficult-to-treat biofilm-related infections [132].

The *S. aureus* biofilm consists of eDNA and protein and is also largely composed of the polysaccharide poly-N-acetyl-β-(1–6)-glucosamine (PNAG). The latter is produced from the *icaADBC* operon found in most strains. However, expression of this operon varies among *S. aureus* strains, and thus the composition of the biofilm also varies among different isolates [133]. Although the *icaADBC* operon is responsible for creating the fundamental building blocks of the biofilm, it is curiously not under control (at least under the conditions tested to date) of the signaling system(s)

that direct biofilm formation in *S. aureus*, namely, the *agr* quorum-sensing signaling cascade ([107]; see the section on daptomycin resistance above). Maturation of the protective *S. aureus* biofilm structure is facilitated by phenol-soluble modulins (PSMs), which has been confirmed to be a signaling requirement for catheter-related biofilm infections in animal infection models ([132, 134]). Despite a plethora of examples of antibiotic-induced biofilm formation in staphylococcal species, most of the regulatory connections remain tenuous at best (for review see Ref. [122]).

The direct association between induction of staphylococcal biofilm formation and antibiotic treatment, especially at subinhibitory concentrations, was first observed in 1940 by Arthur Gardner with the Gram-positive *Clostridium perfringens* (for review Ref. [122]). This effect was then established in several other Gram-positive and Gram-negative pathogens with many additional antibiotics. However, the signaling responsible for induction remains unknown for most Gram-positive cases, with a few recent exceptions with staphylococci. One of these exceptions comes from a report from Michael Otto and colleagues, which demonstrates that in vivo PSMs are key contributors to the *S. aureus* biofilm maturation process [132]. The authors found that PSM placement within the matrix dictates the local structures of the biofilm. Otto and colleagues propose a model by which this local variation is controlled by targeted activity of the *agr* quorum-sensing system (described in the daptomycin resistance section and displayed in Fig. 15.6) [132]. Agr and phenol-soluble modulins (PSMs) also control biofilm detachment. Another report from Schilcher et al. builds upon previous knowledge that at subinhibitory concentrations clindamycin induces higher eDNA content in the *S. aureus* biofilm matrix [135]. This effect was then determined to be triggered by the alternative sigma factor B (σ^B) and its upregulation of known biofilm-associated factors. This report is important because it provided the missing link in staphylococcal signaling between inducible biofilm formation and biofilm-driven resistance. The extent to which σ^B is integrated within other Gram-positive antibiotic-induced biofilm resistance signaling networks remains to be determined.

15.5 Persisters

Despite the application of antibiotics, a subpopulation of bacteria almost always survive, only to regrow and again establish infection [136]. This phenomenon, referred to as "persistence," was first observed in 1944 by Joseph Bigger after it was discovered that penicillin was incapable of sterilizing *S. aureus* cells in culture [137]. As it turns out persistence is ubiquitous among bacterial pathogens, as antibiotic treatments fail to kill a small portion of cells. This problem has contributed greatly to the tolerance of a variety of bacterial infections in the hospital setting, notably those involving *P. aeruginosa*, *E. coli*, *M. tuberculosis*, *Clostridium difficile*, *Salmonella* species, and several other Gram-positive and Gram-negative pathogens [136, 138].

Persister cells appear to be genotypically identical to their susceptible counterparts, which strongly suggests there are specific and reversible signaling mechanisms in place to "weather the antibiotic storm" at the onset of treatment. We have briefly touched on this subject in the biofilm section above. We pointed out that a major reason biofilms are able to resist treatment is that they are comprised of a large percentage of persister cells. Similar to biofilms, the persister state is reversible and therefore regulated through existing signaling mechanisms. Thus, one major question researchers have been struggling to answer is what are the environmental ques that are responsible for this transient state of tolerance? So far, studies have shown that most signals are related to environmental stresses, such as: (i) nutrient limitation – a state that would be observed within the center of biofilms or after engulfment by phagocytes, (ii) diauxie (the lag between metabolizing two different energy sources – usually sugars), (iii) extreme shifts in pH, and lastly (iv) DNA damage [138]. Here we discuss some key bacterial signal transduction cascades that enable pathogens to respond to these environmental ques, enabling the switch into and back out of the dormant-like persister state.

15.5.1 Toxin-Antitoxin (TA) Signaling Systems and Persistence

Bacterial toxin-antitoxin (TA) systems were the first signaling systems discovered to induce persistence through environmental ques. TA systems are ubiquitous in bacteria, with many species possessing multiple versions. TA systems are usually encoded within an operon that consists of two genes, one encoding a toxin that regulates cell growth in some manner and the other encoding a cognate antitoxin that regulates the levels of the toxin (for review see Ref. [139]). In the most simplistic system, the antitoxin, which is normally a DNA-binding domain-containing transcription factor, directly binds to the toxin. In doing so the toxin then acts as a corepressor with the antitoxin to bind DNA and self-regulate repression of the TA operon [138]. Because the antitoxin is usually produced in excess of toxin, the toxin then controls the TA relationship through the toxin-to-antitoxin ratio. In this manner the TA ratio can fine-tune and control the cellular growth and therefore the persistence phenotype. It is interesting to note that the "toxin" in most cases is not actually a toxin in the classical sense, but rather a regulatory protein or RNA whose action indirectly results in cellular toxicity (*e.g.*, through posttranslationally modifying other proteins that actually confer the toxicity). To date there is only one example in which the toxin component of the TA pair directly influences cellular toxicity (Ref. [140] and see below). To add another layer of complexity, it is known that with many TA systems a protease (Lon protease) degrades the antitoxins, which is, in turn, controlled by cellular phosphate levels [136, 138, 139, 141]. One hypothesis for how TA systems are able to direct reversible phenotypic heterogeneity involves the balance the TA module provides between cell growth and arrest. In doing so, it enables a responsive subpopulation to occur only when required. The mechanism for how this balance is controlled is still the subject of much debate [141].

The first identification of a bacterial genetic locus connected to persistence was the discovery of the high-persistence (Hip) mutations within the Hip TA system in *E. coli* [141].

The *hipAB* locus consists of the toxin HipA and antitoxin HipB, HipB being a transcriptional repressor that binds HipA directly. HipA is not a toxin itself, *per se*, but is a bacterial eukaryotic-like serine-threonine kinase (eSTK) that acts indirectly by phosphorylating glutamyl tRNA synthetase to inhibit its function [141]. The accumulation of phosphorylated, uncharged glutamyl tRNA then results in an increase in ppGpp, the cellular alarmone that controls the stringent response and is a general controller of persister formation [141]. ppGpp accomplishes this reprogramming by changing the expression of approximately 500 genes through direct binding of RNA polymerase. Importantly, ppGpp stimulates RpoS expression, which is the master stress-response regulator in many bacteria. Because the ppGpp signaling pathway is a general cellular stress-response signaling pathway, persister-induced signaling often culminates in ppGpp accumulation to produce the persister phenotype [142].

15.5.2 The SOS Response System and Persistence

In addition to the ppGpp signaling system, other general stress-response mechanisms have been associated with persistence. One such signaling system is the SOS response and associated genes (*e.g.*, *lexA*, *recA*, and *recB*), which act in response to DNA damage. When DNA is damaged, single-stranded DNA (ssDNA) accumulates and activates RecA, which then binds to the transcriptional repressor of the SOS response, LexA. LexA then becomes inactivated by self-cleavage, and the SOS response is initiated. Not surprisingly, the SOS signaling system, in particular from studies done in *E. coli*, has been implicated in resistance/tolerance and persister formation in response to DNA-damaging antibiotics such as fluoroquinolones [136]. As it turns out, a TA system called TisAB is responsible. Dorr et al. showed that the TisB toxin component is a small membrane-acting peptide that influences the proton motive force of the bacterial cell. When ciprofloxacin (a fluoroquinolone) is present, the SOS system activates canonical DNA repair enzymes, but it also acts to produce TisB. TisB then binds to the bacterial membrane, decreasing the proton motive force and therefore cellular ATP levels, which initiate growth arrest and thereby the metabolically less active persister phenotype [140]. As TisB is actually a toxin and not a signaling protein, the TisAB TA system is the only TA system currently known to *directly* influence persister formation.

15.5.3 Quorum Sensing and Persistence

In some bacterial species, there is evidence to suggest that persistence is controlled by quorum-sensing mechanisms. In a rare example of a Gram-positive persister model of induction, Leung et al. have shown that competence-stimulating peptide

(CPS), a pheromone produced by the dental pathogen *Streptococcus mutans* and related streptococci species, is a stress-induced alarmone, which activates two TA systems (MazEF and RelEB) to increase the persister population. The CPS competence pheromone appears to work by inducing a LexA homolog in *S. mutans* [143]. These studies are important because they connect the DNA uptake mechanism (induced by CSP) with the SOS response system and persistence in a Gram-positive organism. How ubiquitous this signaling system is in Gram-positive microbes remains to be determined. In a particularly intriguing example of bona fide quorum-sensing induction of persister formation, studies by Vega et al. have shown that when added to the medium, the bacterial cell-cell communication aromatic compound indole induces persister cell formation in *E. coli*, as well as in *Salmonella* Typhimurium. Remarkably, *S.* Typhimurium is not known to produce indole; thus, these data suggest that indole and possibly other low-molecular-weight molecules could act as cross-species inducers of persistence in, for example, polymicrobial biofilms [141]. These recent discoveries illustrate the exciting prospect of quorum sensing controlling persister formation and suggest a feasible path forward to design new antimicrobials.

15.5.4 Persistence Studies In Vivo

Most studies investigating the mechanisms of persister formation have been accomplished using laboratory strains of *E. coli* in test tubes (*i.e.*, in vitro). Although these studies have yielded a plethora of information [136], it is also important, perhaps even more so, to study persister formation in actual pathogens and in the context of their native host environment. Using *S.* Typhimurium single-cell analysis with a murine model of infection, Helaine and colleagues have done just this with surprising results. They found that within 30 minutes after macrophages engulf *S.* Typhimurium in an animal, as much as 20% of the population changes to the persister state, becoming tolerant to antibiotic treatments [144]. This surprising result was in stark contrast to all previous reports from in vitro experiments, which showed that the fraction of persisters is normally no greater than 1% of the total population [138]. The persistence was shown to be aided by Lon protease/ppGpp-dependent TA modules and, interestingly, triggered by the drastic change in pH and nutrient depravation when the bacteria enter the macrophage vacuole [144]. A second paper by the same group describes the mechanism by which this occurs through a new class of uncharacterized TA modules harboring Gcn5 N-acetyltransferase (*i.e.*, acetylation) activity [145]. It was determined that the acetyl transferase, dubbed TacT for "tRNA acetylating toxin," signals for initiation of the persister state by acetylating tRNA, thereby inhibiting tRNA function and simultaneously inducing *lon*-ppGpp-mediated cell growth arrest [145]. Furthermore, this acetylation and therefore growth could be reversed by the *S.* Typhimurium deacetylase CobB [145]. These studies are important, as they show a more comprehensive picture of how TA system signaling can facilitate reversible persister formation that is independent of genotype.

15.6 Concluding Remarks

In this chapter we discussed how bacterial cells sense antimicrobials and respond in a timely manner to tolerate/resist them using signal transduction systems. Although many systems are specific to a particular species or genus, there are also examples of conservation that might elicit the targeting of these pathways for new broad-spectrum antimicrobials. One of these research areas that has recently gained attention involves drugs that inhibit quorum-sensing pathways, which are shared by both Gram-positive and Gram-negative organisms and generally control biofilm formation in many pathogens [146]. In this regard, the most progress has been made with *P. aeruginosa* and related pathogens [147]. Other attempts to subvert antibiotic resistance by inhibition of signaling systems have been made in the area of two-component signaling. Although two-component signaling systems have been deemed too diverse and prone to mutagenesis for the design of serious broad-spectrum inhibitors, recent efforts to target the conserved WalRK system have resuscitated this area of research [148]. An important consideration favoring these approaches is that both quorum-sensing and two-component signaling are completely absent from the mammalian genome. Promising data have also been generated with a recent screening effort for antibiotics that specifically inhibit persister formation [149]. Thus, this work could have broad implications for preventing biofilms and ensuing antibiotic resistance/tolerance. This idea seems rational, as persistence signaling seems to eventually culminate in a finite number of stress-response pathways that are common to many bacterial pathogens. Recent studies have shown that some antibiotics derived from previously "unculturable" bacteria demonstrate great potential to provide a new class of persister-targeting antibiotics [149, 150]. Perhaps the future holds a more multifaceted approach to the problem of resistance and tolerance, such that one antibiotic is given to clear an infection and another to clear the anticipated tolerant subpopulation.

Major Points

- Tolerance and resistance mechanisms can be energetically costly; thus bacteria must have a means to sense a threat, induce a response, and terminate a response.
- Bacterial signal transduction systems provide a means to sense and respond to both their extracellular and intracellular environments.
- Bacterial signaling systems are largely comprised of two-component signaling (TCS) systems, but they can also be serine-threonine kinase (eSTK) and phosphatase (eSTP) systems, along with more specialized signaling such as the *S. aureus* Agr cascade.
- A general response to many cell-envelope targeting antimicrobials is the modification of the envelope charge to electrostatically repel the drug. Some signaling systems that detect and respond to antibiotics in this way, such as the PhoQP and PmrAB TCS systems, are conserved among many bacterial species.
- Bacterial signaling systems, especially cell-cell communication systems (or quorum sensing), are required to regulate biofilm formation and antibiotic tolerance.

- Within the biofilm, a subpopulation of persister cells exist that are metabolically less active than their majority counterparts and whose state in many cases is controlled by toxin-antitoxin (TA) modules.
- Bacterial signaling systems accumulate mutations that contribute to resistance and tolerance; therefore, they are an important component of antimicrobial resistance.

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Chapter 16
Bacterial Type II Topoisomerases and Target-Mediated Drug Resistance

Elizabeth G. Gibson, Rachel E. Ashley, Robert J. Kerns, and Neil Osheroff

16.1 Introduction

Fluoroquinolones are among the most efficacious and broad-spectrum oral antibacterials currently in clinical use [1–4]. They are used as frontline treatments for a wide variety of infections caused by Gram-negative and Gram-positive bacteria [5]. Among the diseases treated with fluoroquinolones are urinary tract infections and pyelonephritis, sexually transmitted diseases, prostatitis, skin and tissue infections, chronic bronchitis, community-acquired and nosocomial pneumonia, and intra-abdominal and pelvic infections [5]. Fluoroquinolones are also the first line of prophylactic treatment for anthrax, the "biological agent most likely to be used" in a bioterrorist attack, according to the Centers for Disease Control and Prevention (CDC) [6]. Furthermore, they are commonly used to treat tuberculosis in cases of resistance or patient intolerance to established regimens [7]. Tuberculosis recently overtook HIV/AIDS as the deadliest disease in the world caused by a single infective agent [8].

Fluoroquinolones kill bacteria by increasing levels of double-stranded DNA breaks generated by enzymes known as type II topoisomerases [2, 9–12]. The vast

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I. W. Fong et al. (eds.), *Antimicrobial Resistance in the 21st Century*, Emerging Infectious Diseases of the 21st Century, https://doi.org/10.1007/978-3-319-78538-7_16

majority of bacteria encode two type II enzymes, gyrase and topoisomerase IV [10, 13–15]. These enzymes are essential for cell survival, and both appear to be physiological targets for fluoroquinolones [2, 9, 10, 14]. In contrast, a handful of species encode only gyrase. This group includes a number of disease-causing organisms, including *Treponema pallidum* (syphilis) [16], *Helicobacter pylori* (stomach and intestinal ulcers) [17], *Campylobacter jejuni* (gastroenteritis) [18], *Mycobacterium leprae* (leprosy) [19], and *Mycobacterium tuberculosis* (tuberculosis) [20]. In these species, gyrase takes on dual characteristics and can fulfill its own functions as well as those of topoisomerase IV [21].

Unfortunately, fluoroquinolone usage is being threatened by an increasing prevalence of resistance, which extends to every bacterial infection treated by this drug class [1, 2]. The most common and clinically relevant form of resistance is target-mediated, which is caused by specific mutations in gyrase and topoisomerase IV [22, 23]. Therefore, it is critically important to understand how this drug class interacts with and alters the activity of its enzyme targets to better guide drug development and to overcome resistance [11]. In this chapter, we will discuss fluoroquinolone action and targeting, resistance mechanisms, and efforts to overcome this resistance.

16.2 Fluoroquinolones

The history of the fluoroquinolones began in 1962, when Lesher et al. made the accidental discovery of nalidixic acid (Fig. 16.1) as a by-product of the synthesis of the antimalarial compound chloroquine [24]. This first-generation quinolone displayed limited efficacy and was used mainly for the treatment of uncomplicated urinary tract infections caused by Gram-negative enteric bacteria [25]. In the 1980s, the second generation of quinolones was established when norfloxacin (Fig. 16.1) was synthesized [1, 25, 26]. This drug featured a fluorine at the C6 position, making it the first true fluoroquinolone, and a cyclic diamine piperazine at the C7 position. The fluorine at the C6 position increased tissue penetration and has been included in every subsequent clinically relevant member of this drug class [1, 25–27].

Even with improved tissue penetration, norfloxacin was still confined to the urinary tract and displayed low serum concentrations [1, 25–27]. However, it broadened the use of quinolones to include sexually transmitted diseases [1, 25–27].

Ciprofloxacin (Fig. 16.1) was the first fluoroquinolone to display efficacy toward both Gram-positive and Gram-negative bacterial species and was the first with sufficiently high tissue penetration and serum concentration to be used outside the urinary tract [1, 25, 26]. The clinical success of ciprofloxacin spawned the development of third-generation fluoroquinolones that include moxifloxacin, gatifloxacin, and levofloxacin (Fig. 16.1) [1, 25–27]. These drugs all exhibit improved half-lives compared to ciprofloxacin [28]. Moreover, they have extended the spectrum of fluoroquinolone activity to include a broader array of Gram-positive bacteria (including a number of respiratory infections), as well as atypical pathogens such as *Legionella pneumophila* and *Chlamydia pneumoniae* [2–5].

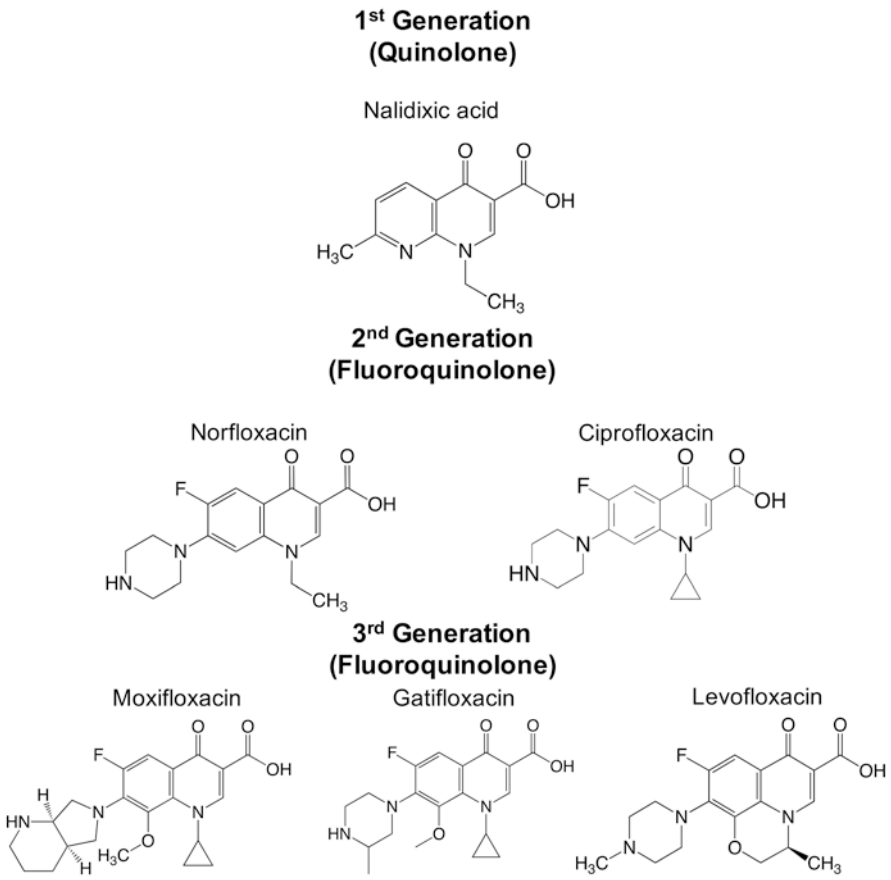

Fig. 16.1 Fluoroquinolone structures. Nalidixic acid, a first-generation quinolone, is the founding member of this drug class. This drug displayed limited efficacy for systemic infections and had a narrow antibacterial spectrum. Norfloxacin and ciprofloxacin, second-generation fluoroquinolones, had improved efficacy, with ciprofloxacin being the more efficacious of the two. Ciprofloxacin displayed an improved antibacterial profile that included additional Gram-positive bacterial infections and improved Gram-negative coverage. Moxifloxacin, gatifloxacin, and levofloxacin, third-generation fluoroquinolones, are the most efficacious and broad-spectrum fluoroquinolones in clinical use today

16.3 Bacterial Type II DNA Topoisomerases

Type II topoisomerases control the topological state of the DNA in the cell [9–11, 29–31]. These enzymes modulate DNA under- and overwinding (i.e., negative and positive supercoiling, respectively) and remove tangles and knots from the genome [11, 12, 31–35]. As discussed earlier, most bacterial species encode two type II topoisomerases, gyrase and topoisomerase IV [9–11, 29–31]. Gyrase was the first type II topoisomerase to be described in any species and was originally reported in 1976 [36]. It is an A_2B_2 heterotetramer in which the two subunit types are GyrA and GyrB [9–11, 29–31]. The A subunits contain the active site tyrosine residues that cleave the DNA (shown in blue in Fig. 16.2). The B subunits form the N-terminal gate of the enzyme and contain the sites of ATP binding and hydrolysis (shown in green in Fig. 16.2) [9–11, 29–31].

The subunits of topoisomerase IV were first identified in Gram-negative species as being required for chromosome partitioning and were named ParC and ParE (blue and green in Fig. 16.2, respectively) [10–13, 31, 37–39]. Sequence analysis revealed that these proteins were homologous to GyrA and GyrB, respectively. In 1990, it was determined that the ParC/ParE complex was a heterotetramer that functioned as a distinct type II topoisomerase [10–13, 31, 37–39]. The enzyme was subsequently named topoisomerase IV. Whereas the subunits of topoisomerase IV are denoted as ParC and ParE in Gram-negative species because of their historic roles in chromosome partitioning, they are called GrlA and GrlB, respectively, (which comes from their initial name gyrase-like proteins) in Gram-positive species.

Gyrase and topoisomerase IV regulate DNA topology by using a double-stranded DNA passage mechanism [10–13, 31, 34]: the enzymes generate a double-stranded break in the gate or G-segment (green in Fig. 16.2) and pass the transport or T-segment (red) through the open DNA gate. The transport helix eventually exits the enzymes when the two subunits at the bottom of the enzyme open to form the exit gate. This reaction takes place at the expense of ATP binding, which opens the DNA gate and induces a conformational change that moves the T-segment through the open gate, and ATP hydrolysis, which drives enzyme turnover.

As a prerequisite for opening the DNA gate, gyrase and topoisomerase IV generate a double-stranded break in the G-segment [10–13, 31, 34]. The scissile bonds on the two strands of the double helix are located across the major groove from one another. Cleavage results in 5′-overhanging termini with a four-base cohesive stagger. In order to maintain genomic integrity during the DNA cleavage event, the enzymes form a covalent phosphotyrosine linkage between active site residues and the newly generated 5′ termini. This covalent enzyme-cleaved DNA complex is critical for the actions of quinolones and is called the *cleavage complex* [10–13, 31, 34].

Despite the sequence and structural similarities between gyrase and topoisomerase IV, differences in the C-terminal domains of GrlA/ParC and GyrA confer these enzymes with unique catalytic activities [10–13, 31, 34]. The C-terminal domain of GrlA/ParC allows topoisomerase IV to interact with two distal DNA segments. Thus, the enzyme uses a “canonical” strand-passage mechanism in which it cap-

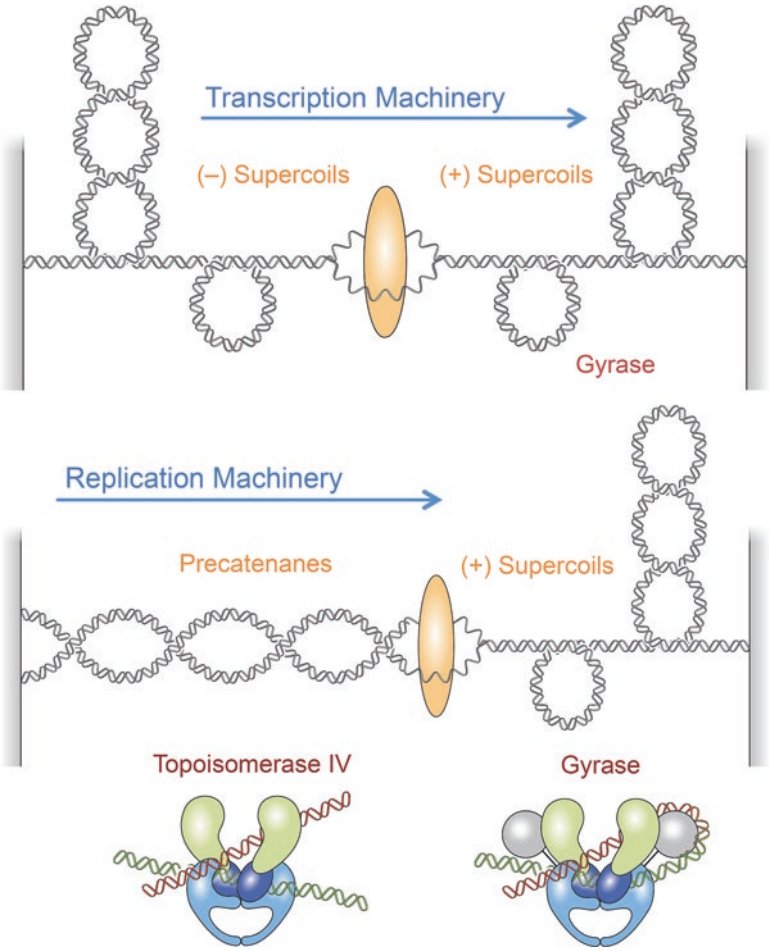

Fig. 16.2 Cellular functions of bacterial type II topoisomerases. Topoisomerase IV (bottom left) uses a canonical double-stranded DNA passage mechanism. The enzyme can remove positive supercoils but acts primarily behind the replication fork (middle) to remove precatenanes and unlink daughter chromosomes. Gyrase (bottom right) uses a DNA-wrapping mechanism that is superimposed upon the double-stranded DNA passage reaction. The enzyme removes positive DNA supercoils ahead of transcription (top) and replication (middle) complexes and maintains the negative superhelicity of the genome. (Artwork by Ethan Tyler, NIH Medical Arts)

tures existing intra- or intermolecular DNA crossovers (Fig. 16.2, left) [10–13, 31, 34]. This allows the enzyme to relax (i.e., remove) positive or negative DNA supercoils and to remove DNA tangles and knots in a highly efficient manner [10–13, 31, 34]. Although topoisomerase IV is able to alleviate torsional stress ahead of DNA tracking systems and appears to play a role in regulating genomic superhelicity, its major function is to remove the precatenanes that form behind DNA replication forks (Fig. 16.2 middle), separate daughter chromosomes following replication, and

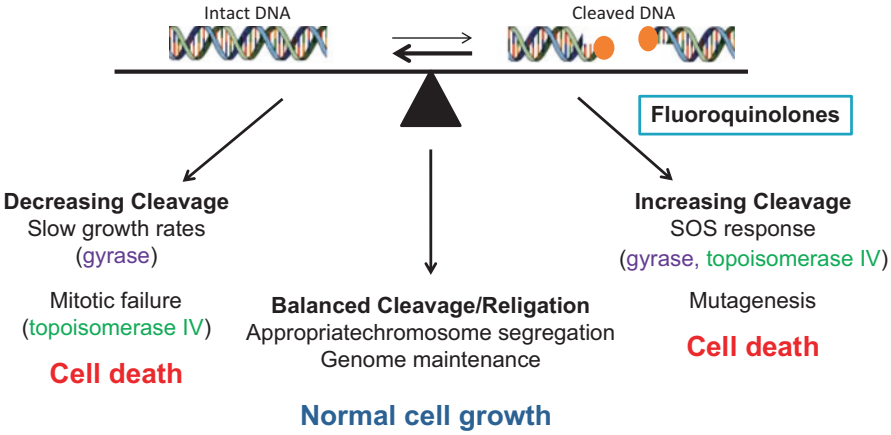

Fig. 16.3 Bacterial type II topoisomerases: the critical balance of DNA cleavage and ligation. The DNA cleavage/ligation activity of bacterial type II topoisomerases must be regulated in the cell. When an appropriate level of cleavage complexes is maintained, topological problems within DNA are resolved, and the cell can grow normally. If the levels of cleavage complexes decrease, slow growth rates and mitotic failure can cause cell death. Conversely, if the levels of cleavage complexes are too high, the resulting strand breaks can block essential nucleic acid functions and induce the SOS response, generate mutations, and lead to cell death. Compounds that increase levels of gyrase or topoisomerase IV cleavage complexes, such as fluoroquinolones, act as topoisomerase "poisons" and convert the proteins to cellular toxins that have the potential to fragment the genome. Compounds that inhibit the catalytic activity of gyrase or topoisomerase IV without increasing levels of DNA cleavage work by robbing the cell of essential enzyme function leading to slow growth rates, mitotic failure, and cell death. (Adapted from Pendleton et al. [46])

remove DNA knots that form during DNA recombination [13, 40–45]. If topoisomerase IV activity drops below threshold levels, cells die of mitotic failure (Fig. 16.3) [10–13, 31, 34].

In contrast to the canonical mechanism used by topoisomerase IV, gyrase uses a mechanism in which the C-terminal domain of the GyrA subunit (gray, Fig. 16.2) wraps DNA, inducing a positive crossover between the G- and T-segments that mimics a positive supercoil [13, 47–49]. Because of this "wrapping" mechanism, the captured G- and T-segments are proximal to one another [50]. As a result, gyrase greatly favors the catalysis of intra- over intermolecular strand-passage reactions. Consequently, the enzyme can efficiently alter superhelical density but is very poor at removing tangles and knots [50, 51]. In addition, because gyrase always acts on the induced positive crossover, it works in a unidirectional manner [13, 52]; in the presence of ATP, the enzyme can remove positive, but not negative supercoils. Furthermore, gyrase is able to induce negative supercoils into relaxed DNA [36, 53]. The major cellular roles of gyrase stem from its DNA-wrapping mechanism. Gyrase functions ahead of replication forks and transcription complexes to alleviate the torsional stress induced by DNA overwinding (Fig. 16.2, middle and bottom) [31, 54]. Furthermore, in conjunction with the ω protein (commonly called topoisomerase I), a type I topoisomerase, gyrase modulates the superhelicity of the bac-

terial chromosome and allows the organism to maintain its genetic material in an underwound state [55]. If gyrase activity in the cell drops, rates of replication/transcription are severely impacted (Fig. 16.3) [9–13, 53]. Furthermore, a number of pleiotropic effects on gene expression are observed due to changes in superhelicity of the bacterial chromosome [56].

Although gyrase and topoisomerase IV are essential enzymes, they also pose a threat to the bacterial cell. Indeed, if a replication fork, transcription complex, or DNA tracking system encounters and attempts to pass through a gyrase- or topoisomerase IV-mediated DNA cleavage complex, it can disrupt the complex and render the enzyme unable to ligate the DNA [40]. This event generates double-stranded DNA breaks that require recombination pathways to repair. Thus, these breaks block essential nucleic acid functions, induce the SOS response, generate mutations, and trigger processes that ultimately impair cell survival [2, 3, 12, 26, 57, 58].

Compounds that increase levels of gyrase or topoisomerase IV cleavage complexes are referred to as "poisons" [59], because they are said to poison these proteins, converting them to cellular toxins that have the potential to fragment the bacterial chromosome [2, 9, 26, 57, 58]. The term "poison" distinguishes these compounds from "catalytic inhibitors," which act primarily by robbing the cell of the catalytic functions of these enzymes [34].

16.4 Fluoroquinolone Mechanism

Fluoroquinolones are potent gyrase/topoisomerase IV poisons [9, 11, 12, 23, 60–62]. These drugs interact with both the protein and DNA within a cleavage complex and intercalate into the DNA backbone at the cleaved scissile bonds [23, 62]. Consequently, two fluoroquinolone molecules are required to stabilize double-stranded breaks induced by the bacterial type II enzymes (Fig. 16.4). The intercalated fluoroquinolones likely produce some distortions within the enzyme active site; however, these drugs act primarily as "molecular doorstops" that form a physical barrier to DNA ligation [23]. Thus, the presence of fluoroquinolones inhibits the rate of gyrase- and topoisomerase IV-mediated DNA ligation. Furthermore, drugs that induce higher levels of enzyme-mediated DNA strand breaks appear to form more stable interactions within the cleavage complex and allow these complexes to persist for longer periods of time [63, 64].

In addition to generating DNA strand breaks in the cell, fluoroquinolones also inhibit the overall catalytic strand-passage activities of gyrase and topoisomerase IV [3, 11, 12]. As a result, there is debate as to whether the inhibition of strand passage contributes to drug efficacy in cells. Although this issue has yet to be definitively decided, a recent study suggests that the deleterious actions of drugs result primarily from the enhancement of DNA cleavage [65]. In this study, the effects of ciprofloxacin on three different fluoroquinolone-resistant mutations of *Escherichia coli* topoisomerase IV that are associated with clinical resistance were examined in vitro. With all three enzymes, ciprofloxacin displayed virtually no ability to enhance DNA

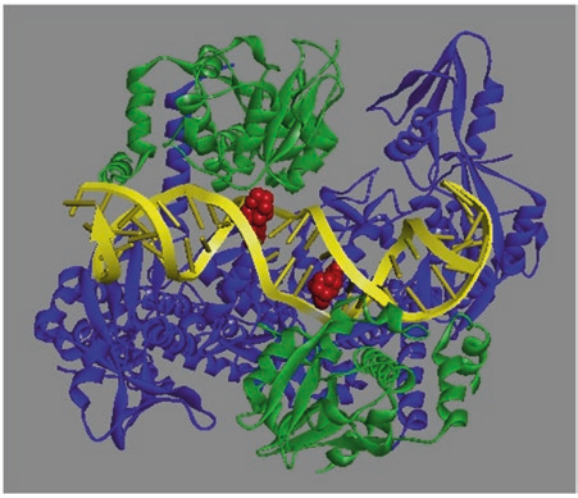

Fig. 16.4 Crystal structure of a topoisomerase IV-DNA cleavage complex formed with *A. baumannii* enzyme in the presence of moxifloxacin. This structure is a top view of the cleavage complex with two fluoroquinolone molecules intercalating four base pairs apart at the cleaved scissile bonds. The presence of the intercalated fluoroquinolones likely produces distortions within the enzyme active site; however, these drugs act primarily as "molecular doorstops" that form a physical barrier to ligation. The catalytic core of the enzyme (blue and green for the A and B subunit, respectively), moxifloxacin (red), and DNA (yellow) are shown. (Adapted from Aldred et al. [11])

cleavage but showed wild-type ability to inhibit DNA relaxation catalyzed by the type II enzymes. Therefore, it appears that the ability to induce DNA cleavage is the primary factor that determines quinolone-induced cytotoxicity.

16.5 Fluoroquinolone Resistance

The World Health Organization (WHO) ranks fluoroquinolones as one of the five "highest priority" and "critically important" classes of antimicrobials [66]. However, due to their widespread use and overuse, resistance has been on the rise since the 1990s [1, 2, 11]. As an extreme example, the CDC has classified *Neisseria gonorrhoeae*, the causative agent of gonorrhea, as one of its top three "urgent level" drug-resistant threats to the United States [67], primarily due to fluoroquinolone resistance. Along with the WHO, it has issued dire warnings that gonorrhea is on the precipice of joining HIV/AIDS and herpes as the third "incurable" sexually transmitted disease [68].

Fluoroquinolones were used routinely to treat gonorrhea starting in 1993 and were used in more than 40% of the cases by the year 2003 [69–71]. However, the use of fluoroquinolones as frontline therapy against this disease was discontinued in 2006 due to the high incidence of resistance; 22.4% of cases reported in the United

States in 2015 were resistant to fluoroquinolones (this value rose to 32.1% among men who have sex with men) [69, 72, 73]. In parts of Asia, fluoroquinolone resistance exceeds 80% [73]. Other infectious bacteria that have raised concerns due to their high level of fluoroquinolone resistance include *Campylobacter* spp., *Salmonella* spp., and *E. coli* [74].

Thus far, three mechanisms of fluoroquinolone resistance have been described [11, 58]. The first is "target-mediated resistance," which results from specific mutations in gyrase or topoisomerase IV [75–77]. The second is "plasmid-mediated resistance," which is caused by the presence of extrachromosomal DNA fragments that encode three different classes of proteins [11, 78, 79]. Some plasmids encode acetylases, which modify and inactivate quinolones and other drugs. Others encode Qnr proteins, which block type II topoisomerases from binding to their DNA substrates or to fluoroquinolones. Still others encode efflux pumps, which decrease the fluoroquinolone concentration in cells. The third mechanism of fluoroquinolone resistance is "chromosome mediated," in which the expression of efflux pumps is elevated or the expression of porins, which play a role in fluoroquinolone uptake, is downregulated [11, 26, 78, 80].

Although the latter two mechanisms contribute significantly to fluoroquinolone resistance, the target-mediated mechanism is generally the form most often associated with clinical resistance [11, 81, 82]. Because target-mediated resistance represents the most common and clinically relevant form of resistance, the remainder of this chapter will focus on this mechanism.

Initial quinolone resistance is almost always associated with specific mutations in gyrase, topoisomerase IV, or both. For example, in a recent clinical study on drug resistance [83], 97% of 60 quinolone-resistant isolates of *E. coli* carried mutations in gyrase, and 90% of these isolates also carried mutations in topoisomerase IV.

In general, the most commonly observed (up to ~90%) fluoroquinolone resistance mutation is in a highly conserved serine residue that was first described as Ser83 in the A subunit of *E. coli* gyrase [84–88]. This residue resides in helix-IV of GyrA. The majority of other resistance mutations usually map to a conserved glutamic/aspartic acid residue that is four amino acids downstream from the serine and also resides in helix-IV. Mutations at these positions often provide a tenfold or higher reduction in susceptibility to clinically relevant fluoroquinolones. Corresponding mutations in *E. coli* topoisomerase IV also result in fluoroquinolone resistance in vitro [85–88].

The prevalence of resistance mutations at the serine residue may reflect the fact that this residue is highly conserved but nonessential. To this point, the common mutations at this residue display no known phenotype, in cells or in vitro, with the exception of fluoroquinolone resistance. It is not clear why this residue is conserved; however, the presence of the serine appears to provide protection against nybomycin, a naturally occurring antibiotic [89]. Thus, it has been proposed there has been natural selection to maintain the serine in the bacterial genome. It is notable that mutations at the glutamic/aspartic residue often decrease the overall catalytic activity of gyrase and topoisomerase IV [77, 90]. This may explain why a higher proportion of resistance mutations are observed at the serine residue.

To determine the contributions of gyrase and topoisomerase IV to fluoroquinolone resistance in cells, *E. coli* strains carrying these mutations in gyrase, topoisomerase IV, or both were analyzed for drug efficacy. Strains carrying mutant gyrase were ~10-fold less susceptible to fluoroquinolones. Although strains carrying mutant topoisomerase IV displayed little, if any, resistance, those carrying mutations in both enzymes had ~100-fold decrease in susceptibility [2, 3, 9, 58]. This pattern of resistance strongly suggests that gyrase is the primary toxic target for fluoroquinolones in *E. coli* (Gram-negative), and topoisomerase IV is a secondary target for the drugs.

Since that initial set of experiments, the primary cellular target for fluoroquinolones in all other species has been identified by mutagenesis studies [11, 91–96]. The enzyme in which the first resistance mutations appear is believed to be the primary toxic target. Surprisingly, when these studies were carried out in *Streptococcus pneumoniae,* a Gram-positive species, the first mutations appeared in topoisomerase IV [94]. Thus, it became dogma in the field that gyrase was the primary target for fluoroquinolones in Gram-negative species and topoisomerase IV was the primary target in Gram-positive species. While this axiom generally holds true, subsequent studies have found that there are often exceptions and that the target has to be determined on a species-by-species and drug-by-drug basis [21, 63, 65, 90, 97, 98].

16.6 Role of the Water-Metal Ion Bridge in Mediating Fluoroquinolone Resistance and Gyrase/Topoisomerase IV Interactions

Although the association of the serine and glutamic/aspartic residues with fluoroquinolone resistance was established in the late 1980s [99–102], the mechanism by which they lead to resistance was described only recently. Ultimately, the mechanistic basis for fluoroquinolone action and resistance turned out to be inextricably linked [11, 63, 65, 103]. Thus, these two important aspects of fluoroquinolone-enzyme interaction will be discussed together.

The initial insight into the roles of the serine and glutamic/aspartic acid residues of fluoroquinolone actions and resistance came from structural studies of cleavage complexes formed with topoisomerase IV or gyrase in the presence of fluoroquinolones [23, 60–62]. Although these studies all localized fluoroquinolones in the same binding pocket, which was proximal to the conserved amino acid residues, there was disagreement regarding drug orientation within the pocket. Furthermore, none of the studies found that the bound fluoroquinolone was close enough to either amino acid to form a direct interaction.

However, one of the structures (which examined the cleavage complex of *Acinetobacter baumannii* topoisomerase IV formed in the presence of moxifloxacin) provided a potential mechanism by which mutations at the serine or glutamic/aspartic residue could lead to fluoroquinolone resistance [23]. It had long been known

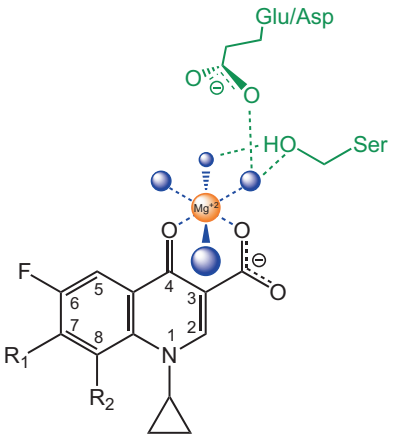

Fig. 16.5 A water-metal ion bridge mediates critical interactions between fluoroquinolones and bacterial type II topoisomerases. A generic fluoroquinolone structure is depicted in black, water molecules are in blue, Mg^{2+} is in orange, and the coordinating serine and glutamic/aspartic acid residues are in green. Blue dashed lines indicate the interaction between the divalent metal ion, four water molecules, and the C3/C4 keto acid of the fluoroquinolone. The green dashed lines represent hydrogen bonds between the serine and glutamic/aspartic acid side-chain hydroxyl groups and the water molecules

that the C3/C4 keto acid of fluoroquinolones chelates divalent metal ions, but the physiological role of these bound metal ions, if any, was unknown. The structure of *A. baumannii* topoisomerase IV was the first to capture this fluoroquinolone-metal ion interaction within a cleavage complex. In this structure, the C3/C4 keto acid of moxifloxacin chelated a non-catalytic magnesium ion that appeared to be coordinated to four water molecules. Two of these water molecules were in sufficiently close proximity to Ser84 and Glu88 (equivalent to *E. coli* GyrA Ser83 and Glu87) to form hydrogen bonds. Thus, the authors suggested that this water-metal ion coordination might play a role in mediating interactions between fluoroquinolones and bacterial type II topoisomerases. A subsequent study that determined the structures of cleavage complexes formed with *M. tuberculosis* gyrase in the presence of moxifloxacin, 8-methyl-moxifloxacin, ciprofloxacin, levofloxacin, or gatifloxacin also observed the chelated metal ion, the associated water molecules, and the protein contacts [64]. A generalized diagram of the proposed water-metal ion "bridge" that facilitates fluoroquinolone interactions with the conserved serine and glutamic/aspartic residues is shown in Fig. 16.5 [22, 65, 90].

The initial functional evidence for the existence and role for the water-metal ion bridge in mediating fluoroquinolone activity and resistance came from biochemical studies on *B. anthracis* topoisomerase IV [90]. These studies utilized wild-type and drug-resistant enzymes that carried mutations in the serine (Ser81) and/or glutamic acid (Glu85) residues. The authors demonstrated that (1) the ability of fluoroquinolones to poison topoisomerase IV relied on the presence of a non-catalytic divalent metal ion; (2) mutations in either the serine or glutamic acid restricted

the metal ions that could be used to support drug activity; and (3) mutations in either amino acid decreased the affinity of the metal ion. Later studies extended these conclusions to topoisomerase IV from *E. coli* and gyrase from *B. anthracis* and *M. tuberculosis* [11, 63, 65]. Thus, it appears that the water-metal ion bridge is used to mediate fluoroquinolone-enzyme interactions in a variety of bacterial species. Furthermore, the loss of one or both of the amino acids that anchor the bridge is sufficient to disrupt these interactions and cause drug resistance [22, 63, 65].

Despite the importance and apparent "universality" of the water-metal ion bridge, it seems to be used differently by enzymes from different bacterial species. Whereas the bridge is critical for the binding of clinically relevant fluoroquinolones to *B. anthracis* gyrase and topoisomerase IV and *M. tuberculosis* gyrase, it is used primarily to align fluoroquinolones in the active site of *E. coli* topoisomerase IV [11, 63, 65, 90].

The divalent metal ion of the water-metal ion bridge interacts with fluoroquinolones through the C3/C4 keto acid of the drug scaffold [63, 65, 90, 97]. This may explain why clinically relevant fluoroquinolones can accommodate such a wide variety of substituents at the N1, C7, and C8 positions. Whereas substituents at the latter positions are unlikely to form critical gyrase or topoisomerase IV interactions, they may contribute minor or species-specific interactions. Furthermore, they may influence the pharmacokinetics of the drugs.

Finally, the water-metal ion bridge appears to be the feature of drug-enzyme interactions that allows discrimination between the bacterial and human type II topoisomerases. Indeed, the amino acids in human topoisomerase IIα that correspond to the serine and acidic residues of the bacterial helix-IV are methionine residues. This likely explains why clinically relevant fluoroquinolones display such poor activity against the human type II enzymes. If these methionine residues in topoisomerase IIα are converted to serine and glutamic acid residues, the activity of ciprofloxacin and moxifloxacin against the human enzyme rises four- to fivefold [90].

16.7 Overcoming Target-Mediated Fluoroquinolone Resistance: Modified Fluoroquinolones and Fluoroquinolone-Like Compounds

16.7.1 C7 Substituents

The influence of the C7 substituent on fluoroquinolone resistance is highlighted by recent studies on quinazolinediones. These compounds are similar in structure to fluoroquinolones but display a strong ability to overcome resistance caused by mutations in the amino acid residues that anchor the water-metal ion bridge [87] (Fig. 16.6). The quinazolinedione scaffold differs from that of fluoroquinolones only at the 2 and 3 ring positions, where the hydrogen at C2 has been replaced with a ketone and the carboxylic acid at C3 has been replaced with an N3 amino group.

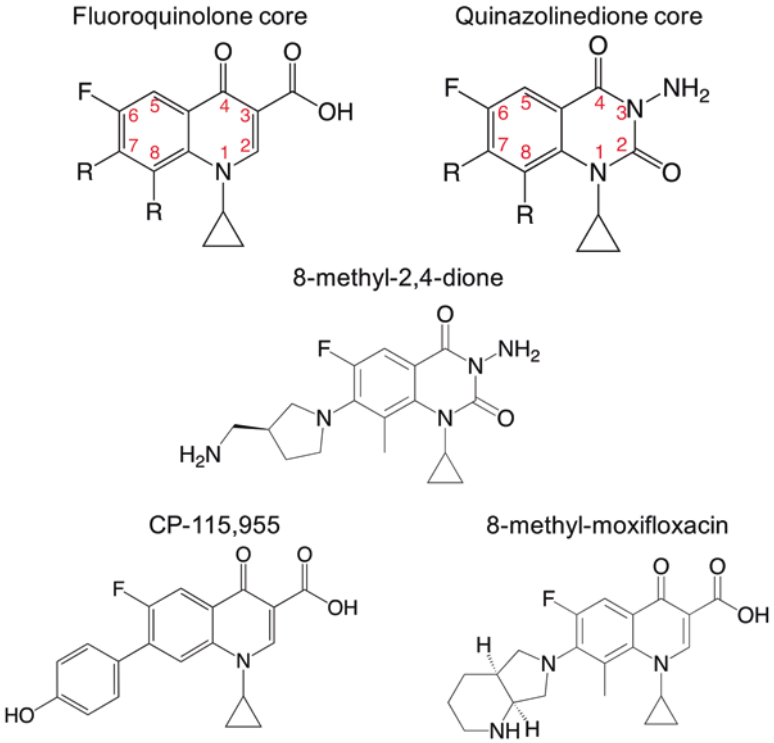

Fig. 16.6 Fluoroquinolone-like compounds with unique properties. The quinazolinedione scaffold is similar to the fluoroquinolone core; however, the loss of the C3/C4 keto acid disrupts the ability to chelate the divalent metal ion used in the water-metal ion bridge. 8-Methyl-2,4-dione, a quinazolinedione, and 8-methyl-moxifloxacin, a fluoroquinolone, overcome resistance by mediating interactions with bacterial type II enzymes through their C7 and C8 substituents, respectively. CP 115,955, a fluoroquinolone, displays high activity against human (through the C7 substituent) and bacterial (through the water-metal ion bridge) type II topoisomerases

These substitutions disrupt the C3/C4 keto acid required to chelate the divalent metal ion used in the water-metal ion bridge. Thus, it is not surprising that interactions between quinazolinediones and bacterial type II topoisomerases are independent of bridge function and are unaffected by resistance mutations in the serine or acidic amino acid residues that serve as bridge anchors. Consequently, it was believed that quinazolinediones represented "fluoroquinolones" with a scaffold that was impervious to classic resistance mutations [87].

However, three lines of evidence lead to the conclusion that the ability of quinazolinediones to overcome resistance results from the substituent at C7 rather than the C3/C4 portion [22, 75]. First, the quinazolinediones reported in the literature most often contained a 3′-(aminomethyl)-1-pyrrolidinyl [3′-(AM)P] (or related) substituent at the C7 position. The 3′-(AM)P moiety is not represented in any clinically relevant fluoroquinolone, opening the possibility that it has properties not pre-

viously ascribed to fluoroquinolone C7 groups. Second, when the C7 piperazine group of ciprofloxacin was replaced with the 3′-(AM)P moiety, the resulting fluoroquinolone overcame resistance caused by mutations in the bridge anchoring amino acid residues in a purified enzyme system [11, 63, 65, 90, 104]. Third, when the C7 3′-(AM)P substituent of the quinazolinedione was replaced with the C7 group of either ciprofloxacin (piperazine) or moxifloxacin (diazabicyclonone), the resulting drugs displayed little activity against either wild-type or resistant bacterial type II topoisomerases [22, 75, 104].

The evidence described above indicates that the quinazolinedione scaffold does not interact with gyrase and topoisomerase IV through metal ion-independent contacts. Rather, these drugs are essentially quinolone derivatives that lack their most important interactions with bacterial type II topoisomerases (i.e., the water-metal ion bridge). These findings ultimately led to a very important conclusion with implications for future design of fluoroquinolones that overcome resistance: it is possible to design C7 substituents for fluoroquinolones that display strong, bridge-independent interactions with gyrase and topoisomerase IV.

The role of the C7 substituent in mediating enzyme interactions has long been known for the interaction of fluoroquinolones with eukaryotic type II topoisomerases [105, 106]. Indeed, fluoroquinolones such as CP-115,953 and CP-115,955 display high activity against type II topoisomerase and are more potent and efficacious against human topoisomerase IIα and IIβ than the anticancer drug etoposide [103]. Both of these compounds rely on a 4′-hydroxyphenyl substituent at the C7 position for their activity [105, 106].

On the basis of structural and modeling studies, it has been proposed that the C7 3′-(AM)P moiety allows quinazolinediones to poison gyrase and topoisomerase IV by interacting with a conserved glutamic acid residue in the GyrB and ParE/GrlB subunit (corresponding to *E. coli* GyrB-Glu466) [87, 104]. Indeed, it appears that the primary amine of the 3′-(AM)P substituent can form both a salt bridge and a hydrogen bond with this acidic residue. Unfortunately, this glutamic acid residue is also conserved among eukaryotic type II topoisomerases, and the quinazolinediones that include the C7 3′-(AM)P moiety display activity against human topoisomerase IIα similar to that of etoposide [22]. Thus, fluoroquinolone substitutions at C7 that overcome resistance should be approached with caution, as they have the potential to crossover into the human system.

16.7.2 C8 Substituents

Recent studies strongly suggest that substituents at the C8 position can have dramatic effects on fluoroquinolone resistance [63]. At the present time, structure-activity relationship studies that examined the effects of C8 substituents on resistance have been confined to relatively minor changes at this position: hydrogen, methyl, or methoxy groups. However, major effects on resistance have been observed. In general, compounds that include a methyl or methoxy group at C8 display higher

activity against enzymes that carry mutations in the bridge-anchoring serine or glutamic/aspartic acid. In some cases, dramatic differences in sensitivity have been reported. For example, converting the C8 methoxy of moxifloxacin to a methyl group results in a fluoroquinolone that poisons *M. tuberculosis* gyrase with twice the potency and efficacy of moxifloxacin and completely overcomes clinically relevant resistance mutations in a purified enzyme system [63, 64]. The fact that such a minor alteration in fluoroquinolone structure can produce such a dramatic difference in the resistance profile of the drug suggests that the C8 position is a ripe target for future drug discovery.

Despite the potential of C8 substituents for overcoming drug resistance, the basis for the high activity of "8-methyl-moxifloxacin" against fluoroquinolone-resistant *M. tuberculosis* gyrase is unknown. Although this compound induces gyrase-mediated DNA strand breaks that are more stable than observed with moxifloxacin, structural studies indicate that 8-methyl-moxifloxacin occupies a space within the cleavage complex that is identical to that of the parent drug [63, 64]. Furthermore, no specific protein or DNA contacts were observed with the C8 substituent. Thus, future chemical studies will need to be combined with strong efforts in mechanistic enzymology and structure in order to fully exploit the C8 substituent as a means to overcome fluoroquinolone resistance.

16.8 Overcoming Target-Mediated Fluoroquinolone Resistance: Novel Compounds

Currently, fluoroquinolones are the only antibacterials in clinical use that target gyrase or topoisomerase IV [9, 11, 23, 60–62]. However, recent drug discovery efforts have resulted in new classes (two of which are in clinical trials) with clinical potential. All of these compounds lack the keto acid that fluoroquinolones use in conjunction with the water-metal ion bridge to interact with their bacterial targets. Consequently, they all display activity against fluoroquinolone-resistant bacterial strains.

16.8.1 Novel Bacterial Topoisomerase Inhibitors (NBTIs)

NBTIs (Fig. 16.7) are napthyridone/aminopiperidine-based compounds that were first reported to have antibacterial activity in 1999 [107]. It was not until 2007 that these compounds were found to have activity against bacterial type II topoisomerases [108]. Early studies demonstrated that at least some of the NBTIs are potent inhibitors of overall catalytic activity [109]. Later studies determined that some of these compounds could also poison the enzymes [60].

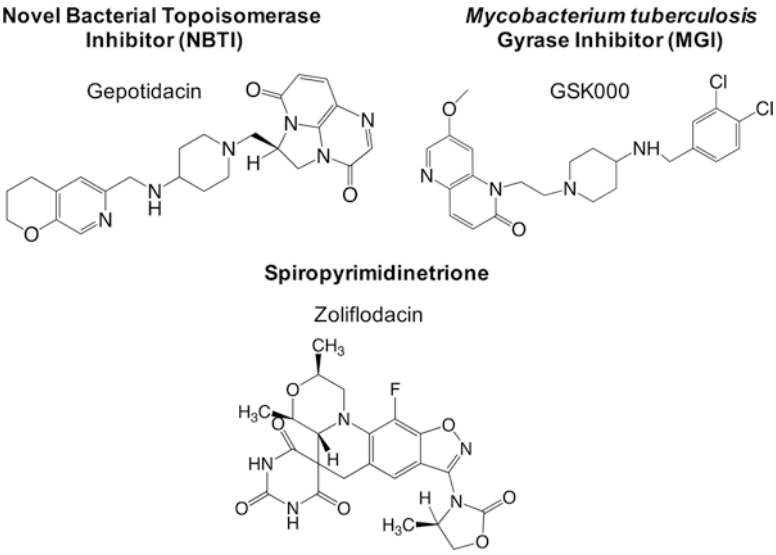

Fig. 16.7 Novel gyrase/topoisomerase IV-targeted compounds. NBTIs, such as gepotidacin, act as gyrase/topoisomerase IV poisons. However, in contrast to fluoroquinolones, they induce only single-stranded DNA breaks. MGIs, such as GSK000, were derived from NBTIs in an effort to optimize activity against *M. tuberculosis.* The founding member of the spiropyrimidinetrione class of antibacterials is zoliflodacin (ETX0914/AZD0914)

Compared to fluoroquinolones, NBTIs are distinct in two major respects. First, structural studies demonstrate that only a single NBTI molecule interacts with the DNA in the active site of gyrase. It binds between the two scissile bonds and elongates the DNA in the active site of the enzyme. This is in contrast to the two fluoroquinolones (one at each cut scissile bond) that interact with DNA in the cleavage complex. Second, whereas fluoroquinolones stabilize double-stranded DNA breaks generated by gyrase or topoisomerase IV, NBTIs that act as gyrase/topoisomerase IV poisons induce only single-stranded DNA breaks. Little else is known about how these compounds interact with gyrase or topoisomerase IV. NBTIs display high activity against bacterial cells that harbor fluoroquinolone-resistant mutations in gyrase and topoisomerase IV. However, no study examining purified fluoroquinolone-resistant mutant enzymes has been reported. One member of the NBTI family, gepotidacin, is currently in phase II clinical trials for the treatment of uncomplicated gonorrhea [110].

16.8.2 Mycobacterium tuberculosis Gyrase Inhibitors (MGIs)

MGIs (Fig. 16.7) were derived from NBTIs in an effort to optimize activity against *M. tuberculosis* [111]. These compounds display high activity against wild-type and fluoroquinolone-resistant strains. On the basis of mutagenesis studies, MGIs are

believed to target gyrase, the only type II topoisomerase in *M. tuberculosis*. No corresponding in vitro study has been reported to date.

16.8.3 Spiropyrimidinetriones

Spiropyrimidinetriones (Fig. 16.7) are a novel class of gyrase/topoisomerase IV poisons. Similar to fluoroquinolones, these enzymes induce enzyme-mediated double-stranded DNA breaks [112]. The founding member of this class, zoliflodacin (ETX0914/AZD0914), maintains activity against multidrug-resistant *Pseudomonas aeruginosa* [113], which contains fluoroquinolone-resistant mutations in the bridge-anchoring residues in both gyrase and topoisomerase IV. The drug is currently in phase II clinical trials for the treatment of uncomplicated gonorrhea [114].

16.9 Conclusions

Fluoroquinolones are one of the most important and widely prescribed classes of antibacterials in clinical use. However, their usefulness is being eroded by the rise of drug resistance. Of the mechanisms that impair fluoroquinolone actions, those that result from mutations in gyrase and topoisomerase IV are the most common and detrimental. The mechanistic studies described above have led to a more complete understanding of how fluoroquinolones interact with their enzyme targets and how mutations alter these interactions. Furthermore, these studies have suggested new strategies for overcoming resistance. Among these strategies are the design of novel fluoroquinolones and the development of new drug classes that do not rely on the water-metal ion bridge for their actions. Hopefully, these approaches will allow gyrase and topoisomerase IV to remain important antibacterial targets in the decades to come.

Major Points

- Although fluoroquinolones are the most efficacious and broad-spectrum oral antibacterials in the clinic, their use is being eroded by resistance.
- Fluoroquinolone binding to gyrase and topoisomerase IV, the cellular targets of these drugs, involves a water-metal ion bridge that is anchored by a keto acid on the drug and a highly conserved serine and glutamic/aspartic acid residue in the enzyme.
- Substitutions in the serine and glutamic/aspartic amino acid residues are responsible for most of the target-mediated fluoroquinolone resistance.
- The development of compounds that interact with gyrase and topoisomerase IV through mechanisms that are independent of the water-metal ion bridge may provide an approach to bypassing existing target-mediated resistance.

Acknowledgments The authors of this article were supported by the US Veterans Administration (Merit Review Award I01 Bx002198 to N.O.), the National Institutes of Health (grants AI87671 to R.J.K., GM33944 and GM126363 to N.O., and GM007628 to E.G.G.), the National Science Foundation (grant DGE-0909667 to R.E.A.), the Pharmaceutical Research and Manufacturers of America Foundation (to E.G.G.), and the American Association of Pharmaceutical Scientists Foundation (to E.G.G.).

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Part III
Finding New Antimicrobials

Chapter 17
Natural Products in Antibiotic Discovery

Fern R. McSorley, Jarrod W. Johnson, and Gerard D. Wright

17.1 History of Natural-Product Antibiotics

17.1.1 Natural Medicinal Therapies

Nature is rich in bioactive molecules that can be used as medicines. The earliest records describing natural medicines are found on clay tablets dating from 2600 BCE in Mesopotamia; these records contain over 1000 plant derived-substances [1, 2]. The most well-known ancient medicinal record is the Egyptian Ebers Papyrus, which dates from 1500 BCE and contains over 700 natural remedies, most of plant origin [3]. Natural/herbal treatments are found throughout history and from all over the world. Examples are the Chinese Materia Medica (Shennong Bencao Jing) (1100 BCE – 659 CE) [3, 4] and the Indian Ayurvedic system (1000 BCE) [3, 5]. These collections of ancient remedies were directed at a range of ailments that included infections; some are still in use today. Such traditional medicines generally consist of complex extracts and mixtures of agents whose bioactive component(s) went unidentified for hundreds of years. Improvements came largely from trial and error efforts that were hindered by confirmational bias and placebo effects.

In the early 1800s, morphine became the first bioactive natural product isolated from a medicinal plant [6]. This milestone led the Western medical field away from

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I. W. Fong et al. (eds.), *Antimicrobial Resistance in the 21st Century*, Emerging Infectious Diseases of the 21st Century, https://doi.org/10.1007/978-3-319-78538-7_17

complex mixtures toward the pharmacology of pure compounds [7]. Old methods based on impure mixtures were unreliable: the variability of growth conditions and extractions from plant materials and microorganisms impacted the concentrations of both beneficial and toxic bioactive compounds present in the mixtures. Isolating the beneficial bioactive compound away from other material found in the natural source, some of which could cause negative effects, allows in-depth analysis of mode of action and efficacy. It also enables physicians to more accurately regulate the dosage of the bioactive compound.

In 1928, just over 100 years after the isolation of morphine, Fleming serendipitously discovered that the fungus *Penicillium notatum* secretes an agent that prevents the growth of bacteria. A decade and a half later, penicillin became the first natural-product antimicrobial to reach widespread clinical use [8]. Selman Waksman coined the term antibiotics to refer to microbially produced compounds that are "against life"; they either halt the growth of (bacteriostatic) or kill (bactericidal) microbes. Among these are small peptides having antibiotic activity [9]; they typically disrupt bacterial membranes in a nonselective manner. Indeed, these peptides are the first line of defense against bacterial infections. A larger suite of highly selective antibiotics is produced by microbes. These compounds have been the main source of our antibiotic drugs thus far [10]. The physiological roles of microbially produced antibiotics are still debated; these molecules evolved either for signaling functions or as chemical warfare agents to ward off neighboring microbes [11]. The density and diversity of resistance elements in microbes are consistent with an evolved detoxifying role to protect against the growth-impeding effects of antibiotics. Regardless of the evolutionary basis for antibiotics, the introduction of penicillin into the clinic in the early 1940s led to the "antibiotic era" of medicine. Natural antibiotics have aided in the treatment and control of infections for the last 80 years (Fig. 17.1). By controlling infections, antibiotics have revolutionized medicine, allowing physicians to perform lifesaving organ transplants and invasive surgeries and to treat cancer using disruptive immune system chemotherapy. The emergence of antibiotic resistance now threatens these breakthroughs, our quality of life, and our life expectancy.

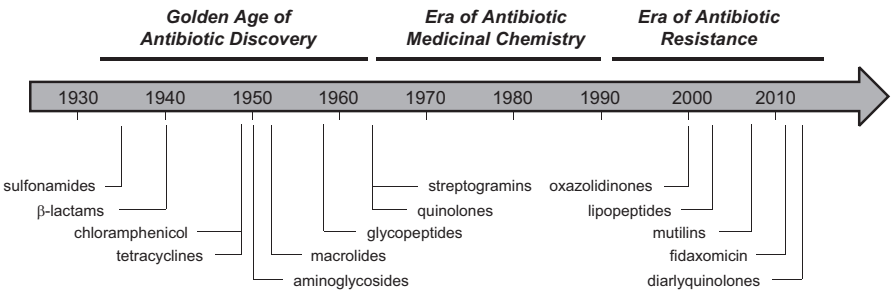


Fig. 17.1 Timeline for the introduction of each class of antibiotic into clinical use. (Adapted from ref. [12])

17.1.2 The Development of Modern Antibiotics

The first antibacterial compounds to enter the clinic were synthetic molecules discovered in the laboratory of Paul Ehrlich [13, 14]. After noticing that some microbes stained differently than others when exposed to synthetic aniline and azo dyes, Ehrlich postulated that a chemical compound could selectively target pathogenic cells and not host cells. His "magic bullet" theory led him to screen hundreds of organoarsenic derivatives for activity. One of these, arsphenamine (Salvarsan), was successfully used to treat syphilis. Salvarsan, although difficult to administer, was the most prescribed antimicrobial drug until it was replaced by penicillin [15].

Four classes of synthetic antibiotics[1] remain successful as drugs in the clinic today: sulfonamides, diaminopyrimidines, quinolones, and oxazolidinones (Table 17.1, Fig. 17.2). The initial class of systemic synthetic antimicrobials was the sulfonamide sulfa drugs [14, 17]. Prontosil, the first sulfa drug for human use, is a prodrug; after administration, it is metabolized into the bioactive agent sulfanilamide. Sulfonamides work by inhibiting dihydropteroate synthase, a critical component in folate synthesis. Humans acquire folate from their diet, while bacteria must biosynthesize this essential nutrient. Consequently, sulfa drugs are selectively active against microbes. Sulfonamides are generally co-administered with the diaminopyrimidine trimethoprim. This synthetic antibiotic also targets folate biosynthesis, inhibiting dihydrofolate reductase. The synergistic combination of trimethoprim and sulfamethoxazole (co-trimoxazole) is sold under a variety of trade names (e.g., Septra, Bactrim).

The quinolone drugs target type II DNA topoisomerases and replication. These agents are potent against both Gram-negative and Gram-positive bacteria. A widely used example is ciprofloxacin (Fig. 17.2), a second-generation fluoroquinolone that is orally available and used to treat urinary tract infections, sinusitis, and many other infections. Several newer generations of quinolones have found a place in the market, making the fluoroquinolones the most successful class of synthetic drugs to date.

The oxazolidinones comprise the fourth class of synthetic antibiotic. These compounds, which block the ribosomal peptidyl transferase center essential for protein synthesis, are effective primarily against Gram-positive bacteria. Linezolid (Fig. 17.2) represents the first generation of this class; it was approved by the US Food and Drug Administration in 2000 and became the first novel chemical scaffold to enter the clinic in several decades.

Fleming's discovery that *P. notatum* secretes a bactericidal substance helped launch the intensive mining of microbes as sources of antibiotics rather than examining synthetic chemical libraries [8]. Florey, Chain, and Heatley's efforts to develop a protocol for isolation of penicillin, followed by in vivo efficacy studies in animals and clinical tests in humans, showed that penicillin, a natural product, was a viable

[1] Here we are deviating from Waksman's definition of antibiotics restricted narrowly to compounds synthesized by microbes to include synthetic and semisynthetic human-made compounds as well.

Table 17.1 Timeline of discovery and introduction of antibiotic classes

Antibiotic class; examples	Discovery	Introduction	Resistance observed	Mechanism of action	Activity; target species
Organoarsenics; salvarsan	1909	1910	1924	Unknown	Bactericidal; antisyphilitic
Sulfadrugs[a]; prontosil, sulfanilamide	1932	1936	1942	Inhibits dihydropteroate synthetase in folate synthesis	Bacteriostatic; Gram-positive bacteria
β-Lactams; penicillins, cephalosporins, carbapenems	1928	1938	1945	Inhibits penicillin-binding proteins in cell-wall biosynthesis	Bactericidal; broad-spectrum
Aminoglycosides; streptomycin, spectinomycin, kanamycin, neomycin	1943	1946	1946	Binds 30S ribosomal subunit inhibiting protein synthesis	Bactericidal; broad-spectrum
Chloramphenicols; chloramphenicol	1946	1948	1950	Binds 50S ribosomal subunit inhibiting protein synthesis	Bacteriostatic; broad-spectrum
Macrolides; erythromycin, clarithromycin	1948	1951	1955	Binds peptidyl transferase center of 50S ribosomal subunit inhibiting protein synthesis	Bacteriostatic; broad-spectrum
Tetracyclines; chlortetracycline, doxycycline	1944	1952	1950	Binds 30S ribosomal subunit inhibiting protein synthesis	Bacteriostatic; broad-spectrum
Ansamycins; rifamycins rifampicin	1957	1958	1962	Binds RNA polymerase β-subunit inhibiting RNA synthesis	Bactericidal; Gram-positive bacteria
Glycopeptides; vancomycin, teicoplanin	1953	1958	1960	Transpeptidase blockade inhibiting cell-wall biosynthesis	Bactericidal; Gram-positive bacteria
Quinolones[a]; ciprofloxacin	1961	1968	1968	Binds DNA gyrase inhibiting DNA synthesis	Bactericidal; broad-spectrum
Streptogramins; pristinamycins	1963	1998	1964	Binds 50S ribosomal subunit inhibiting protein synthesis	Bactericidal, Gram-positive bacteria
Oxazolidinones; linezolid	1955	2000	2001	Binds peptidyl transferase center of 50S ribosomal subunit inhibiting protein synthesis	Bacteriostatic; Gram-positive bacteria
Lipopeptides; daptomycin	1986	2003	1987	Depolarization of cell membrane	Bactericidal; Gram-positive bacteria
Mutulins; pleuromutilin, retapamulin	1951	2007	1999	Binds peptidyl transferase center of 50S ribosomal subunit inhibiting protein synthesis	Bacteriostatic; Gram-positive bacteria
Fidaxomicin (targeting *Clostridium difficile*)	1975	2011	1977	Inhibition of RNA polymerase	Bactericidal; Gram-positive bacteria
Diarylquinolines[a]; bedaquiline	1997	2012	2006	Inhibition of F_1F_O-ATPase	Narrow-spectrum activity, *Mycobacterium tuberculosis*

[a]Synthetic antibiotic classes. Table assembled from references [11, 12, 16]

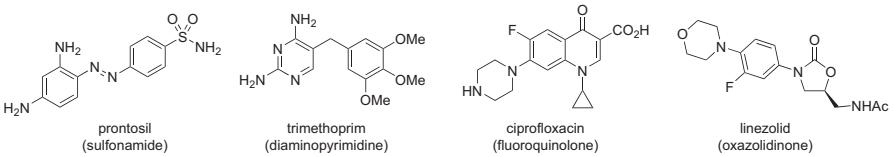


Fig. 17.2 Synthetic antibiotics used in the clinic

antibiotic drug candidate. The discovery of penicillin gave rise to the "golden age" of antibiotic discovery (Fig. 17.1). For the next 20 years, extensive screening of microbes, particularly soil-dwelling actinomycetes, was conducted to identify natural antibiotic compounds. In that time many natural-product scaffolds (aminoglycosides, chloramphenicol, macrolides, tetracyclines, ansamycins, glycopeptides, and streptogramins) were identified and often rapidly developed for clinical use (Table 17.1). By the middle of the 1960s, this simple but effective screening platform [18] appeared to have exhausted its sources, as new antibiotic scaffolds suitable for drug development became harder and harder to find.

The decline of success in isolating new chemical scaffolds from natural sources suitable for drug development, the generally poor pharmacological and toxicological properties of natural products as drugs, and the problematic emergence of resistance ushered in the next phase of antibiotic innovation. The focus shifted to medicinal chemistry efforts as we entered the "medicinal chemistry era." Synthetic chemists prepared and derivatized core antibiotic scaffolds already used in the clinic and screened them for improvements. Chemists were successful in creating so-called "generations" of enhanced synthetic variants that improved pharmacological properties, expanded antibiotic spectra, and evaded resistance. Although many improved drugs and new generations of known drugs emerged from these efforts, no truly novel chemical scaffold entered the clinic from the 1960s to 2000s.

The lack of innovation in antibiotic discovery over the past two decades and the general failure of in vitro target-based drug discovery methods have prompted a renewed interest in natural products [16, 19]. This return to the natural-product compounds that previously dominated antibiotic drug discovery reflects the historic success of these drugs, a growing understanding of the physiochemical properties of small molecules for efficacy against bacterial targets, and new thinking resulting from advances in bacterial genomics, synthetic biology, and the properties of antimicrobial targets.

17.2 Major Classes of Natural-Product Antibiotics and Their Modes of Action

Microbial natural products are the source of most of our antibiotic scaffolds in current clinical use. Brief descriptions of the most prominent, clinically used drugs and their modes of action are outlined below.

β-Lactams

Penicillin falls into the β-lactam category of natural products. Five classes of β-lactams are important in the antibiotic field: penams, cephems, carbapenems, clavams, and monobactams (Fig. 17.3). All β-lactams contain a strained 4-membered β-lactam ring system that in the majority of clinically relevant compounds is fused to a 5- or 6-membered ring system.

Penams, cephems, and carbapenems covalently bind to penicillin-binding proteins (PBPs), essential enzymes that process the D-Ala-D-Ala termini of the pentapeptide portion of the peptidoglycan intermediates in cell-wall metabolism. The electrophilic β-lactam antibiotics mimic the D-Ala-D-Ala substrate (Fig. 17.4) and covalently bind to the PBP [20], preventing the enzyme from facilitating transpeptidation in the final step of peptidoglycan synthesis. This results in a complex series of molecular events that include inhibition of cell division and eventually cell rupture.

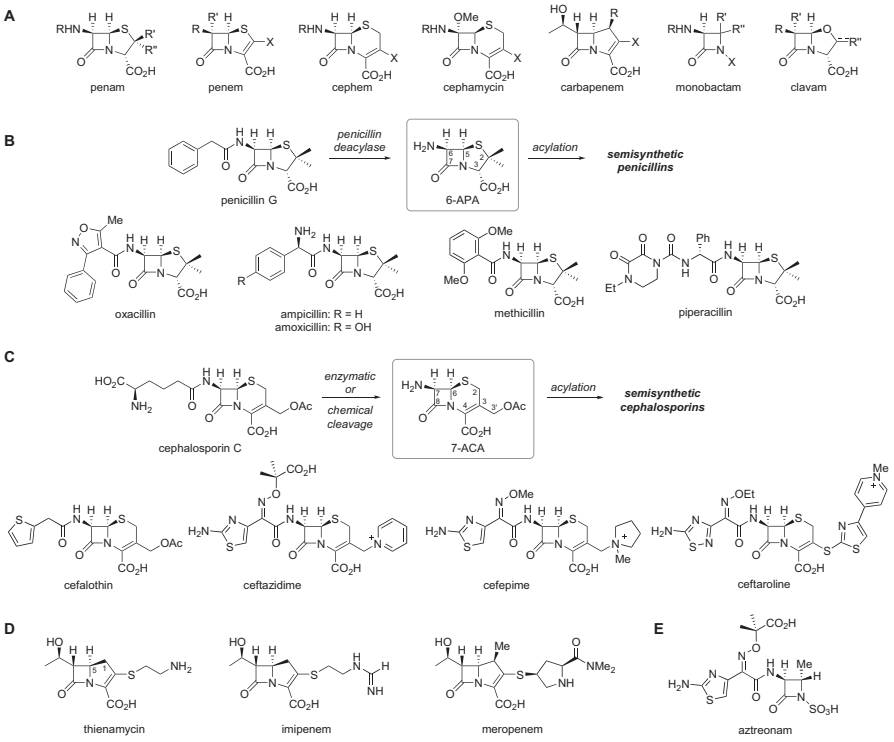


Fig. 17.3 β-Lactam antibiotics. (**A**) Major structural classes of β-lactam antibiotics. (**B**) The deacylation of penicillin G to generate 6-APA opened the door for the synthesis of new semisynthetic penicillins having improved antibiotic potency and spectrum of activity. (**C**) Similarly, 7-ACA has been used as a key intermediate for the preparation of countless semisynthetic cephalosporins having potent, broad-spectrum activity against clinically important Gram-negative bacteria. Ceftaroline has activity against methicillin-resistant *Staphylococcus aureus* (MRSA). (**D**) Natural and synthetic carbapenem antibiotics. (**E**) Aztreonam, a representative monobactam

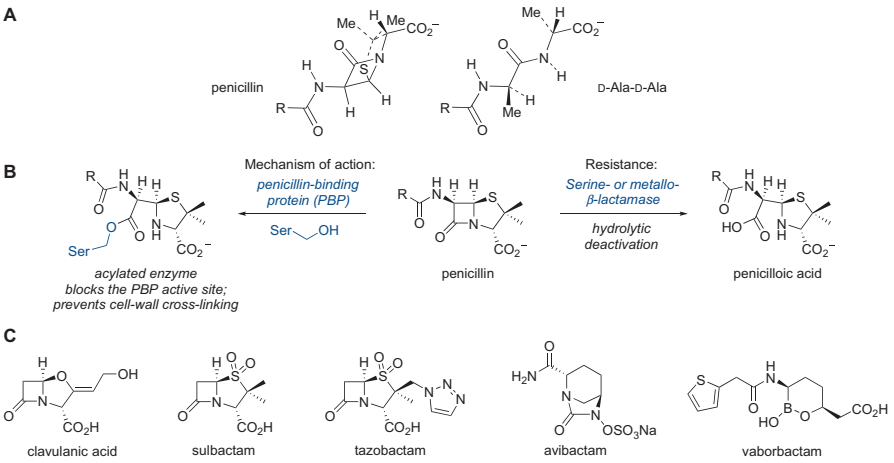


Fig. 17.4 β-Lactams mimic the D-Ala-D-Ala terminus of the peptidoglycan peptide strands and block the cross-linking step in cell-wall biosynthesis. (**A**) Structural analogy of penicillin with the D-Ala-D-Ala portion of peptidoglycan, as proposed by Tipper and Strominger in 1965 [20]. (**B**) Interactions of a penicillin with penicillin-binding proteins (PBPs) and β-lactamases. The acylation of PBP active-site serine prevents cross-linking of the bacterial cell wall and leads to cell lysis. In contrast, β-Lactamases hydrolyze β-lactams rapidly and confer high-level resistance. (**C**) β-Lactamase inhibitors that are used in combination with β-lactams to overcome resistance due to β-lactamases

Thousands of penicillins and cephalosporins have been synthesized through the acylation of 6-aminopenicillanic acid (6-APA) and 7-aminocephalosporanic acid (7-ACA) in order to improve antibiotic potency, the antibiotic spectrum against both Gram-positive and Gram-negative bacteria, and their stability against β-lactamases (Fig. 17.3b) [21]. β-Lactamases are resistance enzymes that rapidly cleave the β-lactam ring and render the antibiotic inactive. Although clavulanic acid and penam sulfones were found to be poor antibiotics, they are potent inhibitors of β-lactamases. Thus, they are combined with β-lactam antibiotics to successfully overcome resistance due to β-lactamases. Augmentin (amoxicillin + clavulanic acid), Unasyn (ampicillin + sulbactam), and Zosyn (piperacillin + tazobactam) are among the most successful combinations [22]. Two other β-lactamase inhibitors, avibactam and vaborbactam (RPX 7009), have been developed and approved recently for use in combinations with β-lactams [23, 24].

The discovery of several monobactams produced by soil-derived Gram-negative bacteria inspired the development of antibiotics based on a more simplified 4-membered lactam ring [25]. Before this discovery chemists had not considered using an *N*-sulfonic acid substituent to stabilize the β-lactam system. This modification eventually led to the successful antibiotic drug aztreonam (Fig. 17.3e) [26]. This example illustrates the importance of natural-product discovery in guiding the synthesis of new scaffolds.

Aminoglycosides

In 1944, Waksman's laboratory discovered streptomycin, the first aminoglycoside antibiotic, as a product of a strain of *Streptomyces griseus* [27]. Over the next two decades, several members of the class were discovered that included kanamycin in 1956 from *Streptomyces kanamyceticus* and gentamicin C in 1963 from *Micromonospora purpurea* [28, 29]. These were the first antibiotics to show efficacy in the treatment of tuberculosis and in infections caused by Gram-negative bacteria. Aminoglycosides consist of a core aminocyclitol ring modified to varying extents by saccharides. Multiple amino groups provide a positive charge at physiological pH and impart high water solubility. Currently, three aminoglycosides are commonly used in the clinic: the natural products, gentamicin C and tobramycin, and the semisynthetic agent amikacin, each of which contains the 2-deoxystreptamine aminocyclitol core (Fig. 17.5a) [30]. Streptomycin, which is unique in this drug class for having a streptamine core aminocyclitol ring, continues to find some clinical use in the treatment of tuberculosis and in combination with penicillins for enterococcal infections that are difficult to treat.

The amino and hydroxy groups of the aminoglycosides interact with the 16S rRNA in the 30S ribosomal unit through a network of hydrogen bonds (Fig. 17.5b). The binding of aminoglycosides to the 16S rRNA results in a conformational change that impedes cognate codon–anticodon validation by the ribosome. The result is the corruption of the genetic code and the synthesis of proteins with incorrect amino acids. This corruption contributes to cell death.

Resistance to aminoglycosides can take many forms. Active efflux can be a significant contributor to resistance in bacteria such as *P. aeruginosa*; however, the main mechanisms of resistance are chemical modification of the drugs or the target [30]. A large number of aminoglycoside *N*-acetyltransferases, *O*-phosphotransferases, and *O*-adenylyltransferases are present in both Gram-positive and Gram-negative pathogens (Fig. 17.5c). Over the past decade, ribosomal methyltransferases that modify the 16S rRNA (e.g., G-1405 and A-1408) and confer high-level aminoglycoside resistance in Gram-negative pathogens have also emerged as significant clinical challenges (Fig. 17.5b).

Macrolides

The term macrolide is a portmanteau that combines macrolactone, a lactone ring containing eight or more atoms, and polyketide. Erythromycin is a first-generation macrolide that was isolated from a soil actinomycete *Saccharopolyspora erythraea*. Erythromycin contains a 14-membered macrolactone framework and a 2-amino sugar (Fig. 17.6). The presence of a ketone at position 9 of the macrolactone can result in formation of a hemiketal with the hydroxyl group at position 6 under acidic conditions (e.g., exposure to gastric acids), thereby decreasing the levels of bioavailable drug [31]. Semisynthetic conversion of erythromycin to clarithromycin or azithromycin removes this possibility and results in improved efficacy and bioavailability. Macrolide antibiotics are most effective against Gram-positive bacteria, but they also have efficacy against some common Gram-negative, upper respiratory tract pathogens.

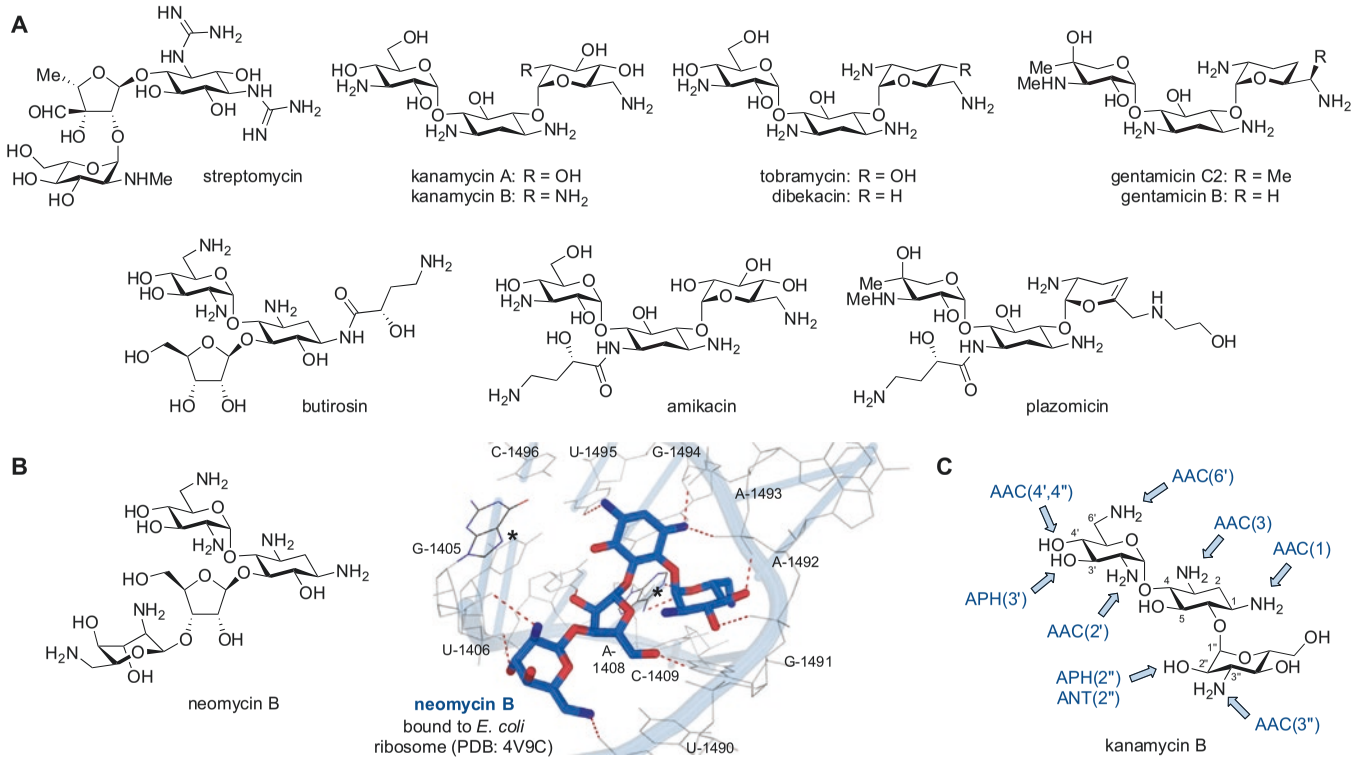


Fig. 17.5 Aminoglycosides. (**A**) Structures of natural and semisynthetic aminoglycosides. (**B**) Neomycin B and its binding mode in the H44 region of the *E. coli* ribosome (PDB: 4V9C). Asterisks at G-1405 and A-1408 indicate sites of methylation by methyltransferases that confer aminoglycoside resistance (e.g., ArmA and NpmA, respectively). (**C**) Major target sites of aminoglycoside-modifying enzymes (AMEs), including *O*-phosphotransferases (APHs), *N*-acetyltransferases (AACs), and *O*-nucleotidyltransferases (ANTs)

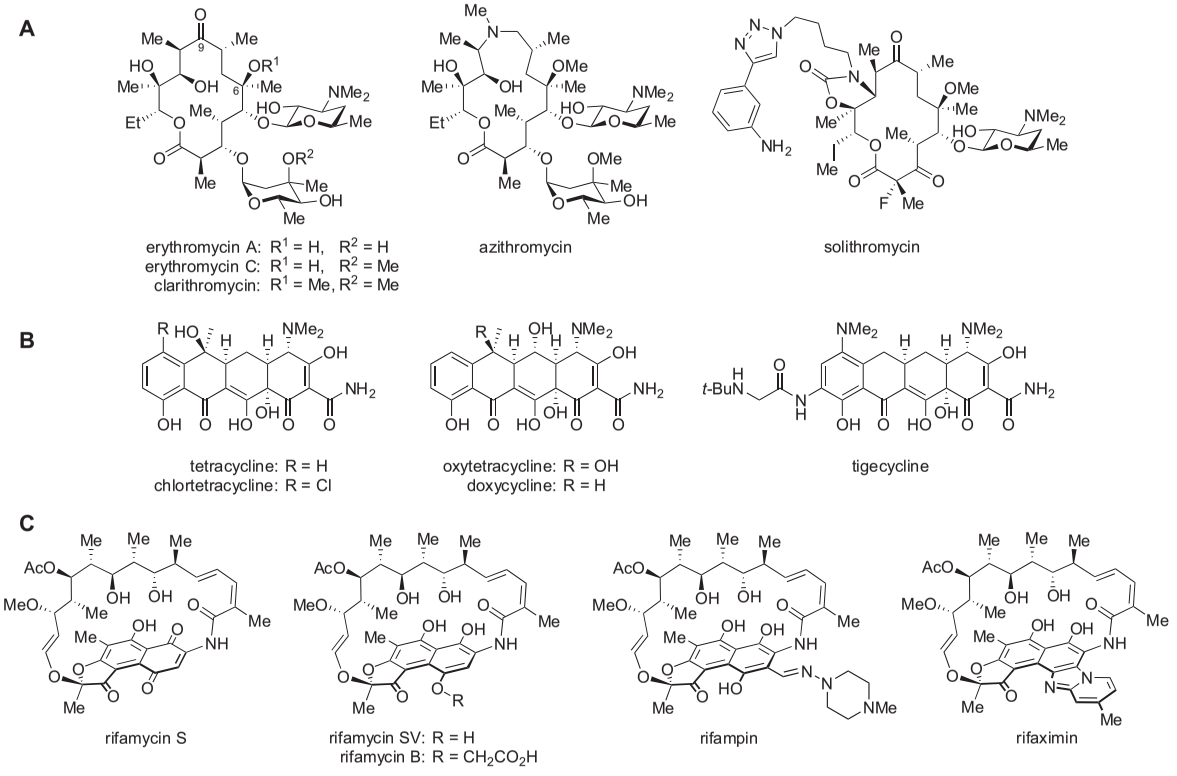


Fig. 17.6 Selected examples of macrolides (**A**), tetracyclines (**B**), and ansamycin antibiotics (**C**)

Macrolides interact with the peptidyl transferase center of the 50S ribosomal subunit, blocking peptide-chain elongation. Specifically, the hydrophobic surface of the macrolide binds to the sidewall of the exit tunnel and causes premature release of short peptidyl-tRNAs [31].

The main causes of clinical macrolide resistance are the 23S rRNA methyltransferases (Erm) [32]. However, a number of macrolide kinases, which modify the desosamine sugar, are increasing in prevalence and diversity. Ring-opening esterases and desosamine glycosyltransferases are also known, but they are less frequently encountered. Active macrolide efflux is common in many Gram-positive pathogens.

Tetracyclines

Chlortetracycline (Aureomycin) and oxytetracycline (Terramycin), discovered in 1948 and 1950, respectively, were the first members of the tetracycline class (Fig. 17.6b). Tetracyclines contain a tetracyclic polyketide core of four fused 6-membered rings; they were the first broad-spectrum antibiotics (i.e., effective against both Gram-positive and Gram-negative pathogens) to enter clinical use. After it was found that the C6-hydroxy group could be reductively removed to form a more stable 6-deoxytetracline, further modification ensued. Multiple generations of tetracyclines have now emerged that include doxycycline, minocycline, and tigecycline [33]. Tigecycline (approved for use in the USA in 2005) has broad-spectrum activity for both Gram-positive and Gram-negative bacteria; it is also effective against methicillin-resistant *Staphylococcus aureus* (MRSA).

Before the emergence of resistance limited their use, the tetracyclines were used for decades to treat infections of the respiratory tract, middle ear, and urinary tract. Like aminoglycosides, tetracyclines bind to the 30S ribosomal subunit. However, they do not cause the production of aberrant proteins. Instead they bind to the aminoacyl-tRNA binding site of the ribosome, thereby competitively preventing translation of mRNA.

Tetracycline resistance in Gram-negative bacteria is most often the result of active efflux. In Gram-positive bacteria, the expression of ribosomal protection proteins lowers the affinity of tetracycline for the bacterial ribosome. Enzyme-mediated inactivation has been reported through the action of TetX, a flavin-dependent monooxygenase that hydroxylates the antibiotic, thereby precipitating a nonenzymatic breakdown of the compounds [34].

Ansamycins

As with the macrolides, ansamycins are polyketide macrocycles; however, ansamycins cyclize to form a macrolactam instead of a macrolactone (Fig. 17.6c). The ansa-bridged macrolactam is formed with an intramolecular amine nucleophile derived from the biosynthetic starter unit, 3-amino-5-hydroxybenzoyl-CoA. The natural ansamycin, rifamycin, is an 18-membered macrolactam that is converted through semisynthesis to the commonly used rifampin. Addition of the piperazinyl hydrazide to the rifamycin naphthyl core in rifampin increases its oral bioavailability and broadens antimicrobial activity. Rifampin is a WHO essential

medicine designated for use in combination therapy for the treatment of tuberculosis.

Rifamycins are inhibitors of transcription [35]. In particular they bind to the mRNA exit site of the β-subunit of RNA polymerase. Resistance mutations in the rifamycin-binding site are common. As a result, rifamycins are most effective when in combination with other antimicrobials [35]. In the treatment of *M. tuberculosis*, which is the main use of the rifamycins, resistance is most often the result of point mutations in the target RNA polymerase [36]. In contrast, many environmental microbes express a wide variety of rifamycin-inactivating enzymes (e.g., kinases, ADP-ribosyltransferases, monooxygenases, and glycosyltransferases).

Glycopeptides

As their name implies, glycopeptides are peptides that are decorated with sugar moieties. Vancomycin and teicoplanin (Fig. 17.7) exemplify this class; both have been developed as Gram-positive-directed antibiotics. Vancomycin, which was discovered in the early 1950s as a product of the actinomycete *Amycolatopsis orientalis,* was used only sporadically for several decades, largely due to difficulties in obtaining pure compound. However, use increased in the 1980s following the widespread emergence of MRSA in hospitals [38]. Emergence of resistance in enterococci (VRE) and then intermediate resistance in *S. aureus* (VISA) spurred the development of second-generation glycopeptides such as telavancin, dalbavancin, and oritavancin that are less susceptible to resistance [39].

Vancomycin and teicoplanin are highly cross-linked pentapeptides that have a high affinity for D-Ala-D-Ala termini of uncrosslinked peptidoglycan chains. Vancomycin forms five hydrogen bonds with the D-Ala-D-Ala terminus of lipid II and prevents the formation of interpeptidyl cross-links by PBPs. That reduces the integrity of the cell wall and leads to cell death. Although glycopeptides and β-lactams both inhibit cell-wall biosynthesis, the glycopeptides sequester the substrate of transpeptidation rather than directly interacting with the PBP catalyst.

Resistance to glycopeptide antibiotics can take two forms. In *Enterococci*, reprogramming of cell-wall biosynthesis to terminate in either D-Ala-D-Lac or D-Ala-D-Ser reduces affinity of the antibiotic. In *Staphylococcus aureus*, acquisition of the

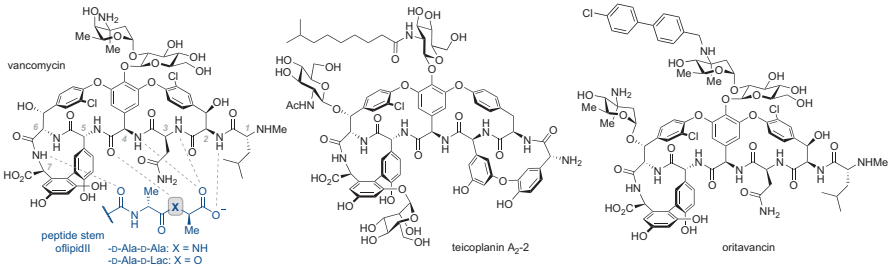


Fig. 17.7 Glycopeptide antibiotics and the interaction between vancomycin and the D-Ala-D-Ala portion of lipid II. In vancomycin-resistant enterococci (VRE), the peptide stem contains a D-Ala-D-Lac terminal region, and the affinity for the glycopeptide is decreased 1000-fold [37]

genes encoding D-Ala-D-Lactate biosynthesis is known but exceedingly rare. Instead, in this organism glycopeptide resistance is primarily the result of increased production of cell-wall polymers that bind the antibiotic.

Streptogramins

Dalfopristin and quinupristin (Fig. 17.8a) are semisynthetic derivatives of virginiamycins/pristinamycins that belong to the A-type and B-type streptogramin families. Type B streptogramins are cyclic hexapeptides, while type A streptogramins are cyclic polyketide–peptide hybrids. This illustrates that a pair of molecules, which have only bacteriostatic activity and are not effective treatments alone, can be combined to work synergistically and form a potent bactericidal drug. The molecular mechanism of synergy is based on the affinity of the compounds for different but adjacent regions of the bacterial ribosome [40]. Dalfopristin binds to the peptidyl transferase center where it reduces the affinity of aminoacyl-tRNAs for the aminoacyl site, which lowers subsequent peptide bond formation and chain elongation in the peptidyl site. In contrast, quinupristin binds in a similar manner to erythromycin at the proximal end of the tunnel, thereby accelerating the release of small oligopeptidyl-tRNAs [41]. When administered together, the two agents form a combination drug known as Synercid. It is used to treat staphylococcal infections [42].

Both type A and B streptogramins are susceptible to efflux-mediated resistance; indeed, the efflux protein Lsa intrinsic to *Enterococcus faecalis* confers resistance to type B streptogramins, limiting Synercid use [40]. A group of *O*-acetyltransferases confer high-level resistance to type A streptogramins, while Vgb is a ring-opening C–O lyase that provides resistance to type B antibiotics.

Lipopeptides

As their name suggests, these compounds are peptides that contain a lipid moiety. Both linear and cyclic, macrolactone and macrolactam lipopeptides exist. Due to the large variations in structure, these molecules have few well-characterized cellular targets. Daptomycin (Fig. 17.8b), initially discovered in the 1980s and discarded at Phase II clinical trials by Eli Lilly due to toxicity, was revisited with a new dosing regimen; it was approved for clinical use in 2003 [43]. Daptomycin has pleiotropic effects on the membrane of Gram-positive bacteria that result in depolarization and physical alteration of the cell membrane that leads to cell death [44]. Daptomycin is effective against most Gram-positive pathogens, including drug-resistant forms such as VRE and MRSA.

Colistin (polymyxin E) is a lipopeptide of the polymyxin class. It is one of the few antibiotics in clinical use that was derived from a non-actinomycete bacterium, *Paenibacillus polymyxa*. Discovered in 1949, colistin has been used sparingly for the treatment of serious infections caused by Gram-negative bacteria due to toxicity issues [45]. As a result of the rise of carbapenem-resistant Gram-negative pathogens, clinicians have been left with few therapeutic options other than colistin. Consequently, its use has increased significantly. The mode of action of polymyxins involves disruption of the outer and inner membranes of Gram-negative bacteria [46].

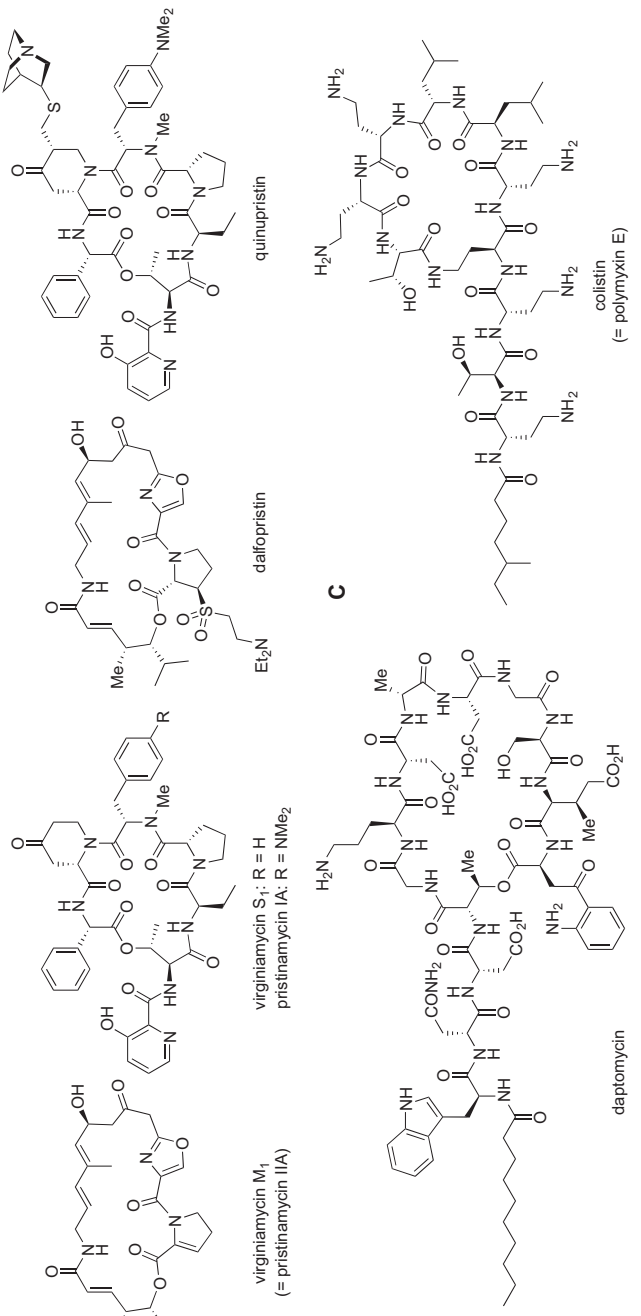


Fig. 17.8 Streptogramins (**A**), daptomycin (**B**), and colistin (**C**)

Resistance to colistin occurs through chemical modification of the lipopolysaccharide component of the Gram-negative outer membrane. Expression of intrinsic aminoarabinose transferases and phosphoethanolamine transferases, which modify lipid A impact the physical properties of the outer membrane, confers colistin resistance [47]. Mobilization of the *mcr-1* phosphoethanolamine transferase gene in Gram-negative pathogens is a growing concern [48]. In contrast, daptomycin resistance remains rare and has not been mobilized. However, resistance mutations in cell membrane and cell-wall structure and function can be selected in vitro and during long-term therapy [49].

Pleuromutilins

In 2007 the first pleuromutilin compound approved for clinical use was the topical antibiotic retapamulin [10]. Although retapamulin represents a new chemical scaffold for clinical use, it is actually a semisynthetic version of the original pleuromutilin, which was isolated from the fungus *Pleurotus mutilis* (renamed *Clitopilus scyphoides* in 1951) [50]. Pleuromutilins contain a fused 8-6-5 tricyclic diterpene architecture and an acyclic tail. It is among the few isoprenoid antibiotics to find clinical use (Fig. 17.9). These antibiotics bind to the peptidyl transfer center of the 50S ribosomal subunit, thereby blocking protein synthesis. 23S rRNA methyltransferases can confer resistance to this class of antibiotic. Retapamulin is a topical agent used for treatment of skin infections caused by Gram-positive bacteria; several other pleuromutilin derivatives are currently in various stages of clinical assessment for systemic use.

Although the isoprenoid class contains the most abundant natural products (over 50,000 known structures), very few are known to exhibit specific antibiotic activity. Other examples include platensimycin, platencin, and fusidic acid.

Chloramphenicol

Chloramphenicol, discovered in 1947 in extracts of *Streptomyces venezuelae*, displays broad-spectrum bacteriostatic activity [51]. This small molecule contains a dichloroacetamide moiety and an aromatic nitro group (Fig. 17.9). The dichloroacetyl moiety is important for activity, as it impedes tRNA from binding to the peptidyl transferase in the 50S ribosomal subunit, thereby preventing elongation. Due to the low-cost production of chloramphenicol, this agent is frequently used in developing countries, even though it has been withdrawn from common use in many areas due to resistance and safety concerns, the latter resulting from

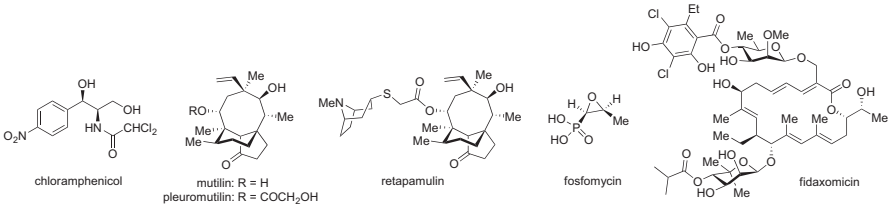


Fig. 17.9 Chloramphenicol, pleuromutilins, fosfomycin, and fidaxomicin

low-frequency association with aplastic anemia. Resistance is primarily the result of *O*-acetyltransferases that modify the antibiotic.

Fosfomycin

The phosphonate fosfomycin is a small (138 Da) antibiotic produced by *Streptomyces fradiae*. It has been in clinical use since the early 1970s for the treatment of urinary tract infections. The rapid emergence of resistance has limited its use, but its high water solubility and low toxicity enables a single dose (3 g of drug/day) or very short course therapy. Fosfomycin targets bacterial cell-wall biosynthesis by inhibition of MurA, an enzyme involved in the first step in the biosynthesis of *N*-acetylmuramic acid [52].

Fosfomycin resistance is readily selected during therapy in the form of mutants in the glycerol-3-phosphate transporter, which is needed for fosfomycin entry into the cell but not essential for bacterial cell growth [52]. A series of enzymes, including glutathione transferases and epoxide hydrolases, are known to inactivate the antibiotic via opening the essential epoxide ring.

Fidaxomicin

Fidaxomicin is the first member of the newest class of natural products to enter the market (2011). It consists of an 18-membered macrolactone polyketide that was discovered independently in Italy (lipiarrmycin), Japan (clostomicin), and the USA (tiacumicin) in the early 1970s [53]. The macrolactone is decorated with two acylated rhamnoses (Fig. 17.9). Fidaxomicin inhibits RNA polymerase by binding to a site distinct from the rifamycin binding site. There, fidaxomicin blocks the conversion of bound promoter DNA to the open single-strand complex that forms the transcription bubble. It has been approved for clinical use for treatment of *Clostridium difficile*-associated diarrhea.

17.3 Natural Products: A Privileged Source of Antibiotics

The vast majority of antibacterials used clinically are natural products, semisynthetic derivatives, or analogues thereof. As mentioned above, the only synthetic classes that are not derived from natural scaffolds are the sulfonamides, diaminopyrimidines, oxazolidinones, and quinolones. Natural products have always been a major source of human medicines and continue to be especially important as leads for antimicrobials – 74 of the 98 small molecules approved as antibacterials from 1981 to 2006 are natural products, semisynthetic derivatives, or natural-product mimics [10].

In the 1980s the arrival of combinatorial chemistry allowed the rapid synthesis of large numbers of synthetic compounds. That transformed the pharmaceutical industry, and companies began to favor screening vast libraries of synthetic compounds over natural-product extracts. With major advances in high-throughput screening (HTS) technologies through the 1990s and 2000s, companies obtained the ability to

quickly screen libraries of millions of compounds. This strategy has been enormously successful in identifying lead compounds having targets in human cells, but it has not been successful for identifying new antibacterials. Extensive high-throughput screening campaigns at GlaxoSmithKline (GSK) [54] and AstraZeneca [55] both failed to identify any candidate structure worthy of further clinical development. The lack of success is due to a combination of several factors, with retrospective analyses of both campaigns pointing to a lack of chemical diversity in the compound libraries.

The chemical libraries of pharmaceutical companies have largely been constructed with guidance from Lipinski's Rules [56], which aim to improve the likelihood of oral bioavailability by keeping molecular weights (MW) under 500, measures of hydrophobicity (log*P* or log*D*) less than 5, and the number of hydrogen-bond donors and acceptors in the molecule less than 10. However, antibiotics have long been known to occupy a different "chemical space" than other drugs, and often they exhibit multiple violations of Lipinski's guidelines. An analysis of physiochemical properties by O'Shea and Moser in 2008 [57] compared a reference set of human drugs against compounds active against Gram-positive and Gram-negative bacteria. Average molecular weights were 338, 813, and 414, and average clog$D_{7.4}$ values were 1.6, −0.2, and −2.8, respectively. Anti-Gram-positive compounds are more polar than reference drugs and can be much larger, especially if their targets are on the cell exterior (e.g., glycopeptides, lipopeptides). Compounds active against Gram-negative bacteria, which must cross the outer membrane, are much more polar and have a strict molecular weight cutoff at 600 Da, likely due to the limitations of transport through porins. Overington pointed out, however, that the bacterial target should be taken into account, since the physiochemical properties of antibiotics targeting the ribosome fall further outside Lipinski's rules than antibacterials that have bacterial protein targets [58].

An analysis of 23 HTS campaigns at AstraZeneca, reported by Brown et al. [59], showed that active antibacterial project compounds were significantly more polar than the screening collection average. Improving biochemical potency through chemical modification of active leads often came with an increase in hydrophobicity and an increased probability of problematic plasma protein binding or cytotoxicity. In cases in which biochemical potency was maintained by increasing polarity, whole-cell activity remained elusive; designing polar compounds was not sufficient for antibacterial activity. Overall, the study highlights the complexities of bacterial cell penetration and efflux systems, especially in Gram-negative bacteria. The authors note that one possibility for improving the antibiotic chemical space of screening libraries would be to return to natural-product screening.

While there is little overlap in the chemical space of compounds in screening libraries with that of antibacterials, there is far more overlap in the physiochemical properties between antibiotics and natural products [60, 61]. In addition to hydrophilicity (i.e., log *P*), other properties, such as the number of rotatable bonds (molecular flexibility), polar surface area, H-bond donors and acceptors, molecular complexity, and 3-dimensionality [62, 63], are better represented in natural-product chemical space.

17.4 Traditional Natural-Product Discovery

Selman Waksman is credited for developing a procedure in which microbial exudates are screened for cell growth inhibition on the surface of solid agar medium plates. This method measures "zones of inhibition" around paper disks to which natural-product samples are applied [16]. The so-called Waksman Platform is much faster and more efficient than systematic testing for antibiotic efficacy in animal disease models, as performed by Ehrlich. When Waksman used the method for high-throughput analysis of soil microbe products, he discovered candicidin, the first polyene antifungal agent; streptomycin and neomycin, the first aminoglycosides; and many other agents that include streptothricin and actinomycin. Many clinically used antibiotics were subsequently found using this method: chlortetracycline (Lederle), chloramphenicol (Parke-Davis), erythromycin (Abbott and Lilly), and tetracycline (Pfizer) [64]. After successfully mining soil-derived bacteria, specifically streptomycetes, the returns dwindled as known compounds repeatedly surfaced in the screens [65]. Consequently, the natural-product screening programs of drug companies slowly shut down, and the focus switched to synthetic chemistry.

Traditionally, antibiotic discovery using the Waksman platform begins with a source of environmental microbes. These have been primarily obtained from soil samples collected by the employees, family, and associates of drug companies across the globe. The microbes in these samples generally focused on the actinomycetes, spore-forming bacteria that over the decades led to collections containing tens to hundreds of thousands of strains. The producer strains are typically grown in a variety of defined media, since the contents of the medium can significantly impact the production of a given compound. Following fermentation, organic solvent extracts or conditioned media samples are prepared and used for screening against a set of pathogenic bacteria. If a natural-product extract elicits antibiotic properties, then activity-guided purification is conducted to isolate the bioactive molecule, and the chemical structure of the active molecule is elucidated. If the chemical hit is promising, then semisynthetic or total synthetic variations of the lead compound are produced and tested. From hundreds of analogs, a therapeutic candidate may emerge. Large-scale production of the optimized lead compound is undertaken, and extensive safety tests are carried out before the candidate enters clinical trials. Three phases of appropriate clinical trials are performed, and if the candidate agent passes, it would proceed to the regulatory approval step. The discovery and development pipeline of an antibiotic can take upward of 10 years and cost hundreds of millions of dollars. Bérdy estimated that ~28,000 antimicrobial natural products from microbial sources have been reported using this approach. It is for this reason, along with the drought in discovery of antibiotics using other chemical matter, that many pharmaceutical companies have withdrawn investments and shut down antibiotic discovery programs [66].

17.5 The Future of Natural-Product Discovery

The time is right for a reevaluation of natural products in antibiotic drug discovery. Their historical success as drugs, the comparative shortcomings of screens of synthetic compound libraries, and the serious need for innovation in securing new antibiotics demand a fresh look at this source of bioactive chemistry. The rediscovery of well-known chemical scaffolds, which prompted a move away from natural products, can be mitigated in several ways. First, previously unsuccessful scaffolds can be reevaluated; second, successful antibiotic drugs can be reinvigorated by combining them with inhibitors of resistance and other antibiotic adjuvants; third, new scaffolds can be sourced from previously neglected genera or through mining of microbial genomes and metagenomes; and finally, "new-to-nature" compounds can be generated through synthetic biology strategies.

17.5.1 Revisiting Discarded Scaffolds

The three most recent natural-product antibiotics to enter the clinic, daptomycin, fidaxomicin, and the pleuromutilins, were all discovered and discarded decades before their successful clinical launch. In the case of daptomycin, off-target human toxicity was deemed a sufficient concern by Eli Lilly to halt clinical development. A decade later, with more careful drug dosing to avoid undesired effects, daptomycin was championed by Cubist Pharmaceuticals, which successfully brought the compound to market [43]. Fidaxomicin was discarded in the 1970s due to poor solubility and narrow spectrum, properties that are advantages in its new incarnation as an orally dosed drug to combat *C. difficile* [53]. These examples offer hope, perhaps even certainty, that new antibiotic drugs can be sourced from known compounds. The estimate that ~28,000 natural-product antibiotics have been reported, while fewer than 500 have entered into clinical use, is encouraging that we can revisit these compounds as antimicrobial sources.

There are challenges to this route, however. A practical consideration is that there is no ready way to obtain these compounds for reevaluation. Most were reported in the scientific or patent literature decades ago, but some remain in the yellowing lab books in the vaults of pharma. Unless the compounds progressed in the development process, the bacterial strains that produce them may not be available in public culture collections. The fate of the extensive libraries of producing organisms held by many companies active during the 1950s–1980s is not widely known. Some have been captured by new entities. For example, the historical Merck collections are now foundational resources of Fundación MEDINA and the Natural Products Discovery Institute. Most strain libraries, however, are not easy to access. This means that interesting chemical scaffolds may be lost until rediscovered by traditional screens. Another challenge is securing intellectual property on known natural compounds and their activities [67]. Nevertheless, a deep reservoir of knowledge and chemistry exists that can be tapped for twenty-first-century antibiotic drug discovery.

17.5.2 Natural-Product Adjuvants, Resistance Inhibitors, and Combination Therapies

All known antibiotic-producing microbes have multiple biosynthetic programs that generate additional natural products. In most cases, these appear to be unrelated to production of the antibiotic of interest, but there are examples in which the additional products are co-expressed to achieve improved antibiotic efficacy. Indeed, coproduction to achieve synergy between molecules may be commonplace in producing microbes [68]. This can include coproduction of nonantibiotic adjuvants that enhance antibiotic activity or inhibit resistance. It can also include co-expression of two (or more) antibiotic compounds that act synergistically. Examples of the latter are the streptogramin antibiotics (described above in Sect. 17.2). Streptogramin producers, such as *Streptomyces pristinaespiralis*, produce type A and B compounds in a ratio of ~7:3. Binding of the type A streptogramin to the peptidyl transferase center of the bacterial ribosome enhances binding of the type B antibiotic to the region of the peptide exit tunnel by ~100-fold, thereby accounting for the observed synergy (reviewed in Ref. 40).

Antibiotic adjuvants have little or no antimicrobial activity themselves, but they enhance antibiotic activity by facilitating transport or blocking resistance [69, 70]. The discovery of clavulanic acid, a potent inactivator of β-lactamases produced by the cephamycin C-producer *Streptomyces clavuligerus*, demonstrated that antibiotic producers can "protect their investments" by co-expressing inhibitors of resistance. Several other cephamycin producers also express clavulanic acid, suggesting that the strategy of producing both antibiotics and inhibitors of resistance may be common. We have prepared a cell-based platform for the screening of resistance inhibitors that can also be used in the rapid identification (and dereplication) of known antibiotic scaffolds [71]. Using this platform, we identified an inactivator of metallo-β-lactamases, including NDM-1 produced by a strain of *Aspergillus versicolor* [72]. This strain also has the biosynthetic machinery to produce a β-lactam antibiotic (unpublished observation). There is little doubt that many other antibiotic–adjuvant pairs exist in nature. Indeed, plant-derived natural products also show efficacy as adjuvants [73].

Screening for lethal synergy is another strategy for extending the life of antibiotics. For example, the combination of bacteriostatic inhibitors of gene expression, such as tetracycline, rifampicin, and chloramphenicol, with the bacteriostatic compound bicyclomycin (Fig. 17.10), an inhibitor of the Rho transcription terminator, caused rapid killing of Gram-negative bacteria [74]. Screens for antimicrobial synergy often use growth inhibition assays and select for increased bacteriostatic activity; however, time–kill assays can be employed to screen for lethal synergy combinations, which cause rapid killing and may diminish the rate of resistance.

The significant challenge in bringing such combinations to market is the need to match pharmacological and dosing properties for each component. This is not trivial and often cited as a complex barrier to systematic exploration of such pairs. Nevertheless, a combination strategy is routine in the treatment of cancer, HIV disease, and even bacterial infections such as tuberculosis.

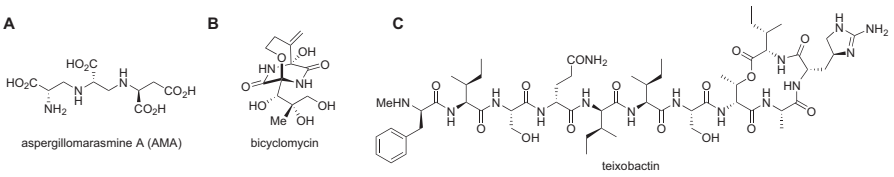


Fig. 17.10 (**A**) Aspergillomarasmine A (AMA) is an inhibitor of metallo-β-lactamases (e.g., NDM-1) and is in preclinical development as an adjuvant with β-lactam antibiotics [72]. (**B**) Bicyclomycin, an inhibitor of Rho transcription terminator, exhibits lethal synergy with inhibitors of gene expression [74]. (**C**) Teixobactin is a cyclic depsipeptide that binds to lipid II [75]

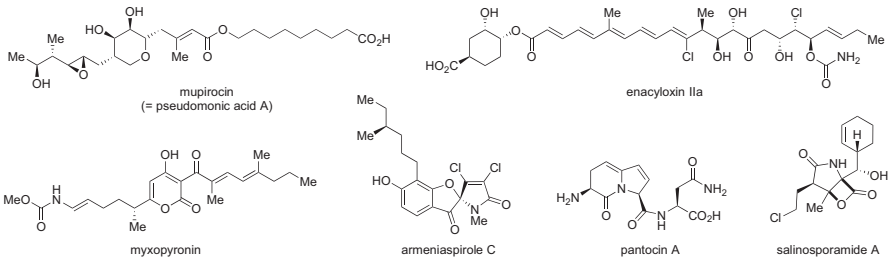


Fig. 17.11 Antibiotics produced by Gram-negative bacteria and marine bacteria

17.5.3 Mining New Sources of Microbial Natural Products

The bulk of the actinomycetes screened by the pharmaceutical industry for antibiotic activities originate from soil environments. These sources are easy to access and offer a wide variety of conditions for enriching various genera. The question of whether such sampling reflects a reasonable representative distribution of microbial natural-product diversity is unresolved. The same common scaffolds can be readily found in samples from around the world, supporting the axiom that "everything is everywhere, the environment selects." However, careful genomic sampling of signature natural-product biosynthetic elements, such as ketosynthase domains from polyketide synthases and adenylation domains of non-ribosomal peptide synthases from a variety of soil environments, suggests that indeed there are significant environmental differences in natural-product potential: there is significant chemical diversity still to be identified [76]. Indeed, marine actinomycetes are sources of several new compounds, many having biological activity (Fig. 17.11).

The fact that most of the focus in antibiotic natural-product discovery has been on the actinomycetes has prompted a search in other orders of bacteria. The Gram-negative betaproteobacteria, such as members of the genus *Burkholderia*, are prodigious producers of antibiotics [77]. The gamma-proteobacteria, pseudomonads, and the deltaproteobacteria, such as the Myxococci [78], also produce numerous natural-product antibiotics (Fig. 17.11). These have only just begun to be mined to discover new chemical scaffolds.

These sources though, are limited to strains that we can readily grow in the laboratory. The "great plate count anomaly" refers to the fact that we are generally limited to growing <5% of the detectable microbes in a soil sample. Strategies to mine this "microbial dark matter" offer ways to access new microbial genetic and chemical diversity [79]. An example of this approach is the iChip, a simple 96-compartment device to capture microbes and grow them in situ, with access to nutrients and growth factors of their natural environment [80]. Using this device and strategy, a new antibiotic, teixobactin, which represents a new scaffold, was identified from a previously uncultured bacterium [75]. Teixobactin, produced by a Gram-negative bacterium, has a Gram-positive-only profile. Its mode of action involves binding to lipid II, which is required for cell wall biosynthesis. Mining other difficult-to-grow bacteria for new chemistry should be possible and therefore offers hope that additional antibiotic scaffolds can be identified.

17.5.4 Genome and Metagenome Mining

Advancements in microbiology and molecular biology techniques have enabled the culture of microbes that previously were difficult to access. Advances in next-generation sequencing (NGS) are providing unequaled access to the genomic details of these organisms. Coupled with automated in silico prediction algorithms, such as antiSMASH, to identify biosynthetic gene clusters [81], this new genomic information has revealed a previously unappreciated and remarkable quantity and genetic diversity of natural products that can be (at least in principle) synthesized by microbes. On average, actinomycetes encode in their genomes 20–40 natural-product biosynthetic gene clusters; fungi encode even more. This new reality offers unprecedented opportunity to mine previously unknown or overlooked chemical diversity. Many of these compounds are difficult to detect and/or are found in low abundance. However, recent advances in mass spectrometry-based sampling and automated compound analysis and identification using artificial intelligence analysis (e.g., [82–85]) enable rapid triage for novelty that was inaccessible from traditional activity-guided purification and characterization methods. Such approaches are yielding new antibiotic scaffolds, such as the telomycins (Fig. 17.12), that target components of the bacterial membrane [86].

Often the expression of biosynthetic gene clusters in the laboratory is challenging, thereby preventing testing or purification of new compounds. Strategies to activate such "silent" clusters are being explored, although none is universal [88–91]. This includes the deletion or overexpression of regulatory genes, addition of chemical perturbants, physical stress (e.g., pH, temperature), and selection of mutants of various genes, such as encoded ribosomal proteins and antibiotic resistance. Failing such strategies, capture of entire clusters and mobilization to surrogate hosts can be used [92, 93].

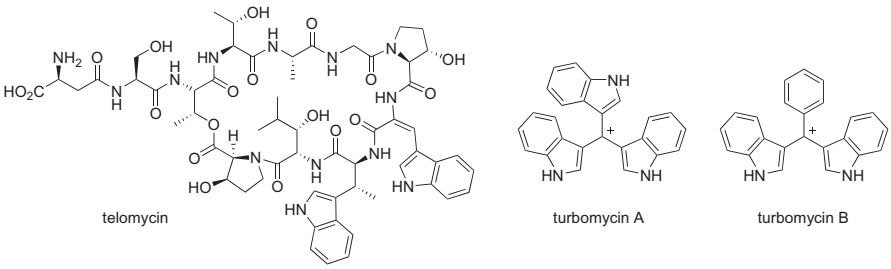


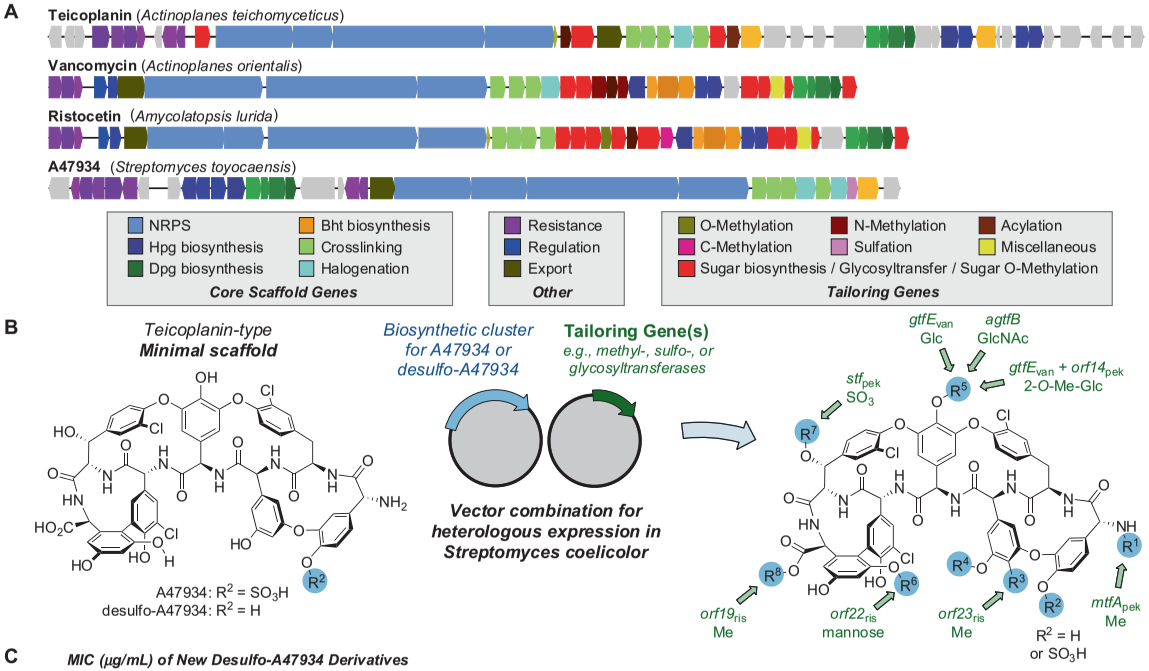
Fig. 17.12 Telomycin and turbomycins A and B [86, 87]

While genome mining has greatly expanded our access to known and new antibiotic scaffolds, the majority of environmental microbes remain difficult to culture. Here, metagenomic strategies in which total DNA is collected from a source (e.g., a soil sample, animal, or plant microbiomes) are being employed. Such strategies are yielding new antimicrobial compounds, such as the turbomycins [87], variants of glycopeptides [94], and colicins [95] (Fig.17.12).

17.5.5 Increasing Diversity Through Synthetic Biology

The long-term future for obtaining antibiotic diversity may be through the generation of nonnatural or synthetic natural products. This oxymoron refers to the engineering of biosynthetic gene clusters to produce novel compounds, not yet known to nature, using synthetic biology concepts [96–98]. The modularity of biosynthetic gene clusters lends itself well to systematic synthetic biology. Biosynthetic gene clusters include a predictable parts list: genes encoding scaffold assembly, tailoring enzymes, supply of components not easily scavenged from primary metabolism (amino acids, sugars, etc.), self-resistance, regulation, and transport (Fig. 17.13). In principle, these elements can be mixed and matched to generate new compounds having novel activities. For example, we have used this approach to generate new-to-nature glycopeptide antibiotics that evade certain forms of resistance in VRE [99] (Fig. 17.13).

As the costs of DNA synthesis continue to drop, one can envision synthesis of large numbers of biosynthetic gene cluster parts, the combinatorial generation of libraries of scaffolds, tailoring enzymes, regulatory elements, etc., and their expression in a suitable heterologous host. The result would be millions of previously untested combinations of biosynthetic genes (Fig. 17.13). With suitable selection, such libraries could deliver hits and lead for new antibiotic drug development.



C

MIC (μg/mL) of New Desulfo-A47934 Derivatives

	vancomycin	desulfo-A47934	R^3 = Me derivative	R^5 = GlcNAc derivative	R^5 = Glc derivative	R^5 = 2-O-Me-Glc derivative	R^6 = mannose derivative	R^7 = SO_3H derivative	R^8 = Me derivative
E. faecalis (ATCC 29212)	4	0.5	0.5	2	4	2	2	1	1
E. faecalis VRE B (ATCC 51299)	64	1	0.5	4	4	4	2	1	1

Fig. 17.13 A synthetic biology approach for increasing chemical diversity in glycopeptide antibiotics. (**A**) Biosynthetic clusters for selected glycopeptides with genes colored according to function. Significant portions of many clusters are comprised of tailoring genes responsible for decorating the glycopeptide backbone (e.g., methyltransferases, sulfotransferases, and glycosyltransferases). (**B**) Novel glycopeptides have been generated by mixing biosynthetic genes. In a recent study by Yim et al. [99], the biosynthetic clusters for A47934 and desulfo-A47934 were expressed in *S. coelicolor* together with a variety of tailoring genes from the biosynthetic clusters of other glycopeptides. Several new A47934- and desulfo-A47934 derivatives were produced and are more potent than vancomycin against *E. faecalis* and VRE B (**C**)

17.6 Concluding Remarks

Natural products, in particular those generated by bacteria and fungi, are the source of the majority of our successful antibiotic drugs. These agents have changed the world of medicine. For the first time in human history, we have good control over infection. With that control has come much of modern medicine. Our natural-product antibiotics have also helped us feed the world by changing the way we raise and care for food animals. It is not hyperbole to suggest that natural-product antibiotics may be the most important scientific discovery of the twentieth century.

Unfortunately, the evolution of antibiotic resistance and its selection in once-susceptible pathogens gravely threatens these advances. We need new antibiotics and alternatives to maintain our control over infectious disease. The advances in our knowledge of how natural products are made by microbes, new and unparalleled access to the genetic determinants of natural-product biosynthesis by NGS of microbial genomes and metagenomes, and the ability to harness this information to identify and exploit this information are growing exponentially.

The proven efficacy of natural products as antibiotics, plus the disappointing results of the past two decades of focus on synthetic compounds, means that we must pivot back to these ancient compounds for leads and inspiration. There is good reason to believe that the era of resistance depicted in Fig. 17.1 will be followed by an era of anti-infective innovation.

Major Points

- Microbial natural products are the source of most of our successful antibiotic drugs.
- These compounds are the result of evolutionary processes that select for optimal penetration and retention in target bacterial cells.
- The chemical diversity and physiochemical properties of microbial natural products cannot yet be effectively matched in most synthetic libraries.
- The re-isolation of known natural-product scaffolds diminished enthusiasm for the natural-product approach in antibiotic discovery.
- Efforts to identify new antibiotic chemical diversity through revisiting discarded compounds, mining of bacterial genomes, isolation of hitherto rare or unsampled microbes, and increasing chemical diversity using synthetic biology strategies offer new routes to identifying antibiotic leads.
- The use of inhibitors of resistance or other adjuvants can also extend the clinical effectiveness of existing antibiotic scaffolds.

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Chapter 18
The New Versus Old Target Debate for Drug Discovery

Alice L. Erwin

18.1 What Debate?

If I had been invited to write on this topic a couple of decades ago, I would not have understood what there was to debate. By mid-2000, complete genome sequences had been published for a dozen or so bacterial pathogens, providing a wealth of potential targets. For those of us whose careers in antibiotic discovery started just before the turn of the century, target evaluation seemed extremely simple. At that time it seemed obvious to me and to my coworkers that new targets were better than old. We argued that for drugs with new mechanisms there would be no pre-existing resistance. A second argument was that while the empiric methods of the past had identified only a small number of antibiotic classes and even fewer targets, new technologies would allow us to cast our net much more widely and be much more productive.

At that time, I saw the need for new antibiotics to replace drugs for which resistance had become common. I had no idea of the limitations of existing antibiotics other than resistance. Moreover, it did not occur to me to wonder whether inhibitors of the new targets (mostly enzymes) would be as effective as existing antibiotics (most of which target the machinery of macromolecular synthesis).

Today, I consider that one of the most important advantages of new targets is the possibility of finding drugs that are not only new but in some way better than current antibiotics. Features that might be considered desirable for new anti-infective drugs include reduced likelihood of resistance emergence, improved activity against persistent infections, or better safety, including less disruption of normal flora.

The sections below will present my view of the advantages and risks of old and new targets. I will illustrate my discussion with examples of antibacterial drugs that

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I. W. Fong et al. (eds.), *Antimicrobial Resistance in the 21st Century*, Emerging Infectious Diseases of the 21st Century, https://doi.org/10.1007/978-3-319-78538-7_18

were recently approved or are currently in clinical trials, as well as some interesting new compounds with efficacy in animals. These can be considered in three groups:

- Inhibitors of three good old targets, including both new members of old chemical classes and first-in-class antibiotics with similar mechanisms to old drugs. Contrary to the idea that old targets were not amenable to modern methods, medicinal chemistry was guided by structural and biochemical data in addition to antibacterial assays for many of these.
- New antibiotics with other, well-defined targets, for which knowledge of the target was used in evaluating analogs. This list is short, as target-directed programs have not yet been very successful.
- Antibacterial drugs with new, often complex mechanisms. Some of these were discovered empirically, with the mechanism of action determined later (if at all). Others are not antibiotics per se but increase the susceptibility of bacteria to host defenses or to other drugs. Some were designed to address aspects of infection not well handled by existing antibiotics.

This review is not intended to provide a complete list of antibacterial drugs in the clinical development pipeline. The reader is referred to recent reviews [12], to the NIH database https://clinicaltrials.gov/, and to the pipeline web pages maintained by the Pew Charitable Trusts Antibiotic Resistance Project (http://www.pewtrusts.org/en/multimedia/data-visualizations/2014/antibiotics-currently-in-clinical-development; http://www.pewtrusts.org/en/multimedia/data-visualizations/2017/nontraditional-products-for-bacterial-infections-in-clinical-development).

18.2 We Have New Antibiotics in the Pipeline with the Good Old Targets

The vast majority of antibiotics inhibit synthesis of macromolecules. Three mechanisms dominate, with several chemical classes of inhibitors known for each. New drugs with the same or similar mechanisms include both new scaffolds and new members of old chemical classes.

18.2.1 Cell Wall: Inhibitors of Transpeptidases and β-Lactamases

Starting with the discovery of benzylpenicillin in 1928, inhibition of the transpeptidases (penicillin-binding proteins, PBPs) involved in peptidoglycan synthesis has been one of the most important antibiotic mechanisms. Until recently, all chemical classes of PBP inhibitors were β-lactams, and the first member of each class to be discovered was a natural product. Additional penicillins, cephalosporins,

carbapenems, and monobactams were often synthetic or semisynthetic. Derivatives of the penicillin precursor 6β-amino-penicillanic acid (6-APA) allowed improvements in ease of administration, broadening of bacterial spectrum, or restoration of activity toward isolates that had become resistant. For example, benzylpenicillin was administered by injection and was active only against Gram-positive bacteria. Ampicillin, first used in 1961, can be taken orally and is active against some Gram-negative species, including *Escherichia coli* and *Proteus mirabilis*, as well as Gram-positive bacteria. By the late 1950s, many isolates of *Staphylococcus aureus* had acquired penicillinase genes, making them resistant to the early penicillins but not to penicillinase-stable semisynthetic penicillins or to the first-generation cephalosporins. For Gram-negative bacteria, the spread of β-lactamase genes was countered by co-administering β-lactamase inhibitors (BLIs) with β-lactam antibiotics. The first such combination, clavulanic acid plus amoxicillin, was approved in 1984 as Augmentin and is still widely used. Two other BLIs, tazobactam and sulbactam, were also developed during the twentieth century. Like clavulanic acid, these are themselves β-lactams and function by forming a stable acyl–enzyme complex, thus poisoning the β-lactamase enzyme. These BLIs have little antibacterial activity of their own and are always used in combination with other β-lactams, though the activity of sulbactam for *Acinetobacter baumannii* has been studied recently [73].

New β-lactamase inhibitor scaffolds The continued evolution of β-lactamases in response to the introduction of new β-lactam antibiotics spurred the search for novel inhibitors of PBPs and/or β-lactamases. The diazabicyclooctane (DBO) class, discovered at Roussel Uclaf, is the first non-β-lactam scaffold to be useful as inhibitors of β-lactamases, with avibactam the first member to be developed [14, 103]. The DBOs differ in mechanism from β-lactam BLI, in that covalent binding of avibactam to the enzyme is described as slowly reversible [25]. The first DBO-β-lactam combination to be approved was Avycaz (avibactam-ceftazidime, in 2015); other avibactam-β-lactam combinations are currently in clinical trials. Relebactam (previously MK-7655) is currently in phase 3 studies, in combination with imipenem and cilastatin (Merck). Two other DBOs, RG6080 (Meiji/Fedora) and zidebactam (Wockhardt), are currently in phase 1.

Another new BLI class is the cyclic boronates, which are not acylase inhibitors but transition-state analogs. The most advanced of the boronates is vaborbactam, being developed by The Medicines Company. A meropenem/vaborbactam combination recently completed a phase 3 study.

Non-β-lactam PBP inhibitors It is remarkable that in decades of natural product research, nearly all PBP inhibitors ever discovered were β-lactams. One exception is the lactivicins, discovered in 1987, which have never been developed for clinical use [103]. More recently, research by the Mobashery laboratory at Notre Dame discovered synthetic PBP inhibitors in a specific search for compounds active against PBP2a, the transpeptidase that mediates methicillin-resistance in MRSA. Medicinal chemistry optimization of the oxadiazole scaffold identified lead

molecules active against both methicillin-susceptible and methicillin-resistant *S. aureus*, with efficacy in a mouse model of *S. aureus* infection [41].

18.2.2 Protein Synthesis: Bind 30S or 50S Ribosomal Subunits

Aminoglycosides, tetracyclines and macrolides The two largest classes of natural product protein synthesis inhibitors, the aminoglycosides and tetracyclines, have a history that is generally similar to that of β-lactam antibiotics. Streptomycin and chlorotetracycline, the first drugs of these classes, are unmodified natural products. Subsequent members included both new natural products with similar chemical structures and semi-synthetic derivatives thereof. Within a chemical class, compounds differed in antibacterial spectrum, pharmacokinetics, and toxicity. As resistance to early members of each class emerged, new antibiotics were developed specifically to address those resistance mechanisms. Discovery of new tetracyclines slowed after the 1960s until the discovery of tigecycline, a glycylcycline, at Lederle in 1993. Plazomicin, derived from sisomicin at Achaogen, and omadacycline, a semisynthetic derivative of minocycline discovered at Paratek, are both very much in line with the previous history of aminoglycosides and tetracyclines, respectively. Both are currently in phase 3 studies. The macrolide class has fewer members but a similarly long history, beginning with erythromycin. Recent members include nafithromycin (Wockhardt), now in phase 2, and solithromycin (Cempra), in phase 3 [29, 33, 106].

A new era of tetracycline research began with the development of chemical methods for synthesizing tetracyclines and related compounds, at the Myers laboratory at Harvard. This technology was licensed to Tetraphase, who have several compounds in the clinic. The most advanced of these, eravacycline, is currently in phase 3. More recent compounds are divergent in structure and have broader antibacterial spectrum, with some active against *Pseudomonas aeruginosa* [20]. The Myers group has also developed methods for synthesis of macrolides [84].

Pleuromutilins The pleuromutilin class of ribosome inhibitors was discovered in 1951, and a couple of semisynthetic derivatives have been developed for topical use. Lefamulin (Nabriva), currently in phase 3, is the first pleuromutilin to be developed as a systemic antibiotic [69].

Oxazolidinones In contrast to most ribosome inhibitors, oxazolidinones are not natural products. The antibacterial activity of the scaffold was first described at DuPont. Linezolid, the first oxazolidinone antibiotic, was discovered at Upjohn through extensive medicinal chemistry. Since the turn of the century, new oxazolidinones have been discovered through research at multiple companies. Tedizolid (previously torezolid, discovered at Dong-A and developed by Trius and Cubist) was the next member of the class to be approved, and several others are currently in clinical trials [3].

18.2.3 DNA Synthesis: Inhibitors of Type II Topoisomerases

The largest class of synthetic antibiotics is the fluoroquinolones, which inhibit two targets, the GyrA subunit of DNA gyrase and the ParC subunit of topoisomerase IV. Many fluoroquinolones have been approved, the most recent being Baxdela (delafloxacin) from Melinta Therapeutics; and there are others currently in various stages of clinical development. Moreover, clinical candidates have been identified for several new scaffolds of Type II topoisomerase inhibitors. Gepotidacin (GlaxoSmithKline) and Zoliflodacin (Entasis Therapeutics), both in phase 2, both inhibit gyrase A with mechanisms different from fluoroquinolones [12].

The lethality of fluoroquinolones is the result of the fact that they not only inhibit the ATPase activity of gyrase but also stabilize the covalent enzyme–DNA complex. Balanced inhibition of both gyrase and topoisomerase IV is important for the characteristically low frequency of spontaneous mutation to high-level fluoroquinolone resistance. Novobiocin has a much higher frequency of resistance, and this has been attributed to its disproportionate inhibition of gyrase (GyrB) vs topoisomerase IV (ParE). This insight inspired programs at both Vertex Pharmaceuticals and Trius Therapeutics aimed at discovery of compounds with balanced inhibition of GyrB and ParE. Both groups employed structure-based drug design and successfully identified dual-active compounds with efficacy in animal models that had the predicted low frequency of resistance. Tricyclic GyrB/ParE (TriBE) inhibitors active against both Gram-negative and Gram-positive bacteria were discovered by Trius before their acquisition by Cubist and then by Merck. Vertex gyrase inhibitors are primarily active against Gram-positive pathogens; their technology was recently licensed by Spero Therapeutics. Using an antisense whole-cell screen, Merck scientists identified a natural product, kibdelomycin, that is also a dual inhibitor of GyrB and ParE. Kibdelomycin is active primarily against Gram-positive bacteria and showed efficacy in a hamster model of *Clostridium difficile* infection [7, 32, 37, 62, 97].

18.3 What Is Good About These Old Targets?

18.3.1 These Targets Work and Keep Working

As noted, there are multiple classes of antibiotics targeting each of the mechanisms described above. Each has been shown to be amenable to rational drug design as well as to the empiric methods that led to the first members of each scaffold. Fluoroquinolones were probably the first class of antibiotics for which data on potency toward the target enzyme as well as antibacterial activity were used to inform design of new analogs [23]. Structural information on the interaction of compounds with their targets was critical for discovery of the dual GyrB/ParE inhibitors and was likely to have been important for the new classes of β-lactamase inhibitors also. Ribosomal crystal structures now allow understanding of binding specificity of new inhibitors but have not yet led to completely novel scaffolds [111].

18.3.2 Low Resistance Frequency

As the impetus for discovery of new antibiotics has usually been the emergence of resistance to existing antibiotics, it may seem paradoxical to describe drugs with these old targets as having a low frequency of resistance. The key distinction is between endogenous resistance, resulting from spontaneous mutations that produce a substantial loss in susceptibility to an antibiotic, and exogenous resistance, resulting from acquisition of genes encoding resistance determinants. Mutations that confer high-level resistance in a single step can lead to rapid selection for resistance, sometimes within a single course of treatment. Drugs for which such mutations are frequent cannot be used as single antibiotics because treatment failure is common. This phenomenon was recognized in the 1950s, leading to the decision that streptomycin and rifampin could safely be used in combination (typically in tuberculosis) but neither could be used as monotherapy. In an influential paper, Lynn Silver described the concept that the reason monotherapy is possible for many of the common broad-spectrum antibiotics is that they effectively have multiple targets [87].

For the classes of antibiotics described above, nearly all resistance is exogenous. Acquired mechanisms of resistance include aminoglycoside-modifying enzymes, β-lactamases, efflux pumps specific for macrolides or tetracyclines, rRNA methyltransferases, alternative PBPs, and other target-modifying enzymes. These resistance determinants nearly always have origins in bacterial populations other than the pathogens being treated by the antibiotic. The existence of transmissible resistance does not prevent a new antibiotic from being useful, sometimes for many decades after the first appearance of a resistance determinant. Understanding the molecular basis of acquired resistance to an existing drug has been extremely valuable, allowing the discovery of new drugs with the same or similar mechanism of action but which avoid resistance.

18.3.3 The Old Targets Gave Us Confidence in New Targets

The success of antibiotics with these old targets has given us confidence that we know how antibiotics work, how to use them, and how to find new ones. Antibiotics are probably the most successful and best understood type of drug. Broadly speaking, all antibiotics have the same, extremely simple mechanism: stop growth of the infecting organism and let the patient recover. Bacterial infections can be considered as either the invasion of the patient by a foreign pathogen or the intrusion of a commensal organism into a normally sterile site. In either scenario, the vast majority of such invasions are easily controlled by the patient's inflammatory system without any problem. If the bacteria somehow get away from the normal host defenses, the result is symptomatic disease. A drug that slows growth of the invader will often allow the patient to regain the upper hand and clear the infection. Although it might seem desirable for antibiotics to kill bacteria outright, in actuality many successful antibiotics are simply bacteriostatic.

Study of the antibiotics discovered during the twentieth century led to standard in vitro methods for determining the antimicrobial susceptibility of bacteria isolated from patients and to understanding how antibacterial activity and pharmacokinetics of an antibiotic contribute to its efficacy in an animal model. The developing field of PK/PD not only allows accurate prediction of effective dosing in animal studies but allows extension of those data to human trials.

For most existing antibiotics, we have a fairly good understanding of mechanism of action, usually inhibition of an essential bacterial process. Collectively, the study of existing antibiotics has given us the feeling that a chemical compound will prevent bacterial growth if it is able to enter bacterial cells and inhibit an essential bacterial process with sufficient potency. If in vitro antibacterial activity is sufficient, the compound will probably have efficacy in mouse models of infection as long as it is not toxic and has appropriate pharmacokinetics to provide sufficient exposure to bacteria at the site of infection.

These features should make antibiotics far easier to discover and develop than drugs for other therapeutic areas. Consider drugs for hypertension, cardiac disease, Alzheimer's, rheumatoid arthritis, etc. Discovery of a new drug often means simultaneously developing new understanding of the molecular basis of the disease and finding compounds that affect that aspect of human biology while leaving the rest of human physiology intact. For many human diseases, animal models are not very useful for testing efficacy of new drugs. If a new drug is approved, it is necessary to teach physicians which patients will benefit and how to use the new drug. In contrast, the principles of antibiotics are well understood by scientists, by physicians, and by regulatory agencies. We know how to evaluate candidate drugs in the lab and in patients, and physicians understand how to use the new drug if it is approved.

18.4 Seeking Antibiotics with New Targets: The Genome Era

At the turn of the century, it was recognized that the so-called Golden Age of Antibiotics had come to an end. Although new members of existing antibiotic classes were being developed, the only new class that had been discovered for decades was the oxazolidinones. Discovery of promising natural products had stalled. The search for new targets was driven by a feeling that as antibiotic resistance was continuing to increase, new experimental approaches would be required.

The explosion of target-directed projects in the early twenty-first century, particularly the widespread use of in vitro enzyme assays for high-throughput screening of synthetic libraries, is often described as the "genome era" of antibiotic research. In addition to the bacterial genome sequences that were appearing, industrial research programs at this time made use of other components of target-directed drug discovery that were already in place in biotechnology and pharmaceutical companies, having been developed for other therapeutic areas. There was increasing interest in enzymes as targets, as it would be possible to screen large chemical libraries with in vitro assays using robotics. If the enzyme could be crys-

tallized, structural data would be available to guide chemistry. Rational drug design seemed feasible and more attractive than the empiric methods by which most antibiotic classes had previously been developed.

The critical value of the genome sequences available by 2000 or so was not the thousands of previously unknown genes but the incredible level of detail on the genes for which functions were known or could be proposed. A gene previously known only in *E. coli* or *Bacillus subtilis* could be amplified from *P. aeruginosa* or *S. aureus* in order to produce recombinant protein. Transposon mutagenesis and other molecular biology techniques allowed genome-wide assessment of genes required for in vitro growth or for full virulence in animals. Research programs focused on targeting enzymes for which in vitro assays could easily be developed would compile a list of 100–150 potential targets that were considered to be essential in vitro, absent from mammalian cells, and conserved across the pathogens of interest. A complementary approach used antisense technology to downregulate individual genes, generating strains that were hypersensitive to inhibitors of the target enzyme (or pathway). Such strains could be used in initial screening or in evaluating compounds that were known to be potent inhibitors of the target [24, 59, 63].

The use of new, genome-wide studies together with robotics and combinational chemistry seemed very exciting at the time but did not lead to the rapid discovery of new antibiotics, as had been hoped. Some of the limitations of these programs will be discussed in a later section.

18.5 New Antibiotics with Other, Well-Defined Targets

Considering antibacterial compounds with novel mechanisms, it is remarkable that very few were discovered by choosing an enzyme target and screening for inhibitors, though this might be considered by some to be the standard approach. Indeed, no antibiotic ever approved was discovered by this approach. This section describes several of the most promising target-directed projects. Not all were initiated by screening for inhibitors of a preselected target. In most cases the target was identified early in the project, and potency toward the target was used in medicinal chemistry. For most of the targets listed below, at least one compound has reached clinical trials; all have inhibitors with reported efficacy in animal models of infection.

18.5.1 Protein Synthesis: Targets Other than the 30S and 50S Ribosomal Subunits

Several inhibitors of protein synthesis with mechanisms different from those discussed above have reached clinical trials.

EF-Tu The thiopeptide LFF571 (Novartis) is a semisynthetic derivative of a natural product, GE2270 A, that binds elongation factor Tu. LFF571 was reported to be effective in treating *C. difficile* infection, based on a phase 2 study [65].

tRNA-synthetases A series of boronate compounds with antimicrobial activity was found to inhibit leucine-tRNA synthetase, leading to discovery of AN3365 (later GSK2251052) at Anacor. This compound appeared very promising because of its Gram-negative activity. However, a phase 2 study for complicated urinary tract infection was halted when resistant mutants were isolated from several patients [70]. A chemical series of methionine-tRNA synthetase inhibitors discovered at GlaxoSmithKline was licensed to Replidyne [40], who discovered the diaryldiamine series, including REP3123 (now CRS3123), being developed by Crestone for *C. difficile* infection [67].

Peptide deformylase (PDF) PDF was a very popular target because it was thought there is no equivalent mammalian enzyme and that initiation of bacterial proteins with formyl-methionine was a universal feature of bacterial proteins. An antibacterial natural product, actinonin, was found to inhibit PDF, providing chemical validation. However, actinonin-resistant mutants of *S. aureus* were easily isolated and found to have inactivated *fmt*, the gene encoding formyl-methionine transferase. Finding that *fmt*-null mutants can be viable suggested that PDF inhibitors would be effective only for species in which *fmt* is essential. These include *Streptococcus pneumoniae* and *Haemophilus influenzae*. There is also some evidence that *fmt*-null mutants of *S. aureus* are reduced in virulence. PDF is a metalloenzyme, so it was expected that finding potent inhibitors would be straightforward. Indeed, at least three PDF inhibitors reached clinical trials [15, 48, 110]. Two of these, BB-83698 and LBM-415, were discontinued after phase 1. GSK1322322 entered phase 2 and has since been discontinued.

18.5.2 Lipid Synthesis

FabI The fatty acid biosynthetic enzyme FabI (enoyl-ACP reductase) has been targeted in several programs. This enzyme had been expected to be conserved across bacterial species. However, the availability of genome sequences made it apparent that some species contain alternative enzymes, designated FabK, FabL, or FabV. These catalyze the same reaction as FabI but are so different in structure that inhibition of all by a single compound appears impossible. Moreover, some pathogens are able to use exogenous fatty acids during infection and are therefore expected to be intrinsically resistant to inhibitors of endogenous fatty acid synthesis [108]. Two FabI inhibitors are currently in clinical trials, CG400549 from CrystalGenomics and Debio 1450 from Debiopharm [12].

LpxC Lipopolysaccharides (LPS) are specific to Gram-negative bacteria. The lipid A moiety is an essential component of LPS and is the most conserved part of the molecule. In principle, most of the enzymes involved in lipid A synthesis could be considered as potential antibiotic targets. In practice, only LpxC (UDP-3-*O*-(*R*-3-hydroxymyristoyl)-*N*-acetylglucosamine deacetylase) has been pursued seriously. Nearly all LpxC programs can be traced back to a chemical series discovered at Merck in the 1980s using a bacterial cell-based screen for inhibitors of any step in the LPS biosynthetic pathway. Activity of the Merck series was limited to *E. coli* and other enterics. A program at Chiron and the University of Washington discovered analogs with activity for both *E. coli* and *P. aeruginosa*. This led to work at many companies, and over a dozen institutions have filed patents on LpxC inhibitors. All of these compounds are hydroxamic acid derivatives, and potency depends on chelating zinc in the LpxC active site. Only one of these has so far reached the clinic: ACHN-975 from Achaogen was discontinued after phase 1. Forge Therapeutics has recently described efficacy of a series of non-hydroxamate LpxC inhibitors, though structures have not yet been disclosed [27].

18.5.3 Antifolates

The antifolates are the oldest synthetic antibiotics still in use today. Sulfonamides were discovered empirically in the 1930s by Gerhard Domagk, with antibacterial activity determinations used to drive medicinal chemistry of compounds derived from azo dyes. After introduction of the first sulfa drugs, it was realized that they mimic *para*-aminobenzoic acid (PABA), thereby inhibiting dihydropterate synthase (DHPS) [92]. Trimethoprim was discovered more rationally. As described by van Miert, George Hitchings hypothesized that analogs of purine and pyrimidine bases might serve as antimetabolite drugs. He discovered several 2,4-diaminopyrimidine inhibitors of the folate pathway that differed in specificity, including trimethoprim for bacteria, methotrexate for cancer, and pyrimethamine for protozoal infections (e.g., malaria); these are all inhibitors of dihydrofolate reductase (DHFR) [102]. The DHFR inhibitor iclaprim was designed to address trimethoprim resistance. Development was stalled in 2008, but it is again in phase 3 clinical trials, sponsored by Motif Bio [12]. Both DHPS and DHFR are being pursued in academic research labs, but no clinical candidates have yet been identified [28, 35].

18.5.4 RNA Polymerase

Discovered in the 1950s, rifamycins are the major antibiotic class targeting RNA polymerase. Fidaxomicin, another inhibitor of RNA polymerase, is a natural product described in 1975 that was approved in 2011 for *C. difficile* infection. A limitation of both these classes is their very high frequency of resistance due to mutations

in *rpoB*. Rifamycins are used primarily in combination with other antibiotics and occasionally as monotherapy in situations where the number of bacteria to be treated is very low, such as for prophylaxis. A recent report described the discovery of pseudouridimycin by screening a library of natural product extracts for inhibitors of RNA polymerase. Pseudouridimycin is active against several Gram-positive species and fastidious Gram-negative bacteria and was reported to be efficacious in a mouse model of *Streptococcus pyogenes* infection. Of note, the frequency of resistance to pseudouridimycin was reported to be tenfold lower than that of rifampin, apparently because it has a different binding site that is less tolerant of mutations [47, 52, 78].

18.6 Did the New-Targets Programs Fail?

One of the risks of any new target is a high likelihood of failure, but the same can be said of any drug discovery effort. Even with well-established antibiotic classes, it is rare for a new clinical candidate to emerge, and the time between discovery and approval is many years. It is thus not surprising that few of the antibiotics approved in the past 5 years came from projects that were initiated after 2000 or so. However, it is surprising that there are so few scaffolds in the pipeline from the target-directed projects started in the genome era.

An influential review from scientists at GlaxoSmithKline described the dismal failure of 67 high-throughput screens of bacterial targets, with very few producing hits that were worth exploratory chemistry. A more recent review from the antibacterial program at AstraZeneca was somewhat more positive, in that they had identified hit scaffolds for 57 of the 65 targets they screened. Lead series were identified for 19 targets. It seems likely that differences in the synthetic libraries at the two pharmaceutical companies accounted for the differing hit rates. Despite initial promising results, the AZ team dropped nearly all their targets when they were unable to find compounds with broad-spectrum antibacterial activity. A contributing factor was that at the time, Gram-negative activity was seen as essential. If they had been primarily interested in drugs for staphylococcal and enterococcal infections, they might have considered a smaller proportion of projects to be failures. Both reviews emphasized, as others have, that pursuing such high numbers of targets might have contributed to failure of the overall programs [74, 99].

The prevailing culture held that all genetically validated targets were equally good and that screening would identify those that were worth pursuing. There was high pressure to run as many screens as possible, leading to an emphasis on targets for which assays would be easy to develop. In practice, these were nearly all enzymes. Knowledge of the biochemical reaction and structure of the active site aided prioritization of “druggable” targets. Despite the idea of pursuing novel targets identified in genome sequences, most work was on genes whose function had already been identified in the previous decades of academic research. Protein–protein interactions were generally not considered druggable.

Hit evaluation and early chemistry focused on improving affinity for the target, sometimes without evidence that any antibacterial activity observed was mechanism-based. The mantra "fail early and often" meant that one project after another was terminated without a chance to learn how to improve the chances of success.

A common experience is that biochemical and structural data could allow the chemists to improve potency of a scaffold substantially but without a corresponding improvement in antibacterial activity. This is in large part because we lack information on how to improve the accumulation of compounds within bacterial cells – an important subject beyond the scope of this review. It is often suggested that chemical libraries in most companies are not well suited for antibacterial discovery and that we would be more successful with different starting points.

Because it is difficult to confer antibacterial activity on chemical scaffolds that lack it, some programs have run target-focused screens that use bacterial cells. A number of approaches using reporter genes, differential growth, morphology, or labeling by a dye or radioisotope have been described [63, 90]. Even in programs that placed a strong emphasis on cell-based assays rather than in vitro enzyme assays, the overall success rate has been low, considered in terms of drugs entering clinical trials.

For some targets, we have some information about why development of a specific compound was halted. Inhibitors of two metalloenzymes (PDF and LpxC) have failed to progress after phase 1, and we can guess that these compounds had toxicity that might have been mechanism-based. Whether toxicity will keep other LpxC inhibitors from reaching the clinic is difficult to determine. The rapid selection of mutants resistant to the LeuRS inhibitor GSK2251052 is consistent with our general understanding of antibiotics with single targets. However, preclinical data suggest that resistance to the MetRS inhibitor CRS3123 may be less of an issue [17]. Perhaps surprisingly, the frequency of resistance to LpxC inhibitors is so far very low [27].

For projects that never reach the clinic, there is rarely any public information about the status of any project or the reason it was terminated. It is therefore impossible to determine why any target has not yielded new drugs, or even to say conclusively that it has failed. A scaffold may be active vs a few isolates of *S. aureus* or *E. coli* and perhaps show efficacy in a mouse model but never reach the desired antibacterial spectrum; or it may have poor pharmacokinetics or show toxicity in preclinical studies. Most of these liabilities are characteristics of the compound or the chemical scaffold but shed no light on the validity of the molecular target. Often resources are diverted from one project to another for reasons that are strategic rather than scientific. Should we conclude in such a case that the target has now been validated, not only genetically but chemically and pharmacologically? If no clinical candidate appears, should we conclude the target is a failure?

18.7 Limitations to Classical Target-Directed Discovery

Today there is increasing recognition that there are two kinds of unmet need for bacterial infections.

Traditional antibiotics We need new traditional antibiotics to replace those for which resistance has become common. The discovery programs described in the previous sections are intended to meet this need. As has been discussed in numerous conferences and white papers, new antibiotics are not emerging at the rate that is required. However, even if we had a full pipeline of broad-spectrum antibiotics for Gram-positive and Gram-negative infections, there would still be a need for other types of drug.

One issue is that the desired target-product profiles change over time. At the turn of the century, a common criticism of LpxC projects was that the resulting drugs would not be useful for treating MRSA. It was difficult to convince senior management that Gram-negative resistance was on the rise. Infectious disease physicians generally did see value in a new antibiotic that would be limited to Gram-negative bacteria – as long as it was active against *P. aeruginosa* as well as enteric bacteria. Today we need drugs for *A. baumannii* as well as for *P. aeruginosa*. At the same time, antibiotic resistance in *Klebsiella* and *Enterobacter* spp. and *E. coli* has increased to the point where an enteric-only drug would be valuable. *C. difficile* infection, long recognized as a complication of treatment with broad-spectrum antibiotics, became a much greater problem as antibiotic-resistant strains became prevalent. It has become common for new Gram-positive agents with poor PK and oral bioavailability to be developed as *C. difficile* drugs. For some of these, microbiome studies are incorporated into clinical trials.

The idea of narrow-spectrum antibiotics is becoming much more attractive. It is thought that resistance to narrow-spectrum drugs may emerge more slowly, as the normal flora will not provide a reservoir for resistance determinants. Further, narrow-spectrum antibiotics are not expected to induce the dysbiosis that is associated with broad-spectrum antibiotics. The development and use of narrow-spectrum antibiotics are far from straightforward but may become more feasible in the next several years [9]. It is therefore reasonable to reconsider some of the targets that were rejected during the genome era because they were not broadly conserved across pathogens.

Second unmet need We also need drugs for prevention and treatment of bacterial infections for which antibiotics have never been fully effective. These include recurrent, latent, or persistent infections, often associated with biofilms. Such infections have plagued humans for millennia but are much more prominent today. Examples are ventilator-associated pneumonia, infections in immunocompromised patients with organ transplants or cancer chemotherapy, infected joint replacements or other orthopedic devices, diabetic foot ulcers, respiratory infections in patients with cystic fibrosis, and recurrent urinary tract infections.

The antibiotics we have today act mainly on growing bacteria. They are probably most effective in treating acute infections in previously healthy individuals. The same is likely to be true for new antibiotics discovered with either the old targets or the enzyme targets of the genome era. We need drugs that are active against non-growing bacteria and against bacteria that have become tolerant to antibiotics.

Some aspects of infection are not affected by antibiotics at all. These include the direct toxic effects of secreted cytotoxins, some of which are treatable with antibodies, and the uncontrolled inflammatory response to lipopolysaccharides or toxic-shock syndrome toxin. Research on sepsis has not yet produced effective drugs, in part because animal models are not very predictive.

Overall, new antibiotics with standard kinds of targets are likely to have the same limitations as existing antibiotics. Different targets, or different experimental approaches, will be needed in order to discover drugs that are not only new but better anti-infective therapeutic agents. Some of these are described in the next section.

18.8 New Drugs with New, Often Complex Mechanisms

A previous section described promising antibacterial compounds that were discovered in target-directed programs, meaning that medicinal chemistry was guided not only by antibacterial activity but by experimental data on the interaction of compounds with the molecular target. For each of the previously described drugs, the mechanism of action is direct inhibition of a process that is essential for bacterial growth.

In contrast, this section describes compounds with a variety of mechanisms, discovered through experimental approaches that do not fit this paradigm. The compounds described below have one or more of the following features: empiric discovery, nonspecific mechanisms, affecting the target at the level of regulation rather than enzymatic activity, and no in vitro antibacterial activity.

18.8.1 Empiric Discovery of New Drugs with New Mechanisms

Although most classes of existing antibiotics were discovered empirically, recent experience with screens for antibacterial activity have been frustrating. Commonly a high proportion of “hit” compounds are nonspecific, affecting both eukaryotic and prokaryotic cells. Often these hits are found to be membrane active (either lysing or depolarizing membranes), behaving like poisons or detergents. Attempts to improve selectivity are rarely successful, so such compounds are routinely discarded [71, 74, 88, 90]. This does not mean that it is impossible to find drugs by tracking antibacterial activity alone. In the examples below, if medicinal chemistry was used, the

driver was antibacterial activity rather than potency vs target. At the time these compounds were discovered, the target was not known (and may still be unknown).

Teixobactin A platform at NovoBiotic for seeking antibacterial compounds produced by previously uncultured microorganisms has revived hope in empiric natural products discovery. In 2015, they described a new Gram-positive antibiotic with efficacy in mouse models of *S. aureus* and pneumococcal infection. Teixobactin inhibits cell wall biosynthesis, binding glycolipids lipid II and lipid III (precursors of peptidoglycan and teichoic acids, respectively). This mechanism of action is similar to that of vancomycin, which binds a different site on lipid II. Because it is not the product of a single gene, it is difficult for spontaneous mutation to alter the structure of lipid II. The frequency of endogenous resistance to vancomycin is thus extremely low, and the same appears to be true for teixobactin. Resistance to vancomycin became common only after a resistance cassette derived from the natural producer was transferred into enterococci, encoding enzymes that produced an altered lipid II. Teixobactin binds to both forms of lipid II and is thus active vs both vancomycin-sensitive and vancomycin-resistant bacteria. Several other antibacterial compounds that bind lipid II are known, including the peptides plectasin and nisin. Apart from the vancomycin derivatives telavancin, dalbavancin, and oritavancin, none of these other lipid II-binding compounds is used clinically [49, 81].

Bedaquiline This review does not otherwise discuss tuberculosis drugs, but two classes of antimycobacterial antibiotics are mentioned here as successful recent examples of using antibacterial activity to guide medicinal chemistry, without knowledge of the molecular target. The recently approved tuberculosis drug bedaquiline targets ATP synthase and is bactericidal for both growing and nongrowing mycobacteria. Bedaquiline was discovered at Janssen by screening for compounds that inhibit growth of *Mycobacterium smegmatis* and then optimizing by medicinal chemistry [1]. It is of interest because of the success of this empiric approach and also because of the unprecedented nature of the target. No other antibacterial drug has a well-defined direct effect on the respiratory pathway.

PA-824 and OPC-67683 A second class of new mycobacterial drugs is the 2-nitroimidazoles, PA-824 (now pretomanid, discovered at PathoGenesis and in development by the TB Alliance) and OPC-67683 (now delamanid, discovered at Otsuka and approved in Europe and Japan in 2014). The scaffold is not entirely new, being related to previously known nitroimidazoles. Metronidazole, a 5-nitroimidazole derived from the natural product azomycin, was first used for anaerobic protozoal infections (e.g., trichomoniasis) and then realized to be active against anaerobic bacteria. Metronidazole is a prodrug, converted within the microbial cell to produce a nitro radical anion and a variety of reactive nitrogen intermediates that kill the cell by damaging cellular components, particularly DNA. In the 1990s, metronidazole was recognized to be active against dormant or anaerobically adapted *M. tuberculosis* but not against actively growing mycobacteria. 2-imidazoles were recognized in the early 1970s to have modest activity against a variety of bacteria. During discov-

ery of PA-824, scientists at PathoGenesis monitored antimycobacterial activity of compounds, resulting in development of the nitroimidazopyran scaffold. PA-824 is active against both actively growing and nonreplicating mycobacteria and has little activity against other bacterial species. Determining potency against the target was not part of compound evaluation, and indeed the mechanism of action is complex and not well understood. Both PA-824 and OPC-67683 are like previous nitroimidazoles in requiring activation within bacterial cells. Activation of PA-824 is thought to produce reactive nitrogen species, similar to metronidazole. However, both PA-824 and OPC-67683 also appear to have a more specific mechanism, affecting synthesis of mycolic acids [64].

Ridinilazole (SMT 19969) A final example of discovering a drug with a novel mechanism by chemical optimization of antibacterial activity is the bis-benzimidazole SMT-19969. The bis-benzimidazole scaffold was initially designed to target specific DNA sequences, by binding in the minor groove of duplex DNA. A subset of compounds were found to possess antibacterial activity, apparently mediated by gyrase inhibition. Optimization for *C. difficile* activity at Summit Therapeutics led to SMT-19969, which lacks activity against other bacterial species, does not inhibit gyrase, and does not bind duplex DNA. Ridinilazole is currently in phase 2 for treatment of *C. difficile* infection, and its target is not known [55].

18.8.2 Empiric Discovery of Compounds That Bind Membranes or DNA

As noted above, disruption of bacterial membranes is not considered a desirable mechanism of action, because it so often is associated with toxicity for eukaryotic cells. However, if it is possible to make the compounds highly specific for bacteria, then membrane activity has some attractive features. The frequency of resistance is usually very low. Also, membrane-active compounds act rapidly and are active vs both nongrowing and growing bacteria.

Brilacidin (formerly PMX-30063) Brilacidin is a non-peptide compound designed to mimic human host-defense peptides. While antimicrobial peptides have so far been limited to topical uses because of their toxicity and poor pharmacokinetics, brilacidin is being developed as a systemic drug [83]. It is currently in a phase 2 study of acute bacterial skin and skin structure infection, sponsored by Cellceutix.

POL-7080 POL-7080, discovered at Polyphor and now in phase 2, is also a peptidomimetic, from a scaffold initially designed to mimic the host-defense peptide protegrin I. Remarkably, during optimization for *P. aeruginosa* activity, the series lost activity against other bacterial species. Its *P. aeruginosa* activity involves binding to the outer membrane protein LptD, and it is thought that its mechanism of action is inhibition of the LPS export system, Lpt [93].

Phage lysins The idea of using lytic peptides derived from phage or bacteria as antibacterial drugs is not new. A key issue is finding lysins with appropriate specificity. Many such peptides are active against only certain isolates of a bacterial species; others, like nisin and gramicidin, are so broad in spectrum that they are generally toxic. Two phage lysins are being developed for staphylococcal infection. N-Rephasin (SAL200) from iNtRON is in phase 2 studies. It was derived from bacteriophage SAP-1 and has broad activity against staphylococci but is not active against other genera [42]. CF-301 (previously PlySs2) from Contrafect is currently in phase 1. CF-301 was derived from a *Streptococcus suis* phage and is broadly cidal for staphylococci and streptococci, including biofilms [82].

Pheromonicins An intriguing approach for generating lytic peptides of desired specificity was proposed by Qiu et al. They fused a staphylococcal AgrD1 pheromone to the channel-forming domain of colicin Ia; the resulting peptide ("pheromonicin") had antibacterial activity toward strains of *S. aureus* able to recognize that pheromone and was able to protect mice from *S. aureus* infection. A fusion protein containing a scrambled pheromone was inactive [75].

Minor groove binders Drugs that bind the minor groove of DNA, such as the antiparasitic drug pentamidine, have a long history. While there is some specificity in the binding sites, these are not generally designed to affect transcription of a particular gene (as for the antisense approach described below). Analogs are evaluated by their antimicrobial activity and by selectivity for the desired target organisms. Minor groove binders have the advantage that they kill rapidly and have low frequency of resistance. However, the nonspecific nature of their activity makes toxicity a concern. MGB-BP-3, currently in phase 1, is derived from the natural product distamycin and is being developed for *C. difficile* infection. A series of bisindole compounds with activity in mouse models of Gram-positive infection discovered at Microbiotix was recently reported to have a mechanism that involves DNA binding [5, 72, 95].

18.8.3 Target-Specific, with a Mechanism at the Level of Transcription or Translation Rather than Binding the Protein Target

Antisense The idea of using antisense molecules to prevent synthesis of a target protein is attractive. Oligonucleotides with high affinity for a specific RNA sequence can be designed, manufactured, and tested much more easily than small-molecule enzyme inhibitors. Specificity for a single bacterial species is feasible. A potential problem is the likely high frequency of resistance due to point mutations that affect binding of the oligonucleotide without impairing fitness. Instability in serum is a problem for RNA drugs that has been addressed using mimics such as phosphoro-

diamidate morpholino oligomers (PMOs). Conjugation of peptides to PMOs can improve their uptake by bacteria. The Greenberg group at University of Texas Southwestern has described peptide-conjugated PMOs for several targets in a variety of bacterial species and in some cases achieved efficacy in mice [38].

Riboswitches A different approach to inhibiting production of a target is using small molecules to bind riboswitches in mRNA. The Breaker lab at Yale first described riboswitches several years ago. Breaker and colleagues recently described riboflavin analogs that bind the FMN riboswitch and are active against *C. difficile* both in vitro and in vivo. Researchers at Merck described a small molecule, ribocil-C, with antibacterial activity for several Gram-positive and Gram-negative species. Like the natural product roseoflavin, ribocil-C acts by binding FMN riboswitches. A recent paper from Merck reported that in *S. aureus*, both these compounds are dual binders of two FMN riboswitches, one controlling riboflavin synthesis and the other riboflavin uptake [8, 104].

18.8.4 Drugs Lacking Antibacterial Activity In Vitro

The idea that studying host–pathogen interaction will lead to discovery of improved therapeutic agents is intellectually attractive but has not yet been very successful with regard to standard antibiotics like those discussed above. In contrast, nearly all the agents discussed below resulted from research on how bacteria cause disease or respond to therapy.

18.8.4.1 Agent Increases Vulnerability of Bacteria to Antibiotics and/or to Host Defenses

Surface-binding mAbs Several monoclonal antibodies that bind to bacterial cell surface antigens of *P. aeruginosa* or *S. aureus* are currently in clinical trials. Antibodies are today considered as alternative or nontraditional approaches because they do not kill bacteria directly, but they are far from new. Administration of pathogen-specific antibodies was used successfully well over a century ago for treatment of pneumococcal pneumonia, meningococcal meningitis, and many other bacterial infections [13].

Three monoclonal antibodies to surface antigens of *P. aeruginosa* are in phase 2 clinical studies. MEDI3902 from MedImmune is a bispecific antibody that binds the exopolysaccharide Psl and also PcrV, the needle of the Type III secretion system [22]. It is being tested for prevention of hospital-acquired or ventilator-associated pneumonia (HAP/VAP) in high-risk patients colonized with *P. aeruginosa.* Aridis has two monoclonal antibodies being tested as adjuncts to antibiotic therapy in

patients with *P. aeruginosa* HAP or VAP. Aerucin binds alginate, and Aerumab (previously AR-101 or Panobacumab) binds LPS of serotype O11 [76].

Two antibody-based agents are being studied in patients with *S. aureus* bacteremia: monoclonal antibody 514G3 from XBiotech and an antibody-antibiotic conjugate (DSTA4637S, RG7861) from Genentech.

Antibiotic potentiators Given the difficulty in discovering new antibiotics, an obvious potential alternative is to find drugs that can either restore the susceptibility of resistant strains or extend the spectrum of Gram-positive antibiotics to Gram-negative bacteria. Apart from the β-lactamase inhibitors discussed in a previous section, no such drugs have reached the market. The only clinical candidate in this category is SPR741.

SPR741(previously NAB741) is a polymyxin derivative being developed by Spero Therapeutics as an antibiotic-potentiating agent, currently in phase 1 [101]. Because its mechanism is permeabilization of the outer membrane, it is possible that SPR741 might also increase the susceptibility of bacteria to host defenses such as defensins and complement.

Many academic and industrial labs have screened for compounds that increase the activity of β-lactams for MRSA. Where mechanisms of active compounds have been identified, they have been surprisingly diverse. A more systematic approach has been taken by Merck scientists, seeking inhibitors of wall teichoic acid synthesis [39, 66, 98, 107].

Efflux pump inhibition is another area that has been extremely frustrating, despite advances in the assembly and function of multidrug efflux pumps and in the interaction of pump components with their substrates [36, 51, 53, 91, 96].

18.8.4.2 Agent Counteracts a Defined Virulence Mechanism

Targeting pathogenesis is often suggested but rarely has been very successful. One issue is that potential targets are identified by demonstrating the inability of mutants to *initiate* infection. It is by no means certain that drugs inhibiting the production or function of those virulence determinants would be able to *reverse* an established function. In addition to these concerns, an additional argument against anti-virulence drugs is that they would be narrow in spectrum. That is now seen as less of a disadvantage than it was several years ago. It must be admitted that there has been very little serious effort by the pharmaceutical industry to discover anti-virulence drugs.

Antitoxin mAbs Like the surface-binding antibodies discussed in a previous section, passively administered toxin-neutralizing antibodies have an extremely long history of successful use. Shigamab, being developed by Taro, consists of two antibodies against Shiga toxin [60]. ASN100 from Arsanis is also a mixture of two antibodies, one active against five cytotoxins and the other active against LukGH leukocidin [2, 21]. ASN100 is being tested as a prophylactic agent in patients colo-

nized with *S. aureus* and at high risk of HAP/VAP, as is MEDI4893, an α-toxin-neutralizing mAb from MedImmune. A second mAb that neutralizes staphylococcal α-toxin is AR-301 (Salvecin) from Aridis, being tested for therapeutic use in HAP and VAP, in combination with antibiotics [31].

MfvR One of the difficulties in targeting virulence determinants is that many pathogens have several mechanisms of virulence. With some exceptions (such as certain secreted toxins and major surface components like pneumococcal capsular polysaccharides), inactivation of a single virulence factor has relatively little effect on infection. In *P. aeruginosa*, several virulence factors are controlled by a single regulatory protein MvfR. In one of the most promising examples of an anti-virulence approach, the Rahme lab at Harvard identified compounds binding to MvfR that are effective in treating infected mice [57, 94].

18.8.4.3 Immunomodulatory Agents

Some aspects of infectious disease result not from the bacteria per se but from an overwhelming inflammatory response to bacterial components. This can persist even if the infection is treated with appropriate antibiotics. Attempts to rescue septic patients with interleukins or other immunomodulators have been largely unsuccessful, even though some such agents appeared to be effective in mice. Two immunomodulatory agents are now in clinical trials.

AB103 from Atox Bio, now in phase 3, is an octapeptide that attenuates the signaling through CD28 that is involved in induction of many proinflammatory cytokines [77]. It is being tested in patients with necrotizing soft tissue infections.

CAL02, now in phase 2 from Combioxin, is a liposomal drug that neutralizes bacterial toxins. It is being tested in addition to standard of care in intensive care unit patients with severe pneumococcal pneumonia [6].

The Kranz lab, University of Illinois, engineered T-cell receptor domains to bind staphylococcal enterotoxins B and C with high affinity and reported efficacy in rabbit models of necrotizing pneumonia and infective endocarditis [56, 86].

18.9 How Well Do We Understand Antibiotics and Resistance?

The previous section described a number of experimental approaches that have the possibility of improving treatment of infectious disease. Several of the antibacterial agents are bactericidal for nongrowing bacteria, making it possible that they will be more effective against persistent infection than current antibiotics are. Many of them were discovered by empiric methods and/or have mechanisms that would not have been considered appropriate for target-directed drug discovery. With the

exception of the tuberculosis drugs, these are all very early in development. It cannot be concluded that these nonclassical approaches will always be more successful than classical target-directed antibiotic discovery.

The target-directed programs of the genome era focused on enzymes. As discussed above, there are a number of reasons why any given project might have failed. There is no obvious reason to think that enzymes as a group should be less suitable as antibiotic targets than the macromolecular synthesis machines that most existing antibiotics inhibit. Indeed, the first broad-spectrum synthetic antibiotics, the antifolates, are enzyme inhibitors. It is puzzling that even for a pathway as well validated as peptidoglycan synthesis, it has been impossible to find good inhibitors of the soluble enzymes (GlmU, MurA-MurF) apart from fosfomycin. One limitation of enzymes as targets is the risk that mutants with endogenous resistance occur at high frequency [87]. This risk is not simply theoretical, as indicated by the failure of GSK2251052 during phase 2 [70]. It is unlikely, however, that high frequency of resistance was the characteristic that killed most of the genome-era projects before a clinical candidate was identified.

It is more likely that after initial identification of a hit series with mechanism-based antibacterial activity, most projects progressed for a while but failed to come close to a solid lead within an allotted period of time. A common experience is that chemistry can produce very potent inhibitors, as assessed in an in vitro biochemical assay, but that potency does not translate into antibacterial activity. The standard explanation is that these compounds "don't get in." This is certainly true to a great extent. Compounds active vs staphylococci and streptococci but not vs Gram-negative bacteria are often active against an efflux-deficient or hyperpermeable mutant of *E. coli* or *P. aeruginosa*. In that case, it is appropriate to conclude that the intrinsic defenses of Gram-negative bacteria are a major limitation to antibacterial activity. Poor antibacterial activity against Gram-positive bacteria is harder to explain, although again failure to cross the cytoplasmic membrane and accumulate against the concentration gradient probably contributes to the problem [89].

We must consider the possibility that we simply don't know enough about bacteria to choose good targets. The assumption of the new-targets programs of the genome era was that we understand antibiotics and infectious disease well enough to be able to do this rationally. Discovery programs at that time were often driven not by new insight into how antibiotics work but by new technology, combined with a feeling that no new insight was needed. I would argue that not all essential genes are equally good targets. Moreover, research into the mechanisms by which bacteria avoid the effects of antibiotics has suggested that drugs with apparently similar mechanisms can have very different effects on bacterial physiology.

One potential problem is that inhibition of the target may have relatively little immediate effect on bacterial physiology. It is very difficult to determine "how essential" a bacterial process is. Will growth stop if catalytic activity is reduced by 25%? Will it be necessary to inhibit 99.9% of activity? Very few studies have attempted to address this, and indeed there are no general methods. Tuberculosis researchers have developed methods for targeted degradation of specific proteins in order to assess the impact on growth and survival of the cell [43, 105]. Extending

these methods beyond mycobacteria would be useful, although reducing the amount of a protein within the cell may have a different effect from chemical inhibition of that protein's activity. For a few enzymes, mutants with partial activity can give us some information as to the level of inhibition that is tolerated. The *envA1* mutant of *E. coli* has an 18-fold reduction in LpxC activity, compared to wild-type strains [109]. Similarly, the *E. coli* mutant *ligA251* has a point mutation that reduces activity of DNA ligase by 20- to 60-fold [46].

Systems biology may allow improved prioritization of targets, with the caveat that we may not yet have the information needed to generate predictive models. One example is the modeling of the lipid A synthetic pathway. A combination of computational and experimental methods led to the suggestion that LpxK would be a better target for inhibition than LpxC if previous knowledge of the pathway's regulation is incorporated into the model. If regulation was not considered, then LpxC appeared to be a better target, as suggested by previous enzymology [26].

While target-directed discovery programs tend to focus on the effect of chemical compounds on the target, it may be more useful to focus on the effect of compounds on the bacterial cell. The most obvious such effects are bacterial stasis or death, morphological changes such as filamentation or spheroplasting, or changes in processes like macromolecular synthesis that can be monitored easily. Bacterial responses to differing antibiotic stresses may also be important. It may be useful to look for targets that, when inhibited, induce the same stress responses as one or more of the well-established existing classes of antibiotics. The SOS response to DNA damage is one example. Fluoroquinolines induce the SOS response, and it is also involved in the thymineless death induced by trimethoprim [30].

Reporters of the cell wall stress response have been used by many groups to screen chemical libraries, finding hits with diverse targets in envelope biogenesis – not only peptidoglycan synthesis but also LPS synthesis and lipoprotein export [19, 68, 100]. The machines involved in export of proteins and lipopolysaccharide to the outer membrane and in maintenance of its permeability barrier could be considered as the only truly novel bacterial pathways that have been discovered in recent decades [58, 79, 80].

The stringent response was first studied decades ago in the context of amino acid starvation. Much more recently it was identified as a critical issue in antibiotic tolerance and biofilm formation. A better understanding of persistence and tolerance may allow us to design more effective antibiotics. One might expect all bacteriostatic inhibitors of protein synthesis to induce similar responses in bacteria. However, a recent study of the effect of bacteriostatic agents on induction of tolerance to β-lactams found that the Met-tRNA synthetase inhibitor mupiricin activates RelA, while the ribosome binders tetracycline and chloramphenicol inhibit induction of the stringent response [45, 54, 85].

Finally, several lines of evidence suggest that the mechanisms of existing antibiotics are more complicated than the simple picture presented above. In 2007, the Collins lab at Boston University proposed that the bactericidal activities of β-lactams, aminoglycosides, and fluoroquinolones have a common mechanism involving production of hydroxyl radicals, as a consequence of the immediate effects of these

antibiotics on their respective molecular targets [44]. This theory is controversial, with not all researchers agreeing with the details of the "common death" pathway [50] (see also Chap. 20). The effects of sublethal concentrations of antibiotics have suggested that halting cell growth has effects that differ from one class of antibiotic to another and are not easily predictable [18, 34]. Similarly, genome-wide studies on changes in bacterial growth and susceptibility when genes are over- or underexpressed have revealed a network of interactions that is not readily explained in terms of single effects of antibacterial drugs [4, 16]. A school of thought suggests that polypharmacology (not promiscuity) is a feature of many successful drugs and should be considered as an advantage [10, 11, 61]. It is possible that in optimizing the potency of a series of antibacterial compounds for a single target, we are removing minor activities that contributed to the antibacterial activity initially observed.

18.10 Concluding Remarks

Old targets, benefits and risks The field has been successful in identifying new antibiotics for the old targets, utilizing current structural biology, and making use of new understanding of mechanisms and resistance determinants as they emerge. Even for the old antibiotic classes, new synthetic methods are allowing increased chemical diversity. However, the side effects and other liabilities of these antibiotic classes must be considered in developing new members. A further problem is that inhibitors of the old targets tend to be broad-spectrum antibiotics, with inherent effects on normal flora. Resistance may be slow to emerge but is likely to spread rapidly, just as for existing antibiotics. Moreover, new inhibitors of the old targets are unlikely to be better than the old drugs at treating persistent infections or toxic effects.

New targets, benefits and risks New targets have the possibility of yielding better drugs – more diverse in chemical structure and mechanism, possibly narrower in spectrum, and/or providing improved treatment of conditions not well handled by existing antibiotics. However, focusing narrowly on targets we think we understand has not been very successful. We need to take a variety of approaches, allowing use of technology and incorporating new knowledge as it becomes available.

We don't know enough about compound uptake One of the major problems in antibiotic discovery is our inability to design compounds with good access to their targets. Aminoglycosides and tetracyclines owe their success in part to their ability to achieve concentrations within a bacterial cell that are higher than external concentrations. β-lactams and other inhibitors of late stages of peptidoglycan synthesis do not need to cross the cytoplasmic membrane, but for Gram-negative bacteria the outer membrane is a barrier to these drugs. Academic research has been very successful in elucidating the structure and synthesis of the outer membrane and of the multidrug efflux pumps that constitute the intrinsic resistance mechanisms of Gram-

negative bacteria. This knowledge has not yet provided concepts or tools to guide medicinal chemists in improving the antibacterial activity of potent compounds.

Commitment is necessary In a recent review, Bisacchi and Manchester made the striking observation that even during the Golden Age of Antibiotics, when many large pharmaceutical companies were heavily invested in anti-infective research, discovery of a new class of antibiotics was a rare event [7].

Major Points

- Most target-directed drug discovery projects focus on seeking inhibitors of individual enzymes
- No antibiotic that has ever been approved was discovered through this approach
- Only a few target-directed projects have yielded clinical candidates
- A critical problem that is not well appreciated is that not all essential bacterial functions are equally good drug targets
- A second important problem is that optimization of a chemical scaffold for potency in a cell-free assay often does not improve its antibacterial activity or drug characteristics
- The antibacterial drug development pipeline is sparse, but includes small molecules and biologics with a wide variety of mechanisms
- Some of these were discovered empirically, others through rational methods including structure-based drug design

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Chapter 19
Non-quinolone Topoisomerase Inhibitors

Anthony Maxwell, Natassja G. Bush, Thomas Germe, and Shannon J. McKie

19.1 Introduction

There is no question that the development of antibiotics has been among the most important advances of the twentieth century, saving a countless number of lives. Not only are antibiotics used in the direct treatment of bacterial infections, but they are also important in surgical situations (e.g., transplant surgery, joint replacement) by preventing infections. However, the twenty-first century has seen increasing concern due to the rise in antimicrobial-resistant bacterial infections [1]. Antibiotic resistance is a growing global threat, with resistance to all classes of antibiotics now reported worldwide. The resistance problem is compounded by a lack of innovation and few new structural classes of antibiotics being brought to the clinic [2–4]. To tackle antibiotic resistance, we need to review our stewardship of existing antibiotics and expand efforts to discover new agents that are not susceptible to known resistance mechanisms.

Quinolones (specifically fluoroquinolones (FQs); Fig. 19.1) are a potent class of synthetic antibiotics that target DNA gyrase and DNA topoisomerase IV, essential enzymes that are ubiquitous among bacterial species. The quinolones were discovered in the early 1960s, and they are now the most successful class of topoisomerase inhibitors; fluoroquinolones are widely prescribed in the USA, Europe, and most regions of the world [5, 6]. This heavy consumption of fluoroquinolones has led to an increase in resistance that derives from a variety of processes including upregulation of efflux pumps, reduced ability to take up the drug, plasmid-based resistance, or mutations in the gyrase and/or topo IV genes [7, 8]. This widespread resistance has resulted in revised stewardship guidelines for quinolones [6] as well as the

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I. W. Fong et al. (eds.), *Antimicrobial Resistance in the 21st Century*, Emerging Infectious Diseases of the 21st Century, https://doi.org/10.1007/978-3-319-78538-7_19

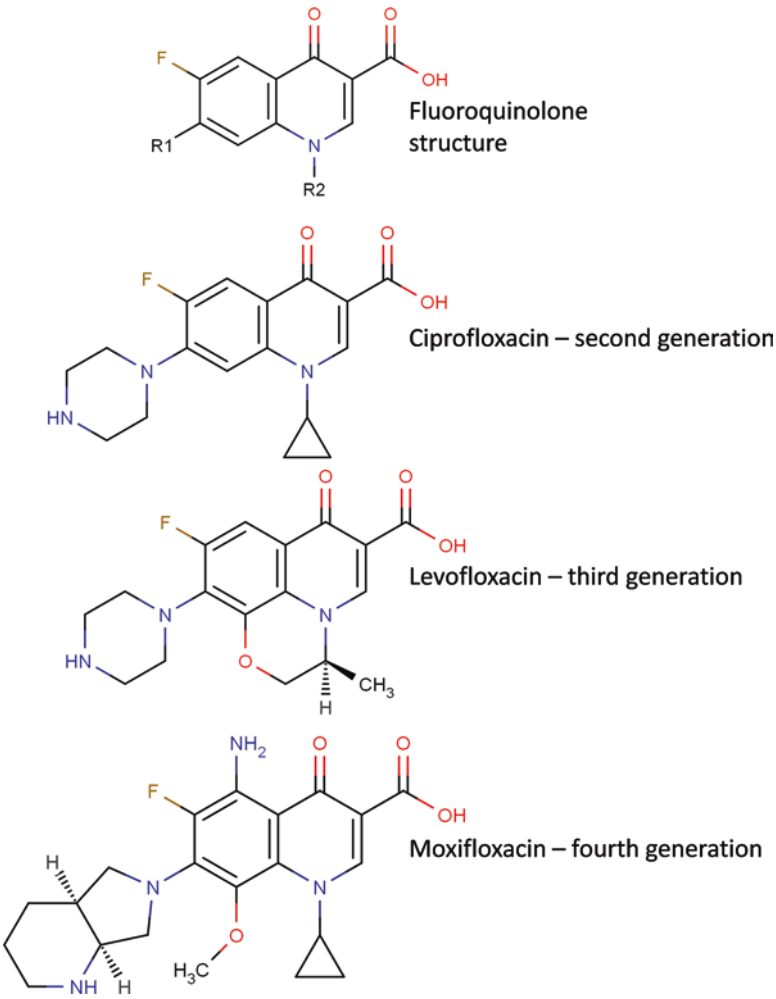

Fig. 19.1 Fluoroquinolone compounds. The basic FQ skeleton is shown along with three FQ compounds in clinical use

WHO categorizing quinolones as highest priority critically important antimicrobials [9].

Due to the success of DNA gyrase (and topo IV) as a clinical target for antimicrobials and to the troubling issues associated with quinolone resistance, other compounds, particularly novel inhibitors of topoisomerases, are logical alternatives to quinolones. These novel agents are the subject of this chapter.

19.2 DNA Topoisomerases

DNA topoisomerases are enzymes that can interconvert different topological forms of DNA; their reactions include relaxation of supercoils, decatenation, and unknotting [10, 11]. They are present and essential in all organisms and are involved in DNA replication and transcription, preventing the buildup of unwanted supercoils and resolving catenated products [12]. Topoisomerases are classified into two types, I and II, depending on whether they catalyze reactions involving single (I)- or double (II)-stranded breaks in DNA; they can also be further divided into subtypes IA, IB, and IC and IIA and IIB, dependent on mechanistic and evolutionary considerations [13, 14]. The subjects of this chapter, DNA gyrase (Fig. 19.2) and DNA topoisomerase (topo) IV, are type IIA enzymes. Type I enzymes catalyze their reactions by transiently breaking one strand of DNA and forming a covalent bond between either the 5′ (IA) or 3′ (IB) phosphate at the break site and the active-site tyrosine of the enzyme. The topoisomerase reaction occurs via a swivel mechanism (type IB) or by strand passage, where a single- or double-stranded segment of DNA is passed through the break (type IA). Type II topoisomerases make a transient double-strand break in DNA, forming covalent bonds with the 5′-phosphates at the break site and passing a double-stranded segment of DNA (the so-called "T" or "transported" segment) through the break [10, 11] in the "G" or "gate" segment. Although this is the case for both IIA and IIB enzymes, the two subtypes are different in several other structural and mechanistic aspects [15]. The principal member of the type IIB subtype is topo VI, originally discovered in archaea and now shown to occur in plants and plasmodial parasites [16, 17]. The members of the IIA subtype, gyrase, topo IV, and eukaryotic topo II, are better known and have been more extensively studied. All these enzymes can relax supercoiled DNA; gyrase is distinct in also being able to catalyze the introduction of negative supercoils into DNA. Structural and mechanistic aspects of these enzymes have been extensively reviewed

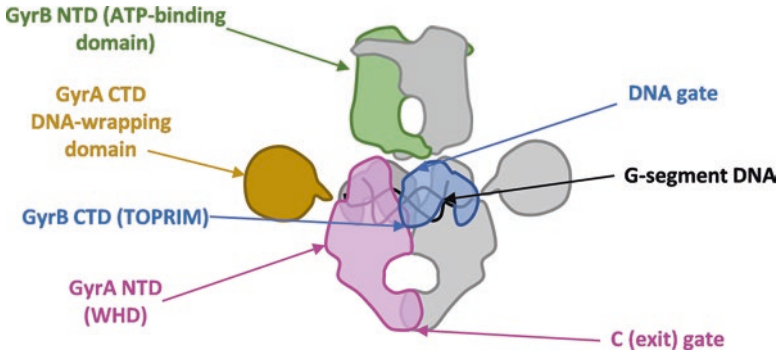

Fig. 19.2 Schematic representation of DNA gyrase (A_2B_2) complexed with G-segment DNA. NTD, N-terminal domain; CTD, C-terminal domain; TOPRIM, topoisomerase-primase domain; WHD, winged-helix domain

elsewhere [10, 11, 18]. The main topic of this chapter concerns their role as targets for antibacterial agents; other recent reviews also address this topic [10, 19–21].

19.3 Topoisomerases as Targets for Antibacterial Chemotherapy

Because of their essentiality and the fact that their reactions proceed via transient breaks in DNA, topoisomerases have become targets for both anticancer and antibacterial chemotherapy [10, 21, 22]. Many topoisomerase-targeted drugs act by stabilizing the DNA-protein covalent complexes that form between the enzymes and DNA during the topoisomerase reaction cycle. However, there are other agents (see below) that act by interrupting the reaction cycle in other ways; these have been dubbed "catalytic inhibitors." Quinolone compounds, and more specifically fluoroquinolones (FQs), such as ciprofloxacin, moxifloxacin, and levofloxacin (Fig. 19.1), are highly successful antibiotics that are used for a wide range of clinical conditions [23, 24]. However, due to resistance to these compounds, new agents that match their clinical characteristics, but are not affected by quinolone-resistance mutations, are urgently needed. This chapter reviews such compounds.

Although, in principle, DNA topo I is a valid target for antibacterial compounds and a number have been investigated [25], there are currently no topo I inhibitors in clinical use, although recent work suggests that there is scope to develop antibiotics targeted to this enzyme [26, 27]; topo I will not be further discussed in this chapter. The scope of DNA gyrase and DNA topo I as targets for tuberculosis therapy has recently been reviewed [20].

There are two main mechanisms of inhibition of topoisomerases: catalytic inhibition and topoisomerase poisons. Catalytic inhibitors arrest enzyme activity and need to bind to their targets with reasonably high affinity to be effective. Poisons are compounds that stabilize the topoisomerase-DNA cleavage complex and tend to be more effective, as relatively low occupancy of the inhibitor bound to its target can lead to cell death, which involves chromosome fragmentation, induction of the SOS response, and possibly the induction of reactive oxygen species [28, 29] (see Chap. 20).

19.4 Cleavage Complex-Stabilizing Agents

Binding at the DNA Cleavage Site

The high degree of success of FQs is at least in part due to their mode of action: the ability to arrest their targets (gyrase and/or topo IV) at the stage in the topoisomerase reaction cycle where the enzyme is covalently bound to a double-stranded DNA break. This so-called cleavage complex results in chromosomal breaks and cell

death; it is also the basis for the mode of action of a number of successful antitumor drugs that target human topo II [30]. Finding agents that act in a similar way that can substitute for FQs has been a major challenge.

As we now know, from X-ray crystallography, how FQs work at the molecular level [31, 32], we can use this information to develop other molecules that act in a similar way but that may avoid the problems of quinolone resistance. As will be seen from some of the examples below, it is possible to find compounds that bind at sites distinct from the quinolone-binding site and that can also stabilize cleavage complexes. This type of compound that can avoid quinolone cross-resistance perhaps represents the best opportunities for finding novel gyrase−/topo IV-targeted antibiotics with clinical potential. The term NBTI (novel (non-fluoroquinolone) bacterial type II topoisomerase inhibitor) has been introduced [31] to encompass compounds that demonstrate these properties.

FQs work through intercalation of two compounds, one at each cleavage site, into the DNA (Fig. 19.3) [31, 32]; the compounds also interact with the enzyme through a water-metal-ion bridge [33]. Mutations affecting the residues involved in contacting the metal ion are found in clinically-isolated strains of FQ-resistant pathogenic bacteria. It is likely that disrupting the water-metal ion bridge destabilizes the ternary complex, mitigating cleavage-complex formation (see Chap. 16). Efforts have therefore been made to develop compounds that do not rely on this water-metal ion bridge. Several compounds share a scaffold with the FQs, for example, the **quinazolinediones** (QZDs) [34–36] and the **imidazopyrazinones** (IPYs) [37, 38]. Compounds of these classes are able to intercalate at the FQ-binding site and stabilize cleavage complexes without relying on the water-metal ion bridge [37, 39, 40]. The water-metal ion bridge is a major contributor to the stable binding of bacterial topoisomerase by the FQs. Removing it requires other contacts to be established by putative intercalating compounds of similar scaffold to afford the same affinity and, by extension, equivalent potency (although the relationship between efficiency of poisoning in vitro and potency against bacterial pathogens is far from straightforward). Indeed in the case of QZDs, efficiency of poisoning tends to be significantly lower than the FQs [41–43], and some of the medicinal chemistry effort has focused on developing alternative contacts with the protein, for instance, by modifying the C7 substituent [44]. In the case of the IPYs, the absence of a water-metal ion bridge is compensated for by direct contact with one of the residues involved in the bridge, which unfortunately affords some degree of cross-resistance between quinolones and these compounds [37]. The IPYs also establish contact with the arginine situated next to the catalytic tyrosine, which is presumably essential for catalytic activity [37]. In the case of the QZDs, attempts have been made to develop such contacts in order to improve the activity of the compounds while minimizing resistance [45].

Structural information on the protein-DNA-drug complexes has shown that they all share a characteristic DNA extension, increasing the distance between the two scissile phosphates compared to an uncleaved “binary” enzyme-DNA complex [31, 33, 37, 46]. In gyrase, this extension is associated with conformational movement involving sliding of the two GyrA subunits against each other and tilting of the

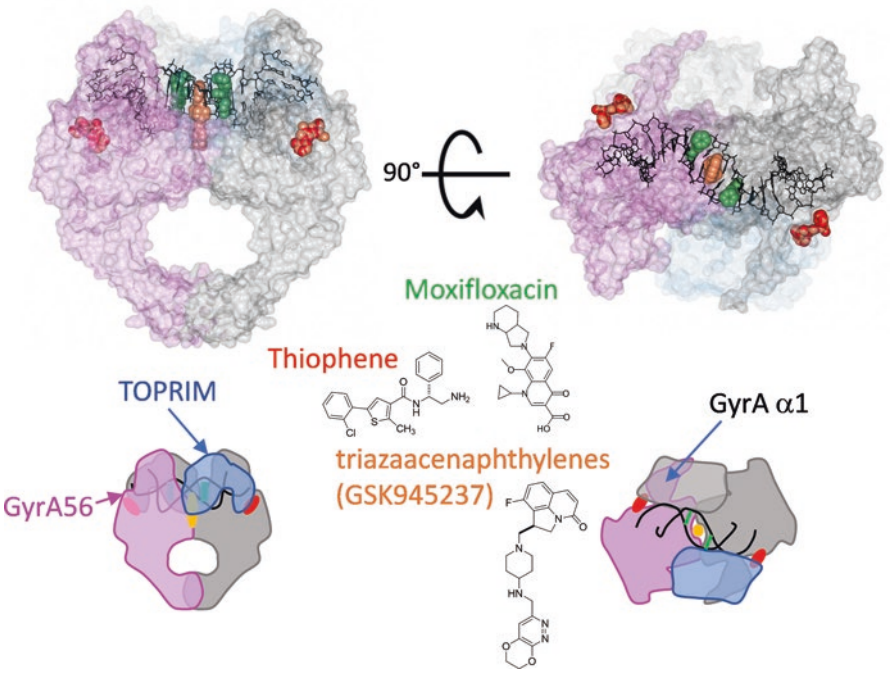

Fig. 19.3 Binding sites of cleavage complex-stabilizing compounds. Two structures are superposed to show binding by moxifloxacin (PDB 5CDQ), GSK945237, and thiophene2 (PDB 5NPP) to the *S. aureus* gyrase core (C-terminal region of GyrB fused to the N-terminal region of GyrA). The TOPRIM domains from 5CDQ are superposed and displayed instead of the ones from 5NPP; the DNA and GyrA domain displayed are from 5NPP. An orientation cartoon shows the color-coded compounds binding at distinct sites on the enzyme. While moxifloxacin intercalates directly at the cleavage site (as with the IPYs and QZDs), the triazaacenaphthylene and thiophene compounds bind at different allosteric sites and stabilize the cleaved intermediate. Triazaacenaphthylenes block the sliding of the GyrA subunits against one another, thereby blocking resealing, whereas the thiophene blocks the TOPRIM domain in a tilted conformation against the DNA

TOPRIM domains toward the dyad axis, suggesting that the enzyme can manipulate DNA geometry to favor cleavage. The dynamic nature of the DNA gate also allows for a variety of structurally unrelated compounds to bind at the FQ site without requiring a water-metal ion bridge and thereby bypassing most FQ-resistance mutations. For instance, **etoposide**, a eukaryotic topoisomerase II inhibitor [46], is also able to stabilize gyrase-DNA cleavage complexes through intercalation into DNA and interaction with residues conserved in the related human topoisomerase II. Likewise, the **spiropyrimidinetriones** (such as QPT-1) can stabilize cleavage complexes by intercalation much like the FQs despite being structurally different [46]. However, unlike etoposide, QPT-1 is not active on human topoisomerase II despite interacting with conserved residues. It was thought that the water-metal ion bridge conferred bacterial specificity to the FQs, but the case of QPT-1 shows that more is at play. This is an important issue, as development of bacterial topoisomerase

II poisons incurs the risk of developing genotoxic compounds by cross-reactivity with human topoisomerase II.

Compounds Binding Outside the DNA Cleavage Site

The extensive conformational changes involved in DNA cleavage open up the theoretical possibility that compounds binding away from the cleavage site affect cleavage by stabilizing the cleavage-prone conformation of the enzyme. Indeed, the **triazaacenaphthylenes** (the original NBTIs), e.g., gepotidacin (GSK2140944 [47]), bind at the interface between the two sliding GyrA subunits and act as a "locking pin" to freeze the enzyme into the DNA-extended state that favors cleavage [31]. Moreover, an allosteric pocket has been identified that is targeted by **thiophene** compounds [48]. Binding to this pocket, which is remote from the DNA cleavage site, results in cleavage-complex stabilization, presumably by allosterically locking the enzyme in the cleavage-prone conformation. This binding pocket is located at the base of the GyrAα1 helix hinging the tilting of the TOPRIM domain [37] (Figs. 19.2 and 19.3). This is similar to the mode of action of the NBTIs but involves the other segment of the enzyme implicated in the conformational transition to the cleaved state (see above). The utilization of novel pockets that exploit the natural ability of the enzyme to transition into the cleaved state has the advantage of bypassing existing resistance to FQs. Moreover, given the conformational changes involved in cleavage, it can be expected that mutations restricting access to these pockets would reduce the ability of the enzyme to cleave DNA and thereby reduce its activity. Consistent with this idea, the frequency of resistance is low for the thiophene compounds [48]. However, it is theoretically possible for a mutation to affect the energy of transition to the cleaved state rather than the binding of the compound. Indeed, mutations conferring resistance to different series of compounds have been located away from the compound's binding site. This raises the possibility of "universal" mutations that would confer resistance to all cleavage-stabilizing agents. This idea remains to be tested. Candidates for such universal mutation include Asp^{82} to Asn (*Escherichia coli* numbering) that confers resistance to both FQs and IPY [37] and the Val^{96} to Ala (in *Bacillus anthracis*) which confers resistance to both FQs and QZDs [49].

This thermodynamic view of poisoning as the stabilization of a natural, cleavage-prone conformation can also help explain the bacterial specificity of compounds like QPT-1 that interact with residues conserved in human topo II. The binding energy of a compound must offset the energetic cost of the transition to the cleaved state for cleavage to be stabilized. Therefore, if the cost of the transition differs between the bacterial and the human enzyme, we can envision a situation whereby a similar binding energy is sufficient to offset the cost of cleavage for one but not the other. This effect could also contribute to the variation in activity of a given compound against different bacterial species.

Non-small Molecule Inhibitors

Although not a small molecule, the bacterial toxin **microcin B17** (MccB17) is also able to stabilize the cleavage complex between gyrase and DNA. MccB17 is a 3.1 kDa posttranslationally modified peptide that contains 8 or 9 oxazole and

thiazole heterocycles [50]. MccB17 targets bacterial gyrase and can stabilize the cleavage complex but in a manner distinct from quinolones [51, 52]. The only known mutation in gyrase that confers resistance to MccB17 is at the C-terminal end of GyrB (Trp^{751} to Arg) [52, 53]; no other drug-resistance mutations map here. Although MccB17 is a potentially attractive option as an antibacterial compound, its poor physicochemical properties have hampered its development as a drug candidate. Despite significant work on this toxin, its binding site and mode of action on gyrase are not known. However, the toxin and fragments thereof have been chemically synthesized, and fragments have also been made using molecular biology/biochemical methods [54–57]. Some of these fragments, with molecular weights <2 kDa, show activity suggesting that it may be possible to generate smaller versions of MccB17 with more attractive physicochemical properties that might have potential as antibacterial agents in the future.

Other non-small molecule inhibitors of gyrase include the phytotoxin albicidin, the CcdB protein toxin, the *E. coli* GyrI protein, and the pentapeptide-repeat proteins, such as Qnr and MfpA; these agents have been reviewed elsewhere [10, 19]. Although the toxin protein CcdB (MW ~12 kDa) is outside the scope of this review, it is interesting to note that peptides based on CcdB as short as 18 amino acids can retain inhibitory activity on gyrase [58].

19.5 Catalytic Inhibitors

The term "catalytic inhibitors" refers to agents that do not inhibit gyrase/topo IV by stabilizing the DNA cleavage complex but affect another aspect of the catalytic cycle. The majority of these are ATPase inhibitors, e.g., aminocoumarins and cyclothialidines, but other types, such as simocyclinones, which inhibit DNA binding, also exist. Although arguably catalytic inhibitors are less likely than cleavage complex-stabilizing compounds to be effective antibiotics, such catalytic inhibition is effective with other antibiotic targets (e.g., rifamycin on RNA polymerase, trimethoprim on dihydrofolate reductase [59]); thus, there is no reason a priori that they should not succeed as antibiotics. Indeed novobiocin, see below, has been utilized as a clinical antibiotic.

Aminocoumarin antibiotics (Fig. 19.4) that target DNA gyrase were discovered as *Streptomyces* natural products in the 1950s; these "classical" agents are **novobiocin**, **clorobiocin**, and **coumermycin A_1** [60, 61]. Early on it was established that these compounds are competitive inhibitors of the gyrase ATPase reaction [62]; they also have activity against topo IV [63]. In this sense, they are classic catalytic inhibitors that affect ATP-dependent topoisomerase reactions without stabilizing the cleavage complex. Specifically, they bind to the ATPase (N-terminal) domain of GyrB and block the binding of ATP, a process that was definitively established using X-ray crystallography [64, 65]. This conclusion was somewhat surprising given that aminocoumarins do not obviously resemble ATP (Fig. 19.4). In fact, it is the sugar ring of the aminocoumarins that overlaps the adenine-binding site in the ATPase

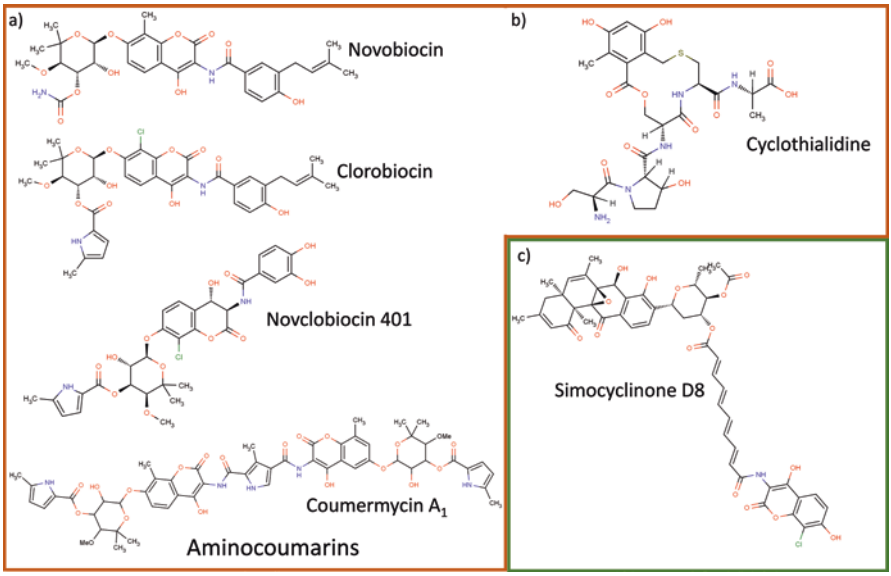

Fig. 19.4 Catalytic inhibitors of gyrase and topo IV. (**a**) Novobiocin, clorobiocin, novclobiocin 401, and coumermycin A_1, (**b**) cyclothialidine, (**c**) simocyclinone D8. ATPase inhibitors are boxed in orange, DNA-binding inhibitors in green

pocket, thereby preventing the binding of ATP. Several crystal structures of gyrase B fragments bound to aminocoumarin compounds now exist (Fig. 19.5 [19, 61]); these fragments have potentiated the design of alternative compounds that can bind at the same site (see below).

Although aminocoumarins are very effective inhibitors of gyrase and topo IV, with K_d values in the 1–20 nM range [66], they have not had a high degree of success as clinical antibiotics. Although novobiocin has been used on its own and in combination as an antibiotic, safety concerns have led to discontinuation of its usage [19]. Its toxicity issues may stem in part from its binding site, the ATPase domain of GyrB/ParE, which is part of the GHKL ATPase/kinase superfamily [67], and secondary eukaryotic targets are therefore likely. Indeed it has been possible to "redesign" novobiocin to target Hsp90 (see below) [68]. Furthermore, the aminocoumarins suffer from solubility issues, making it difficult to develop them as drugs.

To circumvent these problems, attempts have been made to prepare aminocoumarin derivatives with superior properties. These efforts have been made possible by the identification, sequencing, and annotation of the gene clusters for the three "classical" aminocoumarins [69]. This has led to a detailed understanding of the biosynthetic pathways for these compounds [60]. With this information it has been possible to use various technologies: genetic engineering, combinatorial biosynthesis, and mutasynthesis, to generate novel aminocoumarins, which can then be tested for antibacterial activity and for their effects on the target enzymes [70]. A significant amount of this effort has been carried out by Heide and coworkers, who coined

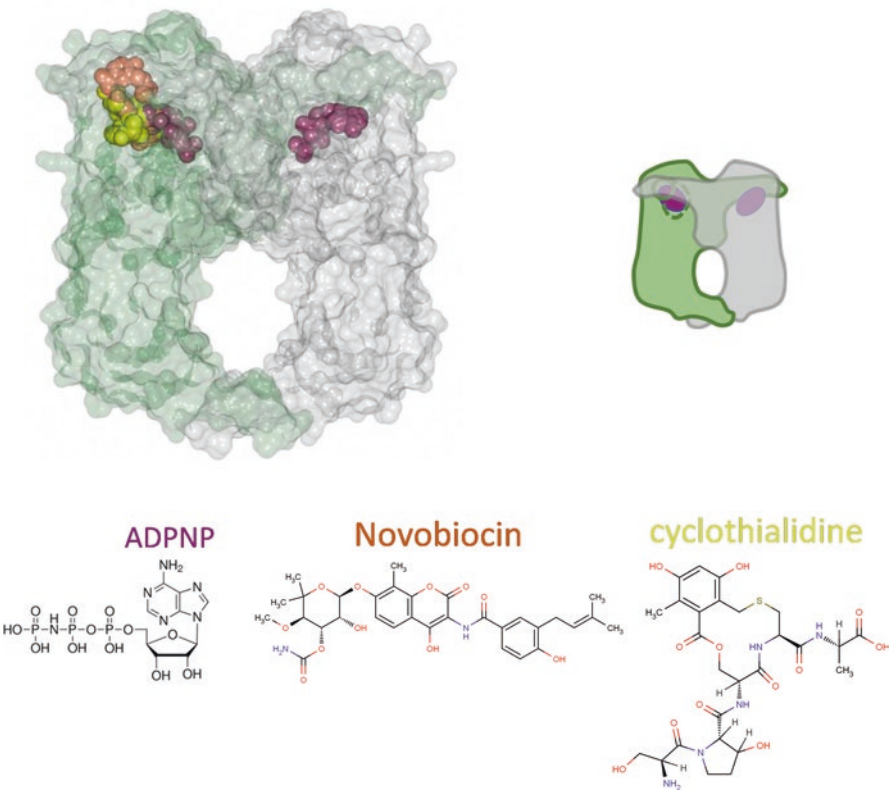

Fig. 19.5 Binding site for aminocoumarins and other ATPase inhibitors. The superposition of structures of the ATP-binding domain of DNA gyrase bound to ADPNP (5′-adenylyl-β,γ-imidodiphosphate), novobiocin, and cyclothialidine. The nucleotide binds inside a furrow and promotes dimerization through binding of a projection from the cognate monomer covering the opening of the furrow. Both novobiocin and cyclothialidine impinge on nucleotide binding as their binding sites overlap with the nucleotide-binding site

the name "novclobiocins" for molecules that are hybrids between clorobiocin and novobiocin [71]. Many novclobiocins have been produced and their activities assessed [69, 72]. One specific example is novclobiocin 401 (Fig. 19.4), which contains the catechol moiety 3,4-dihydroxybenzoic acid, which assists import across the bacterial cell envelope [73]. This modification improved the penetration into *E. coli*, and the analog also retained good activity against gyrases from *E. coli* and *Staphylococcus aureus*. Whether further improvements to the aminocoumarins can generate compounds with clinical potential remains to be seen.

Cyclothialidines (Fig. 19.4) are *Streptomyces*-derived natural products and, like aminocoumarins, are also competitive inhibitors of the gyrase ATPase reaction and bind at essentially the same site in GyrB [65]. Although their antibacterial potency is generally poor, their novel structures and good in vitro activity against gyrase warranted further investigation [74–76]. For example, a chemistry program at

F. Hoffmann-La Roche generated a large number of cyclothialidine analogs, including compounds with improved in vivo efficacy [77, 78]. It remains to be seen whether such compounds can lead to clinically successful antibiotics.

During work on the classical aminocoumarins (described above), the related **simocyclinones** (Fig. 19.4) were discovered [79, 80]. These are also *Streptomyces* natural products and contain an aminocoumarin group, but they also comprise a polyketide moiety and linker. The biosynthetic gene cluster for simocyclinones shares genes related to those found in the gene clusters of classical aminocoumarins [81]. Several simocyclinones were discovered [82, 83], with simocyclinone D8 (SD8) being the best studied. It was expected that simocyclinones would bind at the same site as the aminocoumarins, i.e., the ATPase site of GyrB, and it came as a real surprise when it was found that these compounds did not inhibit the gyrase ATPase reaction but prevented the enzyme from binding DNA [84]. This unexpected result was confirmed by X-ray crystallography, which showed SD8 bound to the N-terminal domain of GyrA at the DNA G-segment binding site [85]. Subsequent mass spectrometry and further X-ray crystallography [86, 87] generated a modified model for the SD8-GyrA complex that satisfied all the mutant data and biophysical analyses (Fig. 19.6). Although simocyclinones are thought not to be particularly potent antibacterials (but see below) and this mode of action is arguably less effective than cleavage-complex stabilization, this work nonetheless showed that there are alternative modes of inhibition of gyrase aside from the quinolone and aminocoumarin mechanisms.

The crystallography work [85, 87], backed up by mutational analysis, firmly established that the simocyclinone-binding site lies in the N-terminal domain of GyrA, but there is evidence from circular dichroism studies of a second binding site in the C-terminal domain of GyrB [88]. The existence of this second site was corroborated by isothermal titration calorimetry experiments [87], but its affinity for SD8 was found to be ~1000-fold weaker than the GyrA site, suggesting that the latter is likely to be the primary target. However, the existence of a second site suggests promiscuousness in simocyclinone binding, which has been reflected in other work (see below).

Although the antibacterial potency of simocyclinones is thought to be relatively weak, particularly against Gram-negative bacteria [82], it has been pointed out that these tests are generally carried out on laboratory strains. Experiments assessing the potency of SD8 against clinical isolates of *E. coli* and *Klebsiella pneumoniae* suggested that these compounds may be more active in a clinical setting [89]. However, the reported activity of simocyclinones against human topoisomerases [90, 91] suggests that they may lack the selectivity required to be effective antibiotics. Perhaps the most promising aspect of simocyclinones is the identification of a novel mode of action that is distinct from those of quinolones and aminocoumarins. Such a mode presents the prospect of developing more "drug-like" molecules that can exploit this binding mode.

As the site of SD8 binding was found to be close to that of the FQs (Fig. 19.6), this raised the possibility of making hybrid compounds, i.e., quinolone-based compounds whose affinity would be enhanced by being further anchored to the

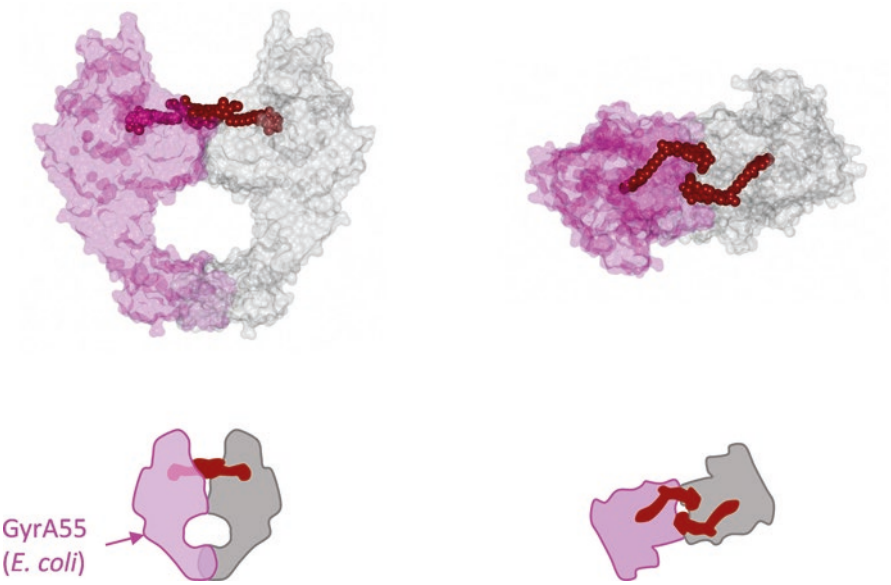

Fig. 19.6 Binding site of simocyclinone. The compound binds in a "saddle" formed by the two GyrA subunits that normally accommodates the G-DNA; two molecules of simocyclinone D8 bind per GyrA dimer. GyrA55 is a truncated version of the GyrA-NTD

aminocoumarin pocket of simocyclinones. A series of ciprofloxacin-coumarin analogs was synthesized, suggesting that it is possible to generate compounds that bind to the FQ-binding site and the coumarin pocket of SD8 and still retain potency [92]. It remains to be seen whether such compounds can be further developed as potential antibiotics. Interestingly, in other work, flavone-based analogs of simocyclinone were synthesized in order to create additional binding opportunities [93]. Two of these compounds were found to inhibit DNA gyrase, but as they also stabilized the DNA cleavage complex, inhibition was probably via a different mechanism; these compounds were shown to be DNA intercalators [93].

Simocyclinones have proved to be fascinating natural product compounds, both in terms of their unexpected mode of action and their biosynthetic pathways. Additional simocyclinones have been discovered from genomic-driven identification using *Streptomyces* and *Kitasatospora* species [94]. These new simocyclinones (D9, D10, and D11) inhibit DNA gyrase, but they show unexpectedly different biosynthetic gene cluster arrangements from simocyclinone D8. The availability of several simocyclinones and the identification of their gene clusters raise the possibility of carrying out engineering experiments to generate novel molecular entities going forward.

Other natural products – aminocoumarins, simocyclinones, and cyclothialidines are natural products produced by *Streptomyces* species. Actinomycetes in general have proven to be rich sources of antibiotic compounds, so it is not a surprise that natural products active against gyrase and topo IV have been found.

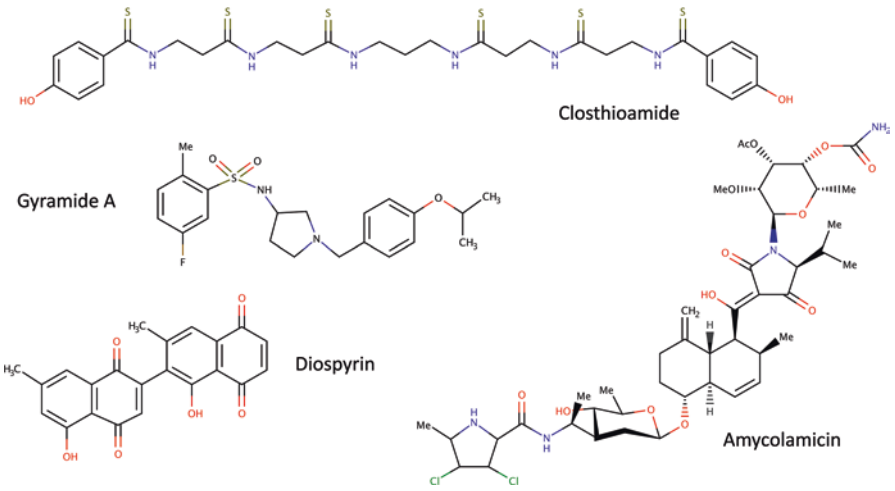

Fig. 19.7 Natural product inhibitors of gyrase and topo IV

However, apart from the limited success of novobiocin, these have yet to be developed as clinically useful compounds. But, given their high degree of chemical diversity and the great success of natural products directed at other targets, it is realistic to expect that gyrase/topo IV-targeted compounds will be successful in the future. There are a whole host of compounds that have been discovered that target gyrase/topo IV and that may have clinical potential going forward. On account of space limitations, only a few examples are given here to illustrate this type of work.

In 2007, a novel inhibitor, identified in an in vivo *E. coli* screen, demonstrated inhibitory effects on cellular division [95]: N-benzyl-3-sulfonamidopyrrolidine, then referred to as "534F6" and now as **gyramide** A (Fig. 19.7). The target of gyramide A was shown to be DNA gyrase, reportedly with a unique binding site and mechanism of inhibition [96]. Gyramides B and C were synthesized through modification of gyramide A. Using an *E. coli* strain having an inactive AcrAB-TolC multidrug efflux pump, gyramide A gave an MIC of 10 μM. The sequencing of spontaneous gyramide A-resistant mutants revealed amino acid substitutions in both GyrA and GyrB that clustered adjacent to the cleavage-religation site and were distinct from those that produce quinolone resistance [96]. Cross-resistance to quinolones was also absent in these gyramide A-resistant mutants. When paired with an efflux pump inhibitor, gyramides A, B, and C demonstrated effective antibacterial action against Gram-negative bacteria, as well as activity against some Gram-positive species [96].

In 2014, gyramide A was suggested to be a competitive inhibitor of ATP hydrolysis [97], in contrast to the earlier report, although this was later withdrawn [98]. It is possible that gyramide A reduces the rate of ATP hydrolysis indirectly by perturbing the binding to DNA. The 2014 paper also showed that gyramide A is a specific inhibitor of gyrase, showing no activity against topo IV in vitro.

Recently, through chemical modifications, gyramide analogs (D–F) have been synthesized and their antibacterial characteristics explored. One drawback with gyramides A, B, and C is that they are readily pumped out of the cell. Thus, to achieve antibacterial activity, these compounds must be used in combination with an efflux pump inhibitor. The new gyramide analogs demonstrate reduced sensitivity to efflux along with an increased inhibitory effect on gyrase (MICs in the 1 μg mL^{-1} range) and an extended spectrum of sensitive species [98]. These promising data, along with the unique binding site and no cross-resistance to ciprofloxacin and novobiocin, suggest that this new class of gyrase inhibitor may have potential as clinically useful antibiotics.

Naphthoquinones have been implicated for usage in the treatment of a variety of human diseases [99, 100]. Extracts from *Euclea natalensis* (the "toothbrush tree"), used in South African traditional medicine for various indications, were found to contain several naphthoquinones, in particular **diospyrin** (Fig. 19.7) [101]. This compound, and related compounds, was found to inhibit DNA gyrase from *E. coli*, *S. aureus*, and *Mycobacterium tuberculosis* [102]. Diospyrin seems to bind at a novel, currently uncharacterized site in GyrB without being an ATPase inhibitor. Although instability issues have hampered further development of this compound (unpublished data), the existence of another potentially exploitable ligand-binding site in gyrase is of potential interest.

Kibdelomycin is a natural product synthesized by *Kibdelosporangium* sp. strain, MA7385, which was isolated from a Central African forest soil sample. Kibdelomycin was discovered using an antisense-induced strain sensitivity (AISS) profiling technique in *S. aureus* [103]. This method involves the reduction of expression of individual genes essential to growth using 245 *S. aureus* strains with inducible antisense RNA. The AISS profile for kibdelomycin showed strong depletions in growth in the strains containing antisense RNA to *gyrB*, *parC* and *parE*, and weak depletions using the antisense *gyrA* strain. This was similar to the profile produced by novobiocin, and much less so to the quinolone profile, although not incomparable. Kibdelomycin has a complex chemical structure (comprehensively described in ref. [104]), which was elucidated using 2D-NMR and mass spectrometry techniques.

Kibdelomycin has demonstrated broad-spectrum potency against aerobic bacteria that include the Gram-positive MRSA, *Streptococcus pneumoniae*, *Enterococcus faecium*, and *Enterococcus faecalis* and the Gram-negative *Moraxella catarrhalis*, *Haemophilus influenza*, and *Acinetobacter baumannii* but not *E. coli* (due to both increased efflux and reduced membrane permeability) or *Pseudomonas aeruginosa* (due to reduced membrane permeability) [105]. Unfortunately, the MIC of kibdelomycin increased 256-fold in the presence of 50% human serum, raising concerns as to its value in vivo as a systemic antibiotic. However, it has been shown to be a potent inhibitor of *Clostridium difficile* growth in an in vivo hamster model, providing 100% protection against infection when dosed orally at 12.5–6.25 mg/kg, twice a day for 4 days [106].

The presence of kibdelomycin has been shown in vitro to potently inhibit *E. coli* gyrase supercoiling (IC_{50} 0.06 μM) and both *S. aureus* gyrase supercoiling and topo

IV decatenation (IC_{50} 0.009 and 0.5 μM, respectively), but it only weakly inhibits *E. coli* topo IV decatenation (IC_{50} 29 μM). Also, producing an effect very similar to novobiocin, kibdelomycin is a potent inhibitor of the ATPase activity of both *E. coli* gyrase and topo IV (IC_{50} 0.011 and 0.9 μM). When crystallized bound to both GyrB and ParE, kibdelomycin exhibited a unique U-shaped binding mode having extensive hydrophobic and polar interactions with surface residues of both proteins, while the pyrrolamide moiety extended deep into the ATP-binding pocket. This behavior is distinct from the binding of aminocoumarins and is consistent in the lack of cross-resistance between the two [107].

Closthioamide, a member of the polythioamide class of DNA gyrase inhibitors (Fig. 19.7), was the first secondary metabolite isolated from the obligate anaerobe *Clostridium cellulolyticum*. While genome mining identified genes involved in polyketide and peptide synthesis, no bioactive compounds were found until the addition of aqueous soil extract to the culture led to the production of closthioamide [108]. Closthioamide is symmetrical in structure with six thioamide groups flanked on either side by a phenol group. It has demonstrated potent activity against Gram-positive strains including MRSA and VRE, giving MICs of 0.14 and 0.4 mg/L, respectively. Only moderate inhibitory effects were found using wild-type *E. coli* strains (MICs of 2.5–3.5 mg/L); however, activity could be increased dramatically using the membrane permeability enhancer polymyxin B nonapeptide (PMBN) or using a drug efflux pump-deficient strain with and without PMBN, to MICs of 0.625, 0.035, and 0.31 mg/L, respectively. These data clearly suggest that the outer membrane and efflux pumps of Gram-negative bacteria are responsible for the reduction of closthioamide's efficacy [109].

Closthioamide's mode of action is unlikely to involve cleavage-complex stabilization, as very little linear DNA is detected when the agent is present in gyrase-DNA reaction mixtures. However, closthioamide did reduce the ATPase activity of gyrase and topo IV by 80% and 60%, respectively. Although the compound also inhibits the ATP-independent relaxation activity of gyrase, it is doubtful that it is a competitive ATPase inhibitor. Rather, it is more likely to allosterically interfere with ATP hydrolysis, inhibiting the enzyme using a novel binding mode, one that has been likened to the mode of action diospyrin [109].

Using the soil-dwelling actinomycete, *Amycolatopsis* sp., **amycolamicin** (Fig. 19.7) was isolated and found to have potent, broad-spectrum antibiotic activity. Its structure, determined using a combination of NMR spectroscopy, chemical degradation, X-ray analysis, and functional group modification, is described in detail in ref. [110]. The compound has shown promise against the Gram-positive MRSA, VRE, and penicillin-resistant *S. pneumoniae* (all with MICs in the range of 0.25–1 μg/mL) as well as against the Gram-negative ampicillin-resistant and beta-lactamase-positive amoxicillin-clavulanate-resistant strains of *H. influenzae* (MIC 0.5 and 2 μg/mL, respectively). The target was determined to be bacterial type II topoisomerases, with amycolamicin inhibiting gyrase and topo IV with IC_{50}s of 0.024 and 6.2 μg/mL, respectively.

The binding region of amycolamicin was explored using an amycolamicin-resistant *S. aureus* mutant. Upon sequencing of both *gyrA* and *gyrB* genes, mutations conferring resistance were found corresponding to substitutions in the B subunit, involving Thr^{173} to Ile and Glu^{201} to Ala changes. Using known novobiocin- and coumermycin-resistant mutations, it was found that some mutations, but not all, affected the binding of amycolamicin, indicating the binding region to be in the vicinity of the GHKL ATP-binding domain [110].

Other GyrB Inhibitors

The crystal structures of the N-terminal domain of GyrB, complexed with ATP analogs [19, 64, 111–113] and with aminocoumarins and cyclothialidines [114–116], have potentiated many drug-design programs using in silico methods, fragment screening, and related approaches that have included work from a number of companies, such as Cubist and F. Hoffmann-La Roche. The result has been a large number of publications describing novel compounds designed to bind to this site [117]. This type of approach has been referred to as bioisosterism [19]. While this is a valid approach, none of these compounds have so far become a clinically useful antibiotic. It is possible that a successful compound may emerge in the future, but it is worth bearing in mind that this ATP-binding site shares similarities with that of a number of other proteins, the GHKL ATPases [67]; thus, mammalian toxicity is always a danger. Indeed, we noted above that it has been possible to engineer novobiocin, the archetypal GyrB ATPase inhibitor, such that it is more specific to the human anticancer target Hsp90 [68, 118], and further modifications have led to compounds that are MAPK pathway inhibitors [119]. In addition, this type of target-based modeling approach does not take into account bacterial permeability and efflux issues. Nonetheless, target-based approaches can be successful (e.g., thiophenes, see above), and modeling may well lead to exploration of new chemical space.

Another potential drawback of targeting the ATP-binding site of gyrase/topo IV is that this mode of inhibition does not generally lead to cleavage-complex stabilization, which is a key feature of the success of topoisomerase-targeted drugs, including the quinolones. However, it has been shown that the ATP analog ADPNP (5'-adenylyl-β,γ-imidodiphosphate) can lead to cleavage-complex stabilization by gyrase [120], and the anticancer compound ICRF-193 stabilizes cleavage complexes with eukaryotic topo II through binding at the ATPase site [121]. Therefore, the possibility of compounds binding at or near the ATPase site and stabilizing the cleavage complex should not be disregarded.

Since this area has been extensively reviewed recently [19, 122], just a few illustrative examples are given here. In one, F. Hoffmann-La Roche embarked on a screen using low-molecular-weight (<300 Da) entities ("needle screening") coupled with a high-throughput gyrase ATPase assay and biophysical validation, followed by a 3D-guided optimization process [123]. Selected "hit" compounds were crystallized with the N-terminal sub-domain (24 kDa) of *S. aureus* GyrB to verify the binding mode. Seven new classes of inhibitor were found, including one compound that was ten times more potent than novobiocin [123].

In other examples, Cubist utilized a fragment-based screening method using NMR to assess the binding of >5000 diverse small chemical entities to the N-terminal sub-domain of *S. aureus* GyrB [124]. Compounds were further evaluated using X-ray crystallography and IC_{50} determination. **Pyrazolopyridones** were developed using this approach [124]; they were subjected to further optimization using medicinal chemistry guided by structure-based drug design [125]. Some of the compounds that emerged showed activity against both gyrase and topo IV, plus antibacterial activity against *S. aureus* [125]. Furthermore, using de novo design based on the GyrB ATPase site, Cubist discovered **azaindole ureas** [126], compounds that show in vitro activity against gyrase and Gram-positive bacteria, including fluoroquinolone-resistant MRSA. AstraZeneca used an NMR screening approach followed by design and synthesis to develop pyrrolamide inhibitors [127]; efficacy of a representative pyrrolamide was demonstrated against *Streptococcus pneumoniae* in a mouse lung infection model.

In terms of academic efforts, a number of laboratories have utilized these types of approaches to discover new gyrase/topo IV inhibitors. For example, Sriram and coworkers (Hyderabad, India) have published extensively on the use of structure-guided approaches to find new molecules, particularly to target *M. tuberculosis* gyrase [128, 129]. This work has yielded a number of molecular scaffolds, e.g., quinolines [130] and phenylthiophene-carboxamide derivatives [131]. Work from Kikelj and others (Ljubljana, Slovenia), using ligand-based and structure-based approaches, has generated a number of different inhibitors targeted to the GyrB ATPase site; these inhibitors include benzothiazole and oxadiazole compounds [132, 133]. In other work, using docking simulations with the structure of the *S. aureus* GyrB ATPase domain, a series of dihydropyrazole compounds have been synthesized and evaluated [134]. Whether these or any other compounds developed from these approaches can be successfully developed into clinically useful antibiotics remains to be seen.

19.6 Concluding Remarks

A clear message that emerges from this review is that gyrase and topo IV are excellent targets for antibacterial chemotherapy. The success of the fluoroquinolones attests to the value of these targets. However, what is clearly needed are new agents, ideally cleavage complex-stabilizing compounds, that can replace the quinolones. The inhibitors described in this chapter have been discovered and developed using a variety of approaches, including screening chemical libraries, following natural product leads, and fragment-based and in silico approaches; they include both target-led and phenotypic-led methodologies. Although arguments can be advanced that favor one or another of these approaches, it is likely that we need to retain a diverse range of approaches to discover the types of agent we seek. Increased chemical diversity, perhaps through investigating novel sources of natural products, is likely to be a key component to success going forward.

While the approaches to new compound discovery are an important issue for discussion, a more challenging question is who will carry out this work? The vast majority of antibiotics available for clinical use have been developed and produced by large pharma companies. While academics and small companies may have discovered and researched compounds, it is only large pharma that has the know-how and resources to bring them to market. However, in the current economic and political climate, large pharma is pulling out of antibiotic R & D, mainly on account of profitability issues, with a consequent reduction in effort in some cases and complete withdrawal in others [135, 136]. We are now faced with potential significant shortcomings in the discovery pipeline [137]. This is leading to the perilous situation of increasing antimicrobial-resistant bacterial infections and a paucity of new agents to treat them [138]. It is probably essential that governments, ideally working in cooperation, confront this challenge and provide the necessary resources and incentives to sustain the antibiotic discovery effort. It is likely that this will be increasingly carried out by the academic and SME sectors, resourced through public financing [139, 140]; it is clear that governments need to take action to address this crisis.

Government action would be starting with a solid base, as some pharma companies are still developing novel quinolones, particularly for niche markets (e.g., delafloxacin produced by Melinta Therapeutics [141]). Moreover, there are many examples of non-quinolone agents being developed, some of which have been described in this chapter. Among the active companies are AstraZeneca (e.g., spiropyrimidinetriones [142]), Cubist (pyrazolopyridones [125]), GSK (NBTIs and thiophenes [31, 48]), Pfizer (quinazolinediones [35]), Sanofi (IPYs [38]), and Vertex (benzimidazoles [143]). It is to be hoped that mechanisms will be found to sustain these efforts and ensure that the considerable expertise in this area of investigation is not lost.

Major Points

- Quinolones are highly successful antibiotics, but resistance is a serious problem.
- DNA topoisomerases (particularly gyrase and topo IV) are important targets for antimicrobial chemotherapy that should continue to be exploited.
- Cleavage-complex stabilization is an excellent mode of action for antibiotics; it is possible to find new, non-quinolone, compounds that work via this mechanism.
- Catalytic inhibitors of topoisomerases can, in principle, be developed as antibiotics of the future.
- It is important to sustain a variety of approaches for discovering new antibiotics.
- Big pharma companies cannot necessarily be relied upon to develop new antibiotics going forward; other ways of developing new drugs need to be explored.

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Chapter 20
Antimicrobial-Mediated Bacterial Suicide

Yuzhi Hong, Karl Drlica, and Xilin Zhao

20.1 Introduction

Controlling antibiotic resistance can be considered from two perspectives: limit the emergence of new resistance and halt the dissemination/horizontal transfer of existing resistance. For both applications, we expect better results from compounds that rapidly reduce bacterial burden. A lower pathogen burden will then reduce our reliance on host immune responses, which are likely to decline as populations age, to clear infection. Lower burden will also help control pathogens as we increase our use of immunosuppressants. Thus, rapid killing by antimicrobials will become increasingly important. The present chapter addresses a common mechanism of rapid killing by focusing on the hypothesis that bacteria respond to severe stress by accumulating toxic reactive oxygen species (ROS) and thereby self-destruct.

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I. W. Fong et al. (eds.), *Antimicrobial Resistance in the 21st Century*, Emerging Infectious Diseases of the 21st Century, https://doi.org/10.1007/978-3-319-78538-7_20

Understanding this process may help us better control bacterial populations. Readers interested in earlier reviews on ROS and on programmed cell death are referred to references [1–4].

We begin by distinguishing between antimicrobial lethality and blocking growth, the usual measure of antimicrobial activity, because the terms are frequently misused. Experimentally, blocking growth is measured with antimicrobial *in the test medium*. The output is expressed as either minimal inhibitory concentration (MIC) or as efficiency of plating. MIC is currently the basis for most antimicrobial considerations, including diagnosis, resistance surveillance, new compound development, and dosing. In contrast, killing is measured as survival after treatment with antimicrobial, usually by scoring colony formation on *drug-free* agar. Input cells that cannot form colonies after removal of drug are commonly considered to be dead.

Several variations exist for measuring killing. One is to determine the concentration of drug that reduces survival by a particular amount following a long incubation, often overnight for rapidly growing bacteria. This measurement, when performed under standardized conditions, is called minimal bactericidal concentration (MBC). Many secondary events can occur during the long incubation required for MBC measurement, which makes it difficult to characterize direct, specific lethal mechanisms using MBC. In contrast, rapid killing, which determines rate and extent of killing occurring within a few hours of drug exposure, is measured to study mechanism and find ways to improve the direct lethal activity of antimicrobials. Work discussed in the present chapter focuses on rapid killing.

A key concept is that rapid antimicrobial-mediated killing occurs in two general ways: (1) the primary lesion is sufficient to directly kill the cell, and (2) the primary lesion induces a lethal, self-destructive stress response (see Fig. 20.1). The contribution of ROS to lethal activity falls in the second category [3]. Since the relevance of ROS action relies on cell death, we begin with a brief discussion of how the importance of lethality is growing as the prevalence of antimicrobial resistance increases.

20.2 Importance of Lethal Action

Traditionally, lethal action has been thought to be important mostly for curing individual infections, particularly for problematic diseases such as endocarditis and meningitis. Dosing decisions have been based on relationships between incubation conditions and killing, largely using measurements of MBC [5]. Compounds fall into two general groups. Members of one group, represented by fluoroquinolones and aminoglycosides, are said to be concentration-dependent killers because increases in drug concentration result in increased killing. Members of the other, which includes β-lactams and macrolides, are time-dependent killers – as long as antimicrobial concentration exceeds MIC by a multiple of 2–5, further increases in drug concentration have little effect on killing [6]. According to this approach, some antimicrobials, in particular fluoroquinolones, are thought to be lethal enough to cure most infections. That perspective questions the need for improving lethality.

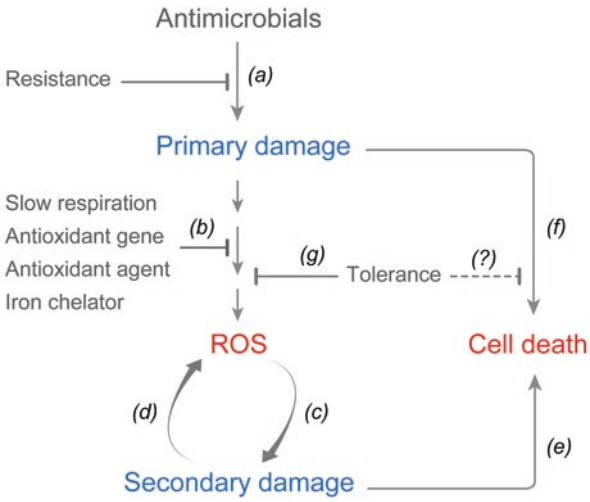

Fig. 20.1 Relationships among resistance, tolerance, and killing. (**a**) Antimicrobials create primary damage that is specific to the compound class. The resulting lesions block growth; the effect is quantified by the minimal inhibitory concentration (MIC). Resistance interferes with the formation of the primary lesion. (**b**) When the initial lesion is sufficiently damaging, a series of events occur that lead to accumulation of ROS; those events can be perturbed in a variety of ways. (**c**) When an ROS threshold is passed, the reactive species cause secondary damage that can then elicit the accumulation of even more ROS (**d**) and cell death (**e**). (**f**) Some antimicrobials produce lesions that are lethal independent of ROS production. Bacterial tolerance (**g**) occurs when lethal action is inhibited even though the primary lesion occurs and growth is still blocked. Tolerant cells may suppress lethal pathway (**e**) by inhibiting respiration and thereby ROS production/accumulation. Whether tolerance also arises from blockage of lethal pathway (**f**) is likely compound-specific

The traditional approach worked fine before antimicrobial resistance became a major problem. Now, as we move deeper into the era of antibiotic resistance, attention must shift to restricting the emergence and spread of resistance. Simply curing most patients is unlikely to be adequate when many millions of doses are consumed, especially with antimicrobials that are mutagenic and thereby lead to induced resistance. Induced resistance is especially noticeable with the quinolone-class antibacterials and other DNA-damaging agents that derepress the mutagenic SOS response (β-lactams also induce the SOS response [7]). The mutagenic action of quinolones is readily demonstrated by adding the agent to agar, plating bacteria, and then counting colonies daily during a 2-week incubation. A marked increase in colony number occurs that is blocked by a mutation preventing induction of the SOS response [8, 9]. In this type of experiment, greater lethal activity correlates with reduced recovery of induced mutants [8]. Rapid lethal action is needed to reduce the number of induced mutants.

A related but distinct concept is that killing may lower antimicrobial concentrations needed to suppress the enrichment of resistant mutant subpopulations present before drug exposure [10, 11]. If concentrations can be kept above the MIC of the

least susceptible mutant subpopulation, resistance is less likely to emerge than if the goal is only to keep concentrations above the MIC of the major portion of the population [10–12]. Unfortunately, with most pathogen-antimicrobial combinations, keeping antimicrobial concentrations high enough and exposure time long enough to restrict the emergence of resistance is likely to have adverse effects on patients. Thus, it is encouraging that studies with animal models show that some highly lethal compounds restrict the emergence of resistance without needing to keep concentrations above the MIC of mutant subpopulations throughout treatment [13–16].

Lethal activity is also an important consideration with drug-tolerant cells (see Chap. 13 for discussion of tolerance). Such bacteria, when exposed to highly lethal antimicrobials, fail to grow, but they are not killed (in contrast, resistant cells grow in the presence of drug; see Fig. 20.1 for relationships). Tolerant cells (persisters), which are usually a small fraction of the population, are a problem because they may survive treatment and subsequently cause relapse. Since many antimicrobials are active primarily with replicating cells, non-growing cells can display a form of tolerance, as seen with ampicillin and first-generation quinolones. Antibiotic tolerance can also arise from mutation, and it can facilitate the emergence of resistance [17]. Thus, finding ways to overcome tolerance, i.e., weakened lethal activity, is of major importance for restricting the emergence of resistance.

20.3 Experimental Manipulation of ROS

20.3.1 Genetic Perturbations

Key evidence for the contribution of ROS to lethal antimicrobial action derives from manipulating ROS levels and correlating those changes with bacterial survival. One type of genetic approach involves increasing or decreasing the expression of catalase/peroxidase. For example, deletion of *katG* increases ROS accumulation and lethality arising from treatment of *E. coli* with quinolones [18–20], from thymine starvation [21], and from exposure to UV irradiation (Y. Hong & X. Zhao, unpublished observation). Similarly, deletion of *ahpCF*, which encodes a peroxidase, increases the lethal activity of kanamycin and ampicillin [18]. In a reciprocal experiment, overexpression of *katG* suppresses antimicrobial lethality [20].

Many other genes that protect from oxidative stress have more complex effects. Among these are *sodA* and *sodB*, genes that encode superoxide dismutases (enzymes that convert superoxide to peroxide). A deficiency of either gene has little effect on the lethal activity of norfloxacin [18]; however, a *sodA sodB* double mutant protects from the lethal stress arising from norfloxacin, ampicillin, and kanamycin, as if elevation of superoxide concentration is protective (wild-type genes would reduce superoxide levels). A similar conclusion emerged from a study of the DNA-damaging agent bleomycin [22]. In this case, a *sodA sodB* double mutant exhibited reduced killing from bleomycin, while overexpression of superoxide dismutase

increased lethal activity. Likewise, treatment with a low concentration of plumbagin, a metabolic generator of superoxide, protected from the lethal activity of bleomycin [22]. The protective effect of elevated superoxide was confirmed by a subsequent finding in which low concentrations of plumbagin or paraquat, another metabolic generator of superoxide, reduced the lethal activity of oxolinic acid, kanamycin, and ampicillin [23]. When overall ROS levels were examined using a fluorescent probe, the increase associated with oxolinic acid treatment was muted by plumbagin and paraquat [23]. Apparently, spontaneously elevated concentrations of superoxide in the *sodA sodB* double mutant induce genes that lower levels of other ROS during antimicrobial treatment and thereby protect from lethal stress. The identity of those genes is unknown.

Not every mutant has provided a simple yes or no answer to whether it stimulates or restricts the lethal stress response. For example, preinduction of many protective, oxidative stress genes may account for an *oxyR* deficiency and for the Hpx$^-$ triple mutant (Hpx$^-$ contains deficiencies in *ahpCF*, *katE*, and *katG*) showing no effect on antimicrobial lethality [24] – if these mutants have elevated levels of anti-oxidative defenses before addition of antimicrobial, subsequent ROS production could be dampened when stressor is added. In other cases, discussed below, a gene can appear to be protective at low levels of stress and destructive at high levels. Thus, stressor concentration or exposure time can be a crucial variable in antimicrobial-ROS experiments.

Another idea emerging from early work was that iron is released from iron-sulfur clusters during lethal antimicrobial treatment. That iron would then become available for conversion of peroxide to hydroxyl radical via the Fenton reaction [25]. The hypothesis was based largely on a deficiency in *iscS* protecting from the lethal action of norfloxacin, ampicillin, and kanamycin. The deficiency also suppressed the increase in the hydroxyl radical signal from a fluorescent dye. However, this conclusion needs to be revisited, because follow-up work with gentamicin, an aminoglycoside, argued that perturbing iron-sulfur clusters interferes with drug uptake [26]. Since lethality cannot be studied if drug uptake is blocked (see Fig. 20.1), care must be taken to assure that the lethal response is being studied, not the many steps that lead to formation of the primary, stress-mediated lesion.

20.3.2 Chemical Perturbations

One chemical approach involves treating bacterial cells with antioxidants, such as thiourea, dimethyl sulfoxide, ascorbic acid, glutathione, or resveratrol. Such compounds are expected to scavenge hydroxyl radical. With chemical perturbation, an important consideration is the concentration of the perturbing agent, since that concentration needs to be adjusted to avoid interfering with bacterial growth – growth inhibition can create a type of tolerance. Another consideration is whether antioxidants actually scavenge hydroxyl radical, since the rapid reaction of hydroxyl radical with other biomolecules might obscure any reaction with thiourea [24]. That

would make the observed protection from killing by thiourea [25] an off-target effect. To our knowledge, no off-target effect of antioxidants has been identified.

Another chemical perturbation uses iron chelators, such as 2,2′ bipyridyl. These agents are expected to act in two ways. One is by inhibiting the iron-requiring Fenton reaction in which hydrogen peroxide produces hydroxyl radical. A second involves iron-requiring proteins involved in respiration, a source of superoxide and ultimately hydroxyl radical. Experiments employing bipyridyl have consistently supported the conclusion that lethal activity arising from a variety of stressors involves ROS [18, 25].

Ruling out off-target effects due to chelators and antioxidants is difficult. One argument is that antioxidants that interfere with antimicrobial lethality are chemically diverse and unlikely to have the same off-target effect. Another argument derives from consideration of catalase as an antioxidant. We found that *E. coli* cells, thought to be killed by the lethal antimicrobial trimethoprim and then plated on drug-free agar, are revived by addition of catalase to the plated cells (Y. Hong and X. Zhao, unpublished observations). Since hydrogen peroxide readily enters and exits from cells, extracellular catalase would lower the overall peroxide concentration and provide a specific protective effect that would not be attributed to either off-target effects or growth inhibition. Overall, effects of chelators and antioxidants fit well with their suppression of ROS accumulation.

20.4 Bactericidal Activity of Antimicrobials Mediated by ROS

20.4.1 Development of the ROS-Lethality Hypothesis

Credit for the general nature of the ROS-lethality hypothesis is usually attributed to a 2007 report from the Collins laboratory [25], although involvement of ROS with lethal activity had been proposed earlier. For example, activation of the SoxRS regulon conferred resistance to multiple antimicrobial classes [27–29]; moreover, antioxidants, such as vitamin C and glutathione, raised minimal inhibitory concentration (MIC) and efficiency of plating for quinolones and aminoglycosides [30, 31]. In addition, elevated levels of oxidative stress signals were detected in cells treated with antimicrobials [32, 33]. However, such observations do not establish a connection between ROS and cell death, because the measurements reported inhibition of bacterial growth, not cell death. Thus, the work by Kohanski et al. [25] was a qualitative advance because killing was measured. We later showed that the killing was separate from bacteriostatic effects by normalizing killing to MIC [18].

Among the central observations from the Kohanski et al. study [25] was that killing caused by norfloxacin, ampicillin, or kanamycin was suppressed by thiourea and 2,2′ bipyridyl. Although the experimental conditions also suppressed bacterial growth, which itself was known to interfere with killing, subsequent work [18] showed that subinhibitory concentrations of thiourea and bipyridyl protect from

antimicrobial lethality. The latter study also described genetic perturbations that supported the hypothesis [18].

It soon became clear that not all derivatives of an antimicrobial class depend on ROS to kill cells [34]. For example, the quinolones were known to kill by two general pathways, one that was blocked by chloramphenicol, an inhibitor of protein synthesis, and one that was not [35–37]. This dichotomy also applied to the contribution of ROS: 2,2′ bipyridyl treatment [34] and anaerobic conditions [38] paralleled the behavior of chloramphenicol. Overall, the quinolone experiments established that first-generation compounds, such as nalidixic and oxolinic acids, kill *E. coli* by a mechanism that relies heavily on ROS; the fluoroquinolones additionally kill bacteria by a pathway that appears to rely more on chromosome fragmentation than on ROS, although fluoroquinolones still trigger ROS accumulation [34]. Within this scheme, norfloxacin is an outlier having an intermediate, concentration-dependent activity [38]. As pointed out below in Sect. 5, subsequent challenges to the ROS-lethality hypothesis relied in part on work with norfloxacin.

20.4.2 Factors Involved in ROS Accumulation

Steps leading from stress to accumulation of ROS have been investigated by identifying genes that, when defective, alter ROS levels and stress-mediated cell death. The action of the gene products can be fit into a scheme (Fig. 20.2). One of the factors is the MazEF toxin-antitoxin pair. The MazF protein is an endoribonuclease that during stress cleaves mRNA and thereby blocks protein synthesis. At low, bacteriostatic concentrations of antimicrobial, blocking gene expression and bacterial growth by MazF would allow time for cells to efflux noxious molecules and repair damage. However, at high, lethal levels of stress, MazF would produce toxic levels of truncated mRNA and misfolded proteins that perturb cell membrane function and elevate ROS levels. Thus, MazF is expected to be bifunctional with respect to stress. Indeed, bifunctionality has been observed with *Bacillus subtilis*. For example, at low doses of UV irradiation or low concentrations of moxifloxacin, a Δ*ndoA* (*mazF*) mutant is more readily killed than wild-type cells, but at high doses the opposite is seen [39]. Bifunctionality is a key feature of a stress response that either allows repair of minor damage or causes self-destruction when damage is severe.

EF4 is another factor that contributes to ROS accumulation. This ribosomal elongation protein is normally sequestered in the cell membrane, but during stress, the protein enters the cytosol, binds to stress-stalled ribosomes, and stimulates ribosomal back-translation. These events allow protein synthesis to recover from moderate stress. But EF4 also blocks tagging of truncated proteins for degradation by tmRNA – this activity would facilitate the accumulation of toxic peptides, presumably arising from MazF action. Indeed, the absence of EF4 *protects E. coli* from being killed by quinolones (wild-type protein would be destructive) [40]. Thus, EF4 and MazEF appear to be bifunctional proteins that protect from moderate stress but promote death when stress is high. How the action of MazF and EF4 is connected to the initial lesion is unknown.

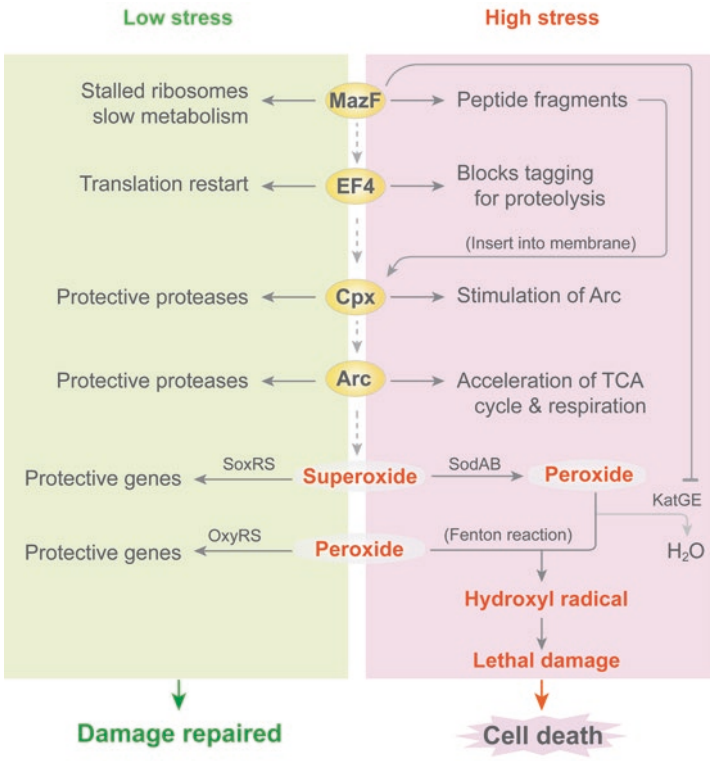

Fig. 20.2 Scheme describing bifunctional nature of factors involved in the live-or-die stress-response pathway. Low to moderate levels of stress result in a protective stress response. The stressors, such as quinolones, generate specific primary lesions that in an unknown way stimulate the MazF toxin to cleave mRNA, thereby halting translation and allowing cells time to repair damage. The protein fragments resulting from translation or MazF-mediated mRNA cleavage are tagged for degradation by tmRNA and EF4, a ribosomal protein that facililtates restart of stalled ribosomes. Truncated, misfolded peptides that enter cell membranes stimulate the Cpx two-component system to induce genes involved in membrane protein repair. Safety valves YihE kinase and KatG catalase negatively regulate MazF and detoxify peroxide, respectively. At high stress levels, mRNA cleavage by MazF is extensive, and EF4 blocks the tagging of truncated peptides by tmRNA. Thus, high levels of protein fragments accumulate and enter cell membranes. That causes the Cpx system to induce the Arc two-component system, which in turn leads to high-level production of superoxide. Superoxide dismutates to peroxide; the Fenton reaction then converts peroxide to hydroxyl radical. Hydroxyl radical damages nucleotides and many macromolecule types, causing mutations and cell death. To assure death, MazF cleaves *katG* mRNA, which lowers the level of KatG, a protein that would otherwise reduce peroxide levels. Other protective functions, such as induction of membrane repair by Cpx/Arc and induction of the SoxRS/OxyRS regulon by superoxide/peroxide, are overwhelmed

Insertion of truncated, misfolded proteins into the cell membrane activates a two-component membrane stress-response system called Cpx [41, 42]. When stress is high, activation of Cpx stimulates another two-component system (Arc) that then contributes to the generation of elevated levels of ROS [43] as discussed below in Sect. 6. The destructive feature of Cpx is revealed by a CpxR deficiency protecting

from nalidixic acid-mediated cell death [19]. The Cpx system also serves to repair membrane protein damage [44] and to mitigate MazF toxicity [19] when stress is moderate. Thus, Cpx has both destructive and protective functions; it represents a third bifunctional system involved in lethal antimicrobial action. Arc also exhibits protective and destructive properties (*arc* is discussed in more detail in Sect. 6.2).

Superoxide occupies a central position in the lethal stress response. At moderate stress levels, superoxide is thought to accumulate and stimulate protective gene responses, such as induction of the SoxRS regulon. During periods of harsh stress, superoxide may accumulate to high levels and rapidly dismutate, thereby creating elevated levels of hydrogen peroxide (superoxide dismutases are 1000-fold more efficient than catalase/peroxidase, enzymes that detoxify hydrogen peroxide by converting it to water). The result is accumulation of hydrogen peroxide, which can then be converted to hydroxyl radical, a compound whose toxic effects may overwhelm the protective functions stimulated by superoxide. As pointed out above, such a bifunctional nature of superoxide would explain why low concentrations of metabolic generators of superoxide, such as plumbagin and paraquat, reduce the lethal action of bleomycin and the quinolones [22, 23, 45], even though high concentrations of plumbagin kill bacteria.

Several safety valves operate within this scheme of bifunctional factors. One involves a protein kinase called YihE. The absence of YihE elevates the lethal action of nalidixic acid by 100-fold but only if the MazEF toxin is present [19]. Thus, YihE appears to be a negative regulator of MazF. Another safety valve is the *katG* catalase/peroxidase, which, as mentioned above, detoxifies peroxide by converting it to water. Deletion of *katG* increases norfloxacin lethality by 20-fold *without* affecting MIC [18]. Similarly, deletion of *ahpCF* increases lethality of both ampicillin and kanamycin, although this deletion has no effect on quinolone-mediated killing. Among the many protective genes (safety valves) induced by oxidative stress are efflux pumps that remove noxious stressors [23, 45]; presumably the pumps reduce the signal leading to ROS accumulation.

Variations on the theme developed above are likely to emerge as more is learned about individual stressors. For example, each stressor generates a unique lesion that is likely to be recognized in a unique way, and many pathways may connect the lesion to the accumulation of ROS. Thus, the scheme in Fig. 20.2 should be considered only as a framework for future testing.

20.4.3 ROS-Mediated Toxicity

The most toxic of the reactive oxygen species is hydroxyl radical. It readily damages DNA by creating single-strand breaks and by converting single-strand DNA lesions into double-strand breaks [46]. Hydroxyl radical also carbonylates proteins [47] and peroxidates membrane lipids [48]. In addition to directly breaking DNA, hydroxyl radical oxidizes the guanine nucleotide pool, thereby producing 8-oxo-guanine. When 8-oxo-guanine is incorporated into DNA, it is expected to lead to double-strand breaks. The DNA breaks are thought to derive from excision of the

8-oxo-dG, since deficiencies in the excision enzymes protect (10–20 fold) from the lethal action of norfloxacin, ampicillin, and kanamycin [20, 49]. Overexpression of *mutT*, which "sanitizes" 8-oxo-guanine from the nucleotide pool, reduces the rate of killing for the three antimicrobial classes [49]. Thus, lethal DNA damage is a common theme associated with killing, even by antimicrobials that do not have DNA as their primary target. That explains why ampicillin induces the SOS response [7].

DNA damage leads to the interesting possibility that ROS is self-amplifying. When a threshold concentration of ROS is reached, DNA damage could be sufficient to stimulate the production of even more ROS. Like a nuclear reaction, ROS accumulation would be unstoppable, and death would be assured. In this sense, bacterial cells self-destruct when faced with severe lethal stress [19]. To our knowledge, stress-mediated ROS accumulation due to self-amplification has not been demonstrated. One prediction from the self-amplification idea is that primary stress of one type, such as DNA damage by a quinolone, will cause ROS-mediated damage of a second type, such as protein carbonylation. Repair of protein damage, if specifically blocked by a *lon* or *hslV* deficiency, would exacerbate damage of a third type, such as lipid peroxidation. Our unpublished work supports this scenario (X. Wang & X. Zhao).

20.5 Challenges to the ROS-Mediated Stress-Response Hypothesis

The initial proposal for ROS-mediated antimicrobial lethality [25] required modification with respect to *sod* effects (Sect. 3.1), reconsideration of mutations affecting iron-sulfur proteins (Sect. 3.1), and complexities with respect to fluoroquinolones having at least two lethal mechanisms [34, 38]. However, the overarching idea was independently solidified [18, 19, 23]. At about the same time, two laboratories challenged the idea that ROS contribute to the lethal action of multiple antimicrobial classes. Below we discuss the resulting controversy.

One of the challenging studies [24] reported failure to confirm ROS involvement in the lethal action of norfloxacin, kanamycin, and ampicillin under conditions that were similar to those reported by Kohanski et al. [25]. Those conditions, which involved use of a single drug concentration, appear to have been too narrowly defined to take into account differences in conditions between laboratories. For example, norfloxacin concentration was known to be important for observing lethality [38], so it needed to be varied. In the other work, Keren et al. [50] focused on whether killing depends on ROS (requires ROS). By examining high levels of stress, they confirmed that fluoroquinolones have an ROS-independent mode of killing [34, 38] that is insensitive to anaerobiosis [38]. The issue of whether ROS *contribute* to killing, as proposed by Collins [25] and extended by our work [18], was actually supported by Keren et al. [50] at low levels of stress. The high levels examined were outside the range of discrimination: only residual "persisters" survived (such cells

are expected to be insensitive to ROS-mediated killing due to lack of respiration). Moreover, many of the experiments involved long incubation times, a feature that is problematic, since it was known that ROS effects are largely kinetic [51]. Thus, ROS are not required for killing, as was previously known, and whether they contribute to lethal action was not experimentally challenged. Subsequent work from Collins [20] solidified the idea that ROS contribute to antimicrobial killing by addressing other issues raised by Keren et al. and Liu and Imlay (discussed below).

A follow-up review article by Imlay suggested that in bacterial cells, endogenous ROS concentrations are not high enough to kill cells [52]. In studies of thymineless death [21] and quinolone-mediated killing (G. Luan, unpublished observations), we found that lethal stress creates a substrate that is hypersusceptible to ROS attack, thereby making generation of extreme levels of endogenous ROS unnecessary to kill cells. Overall, the challenges have led to reexamination and solidification of the ROS-lethality hypothesis.

Two other reports also contained objections to the idea that lethal antimicrobials kill bacteria by a common mechanism involving ROS. One [53] showed that *cpx*-mediated resistance applies to some (aminoglycoside, hydroxyurea) but not all (fluoroquinolone, β-lactam) antimicrobials. Since resistance is a bacteriostatic phenomenon (see Fig. 20.1), the work failed to focus on the lethal stress response. The second report [26] showed that for gentamicin, an iron requirement concerns drug uptake, which would supersede iron effects on ROS formation (similar issues apply to respiration and drug uptake, as discussed in Sect. 6). Additional work is required to separate drug uptake from a lethal stress response.

The issues raised above elicited several sets of follow-up experiments [20]. One set involved additional dyes to detect a variety of ROS. In general, ampicillin and norfloxacin were very active; gentamicin was less striking, but the results were clear. Another set addressed the failure [24] to detect extracellular peroxide following antimicrobial treatment. When an intracellular assay was introduced, peroxide was readily detected. A third point involved induction of promoters of genes known to be sensitive to peroxide (pOxyS) and superoxide (pSoxS). Both were induced by norfloxacin and ampicillin. These tests, which gave signals equivalent to 10 μM exogenous hydrogen peroxide, produced gene expression patterns that were similar for this peroxide concentration and for norfloxacin or ampicillin treatment. In a fourth point, lethal activity for the three antimicrobial classes was lower under anaerobic conditions. Thus, the key issues raised by the challenges to the ROS-lethality hypothesis are accommodated by experimental considerations [2, 3, 20] and by reiterating that ROS contribute to, rather than completely account for, lethal activity [34, 38].

Among the necessary clarifications were experimental definitions of killing and the lethal stress response (Fig. 20.1). The lethal stress response must occur after formation of the primary lesion. Consequently, studies that focus on factors acting at or before primary lesion formation, such as drug uptake, efflux, and target interactions, are largely uninformative. The effects of these factors can be removed from consideration by normalizing lethal activity to growth inhibition (MIC) [18], a feature often absent from studies of ROS and killing.

20.6 Role of Respiration in ROS Accumulation and Cell Death

20.6.1 Source of ROS

The respiratory chain is a major source of superoxide and hydrogen peroxide [54, 55], as these ROS form when molecular oxygen oxidizes redox enzymes, such as fumarate reductase (Frd), succinate dehydrogenase (Sdh), and aspartate oxidase (NadB) [56]. Indeed, about a quarter of the cytoplasmic H_2O_2 derives from NadB [57]. The electrons involved in formation of superoxide and hydrogen peroxide are thought to derive largely from the tricarboxylic acid (TCA) cycle, which we discuss below. In principle, dismutation of superoxide can also serve as a source of H_2O_2, which can then form highly toxic hydroxyl radical via the Fenton reaction.

TCA cycle. Several lines of evidence connect the TCA cycle with the lethal action of antibiotics. One is that treatment of *E. coli* cultures with bactericidal antibiotics leads to upregulation of genes involved in central metabolism, including the TCA cycle and respiration [25, 43, 58]. A second line links the efficacy of bactericidal antibiotic therapy to carbon flux through the TCA cycle [59, 60]. A third line showed that mutations in genes involved in the TCA cycle reduce stress-mediated lethality. Surprisingly, antibiotic classes, represented by norfloxacin, ampicillin, and kanamycin, differ in the TCA cycle genes involved. For example, norfloxacin-mediated lethality is reduced by mutation of *icdA* and *acnB*, while killing by ampicillin is reduced by mutation of these two genes (*icdA* and *acnB*) and *sucB*. Killing by kanamycin is reduced by mutation of four genes, *icdA, acnB, sucB*, and *mdh* [25]. Additional work is needed to determine whether these differences are characteristic of drug mechanism or whether they result from differences in the concentration of the three drugs examined (normalized to MIC).

Respiration. *E. coli* encodes three cytochrome terminal oxidases: *bd*-I (CydAB), *bd*-II (AppAB), and *bo* (CyoABCDE). Reduction of stress-mediated lethality by a deficiency of *cydB*, which is associated with decreased ROS levels during hydroxyurea treatment [61] and thymineless death [21], establishes the importance of the respiratory chain. Moreover, the rate of oxygen consumption serves as a measure of respiration that can be compared with antimicrobial-mediated cell death [20, 62]. For example, a deficiency of *atpA* increases oxygen consumption, ROS level, and killing during treatment of cells with ampicillin or norfloxacin [62]. Conversely, a cytochrome oxidase null mutant fails to show an acceleration of respiration or cell death when treated with norfloxacin, ampicillin, or gentamicin [62]. Treatment of wild-type cells with the bacteriostatic agent chloramphenicol rapidly attenuates cellular respiration and the lethality associated with lethal doses of norfloxacin or ampicillin [62]. In yet another example, NADH-coupled electron transport (NADH dehydrogenase I) is a common upregulated pathway for all three bactericidal drugs [25]. Such data fit with upregulation of genes involved in central metabolism and respiration being associated with exposure to lethal antimicrobials [25, 43].

Complexities of drug uptake and killing. We have proposed that ROS contributes to the lethal activity of aminoglycosides (kanamycin), because lethal action, when normalized to MIC to rule out effects of drug uptake, efflux, and target interactions, was decreased by mutation of superoxide dismutase genes and increased by a deficiency in a catalase/peroxidase [18]. Other works implicated the TCA cycle in aminoglycoside lethality [25, 43], and recently fumarate or glyoxylate, used to activate or inhibit the TCA cycle, potentiated or suppressed, respectively, the lethal activity of tobramycin with *Pseudomonas aeruginosa* [63]. Complexity arises because proton-motive force, which derives from respiration and is necessary for aminoglycoside uptake [26], is a required precursor to lethal activity. Moreover, uptake and killing are stimulated by the same factor (e.g., respiration). Thus, normalization to MIC is required to remove uptake from consideration before the effect of respiration on ROS and killing can be assessed.

20.6.2 Role of the Arc Two-Component System in the Response to Oxidative Stress

The *E. coli* ArcAB two-component system participates in the regulation of multiple operons that are involved in central metabolism, such as the enzymes of the TCA cycle, pyruvate dehydrogenase, cytochrome *o* ubiquinol oxidase, and NADH-quinone oxidoreductase I [64]. In general, Arc represses these genes during anaerobic growth. A connection to antimicrobial lethality was made through a gene expression study in which the effects of a lethal aminoglycoside (gentamicin) were compared to those of the bacteriostatic derivative spectinomycin – a spike in the expression of *arc*-associated TCA cycle genes was specific to gentamicin treatment [43]. Deletion of *arc* then reduced gentamicin-mediated accumulation of hydroxyl radical and increased survival [43]. The ability of an *arc* deficiency to protect from the lethal activity of norfloxacin and ampicillin suggested a position for wild-type *arc* in the pathway stimulating self-destruction [43] (the connection still requires tests in which lethal action is normalized to MIC, as pointed out above for other experiments involving the TCA cycle, respiration, and aminoglycoside-mediated killing).

The wild-type *arc* system also appears to protect from oxidative stress, an effect opposite to the stimulation of killing described above. The DNA-binding activity of ArcA is controlled by reversible phosphorylation through ArcB, whose kinase activity is governed by the redox states of quinone pools. Those pools are linked to the NADH/NAD+ redox couple through respiration [64]. Under some conditions, disruption of ArcAB leads to reduced survival following oxidative stress generated by hydrogen peroxide [65, 66].

The *arc* system also upregulates proteases involved in removal of misfolded membrane proteins [43], which would be protective. This observation fits with cross talk between Arc and Cpx, which repairs damage to membrane proteins. As pointed

out in an earlier section, Cpx protects from low levels of lethal stress and contributes to death when stress is high [19]. Indeed, Cpx may contribute to the accumulation of ROS by stimulating Arc to accelerate the TCA cycle [43]. We reiterate the general theme in which a set of proteins (MazEF, EF-4, Cpx, and ArcAB) protect from moderate levels of lethal stress, but when stress is severe, the proteins contribute to the accumulation of ROS and cell death.

20.7 ROS-Mediated Programmed Cell Death

Among the important questions concerning the ROS-lethality hypothesis is whether ROS accumulation and toxicity cause death or whether dead cells are simply the source of ROS. One type of support for ROS causality is that pretreatment with a low dose of peroxide causes a 30-min lag in killing by norfloxacin, ampicillin, and gentamicin [20], consistent with protective systems being induced.

The question of causality can also be addressed by determining whether bacterial cells continue along an ROS-dependent death pathway even after removal of the initial stressor. As a test, we treated *E. coli* cultures with quinolones (nalidixic acid or ciprofloxacin) at lethal concentrations for times sufficient to reduce survival when assayed by colony formation on drug-free agar. When we included 0.5 x MIC thiourea (an ROS scavenger) in the agar to block ROS action occurring after removal of quinolone, thiourea increased survival by 5–10- and ~100-fold for wild-type and Δ*yihE* cells, respectively [19]. These data show that the toxic action of ROS continues even after removal of the initial stressor in cells that are not yet dead (they still form colonies if treated with thiourea).

In follow-up work, we took advantage of the finding that when a *dnaB*-TS mutant is shifted to nonpermissive conditions, cells die if the medium is rich in nutrients (they live if the medium contains only minimal salts and glucose) [37]. We reasoned that cell death would occur in rich medium because higher levels of ROS would be produced by the stress of replication inhibition, as we observed in a study of thymineless death [21]. Thus, temperature shifts with a reversible *dnaB*-TS mutant provided another way to rapidly remove the primary stressor. We found that killing was blocked by the presence of catalase or an ROS scavenger (thiourea) in the agar plates used for determination of survival (Y. Hong & X. Zhao, unpublished observation). Thus, two types of stressor appear to stimulate bacterial programmed cell death (PCD), which we define as ROS-dependent death arising after removal of the primary stressor.

PCD, which is known to require metabolic energy, has previously been associated with physiological or developmental signals (reviewed in [4]). For example, with eukaryotic cells, PCD includes apoptosis, autophagy, programmed necrosis, tissue homeostasis, immune function, stress responses, and processes that are critical for embryogenesis. With bacteria, the concept of PCD has been controversial, because the molecular events in bacterial death have not been identified [67, 68] and because it has not been obvious how suicide by some members of a bacterial popu-

lation would benefit other members [69]. Nevertheless, the existence of bacterial PCD is supported by studies of sporulation and by detection of apoptosis biomarkers previously reported for eukaryotic systems (with antibiotic stress, *E. coli* cells exhibit DNA fragmentation, chromosome condensation, and membrane depolarization [70]). Among the examples of bacterial development are mother cell lysis during spore formation, which is seen with *Bacillus subtilis* and *Myxococcus xanthus* (as reviewed in [4]). With *Xanthomonas campestris*, apoptosis-like phenotypes, as mentioned above, are seen during death caused by incubation in rich Luria-Bertani (LB) medium [71, 72]. However, in none of these examples have cells been shown to continue along a documented death pathway after withdrawal of the primary stressor – in each case, the primary stressor was present throughout the experiment. Thus, data showing that lethal stress propels bacteria along an ROS-requiring death pathway, described above, add key support to the idea that bacteria undergo a self-destructive process in response to stress.

20.8 Paradoxical Tolerance at High Quinolone Concentration Involves ROS

A different type of ROS-related phenomenon is observed with the paradoxical loss of killing that occurs at very high concentrations of quinolone. At moderate concentrations, quinolones are very lethal, but with some derivatives and bacterial species, 100% of cultured cells survive when quinolone concentrations are very high. The phenomenon is observed with a variety of bacterial species and with many different quinolones, most notably with nalidixic acid, a first-generation member of the quinolone-type compounds (nalidixic acid is formally a naphthyridine rather than a quinolone, as are some other clinically used agents, such as enoxacin and gemifloxacin). We reasoned that if ROS contribute to quinolone lethality, a drop in ROS accumulation could explain the loss of killing seen at very high nalidixic acid concentrations.

When *E. coli* cultures are treated with various concentrations of nalidixic acid, followed by measurement of ROS using an ROS-sensitive dye, high, nonlethal concentrations of the drug induce lower levels of ROS than moderate, lethal concentrations when measured by fluorescence microscopy (individual cells) and flow cytometry (batch cultures) (G. Luan et al., unpublished observations). At the high, nonlethal quinolone concentrations, sublethal doses of exogenous hydrogen peroxide become lethal and eliminate nalidixic acid-associated paradoxical survival. Thus, the quinolone-mediated lesions needed for ROS toxicity persist at high, nonlethal quinolone concentrations. That leaves a drop in ROS as the most likely explanation for loss of killing.

Nalidixic acid-induced accumulation of ROS and death are blocked by inhibitors of protein synthesis, such as chloramphenicol ([73] and G. Luan et al., unpublished observations). Among the effects of chloramphenicol is inhibition of respiration, the source of ROS [62]. We found that a deficiency of catalase (Δ*katG*) raised nalidixic

acid-induced ROS levels and overcame the inhibitory effect of chloramphenicol on quinolone-mediated killing. Thus, the inhibitory effects of chloramphenicol must not be absolute. As expected, stimulation of nalidixic acid lethality by Δ*katG* was blocked by additional treatment with a combination of thiourea and bipyridyl.

Nalidixic acid also inhibits protein synthesis [73], a finding that leads to the following explanation for high-concentration tolerance. At high concentrations, nalidixic acid causes chromosome breakage [36, 74] that leads to loss of DNA supercoiling. Initiation of transcription and therefore translation are expected to decline when supercoiling is lost, which will lead to a reduction in respiration and ROS. Since DNA damage can be repaired during the long incubation period required to measure survival (X. Wang & X. Zhao, unpublished observations), a reduction of ROS would allow bacterial survival. Thus, the ROS-lethality hypothesis provides an explanation for a decades-old quinolone mystery.

20.9 Thymineless Death and Related Antimicrobials

A study of thymineless death provides insight into one way in which ROS kill bacteria. Thymineless death refers to the rapid loss of bacterial viability that occurs during starvation for thymine or thymidine. The phenomenon is of considerable interest, because the underlying molecular events also apply to the action of several antibacterial (trimethoprim, sulfamethoxazole), antimalarial (pyrimethamine, sulfonamide), anticancer (methotrexate, fluorouracil), and immune-modulating (methotrexate) agents. Many explanations have been proposed to explain thymineless death. Among these are unbalanced growth, toxin-antitoxin module action, nucleotide misincorporation, induction of the SOS regulon, destruction of replication forks, and partial degradation of DNA at *oriC* [75, 76]. None of these explanations have satisfied all of the experimental observations. Recent attention has focused on proteins involved in recombinational repair, as some appear to contribute to death while others facilitate survival [77–80].

Since DNA replication is severely slowed by withdrawal of thymidine and since other means of inhibiting DNA replication (fluoroquinolone or hydroxyurea treatment) cause the accumulation of ROS, we examined the possibility that ROS contribute to thymineless death. Two processes appear to be required for rapid death: generation of persistent single-strand DNA regions and accumulation of ROS [21]. The attack of single-strand DNA regions by ROS then leads to lethal double-stranded DNA breaks. Interference with either production of single-strand DNA or accumulation of ROS inhibits thymineless death. Our current view is that proteins involved in recombinational DNA repair, such as RecF and RecQ, expand the single-strand DNA substrate for ROS attack (the absence of these proteins reduces thymineless death). Other DNA repair proteins, such as RecBC, are involved in surviving the damage, and the SOS response increases expression of SulA, which serves as a checkpoint that halts cell division until the damage to DNA is repaired in cells that do not die [77].

Although both quinolone treatment and thymine starvation block replication, which is presumably a signal for initiating ROS accumulation, subsequent events must differ, because different genes are involved. For example, ROS accumulation and thymineless death are unaffected by the absence of toxin-antitoxin modules, and disruption of *arcA/B*, *lon, clpA/P, cpxA/R*, or *ssrA* has little effect on thymineless death [21]. Nevertheless, surges in ROS and involvement of the respiratory chain are common features [25, 61, 62]

20.10 Potential Consequences of Antioxidant Consumption

Part of the evidence supporting a role for ROS in antimicrobial lethality stems from the protective effects of antioxidants. For example, thiourea, glutathione, and vitamin C reduce fluoroquinolone lethality by orders of magnitude [18, 25, 51, 81]; thiourea and glutathione reduce lethality of daptomycin and oxacillin by 10–100-fold [51]. Since human consumption of antioxidants is large [82], the potential for interference with antimicrobial action exists. One aspect may involve food products. In a recent study, Marathe et al. [83] examined effects of the antioxidant curcumin on ciprofloxacin-mediated lethality with *Salmonella*. Curcumin is a common food ingredient in Southeast Asia, and it is often used medicinally. Curcumin reduces the lethal activity of ciprofloxacin with cultured bacteria, with bacteria infecting macrophages, and with bacteria infecting mice. Since curcumin also suppresses the antibacterial activity of the immune response, it may act in two ways to increase bacterial survival [83]. Thus, a cautionary note has been raised concerning antibiotic therapy and consumption of foods having antioxidant activity [83].

Antioxidants are also consumed as nutritional supplements. One of the popular agents is resveratrol, a natural polyphenol antioxidant [81]. Resveratrol is thought to have beneficial effects for ailments such as cardiovascular disease [84], neurodegenerative disorders [84, 85], and some forms of cancer [84, 86]. To address whether antioxidant nutritional supplements are likely to interfere with the ability of antimicrobials to kill bacteria, we added resveratrol to cultures of *E. coli* and *S. aureus*, treated them with antimicrobial, and assayed for bacterial survival [87]. Resveratrol, at concentrations likely to be present during human consumption, reduced killing by two- to threefold during a 2-h exposure to moxifloxacin or kanamycin. At higher but still subinhibitory concentrations, resveratrol lowered antimicrobial lethality by more than 1000-fold. Resveratrol also reduced the accumulation of ROS characteristic of treatment with oxolinic acid, a first-generation quinolone. Collectively these observations support the general idea that the lethal activity of some antimicrobials involves ROS.

Subinhibitory concentrations of resveratrol also promoted (two- to sixfold) the recovery of rifampicin-resistant mutants arising from the action of ciprofloxacin, kanamycin, or daptomycin. This finding can be explained by resveratrol lowering ROS to sublethal levels that are still mutagenic, while the absence of resveratrol allows ROS levels to be high enough to kill mutagenized cells. Suppression of kill-

ing by antimicrobials and promotion of mutant recovery by curcumin and resveratrol suggest that antioxidant consumption may contribute to the emergence of antimicrobial resistance, especially if new derivatives and/or formulations of resveratrol markedly raise bioavailability. One approach to preserving antibiotic activity would be to suspend antioxidant consumption during treatment with lethal antimicrobials.

20.11 Unresolved Issues

20.11.1 In Vitro Systems

Several aspects of the ROS-lethality hypothesis require additional work. A central issue is to understand how a bacterium communicates information about an antimicrobial lesion to the apparatus that produces ROS. How the initial lesion is detected is not known for any system. A second issue concerns events that occur during anaerobic growth: some antimicrobials are lethal in the absence of oxygen, which would preclude involvement of ROS. Although killing may proceed through the direct action of primary lesions, we postulate that under anaerobic conditions, non-oxygen centered radicals perform the same function as ROS during aerobic growth. That hypothesis is currently being tested.

Chemical probes present a different type of challenge. While both thiourea and bipyridyl protect from antimicrobial lethality, off-target effects are difficult to eliminate. In the case of iron chelators, such as 2,2′-bipyridyl, action could occur in many ways, since bacterial cells contain a variety of iron-containing proteins that might affect a crucial activity unrelated to ROS accumulation. Excluding off-target effects using mutants is a challenge for events, such as the Fenton reaction, that are not mediated by protein or RNA, because resistant mutants are not available as controls. Nevertheless, ROS do not accumulate when nalidixic acid-treated cells are administered bipyridyl (G. Luan et al., unpublished observation).

20.11.2 Contribution of Infection

During infection, bacteria experience stress from host defense systems. One consequence may be preparation of the pathogen for antimicrobial-mediated stress. For example, host-derived reactive oxygen or reactive nitrogen species may induce bacterial protective systems that would counter bacterial production of ROS induced by antimicrobials. According to the scheme presented in Fig. 20.1, suppression of ROS-mediated killing would constitute a form of bacterial tolerance.

Heritable, genetic changes that increase antimicrobial tolerance can be selected both in vitro and in vivo. Indeed, genome-wide maps of tolerance-associated genes, the “tolerome,” have been described [88] that include numerous genes and pathways

that slow bacterial growth. Particularly interesting are mutations in global transcriptional regulators, because they can produce a rapid, pleiotropic phenotypic effect: they may constitute a prominent mechanism underlying tolerance. A clinically relevant example of a global regulator of tolerance is seen with *S. aureus* mutants that are deficient in the quorum-sensing *agr* regulon (see also Chap. 14). Paradoxically, defects in this virulence factor are selected during serious hospital infection and are associated with worse outcome [89, 90]. Recent work indicates that Δ*agr* mutants are less readily killed by gentamicin, ciprofloxacin, and several other lethal stressors without affecting MIC [91], thereby conforming to a tolerance phenotype. Wild-type *agr* normally downregulates a peroxidase that would otherwise protect from ROS-mediated quinolone lethality. In the absence of *agr* and downregulation, ciprofloxacin loses some lethality. With gentamicin and daptomycin, modulation of *S. aureus* tolerance by Δ*agr* involves leakage of bacterial components that affect antimicrobial lethality [91, 92]. Thus, mutation of global regulators during clinical infection can result in multiple phenotypes associated with tolerance that are not detected as resistance.

20.12 Concluding Remarks

The initial assertion that ROS contribute to the lethal action of multiple antimicrobial classes [25] has expanded to include a contribution of ROS to several severe stress conditions. The idea that bacteria produce toxic ROS supports the more general concept that bacteria respond to lethal stress by self-destructing. Existing data, both direct and indirect, lead to the following scenario: when bacteria experience severe stress, they make a live-or-die decision based on the response of several bifunctional genes that are protective at low levels of stress but destructive at high levels (Fig. 20.2). Destruction is achieved by ROS accumulation exceeding a threshold beyond which genes designed to protect from oxidative stress fail to halt the self-amplification of ROS. How bacterial suicide may confer a selective advantage upon bacterial populations is currently unknown (see [4]).

The bifunctional nature of the lethal stress-response system makes application of the principles challenging, because lethal treatment may be either enhanced or diminished by increasing oxidative stress. For example, elevation of intracellular superoxide levels prior to drug exposure may be protective, because the cells are less susceptible to antimicrobials due to induction of protective genes. However, if stress is severe, ROS accumulation will lead to cell death. Collectively, these observations suggest that superoxide concentration, localization (intracellular, extracellular), and regulation are complex. They may even change during the course of drug exposure and infection. Nevertheless, several practical applications emerge from ROS contributing to antibacterial lethality. One is to avoid consuming antioxidants during therapy with lethal antimicrobials, as antioxidants quench ROS. Another is that ROS accumulation may represent a new form of interfering cross-reaction for tolerance

among seemingly unrelated drugs: drugs that reduce metabolism could inhibit the production of ROS needed for full lethal activity of other antimicrobials.

Overall, consideration of ROS enriches our understanding of how bacteria respond to severe stress and opens new ways to control bacterial populations.

Major Points

- Although lethal activity of current antimicrobials may be sufficient to clear most infections, antimicrobial lethality needs to be even more rapid and extensive to restrict the induction of resistance.
- Bacteria exhibit a lethal, ROS-mediated stress response that contributes to the lethal activity of multiple antimicrobial classes; bacteriostatic assays of antimicrobial activity are often insensitive to perturbation of lethal stress responses.
- The effects of ROS are transient, which makes measurements of MBC uninformative with respect to rapid killing and suppression of induction of new resistant mutants.
- Suppression of the ROS-mediated lethal stress response with antioxidants may contribute to the emergence of resistance.
- Modulation of the ROS-mediated lethal stress response offers a new way to control bacterial populations.

Acknowledgments We thank the following for critical comments on the manuscript: Marila Gennaro and Bo Shopsin.

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Chapter 21
PK/PD-Based Prediction of "Anti-Mutant" Antibiotic Exposures Using In Vitro Dynamic Models

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21.1 Introduction

Antibiotic resistance is emerging among a wide variety of pathogens responsible for both common and infrequent infections. This has stimulated a search for so-called "anti-mutant" antibiotic dosing regimens, i.e., regimens that might prevent or severely restrict the enrichment of resistant mutant subpopulations that can be designed from drug concentration-resistance relationships. Clinical reports concerning the selection of antibiotic-resistant mutants and/or loss in susceptibility of bacterial pathogens during treatment are too sparse to allow delineation of these concentration-resistance relationships. For this reason, PK/PD (pharmacokinetic/pharmacodynamic) relationships with the emergence of bacterial resistance have been studied in vitro using dynamic models and, to a lesser extent, in vivo, using animal models. Comprehensive reviews of these studies published recently [1, 2] cover most of the in vitro and in vivo studies on this topic. Both reviews indirectly reflect discrepancies and even contradictions among results reported from various study groups. Given this situation and, especially, controversial interpretations of resistance data by some authors, we address whether the inconsistencies among the reported findings are real or apparent. We also highlight shortcomings and/or limitations in study design and analysis that might have led to unjustified conclusions. Since the contribution of in vitro studies to the current knowledge of concentration-resistance relationships seems precise, data obtained in dynamic models are analyzed in this chapter, with special emphasis on the mutant selection window (MSW)

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I. W. Fong et al. (eds.), *Antimicrobial Resistance in the 21st Century*, Emerging Infectious Diseases of the 21st Century, https://doi.org/10.1007/978-3-319-78538-7_21

hypothesis [3, 4]. In this regard, the present chapter is an extension of an earlier publication [5].

21.2 Antibiotic Concentration-Bacterial Resistance Relationships

Targeted bacterial resistance studies using in vitro dynamic models [6–16] were launched at the turn of the new millennium. In most of the resistance studies, loss in susceptibility of antibiotic-exposed pathogens and/or the enrichment of resistant mutant subpopulations was related to the ratio of 24-h area under the concentration-time curve (AUC_{24}) to the MIC. The fact that AUC_{24}/MIC ratio rather than AUC_{24} per se related to susceptibility and/or population analysis data implies that AUC_{24}/MIC is an inter-strain resistance predictor that allows generalization of findings obtained with different bacterial strains.

Contrary to expectations, most of these early works did not reveal clear relationships between antibiotic exposure, expressed by the AUC_{24}/MIC ratio, and the loss in susceptibility of antibiotic-exposed pathogen cultures and/or the enrichment of resistant mutants. For example, similar low resistance frequencies were reported in an in vitro staphylococcal study at AUC_{24}/MICs for ciprofloxacin that varied by a factor of 16 [7]. Moreover, in the same study, frequency of resistance in *Staphylococcus aureus* exposed to norfloxacin was paradoxically more pronounced at a relatively high AUC_{24}/MIC ratio (55 h) than at a low value of AUC_{24}/MIC (3 h). Obviously, in the absence of relationships between AUC_{24}/MIC and loss in susceptibility of antibiotic-exposed pathogens and/or the enrichment of resistant mutant subpopulations, the AUC_{24}/MIC thresholds reported to protect against the enrichment of resistant mutants [8–15] are questionable.

These failures result from shortcomings in study design summarized elsewhere [5], among which are the absence of resistant mutants in the starting inoculum, insufficient duration of simulated treatments, simulation of AUC_{24}/MIC ratios that provide either sub-optimal or super-optimal effects of antibiotics on resistant subpopulation(s) but not intermediate AUC_{24}/MICs ratios, and inappropriate data analysis. In particular, simple AUC_{24}/MIC relationships with the emergence of resistance – the greater the AUC_{24}/MIC ratio, the less pronounced the enrichment of resistant mutants or loss in susceptibility of antibiotic-exposed bacteria – were reported in some studies, although more complex relationships between antibiotic concentration and bacterial resistance are expected based on the “mutant selection window” (MSW) hypothesis [3, 4]. According to this idea, resistant mutants are selectively enriched at antibiotic concentrations between the MIC and the mutant prevention concentration (MPC) but not at concentrations below the MIC or above the MPC.

Strong support for the window hypothesis was first provided by an in vitro dynamic model study in which *S. aureus* was exposed to 3-day dosing with

ciprofloxacin, gatifloxacin, levofloxacin, or moxifloxacin such that peak concentrations were equal to the MIC, between the MIC and MPC and above the MPC [17]. Loss in fluoroquinolone susceptibility, expressed by the ratio of postexposure MIC (MIC_{final}) to the pre-exposure MIC ($MIC_{initial}$), was observed at concentrations that fell inside the MSW but not at concentrations below the MIC or above the MPC. By using the combined data for the four fluoroquinolones administered over a wide range of the AUC_{24}/MIC ratio, the AUC_{24}/MIC relationship to $MIC_{final}/MIC_{initial}$ was seen as a bell-shaped curve fitted by a Gaussian-type function. The loss in susceptibility of *S. aureus* occurred at AUC_{24}/MIC ratios of 25–100 h but not at AUC_{24}/MICs <15 h or > 200 h.

Given the bell-shaped pattern of the AUC_{24}/MIC relationship with $MIC_{final}/MIC_{initial}$, the previous failures to correlate resistance with AUC_{24}/MIC using linear or log-linear regression are understandable. Moreover, the bell-shaped AUC_{24}/MIC-resistance relationships established with fluoroquinolone-exposed *S. aureus* [17] allowed us to take a fresh look at earlier studies that failed to link bacterial resistance manifestations with antibiotic exposures. For example, despite the use of different endpoints of resistance (resistance frequency in a staphylococcal study with norfloxacin and ciprofloxacin [7] and susceptibility measurements in a study with four fluoroquinolones [17]), data were consistent with a bell-shaped curve when $MIC_{final}/MIC_{initial}$ was plotted against AUC_{24}/MIC. Indeed, the higher resistance frequency seen at a relatively high AUC_{24}/MIC ratio for norfloxacin (55 h) compared to the lower frequency at a low AUC_{24}/MIC (3 h) can be explained by the former AUC_{24}/MIC value corresponding to the maximum on the bell-shaped curve with the latter AUC_{24}/MIC value corresponding to the beginning of the ascending portion of the $MIC_{final}/MIC_{initial}$-$AUC_{24}$/MIC curve [17]. Low ciprofloxacin resistance frequencies at AUC_{24}/MIC of 11 and 178 h are also explainable: the former value corresponds to the beginning of the ascending portion of the curve and the latter to the end of descending portion of the bell-shaped $MIC_{final}/MIC_{initial}$-$AUC_{24}$/MIC curve. Thus, apparently paradoxical data reported with ciprofloxacin and norfloxacin [7] are explained by the specific shape of the AUC_{24}/MIC-resistance curves.

Bell-shaped curves that reflect AUC_{24}/MIC-$MIC_{final}/MIC_{initial}$ relationships also have been observed with *S. aureus* exposed to levofloxacin [18, 19] and the investigational fluoroquinolone ABT 492 [18]. Unlike simulations of the pharmacokinetics of the four fluoroquinolones described above [17], when AUC_{24}/MIC-$MIC_{final}/MIC_{initial}$ curves were superimposed, similar curves for *S. aureus* exposed to levofloxacin and ABT 492 did not superimpose. Bell-shaped AUC_{24}/MIC relationships have also been observed with 48-hour colony counts of resistant *S. aureus* in a garenoxacin study [20]. Fragments of a bell-shaped curve of the AUC_{24}/MIC-dependent population analysis profile (PAP) index were observed in a study that exposed *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* to moxifloxacin [21], and a bell-shaped curve has been reported with moxifloxacin-exposed *S. pneumoniae* [22]. As with *S. aureus* [17], the most pronounced loss in susceptibility of *S. pneumoniae* to the fluoroquinolone was observed at the intermediate AUC_{24}/MIC ratios of 40–50 h. Using population analysis data, a similar bell-shaped relationship was established between AUC_{24}/MIC and the ratio of mutation frequency (f)

observed with moxifloxacin-exposed *S. pneumoniae* (f_{final}) to the frequency of mutation before antibiotic dosing ($f_{initial}$). The $f_{final}/f_{initial}$ ratio (mutants resistant to 4 × MIC) was maximal at the intermediate value of AUC_{24}/MIC of 40 and 60 h.

Qualitatively similar AUC_{24}/MIC-resistance relationships have been established with fluoroquinolone-exposed Gram-negative bacteria [20, 23–28]. Unlike resistance studies with Gram-positive bacteria, population analysis data were used in most Gram-negative studies. With population data, not only posttreatment sizes of resistant subpopulations [20], which are insufficiently informative [29, 30], but integral evaluation of the time courses of resistant mutant enrichment over the entire simulated treatment [24–28] was used. The area under the mutant concentration-time curve ($AUBC_M$) [29] served as an endpoint of bacterial resistance and was related to simulated AUC_{24}/MIC ratios. With $AUBC_M$-based analysis, bell-shaped relationships were observed with ciprofloxacin-exposed *E. coli* (four strains: [24, 25]), *K. pneumoniae* (three strains [27]), and *P. aeruginosa* (four strains [26, 28]). A similar pattern was observed with the AUC_{24}/MIC relationship using 48-hour colony counts for resistant *P. aeruginosa* in the above-mentioned garenoxacin study (one strain) [20] and with the $MIC_{final}/MIC_{initial}$ ratio as an endpoint in a study that exposed *E. coli* to two veterinary fluoroquinolones – enrofloxacin and marbofloxacin (data presented for one strain [23]). Also, bell-shaped relationships between $MIC_{final}/MIC_{initial}$ and AUC_{24}/MIC could be reconstructed [17] based on data reported in a study with levofloxacin- and trovafloxacin-exposed *Bacteroides fragilis* [15]. Thus, fluoroquinolones clearly exhibit a bell-shaped relationship between drug exposure and mutant enrichment or loss in bacterial susceptibility for both Gram-positive and Gram-negative bacteria.

Although most resistance studies have been performed with fluoroquinolones, bell-shaped curves showing AUC_{24}/MIC-dependent changes in $MIC_{final}/MIC_{initial}$ and in the ratio of maximal-to-initial numbers of resistant mutants ($N_{max}/N_{initial}$) have been reported with daptomycin- and vancomycin-exposed *S. aureus* (two strains) [31]. Similar curves were observed when plotting $AUBC_M$ against AUC_{24}/MIC for daptomycin [32]. A bell-shaped relationship was also observed with resistant *E. coli* subpopulation densities after a 24-h exposure to q.i.d fosfomycin [33]. Moreover, a bell-shaped relationship between $AUBC_M$ and AUC_{24}/MIC was recently established with linezolid-exposed *S. aureus* (three strains) [34]. It is worth noting that for linezolid studies, it was necessary to include a small number of resistant cells in the starting inoculum [35], since multiple copies of the linezolid target are likely to exist and therefore require spontaneous mutants to have multiple mutations, a rare event.

Overall, the findings mentioned above indicate that the relationship of AUC_{24}/MIC with resistance is bell shaped for antibiotics of several classes and with both Gram-positive and Gram-negative pathogens. Thus, AUC_{24}/MIC can be used to predict the emergence of resistance.

21.3 Alternative Predictors of Resistant Bacteria Enrichment

Although the AUC_{24}/MIC ratio has been related to the enrichment of resistant bacterial mutants, it is not the only predictor for the emergence of antibiotic resistance. Other predictors that emerge from the mutant window hypothesis are the ratio of AUC_{24} to the MPC, the time during which antibiotic concentration is inside the MSW (T_{MSW}), and the time when drug concentration is above the MPC ($T_{>MPC}$). Each is briefly discussed below.

21.3.1 AUC_{24}/MPC

From the standpoint of the MSW hypothesis, the AUC_{24}/MPC ratio rather than the AUC_{24}/MIC might better predict the enrichment of resistant mutants and/or loss in susceptibility of antibiotic-exposed bacteria [36–38] because MPC directly measures mutant susceptibility, which in many cases does not correlate well with bulk culture MIC. Nevertheless, AUC_{24}/MIC- and AUC_{24}/MPC-resistance relationships for a given organism can differ only in quantitative, not in qualitative terms (bell-shaped curves shifted along the *x*-axis). Consequently, the predictive potentials of AUC_{24}/MIC and AUC_{24}/MPC ratios can be distinguished most clearly by their ability to serve as inter-strain predictors of resistance.

Obviously, bacterial resistance studies that utilize at least two bacterial strains with different MPC/MIC ratios are needed to distinguish between AUC_{24}/MPC and AUC_{24}/MIC as potential predictors of resistance. To compare the abilities of AUC_{24}/MPC and AUC_{24}/MIC as inter-strain predictors of resistance, two strains of *S. aureus* with distinctly different MPC/MIC (4 versus 16) were used in a study that simulated twice-daily dosing of ciprofloxacin for 3 days [29]. When comparing the descending portions of the AUC_{24}/MPC and AUC_{24}/MIC relationships with $AUBC_M$ over a wide range of drug exposure, the AUC_{24}/MIC plots were more stratified than the respective AUC_{24}/MPC plots. For example, with mutants resistant to 4 × MIC ciprofloxacin, the square correlation coefficient for the $AUBC_M$ against log AUC_{24}/MPC relationship was 1.6-fold greater (r^2 0.70) than for the $AUBC_M$ against log AUC_{24}/MIC relationship (r^2 0.43). Even greater differences between AUC_{24}/MPC and AUC_{24}/MIC relationships were reported with mutants resistant to 8 × MIC of antibiotic (r^2 0.72 versus 0.35). Figure 21.1 shows a systematic increase in the predictive power of AUC_{24}/MPC and a concomitant decrease in the predictive power of AUC_{24}/MIC with an increase in culture MIC. These findings suggest that the AUC_{24}/MPC ratio is a more potent inter-strain predictor for staphylococcal resistance to the fluoroquinolone than the AUC_{24}/MIC ratio. This implies lower strain-to-strain variability in AUC_{24}/MPC thresholds that prevent mutant enrichment. Less variation in the "anti-mutant" thresholds is desired because clinical recommendations need to be suitable for many strains.

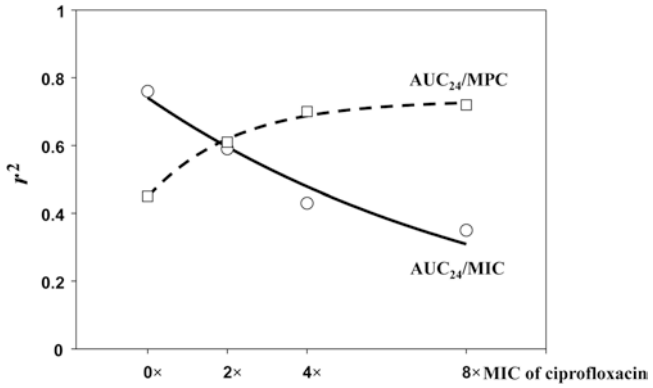

Fig. 21.1 MPC- and MIC-related pharmacokinetic variables as predictors of the enrichment of ciprofloxacin-resistant mutants of *S. aureus*. (Reconstructed from Ref. [29])

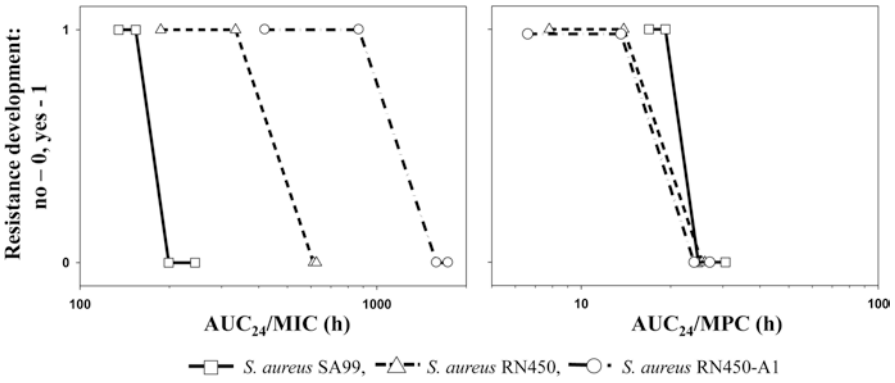

Fig. 21.2 AUC_{24}/MIC- and AUC_{24}/MPC-dependent resistance of levofloxacin-exposed *S. aureus*. (Reconstructed from Ref. [19])

The distinct advantages of the AUC_{24}/MPC over the AUC_{24}/MIC ratio were demonstrated subsequently in a similarly designed study with levofloxacin-exposed *S. aureus* [19]. In this work three strains with the same MIC for levofloxacin but with distinctly different MPCs (MPC/MIC from 8 to 64) were treated with once-daily fluoroquinolone for 3 days. According to our analysis, plotting $MIC_{final}/MIC_{initial}$ against either AUC_{24}/MPC or AUC_{24}/MIC did not allow combination of data obtained with individual *S. aureus* strains: both AUC_{24}/MPC and AUC_{24}/MIC relationships with $MIC_{final}/MIC_{initial}$ were too stratified to be combined. However, qualitative characteristics of resistance, i.e., the loss in susceptibility (posttreatment MIC elevation) or the absence of such a loss, were better correlated to AUC_{24}/MPC than to AUC_{24}/MIC in a strain-independent manner. Reconstructed from reported data [19], Fig. 21.2 demonstrates bacterial strain specificity of the AUC_{24}/MIC-resistance relationships in contrast to the strain-independent AUC_{24}/MPC-resistance

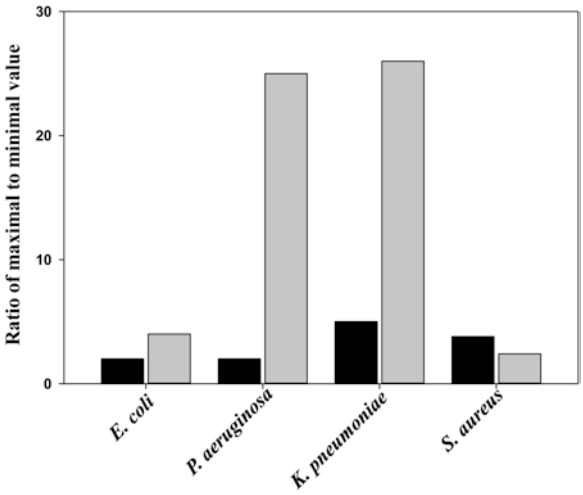

Fig. 21.3 Strain-to-strain variability in the "anti-mutant" thresholds of AUC_{24}/MIC (■) and AUC_{24}/MPC (□). (Reconstructed from Ref. [24, 27–29])

relationship. As seen in the figure, unlike the stratified AUC_{24}/MIC plots observed with individual strains, the respective AUC_{24}/MPC are virtually superimposed.

In contrast to fluoroquinolone-exposed *S. aureus*, with Gram-negative bacteria, correlations between $AUBC_M$, which reflects the enrichment of ciprofloxacin-resistant mutants, and simulated AUC_{24}/MPC were not as strong as between $AUBC_M$ and AUC_{24}/MIC. The respective r^2s with ciprofloxacin-exposed *Escherichia coli* were 0.69 versus 0.86 [24], with *Klebsiella pneumoniae* they were 0.72 versus 0.76 [27], and with *P. aeruginosa* they were 0.65 versus 0.75 for the AUC_{24}/MPC and AUC_{24}/MIC ratios [28]. This difference between Gram-negative and Gram-positive bacteria could reflect strain-to-strain variability in the "anti-mutant" thresholds. As seen in Fig. 21.3, with each Gram-negative situation, the scattering of the "anti-mutant" AUC_{24}/MPC was more pronounced than with AUC_{24}/MIC: with *E. coli* it was 4-fold versus 2-fold, with *K. pneumoniae* it was 25-fold versus 2-fold, and with *P. aeruginosa* it was 26-fold versus 5-fold differences. Unlike the Gram-negative bacteria tested, *S. aureus* strains exhibit less variable "anti-mutant" AUC_{24}/MPC ratios than the respective AUC_{24}/MIC ratios (2.4-fold versus 3.8-fold differences).

In a recent study that exposed *S. aureus* strains to 5-day treatment with linezolid (MPC/MIC from 2.5 to 5), a lower r^2 (0.79) was also reported for the $AUBC_M$ relationship with AUC_{24}/MPC than with AUC_{24}/MIC (r^2 0.91) [34]. However, strain-to-strain variability for the "anti-mutant" AUC_{24}/MPC and AUC_{24}/MIC ratios was identical: twofold differences (from 48 to 96 h and from 120 to 240 h, respectively). In another study that simulated 5-day treatments of two strains of *S. aureus* with daptomycin (MPC/MIC from 3 to 5) and vancomycin (MPC/MIC from 3 to 8) [31], the AUC_{24}/MPC ratio was less predictive for *S. aureus* resistance than the AUC_{24}/MIC ratio. In contrast to reasonable AUC_{24}/MIC relationships with $N_{max}/N_{initial}$ (r^2 0.68 for mutants resistant to 2 × MIC and r^2 0.66 for mutants resistant to 4 × MIC of the tested antibiotics) and $MIC_{final}/MIC_{initial}$ (r^2 0.64), there was no correlation between AUC_{24}/MPC and either population analysis or susceptibility data. Based on

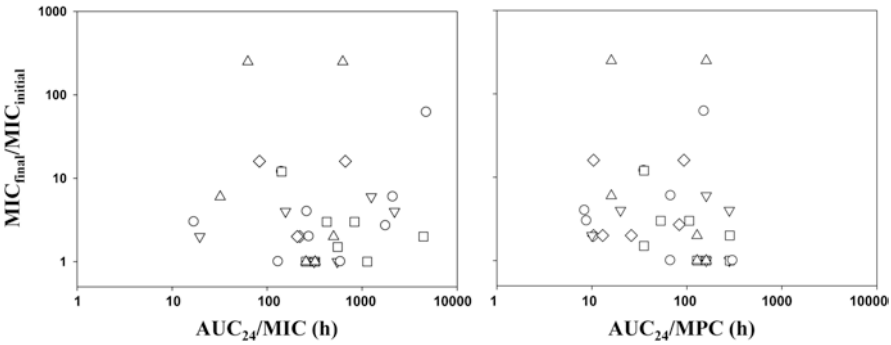

Fig. 21.4 AUC$_{24}$/MIC- and AUC$_{24}$/MPC-dependent resistance of *S. aureus* to five fluoroquinolones: ciprofloxacin (◊), gatifloxacin (▽), gemifloxacin (△), levofloxacin (□), moxifloxacin (○). (Reconstructed from Ref. [39])

data obtained with two *S. aureus* strains exposed to daptomycin and vancomycin, MIC$_{final}$/MIC$_{initial}$ plots against AUC$_{24}$/MPC were characterized by widely scattered points. Thus, preference for a particular parameter may vary according to the antibiotic-pathogen pairs studied.

In this section we confined ourselves to studies containing clear evidence on the advantages or disadvantages of AUC$_{24}$/MPC over AUC$_{24}$/MIC. There are other studies in which the conclusion that one of the predictors of bacterial resistance (usually AUC$_{24}$/MPC ratio) is preferable was not supported by the experimental findings. One example is a resistance study with two strains of *S. aureus* exposed to five fluoroquinolones (three AUC$_{24}$/MPC ratios per one antibiotic-pathogen pair, MPC/MIC ratios from 2 to 15) [39]. According to the author's statement, AUC$_{24}$/MPC was recognized as "the only parameter to correlate with the development of resistance" although this correlation was extremely weak (r^2 0.2). As seen in Fig. 21.4, when plotting MIC$_{final}$/MIC$_{initial}$ against simulated AUC$_{24}$/MPC or AUC$_{24}$/MIC ratios, a cloud of scattered points is observed using both potential predictors for emergence of staphylococcal resistance. It is possible that this scatter could be avoided by using a wider range of simulated AUC$_{24}$/MPC or AUC$_{24}$/MIC ratios and treatments longer than 48 h. At least 72-hour treatments were recommended in our resistance studies with fluoroquinolones [32]. Short observation times (24 h) could have affected the results even more for a single-dose study with ciprofloxacin-exposed *E. coli* (three strains with MPC/MIC from 4 to 16) [40]. For this reason, the authors' conclusion that "AUC/MPC ratio was the single pharmacodynamic index that predicted prevention of resistant mutant development" should be taken with caution.

Thus, contrary to expectations, use of AUC$_{24}$/MPC as an inter-strain predictor of resistance has an advantage over AUC$_{24}$/MIC only with some antibiotic-pathogen pairs (fluoroquinolone-exposed *S. aureus*) but not with other pairs (fluoroquinolone-exposed *E. coli, K. pneumoniae, P. aeruginosa*, daptomycin- and vancomycin-exposed *S. aureus*). Further studies are needed to understand apparent differences among bacterial species and antimicrobials.

21.3.2 T_{MSW}

Unlike AUC_{24}/MIC, which is commonly accepted as a predictor for the emergence of bacterial resistance, T_{MSW} (time in the mutant selection window) has been used only infrequently, even though this parameter is closely linked to the MSW hypothesis. A sigmoid T_{MSW} relationship with resistance was discovered in the study, described above, that exposed *S. aureus* to four fluoroquinolones [17]. The MIC_{final}/$MIC_{initial}$ increased systematically with increases in T_{MSW} when expressed as a percentage of the dosing interval. Subsequently, similar relationships were observed with gatifloxacin-exposed *S. aureus* when simulating normal and impaired elimination pharmacokinetics (half-lives 7 and 31 h, respectively) [41]. At both half-lives, the T_{MSW} plots of the MIC_{final}/$MIC_{initial}$ ratio were sigmoid in shape, but they were different for normal and impaired elimination of gatifloxacin: at a given T_{MSW}, the loss in susceptibility of antibiotic-exposed *S. aureus* was more pronounced in the normal than in the impaired case, showing pharmacokinetic profile-dependent emergence of resistance. Similar relationships were also observed with daptomycin- and vancomycin-exposed *S. aureus* [31] using population analysis and susceptibility data: a systematic increase in the MIC_{final}/$MIC_{initial}$ and N_{max}/$N_{initial}$ was associated with longer T_{MSW}s. These findings suggest that the T_{MSW} may be an additional predictor of bacterial resistance.

Against this background, other studies [40, 42–44] called into question the relevance of T_{MSW} for predicting loss in susceptibility and/or the enrichment of resistant mutants. Our analysis, described below, indicates that conclusions drawn in these studies are incorrect, and/or they are unsupported by the reported data. For example, the false impression that T_{MSW} does not predict *S. aureus* resistance [42] resulted from the unjustified combination of data obtained in simulations of conventional dosing regimens in which ciprofloxacin concentrations exceeded the MIC plus constant rate infusions in which antibiotic concentrations were close to the MIC (1.2 × MIC), a situation that may be described as providing T_{MSW} of either 100% or 0% of the dosing interval. Meanwhile, by plotting MIC_{final}/$MIC_{initial}$ against T_{MSW} achieved in simulations of only conventional, intermittent ciprofloxacin dosing, a reasonable T_{MSW}-resistance relationship could be established (Fig. 21.5). Therefore, the authors' conclusion for the lack of "a clear relationship between T_{MSW} and the degree of resistance" [42] contradicts their own data. A similar relationship can be seen with ciprofloxacin-exposed *E. coli* [40], at least for one of three strains for which quantitative resistance data were presented (Fig. 21.6). Consequently, the conclusion drawn by the authors about the lack of "a simple relationship between T_{MSW} and the prevention of the emergence of resistance" is not supported by the experimental findings. We conclude that the data reported in these two studies [40, 42] are more in support of, rather than against, using T_{MSW} as a predictor of bacterial resistance.

In another study, measurements with isoniazid-exposed *Mycobacterium tuberculosis* led to the conclusion that "T_{MSW} does not predict the emergence of resistance" [43]. Given the antibiotic half-life-dependent relationships of T_{MSW} with resistance

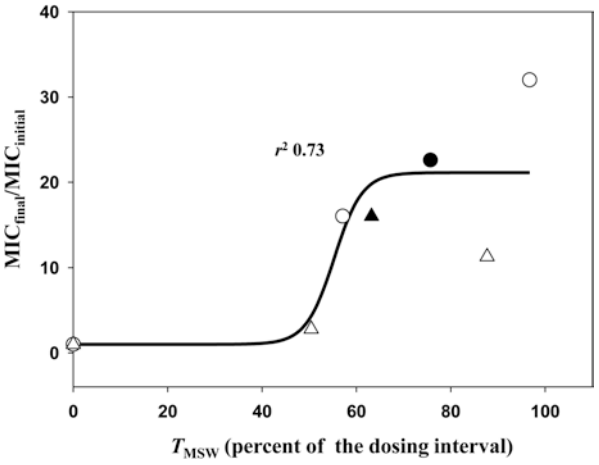

Fig. 21.5 T_{MSW}-dependent loss in susceptibility of *S. aureus* 8043 (◯) and 8282 (△) exposed to twice-daily (white symbols) and thrice-daily (black symbols) ciprofloxacin. (Reconstructed from Ref. [42]) fitted by equation: $Y = Y_0 + a/\{1 + \exp[-(x - x_0)/b]\}$ (Eq. 1). $Y_0 = 1$, $a = 20.13$, $b = 3.000$, $x_0 = 55.26$

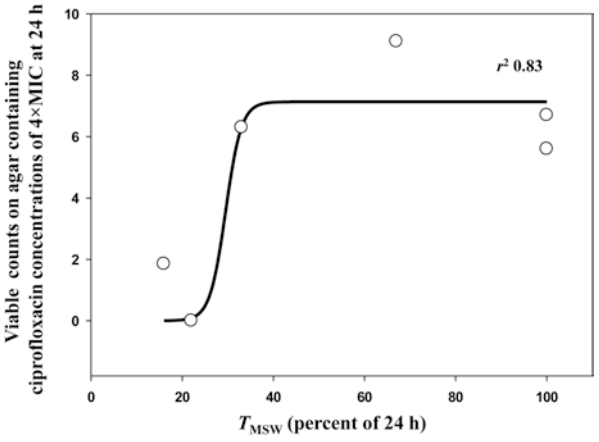

Fig. 21.6 T_{MSW}-dependent resistance of *E. coli* Nu14 to ciprofloxacin (half-life 4 h). (Reconstructed from Ref. [40]) fitted by Eq. 1: $Y_0 = 0$, $x_0 = 29.52$, $a = 7.134$, $b = 1.727$

[41], this conclusion was the result of inappropriate combination of data obtained in simulations of isoniazid pharmacokinetics in fast and slow acetylators (half-lives 1.8 and 4.2 h, respectively). Quite possibly fast and slow acetylator data might relate to different T_{MSW}-resistance relationships. On the other hand, there were insufficient data to establish a specific relationship for each type of pharmacokinetic profile (two points per one profile only). Moreover, in both cases, T_{MSW} varied over very small ranges: from 30% to 54% (fast acetylator simulations) and from 80% to 100% (slow accelerator simulations) of the dosing interval. Based on these limited data, delineation of a relationship or lack of a relationship is questionable.

A less clear situation is found with a study [44] in which T_{MSW} failed to be predictive for moxifloxacin and levofloxacin resistance with *S. pneumoniae* (four strains exposed to 3-day treatments with the fluoroquinolones). In this case the unsuccessful attempts to relate susceptibility of antibiotic-exposed bacteria with T_{MSW} might

have resulted from the overestimation of MPCs, at least for a *S. pneumoniae* strain that exhibited a biphasic pattern in the frequency-concentration curve. As shown in our study with ciprofloxacin-exposed *E. coli* [24], the higher MPC derived from the second phase of such curves describes the second-step mutations that might or might not be present in pharmacokinetic simulations. As a result, the true value of T_{MSW} could be overestimated, and the value of $T_{>MPC}$ could be underestimated. Moreover, in most simulations (e.g., in five out of six experiments with moxifloxacin), there was no loss in susceptibility of *S. pneumoniae*. Because of the unbalanced study design, T_{MSW} was used to explain the lack of resistance rather than the emergence of resistance. We conclude that arguments against the predictive value of T_{MSW} are at best weak.

However, even in studies in which relationships between T_{MSW} and emergence of resistance were demonstrated, the predictive power of T_{MSW} was always lower than the AUC_{24}/MIC ratio. For example, in the studies described above, with *S. aureus* exposed to four fluoroquinolones, the respective r^2s were 0.72 versus 0.90 [17], with ciprofloxacin-exposed *E. coli* the r^2s were 0.61 versus 0.84 [25], and with ciprofloxacin-exposed *P. aeruginosa* they were 0.56 versus 0.80 [45]. With doripenem-exposed *P. aeruginosa* r^2s were 0.69 versus 0.80 [45], and with glycopeptide-exposed *S. aureus* they were 0.60 versus 0.68 ($MIC_{final}/MIC_{initial}$ data) or 0.50 versus 0.64 (N_{max}/N_{min} data) [31].

The differences in predictive power described above may be due to relating AUC_{24}/MIC to T_{MSW} while ignoring information about the *position* of simulated antibiotic concentrations inside the MSW, a feature that is likely to be very important with respect to mutant amplification. To test this hypothesis, the enrichment of ciprofloxacin-resistant *S. aureus* was examined at drug concentrations that oscillated near the MPC, i.e., close to the top of the MSW ("upper case"), or close to the MIC, i.e., at the lower limit of the MSW ("lower case"). In both cases the T_{MSW} was the same [46]. In this study, two methicillin-resistant strains of *S. aureus* (MPC/MIC 4 and 16) were exposed to twice-daily ciprofloxacin for 3 consecutive days. The simulated AUC_{24}/MIC were 50 h ("lower case") and 260 h ("upper case") to provide T_{MSW} of 75% of the dosing interval with one strain and 30 h ("lower case") and 100 h ("upper case") to provide T_{MSW} of 56% with another strain. With each strain, $AUBC_M$ (a measure of mutant enrichment) observed in the "lower case" was much greater than in the "upper case," thereby showing less pronounced enrichment of ciprofloxacin-resistant staphylococci at antibiotic concentrations oscillating near the MPC than near the MIC, even though for each strain T_{MSW} was the same.

Heterogeneity of the MSW was further examined in a study that exposed four *Escherichia coli* strains to twice-daily ciprofloxacin dosing for 3 days [25]. To explore the different predictive powers of T_{MSW} and AUC_{24}/MIC, the enrichment of ciprofloxacin-resistant *E. coli* mutants was studied at wide ranges of T_{MSW}s and AUC_{24}/MICs (up to eight points per strain). Peak antibiotic concentrations were simulated to be close to the MIC, between the MIC and MPC, and above the MPC; T_{MSW} varied from 0% to 100% of the dosing interval. The amplification (enrichment) of resistant mutants was monitored by plating on media with 8 × MIC of the antibiotic. With each organism, T_{MSW} plots of the $AUBC_M$ split into two portions,

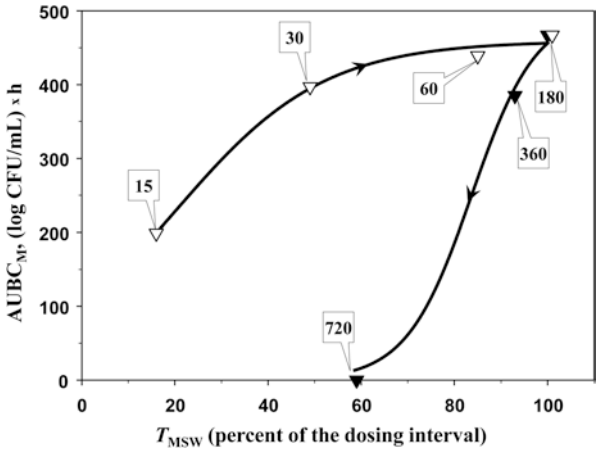

Fig. 21.7 T_{MSW}-dependent changes in the $AUBC_M$ reflecting the enrichment of *E. coli* mutants resistant to 4 × MIC of ciprofloxacin fitted by Eq. 1, separately for points that belong to the ascending portion ($Y_0 = 0$, $x_0 = 20.18$, $a = 459.1$, $b = 15.86$) and descending portion ($Y_0 = 0$, $x_0 = 83.40$, $a = 500.1$, $b = 6.969$) of the $AUBC_M$-AUC_{24}/MIC curve. AUC_{24}/MIC values are shown in callouts. (Reconstructed from Ref. [25])

one for antibiotic concentrations below the MPC ($T_{>MPC} = 0$) and the other for concentrations consistently above the MPC ($T_{>MPC} > 0$). The result was a hysteresis loop. Figure 21.7 illustrates a T_{MSW} relationship with $AUBC_M$ observed with one of the *E. coli* strains examined. As seen in the figure, when antibiotic concentrations were below the MPC (points corresponding to the ascending portion of the bell-shaped $AUBC_M$-AUC_{24}/MIC curve – AUC_{24}/MIC ratios of 15, 30 and 60 h), the $AUBC_M$ at a given T_{MSW} was greater than at the same T_{MSW} relevant to the descending portion of the $AUBC_M$-AUC_{24}/MIC curve (AUC_{24}/MIC ratios of 360 and 720 h gave the same T_{MSW}).

The distinct $T_{>MPC}$-dependent splitting of the $AUBC_M$-T_{MSW} curves (Fig. 21.7) prevents consideration of data obtained at $T_{>MPC} = 0$ and at $T_{>MPC} > 0$ as a single data set. When the data with the four *E. coli* strains were combined, a sigmoid function fits well with $AUBC_M$ versus T_{MSW} data sets taken separately at $T_{>MPC} = 0$ and $T_{>MPC} > 0$ (r^2s 0.81 and 0.92, respectively). In both cases, correlation of T_{MSW} with resistance appeared to be of the same power as observed with the AUC_{24}/MIC ratio (r^2 0.84). In contrast to the separated analysis of the T_{MSW} data referring to the conditions of $T_{>MPC} = 0$ or $T_{>MPC} > 0$, fitting the whole data pool while ignoring $T_{>MPC}$ exhibited a weaker correlation between T_{MSW} and mutant enrichment (r^2 0.61).

Hysteresis loops have also been reported for T_{MSW} relationships with *S. aureus* resistance to linezolid [47]. Using inocula of three methicillin-resistant *S. aureus* strains (MIC of linezolid = 2 mg/L), spiked with low concentrations of previously selected resistant mutants (MIC, 8 mg/L), AUC_{24}/MIC- and T_{MSW}-dependent mutant enrichment was observed in 5-day treatments with twice-daily linezolid. With each strain, T_{MSW} relationships with the $AUBC_M$ (for mutants resistant to 4 × MIC) exhib-

ited a hysteresis loop, with the upper sigmoid corresponding to $T_{>MPC} = 0$, and the lower one to the $T_{>MPC} > 0$. Based on combined data obtained with the three bacterial strains, $AUBC_M$ correlated better with T_{MSW} data taken separately when $T_{>MPC}$ was zero or exceeded zero (r^2 0.99) than with pooled data ignoring $T_{>MPC}$ (r^2 0.24).

We conclude that a hysteresis loop is inherent in the T_{MSW} relationships with mutant enrichment. It is very likely that the incorrect combination of data obtained at $T_{>MPC} = 0$ and at $T_{>MPC} > 0$ is among the reasons for an underestimation of the true role of T_{MSW} as a predictor of the emergence of bacterial resistance. For example, in a resistance study with meropenem-exposed *Acinetobacter baumannii* [48], the conclusion that T_{MSW} is not a suitable parameter relating to mutant enrichment might result from inappropriate combining T_{MSW}s belonging to the upper ($T_{>MPC} = 0$) and lower ($T_{>MPC} > 0$) portions of the hysteresis loop. With each *A. baumannii* strain, the T_{MSW}s observed at the minimal antibiotic exposure met the condition $T_{>MPC} = 0$, whereas the T_{MSW}s at the maximal exposure met the situation in which $T_{>MPC} > 0$.

Overall, although T_{MSW} is mutually related to the MSW, the appropriate use of this parameter requires consideration of the $T_{>MPC}$ data.

21.3.3 $T_{>MPC}$

The available reports on the use of $T_{>MPC}$ as a predictor of bacterial resistance are much less frequent than those that report AUC_{24}/MIC, AUC_{24}/MPC, and T_{MSW}, in part because antibiotic concentrations simulated in these studies exceeded the MPCs for only a short time or did not reach the MPCs. Even in cases in which $T_{>MPC}$s were positive, the reported data [39, 40] are too limited to delineate quantitative $T_{>MPC}$ relationships with the enrichment of resistant mutants. However, unlike the staphylococcal study with five fluoroquinolones [39], a reasonable link between the emergence of bacterial resistance (qualitative characteristics only) and $T_{>MPC}$ can be seen from the *E. coli* study using ciprofloxacin [40]. With each of three *E. coli* strains, at least in simulations of ciprofloxacin pharmacokinetics having a half-life of 4 h, the emergence of resistance was consistently associated with lower $T_{>MPC}$. Apparently, the authors' conclusion that the emergence of bacterial resistance cannot be predicted by the $T_{>MPC}$ reflects the inability to combine data obtained with different *E. coli* strains: at the same $T_{>MPC}$, resistance to ciprofloxacin developed with one strain but not with another.

In another study, suppression of *A. baumannii* resistance to meropenem (again, qualitative characteristics – the presence or absence of resistant mutants of antibiotic-exposed bacteria) was achieved for two strains with MPC/MIC ratios of approximately 15 and 60 [48] at similar $T_{>MPC}$s. It is noteworthy that strain-independent $T_{>MPC}$-resistance relationships could be established for each mode of antibiotic administration (0.5- and 3-h infusions). These relationships are specific for the type of simulated pharmacokinetic profile: the protective $T_{>MPC}$ was lower in the longer

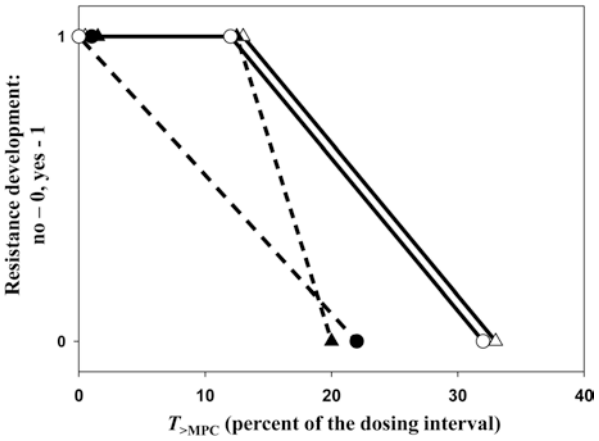

Fig. 21.8 $T_{>MPC}$-dependent resistance of *A. baumannii* CSRA24 (◯) and CSRA91 (△) exposed with meropenem (0.5-h infusion – open symbols, solid lines 3-h infusion – filled symbols, dotted lines). (Reconstructed from Ref. [48])

than in shorter meropenem infusions (Fig. 21.8). Such data are further evidence for pharmacokinetic profile-dependent emergence of bacterial resistance.

A quantitative resistance index, $AUBC_M$, was first related to $T_{>MPC}$ in the above-mentioned ciprofloxacin study with *E. coli* [25]. When $AUBC_M$ versus $T_{>MPC}$ data sets for four strains of *E. coli* were combined, a mono-exponential decay function fits these data with a relatively high r^2 (0.71). Using the points that met the condition of $T_{>MPC} > 0$, similar correlations between $AUBC_M$ with AUC_{24}/MPC (r^2 0.74) and with AUC_{24}/MIC (r^2 0.81) were observed. Thus, the predictive power of $T_{>MPC}$ was not inferior to AUC_{24}/MPC or to AUC_{24}/MIC ratios.

Even stronger correlations were reported recently between $AUBC_M$ and $T_{>MPC}$ with linezolid-exposed *S. aureus* [47]. A sigmoid function fits combined data for three *S. aureus* strains with a high r^2 (0.99). For the points that meet the condition $T_{>MPC} > 0$, the sum of T_{MSW} and $T_{>MPC}$ equals 100% of the dosing interval, and the $T_{>MPC}$ plot of $AUBC_M$ is a mirror image of the T_{MSW} plot at $T_{>MPC} > 0$ with the same r^2. In this study, both $T_{>MPC}$ and T_{MSW} at $T_{>MPC} > 0$ exhibited stronger correlations with $AUBC_M$ than did AUC_{24}/MPC (r^2 0.80) and AUC_{24}/MIC (r^2 0.85).

Thus, together with AUC_{24}/MIC, AUC_{24}/MPC, and T_{MSW}, $T_{>MPC}$ can be considered as a strain-independent predictor for the emergence of bacterial resistance.

21.4 Clinical Relevance of In Vitro Resistance Studies: Predicted "Anti-Mutant" AUC_{24}/MIC Ratios Versus Clinically Attainable AUC_{24}/MICs

Predicting the "anti-mutant" AUC_{24}/MIC ratios relative to clinically attainable AUC_{24}/MICs is a primary goal of bacterial resistance studies with dynamic models. Obviously, such predictions can be ensured only when reasonable AUC_{24}/MIC relationships were established with mutant enrichment and/or changing susceptibility

of antibiotic-exposed bacteria. However, an "anti-mutant" AUC_{24}/MIC ratio predicted from in vitro studies always represents a conservative target for dosing adjustment because dynamic models do not consider host defense factors. Moreover, unlike AUC_{24}/MIC breakpoints used to determine the potential for an antibacterial to kill susceptible subpopulations [49], the "anti-mutant" AUC_{24}/MIC ratios predicted with in vitro studies cannot be referred to clinically established protective AUC_{24}/MICs because they have not been reported. Therefore, these predictions are more conditional than are those with antibiotic effects on susceptible bacterial subpopulations.

21.4.1 Monotherapy

The "anti-mutant" AUC_{24}/MIC ratios were established when *S. aureus* was exposed to fluoroquinolones [17]. Based on the bell-shaped AUC_{24}/MIC relationships with $MIC_{final}/MIC_{initial}$, predicted "protective" AUC_{24}/MICs appeared to be similar for levofloxacin (201 h), moxifloxacin (222 h), gatifloxacin (241 h), and ciprofloxacin (244 h). However, these thresholds are clinically attainable only with moxifloxacin. With a 400 mg dose of moxifloxacin, the "anti-mutant" AUC_{24}/MIC ratio is 66% of the clinically attainable value, whereas with two 500 mg doses of ciprofloxacin, a 500 mg dose of levofloxacin, or a 400 mg dose of gatifloxacin, the respective anti-mutant AUC_{24}/MICs are 420%, 220%, and 190% of the clinically attainable values. Thus, at least against *S. aureus*, moxifloxacin is expected to protect against resistance development in a clinical setting, whereas the three other fluoroquinolones will likely enrich mutant subpopulations.

Resistance thresholds reported in vitro studies from different research groups exhibit considerable variability. For example, with grepafloxacin-exposed *S. pneumoniae*, "protective" AUC_{24}/MIC ratios varied from 32 h [14] to 80 h [10] while those of levofloxacin were from 9 h [14] to 26 h [9] and 35 h [11]. Furthermore, although moxifloxacin-resistant *S. pneumoniae* were not found at AUC_{24}/MIC ratios of 60 h [11] and 107 h [10], significant losses in susceptibility were seen at AUC_{24}/MICs as high as 43,500 h [14]. Analysis of these findings [5] indicates that different estimates of the "anti-mutant" AUC_{24}/MIC ratio can be attributed to differences in study design and data processing. For this reason, it is of particular interest to compare "anti-mutant" AUC_{24}/MICs obtained under the same experimental conditions. Based on data reported in ciprofloxacin resistance studies that determine "anti-mutant" AUC_{24}/MIC ratios using the descending portion of the $MIC_{final}/MIC_{initial}$ or $AUBC_M$ versus AUC_{24}/MIC curve [17, 29, 50], lower resistance thresholds were established with Gram-positive than with Gram-negative bacteria. The "anti-mutant" AUC_{24}/MIC ratios were 125–244 h with *S. aureus* (three strains) [17, 29], 700–1100 h with *E. coli* (four strains), 1300–2600 h with *K. pneumoniae* (three strains), and 300–1400 h with *P. aeruginosa* (four strains) [50]. However, when related to clinically attainable AUC_{24}/MIC ratios for each individual strain, different distributions emerge for ciprofloxacin "anti-mutant" potentials for different species.

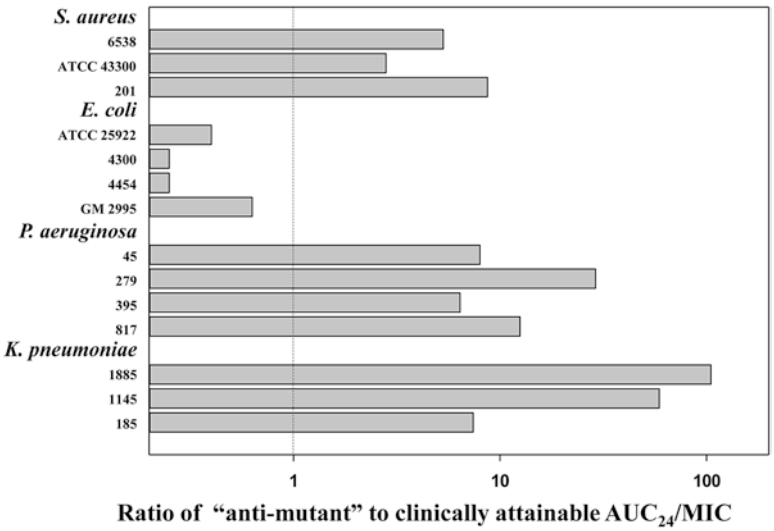

Fig. 21.9 "Anti-mutant" thresholds of AUC_{24}/MIC related to the clinically attainable AUC_{24}/MIC ratios: ciprofloxacin against Gram-positive and Gram-negative bacteria. (Reconstructed from Ref. [17, 29, 50])

As shown in Fig. 21.9, with *E. coli* but not with *S. aureus*, *P. aeruginosa*, and *K. pneumoniae*, the predicted "anti-mutant" AUC_{24}/MICs are achieved in a clinical setting (ratio of the resistance threshold to the clinically achievable AUC_{24}/MIC <1).

Our resistance study with daptomycin- and vancomycin-exposed *S. aureus* [31] provides an example of a more favorable situation, at least for daptomycin. Based on combined data obtained with two *S. aureus* strains, the predicted "anti-mutant" AUC_{24}/MIC ratio (200 h) was smaller than the clinically attainable AUC_{24}/MIC_{90}s: 380 h for a 4 mg/kg dose of daptomycin and 570 h for a 6 mg/kg dose of the antibiotic. Unlike daptomycin, the "anti-mutant" target for vancomycin virtually coincided with the clinically attainable AUC_{24}/MIC_{90} ratio (200 h for 1 gm twice-daily dosing). Reasonably optimistic predictions were made in a linezolid study with *S. aureus* [34]: with two of three studied strains, the "anti-mutant" AUC_{24}/MIC ratios were equal to the clinically attainable value (120 h for a 600 mg dose twice a day). However, with the third *S. aureus* strain, the "anti-mutant" threshold was twofold greater.

Overall, these examples show that clinically achievable AUC_{24}/MIC ratios may not always overlap the "anti-mutant" AUC_{24}/MIC thresholds. In conjunction with extensive MPC testing of various antibiotic-pathogen pairs [51], these findings predict emergence of bacterial resistance with the use of many existing antibiotics as usually prescribed. As dose escalation is rarely possible due to limited patient tolerability, combined antibiotic therapy provides an alternative to the replacement of a less "protective" agent by a more "protective" agent.

21.4.2 Combined Therapy

Resistance studies that expose bacteria to antibiotic combinations using in vitro dynamic models have been relatively infrequent. In one example, to determine whether doxycycline can minimize or prevent the emergence of staphylococcal resistance to moxifloxacin, 5-day treatments with once-daily moxifloxacin or doxycycline given alone or in several combinations were simulated over a threefold AUC_{24}/MIC range for each antibiotic [52]. Combined use of moxifloxacin and doxycycline delayed the loss in susceptibility of *S. aureus* to both antibiotics at moderate AUC_{24}/MIC ratios or completely prevented the loss at relatively high AUC_{24}/MIC. In another study [53], a combination of twice-daily linezolid with once-daily doxycycline against *Enterococcus faecium* was tested in three-day treatments. Unlike linezolid alone, neither growth on linezolid-containing media (4×, 8× and 16 × MIC) nor changes in susceptibility occurred when combined use of these antibiotics was simulated. The presence of linezolid decreased the numbers of doxycycline-resistant enterococci present without any loss in susceptibility.

More recently, due to renewed interest in colistin, a series of papers on colistin combinations with other agents have been published. For example, colistin combinations with doripenem are protected against the emergence of colistin resistance in colistin-susceptible and hetero-resistant but not in multidrug-resistant *K. pneumoniae* [54]. Also, combinations of colistin with fosfomycin reduced the probability of development of *Enterobacteriaceae* resistant to both antibiotics [55]. Moreover, colistin prevented the enrichment of meropenem-resistant *A. baumannii* in simulations of combined treatments with colistin + carbapenem [56] and the loss of tigecycline susceptibility in *A. baumannii* treated with a tigecycline-colistin combination [57]. With an endocardial vegetation model, ceftaroline and ceftriaxone but not gentamicin prevented the enrichment of daptomycin-resistant *Streptococcus mitis* [58]. With *M. tuberculosis*, linezolid-rifampicin combinations exhibited greater "anti-mutant" power than the single agents in simulations of antibiotic pharmacokinetics over wide dose ranges [59].

These combination studies may be useful for designing regimens to prevent and/or restrict the emergence of bacterial resistance, but none provides a way to predict these effects from independent tests that do not take into account antibiotic pharmacokinetics. One such way has been recently proposed in a resistance study with *S. aureus* exposed to linezolid-rifampicin combinations [60]. Using antibiotic concentration ratios that correspond to linezolid/rifampicin AUC_{24} ratios to be simulated in a dynamic model, the MPCs of each agent in combination were determined. For example, to determine the MPCs necessary for the prediction of the "anti-mutant" effects of simulated antibiotic pharmacokinetics using a combination of clinical daily doses of linezolid (1200 mg) and rifampicin (600 mg) providing AUC_{24} ratio of 240/60 = 4, the respective "pharmacokinetically derived" antibiotic concentration ratio was 4 to 1. MPC testing at pharmacokinetically derived concentration ratios showed a 2.5–3.1-fold decrease of linezolid MPC when in combination with

rifampicin and a 760–6400-fold decrease of rifampicin MPC in the presence of linezolid.

Using a mixed inoculum of linezolid-susceptible and -resistant cells [35] for pharmacokinetic simulations, combinations of linezolid with rifampicin completely suppressed the enrichment of linezolid-resistant *S. aureus* mutants and restricted the development of rifampicin resistance in *S. aureus* [60]. In contrast to simulated combined treatments, mutants resistant to both antibiotics were enriched when linezolid and rifampicin were administered separately. These effects were likely due to lowering the MPC of linezolid and rifampicin in the combinations relative to MPCs of the single antibiotics. In essence, linezolid-rifampicin combinations provided much longer times above the antibiotic MPCs (73–100% of the dosing interval for linezolid and 42–58% for rifampicin) compared to the $T_{>MPC}$s in mono-treatments (0–44% for linezolid and 0% for rifampicin). Thus, the $T_{>MPC}$s for antibiotic combinations provided a quantitative description of how combined use of linezolid and rifampicin restricts the enrichment of linezolid-resistant relative to rifampicin-resistant mutants.

It is possible that an MPC-based prediction of the "anti-mutant" potential for linezolid and rifampicin combinations was successful due to pharmacokinetically derived concentration ratios used to determine MPC of the antibiotics given in combination. For each simulated dosing regimen, including clinically relevant dosing, the MPC of each antibiotic was determined at the concentration ratio that strictly corresponded to the ratio of AUC_{24}s provided by a given linezolid-rifampicin combination. As seen in Fig. 21.10, the MPC of linezolid, combined with rifampicin, was independent of the antibiotic concentration ratio, whereas the MPC of rifampicin, combined with linezolid, decreased systematically with increases in linezolid concentrations in the combination. Therefore the MPCs reported in studies with other antibiotic combinations at arbitrarily chosen concentration ratios [61–65] might be insufficiently predictive for the "anti-mutant" effects.

Overall, the linezolid-rifampicin study [60] suggests that "anti-mutant" antibiotic combinations can be predicted by the MPCs determined at pharmacokinetically-based antibiotic concentration ratios. This approach avoids uncertainties about the optimal choice of antibiotic concentration ratios, as occurs with checkerboard techniques for susceptibility testing when the optimal concentration ratio may or may not have any relationship to human antibiotic pharmacokinetics.

21.5 Conclusions

Analysis of the enrichment of resistant bacterial subpopulations using in vitro dynamic models shows the usefulness of this approach to better understand PK/PD-mediated enrichment of resistant mutants with concomitant loss in pathogen susceptibility. These studies have contributed to the delineation of AUC_{24}/MIC, AUC_{24}/MPC, T_{MSW}, and $T_{>MPC}$ relationships with resistance and to the prediction of "anti-mutant" thresholds and dosing regimens. However, current knowledge of

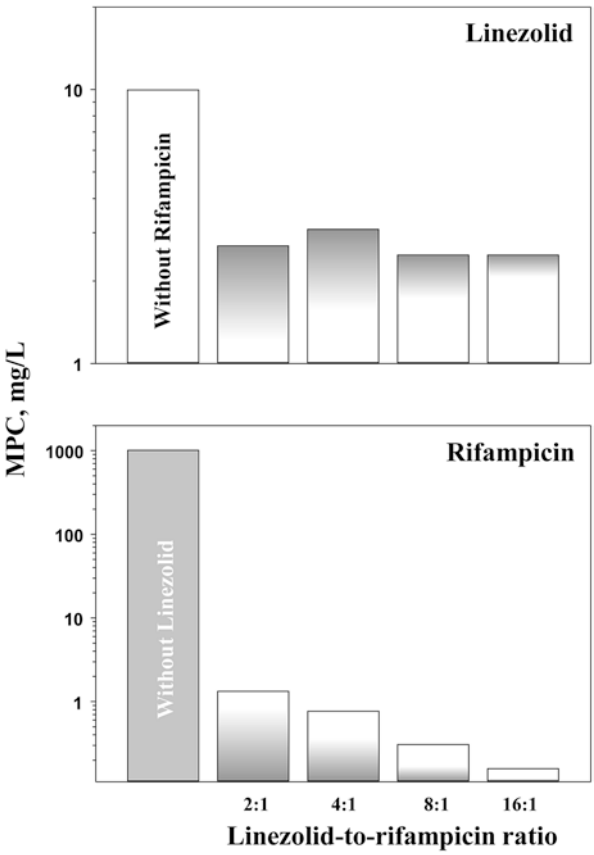

Fig. 21.10 MPC values of linezolid and rifampicin alone and in combinations. (Reconstructed from Ref. [60])

these relationships and their clinical relevance remain limited because of the scarcity of dynamic resistance studies with many antibiotic classes and diverse pathogens. Indeed, only a few bacterial species have been examined; quantitative findings reported with a limited number of pathogens will remain applicable only to those antimicrobial-pathogen pairs and not to other strains of the same species until the data are generalized. Moreover, further studies that compare inter-strain predictions of mutant enrichment using AUC_{24}/MIC and AUC_{24}/MPC are particularly needed, due to apparent differences between fluoroquinolone-exposed Gram-positive and Gram-negative bacteria. Nevertheless, bacterial resistance studies using dynamic models provide notable progress in understanding of the mutant selection window as a framework for predicting the selective enrichment of resistant mutants.

Major Points

- Relationships between PK/PD (pharmacokinetic/pharmacodynamic) indices and the emergence of bacterial resistance are a basis for designing "anti-mutant" antibiotic dosing regimens, i.e., regimens that are expected to prevent or restrict the enrichment of resistant mutant subpopulations.

- In vitro dynamic models provide a way to study the enrichment of resistant mutants while simulating human antibiotic pharmacokinetics.
- Using these models, bell-shaped relationships between the ratios of the area under the concentration-time curve (AUC) to the MIC or MPC (mutant prevention concentration) and the enrichment of resistant mutants and/or loss in susceptibility of antibiotic-exposed bacteria are established.
- The general pattern of these relationships is consistent with the mutant selection window (MSW) hypothesis that predicts that the selection of resistant mutants occurs largely at antibiotic concentrations between the MIC and MPC.
- Together with AUC/MIC and AUC/MPC ratios, times inside the MSW and above the MPC can be predictive for the emergence of bacterial resistance.
- Based on the AUC/MIC-resistance relationships, the "anti-mutant" thresholds were predicted for various "antibiotic-pathogen" pairs.
- For most cases examined, doses used clinically expose bacterial pathogens to concentrations inside the MSW for much of the dosing interval, a feature that reveals a fundamental dosing flaw with respect to the emergence of resistance.

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Part IV
Bringing Compounds to Market

Chapter 22
The Role of Pharmacometrics in the Development of Antimicrobial Agents

Justin C. Bader, Elizabeth A. Lakota, Brian VanScoy, Sujata M. Bhavnani, and Paul G. Ambrose

We live in a world teeming with antimicrobial-resistant pathogens. For a number of pan-resistant pathogens, our once plentiful antimicrobial armamentarium is now quite limited. There is a critical need for new antimicrobial agents to treat patients with infections due to these highly resistant organisms such as Gram-negative bacilli [1]. The need for new agents is especially great for the treatment of patient populations at great risk for morbidity and mortality, such as those with hospital-acquired and ventilator-associated bacterial pneumonia (HABP and VABP, respectively) arising from resistant pathogens.

Pharmacokinetic-pharmacodynamic (PK-PD) principles have recently become an important cornerstone for antimicrobial agent assessment. The use of these principles together with the broader science of pharmacometrics, a branch of science that includes population pharmacokinetic (PK) and PK-PD analysis, has enabled both early- and late-stage analyses supporting antimicrobial dose selection. These data have served to greatly de-risk antimicrobial drug development and increase the likelihood of regulatory success [2]. Our confidence in pharmacometric data stems, in large measure, from the general concordance that exists between the results from PK-PD analyses based on data from preclinical models of infection and those from randomized clinical trials [3, 4]. Recent US Food and Drug Administration (US FDA) and the European Medicines Agency guidance documents recommending the use of PK and PK-PD analyses throughout the drug development process for a number of indications [5–11] demonstrate the reliance on pharmacometric data for regulatory pathways to develop antimicrobial agents.

The benefits of a pharmacometric approach are even more relevant when developing antimicrobial agents for the treatment of patients with multiple or extensively drug-resistant (MDR and XDR, respectively) pathogens. Given the rarity of such

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I. W. Fong et al. (eds.), *Antimicrobial Resistance in the 21st Century*, Emerging Infectious Diseases of the 21st Century, https://doi.org/10.1007/978-3-319-78538-7_22

pathogens, enrollment of patients in clinical trials for these agents can be slow and often requires several years to accrue a modest level of enrollment. This severely limits the amount of information available to conduct traditional statistical analyses of clinical data. Moreover, it is unethical to enroll patients into a randomized clinical trial of any design for which one treatment arm is not reliably active against MDR or XDR pathogens. Consequently, if we are to develop antimicrobial agents for the treatment of seriously ill patients infected with MDR and XDR pathogens, the normal paradigm of basing antimicrobial approval on the results of multiple randomized clinical studies is difficult to impossible. This is ultimately due to the lack of comparators with suitable efficacy and low numbers of patients with these infections. In this context, we must therefore consider other data to supply the evidence necessary for antimicrobial drug approval. The focus of this chapter is not only on basic pharmacometric concepts in the setting of pathogens with usual drug resistance (UDR) but also on how pharmacometric analyses can be used to leverage limited clinical data packages in order to support antimicrobial drug approval for the treatment of patients with infections due to specified MDR or XDR pathogens.

22.1 The Bottom Line Upfront

The certainty that pharmacometric data provides to support antimicrobial agent drug development begins in the laboratory. The answers to three critical preclinical questions can be used to forecast the clinical efficacy and durability of an antimicrobial drug regimen. The questions posed, which can be answered using in vivo and in vitro PK-PD infection models, include the following:

1. What is the PK-PD index that is most associated with efficacy?
2. What is the magnitude of the PK-PD index necessary for efficacy?
3. What is the relationship between antimicrobial drug exposure and time to emergence of drug resistance?

An important goal for the drug development scientist is to leverage the relevant preclinical PK-PD infection models to answer each question using an appropriate model in the most time and cost-efficient manner. As discussed in Sect. 22.2, there are a number of standard preclinical PK-PD infection models, including the neutropenic murine-thigh and murine-lung infection models and the one-compartment in vitro infection model, which have been used to characterize the PK-PD of antimicrobial agents. The model chosen should be most appropriate to answer the scientific question posed (Fig. 22.1). While a number of in vivo and in vitro PK-PD infection models can be used to identify the PK-PD index most associated with efficacy, infection models that can be used to characterize the relationship between drug exposure and time to emergence of resistance are limited. The evaluation of PK-PD relationships for emergence of resistance is best accomplished using the in vitro hollow-fiber infection model.

In Sect. 22.3, the importance of developing and refining a population pharmacokinetic (PK) model in order to inform and support programmatic decisions is

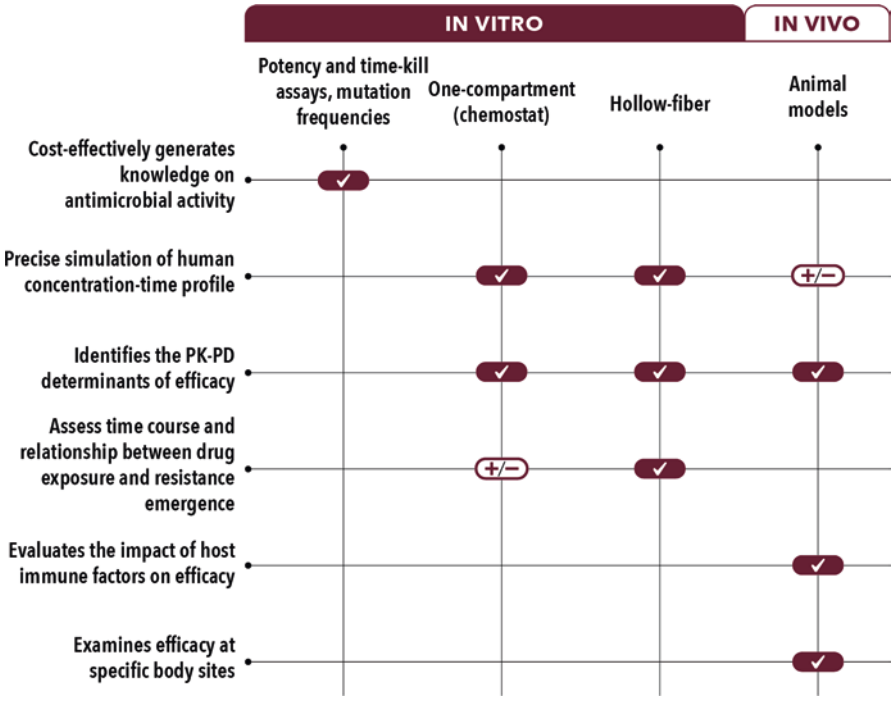

Fig. 22.1 The preclinical toolbox for antimicrobial drug development

reviewed. The value of these models and breadth of questions they are able to answer will depend largely upon the richness of the data upon which they are built and refined. This section will present the consideration needed for ensuring that one is collecting data which are relevant for answering pivotal questions.

Section 22.4 describes the iterative process of dosing regimen selection. Analyses to support early-stage dosing regimen selection integrate the aforementioned preclinical information with healthy volunteer PK data using Monte Carlo simulation in the context of the minimum inhibitory concentration (MIC) distribution(s) for the target pathogen(s). These analyses should explicitly account for between-species differences in PK, protein binding, and effect site exposures. The underlying population PK model used for the simulations should be developed using a robust clinical dataset, which initially includes data from healthy subjects after receiving single and multiple doses, and is ultimately refined using data from special populations and target patient populations treated with dosing regimens intended for labeling.

Finally, Sect. 22.5 will discuss the value of PK-PD analyses based on clinical data in the context of clinical data packages in the setting of UDR or MDR and/or XDR. The use of these data to confirm that adequate drug exposures relative to nonclinical PK-PD targets for efficacy are achieved in the context of both robust and limited clinical data packages is reviewed. The opportunities for evaluating PK-PD relationships for safety endpoints and use these data to guide labeling and/or clinical practice guidelines will be addressed. Finally, the concept of Bayesian analyses, which integrate preclinical and clinical PK-PD information to inform clinical trial design questions, will be discussed.

22.2 Assembling a Robust Preclinical PK-PD Data Package

The preclinical PK-PD package for a new drug application (NDA) serves as the foundation for selecting and supporting dosing regimens for clinical study. These data are vital to ensuring the success of any drug development program but should be held in higher regard when developing antimicrobial agents to treat patients with infections due to MDR and XDR organisms. As will be discussed in greater detail in Sect. 22.5, clinical data are likely to be limited in such programs; thus, we must put greater weight on preclinical data to increase regulatory certainty. Consequently, as described herein, the selection, design, and execution of preclinical studies must be thoughtfully planned to ensure a robust PK-PD data package is obtained. Such data will then allow for more informative preclinical inputs for dose selection analyses as described in Sect. 22.3.

22.2.1 Determining the PK-PD Index Most Associated with Efficacy

To begin formulating a preclinical PK-PD data package, the first question which must be asked and answered is in regard to the PK-PD index which is most associated with efficacy for a given antimicrobial. Antimicrobials are typically said to exhibit concentration- or time-dependent patterns of killing activity [12]. In the case of antimicrobials with concentration-dependent activity, the rate and extent of killing increase in tandem with drug concentrations. This pattern of activity is best described using the ratios of the area under the concentration-time curve (AUC) or maximum concentrations (Cmax) over the MIC (AUC:MIC and Cmax:MIC ratios, respectively). The objective when dosing concentration-dependent antimicrobials is to achieve exposures in patients which maximize the killing of pathogens while minimizing the likelihood of witnessing drug-induced toxicities.

Alternatively, the objective when administering antimicrobials which exhibit time-dependent killing is not to maximize drug exposures but rather to optimize dosing to maintain drug concentrations above a target threshold such as an MIC. Accordingly, this pattern of activity can be characterized by the percentage of time drug concentrations remain above an MIC or other threshold (%T > MIC and %T > threshold, respectively). Jointly, the AUC:MIC ratio, Cmax:AUC ratio, and %T > MIC comprise the three most commonly utilized PK-PD indices to describe antimicrobial activity (Fig. 22.2). Determining the PK-PD index which best describes efficacy for a given antimicrobial can be challenging if one does not take extra precautions. Given that the magnitude of drug introduced into a system impacts all of the aforementioned PK-PD indices, significant collinearity is observed when attempting to differentiate these indices on the basis of dose alone. That is to say AUC:MIC ratio, Cmax:AUC ratio, and %T > MIC all increase with dose. Dose-fractionation studies are used to mitigate the impact of this col-

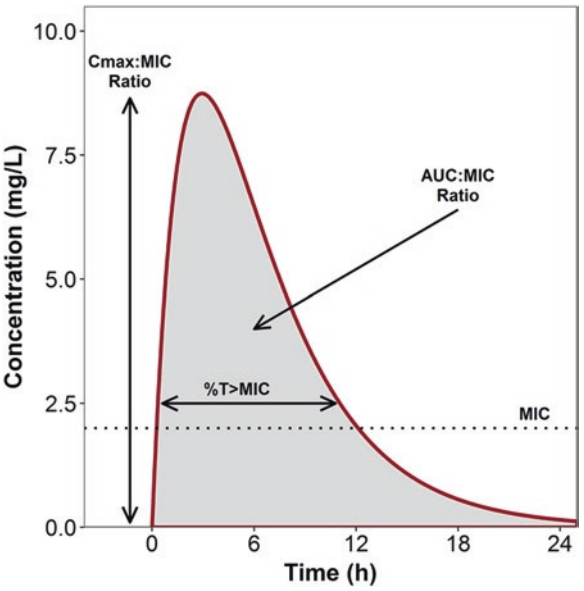

Fig. 22.2 PK-PD indices depicted utilizing a plasma concentration-time curve

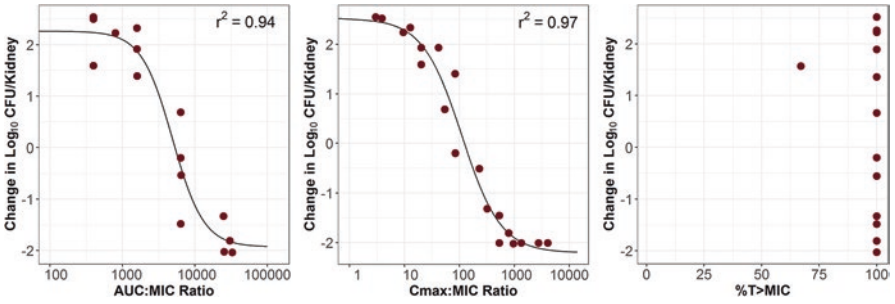

Fig. 22.3 Relationships between change in $\log_{10}$ CFU from baseline at 24 h and total-drug AUC:MIC ratio, Cmax:MIC ratio, and %T > MIC for anidulafungin against *C. glabrata* based on data from a neutropenic murine candidiasis model [13]. (Reproduced from Ref. [14] with permission from J Antimicrob Chemother. Copyright © 2017 British Society for Antimicrobial Chemotherapy, [Journal of Antimicrobial Chemotherapy, 2018; 73 (suppl 1):i44-50.])

linearity through the administration of dosing regimens which utilize the same total dose of an antimicrobial agent but which are differentiated in their frequency of dosing (e.g., 600 mg once daily, 300 mg twice daily, 150 mg four times daily, etc.). A range of exposures is obtained by administering regimens in a similar manner over multiple dose levels.

Using data obtained from a dose-fractionation study that was conducted using a neutropenic murine candidiasis model [13], Fig. 22.3 shows relationships between change in $\log_{10}$ CFU from baseline at 24 h and total-drug AUC:MIC ratio, Cmax:MIC ratio, and %T > MIC for anidulafungin against *C. glabrata* [14].

In this study, neutropenic mice were infected with *Candida glabrata* and administered 1 of 20 anidulafungin dosing regimens. Total doses of 1.25, 5, 20, 80, or 320 mg/kg were administered over a 96-h period in the form of one, two, four, or six divided doses (i.e., doses were given every 96, 48, 24, or 16 h, respectively). Hill models were used to characterize the relationships between changes in fungal density (i.e., colony-forming units [CFU]) in homogenized kidney tissue at 96 h relative to baseline and the three aforementioned PK-PD indices. The data presented demonstrated that changes in fungal density were most closely associated with anidulafungin AUC:MIC and Cmax:MIC ratios, indicating that this agent exhibits a concentration-dependent pattern of fungicidal activity. However, unlike AUC values, the Cmax is achieved at a transient time point, making it difficult to accurately capture in studies and apply to support dose selection. Therefore, in such situations, AUC:MIC ratio serves as a more reliable and predictable PK-PD index than Cmax:MIC ratio.

22.2.2 Identifying PK-PD Targets for Efficacy

Once the PK-PD index most associated with efficacy is known, the next step is to determine the magnitudes of this index which are associated with various levels of pathogen killing. These thresholds are commonly known as PK-PD targets for efficacy, and they provide crucial information to assist in estimating the likelihood of achieving efficacious drug concentrations in patients following the administration of a given antimicrobial dosing regimen. Dose-ranging studies are used to derive these PK-PD targets, wherein changes in microbial density are evaluated across a wide range of antimicrobial doses. Given that the PK-PD index most associated with efficacy is known by this point in time, there is no longer a need to account for potential collinearities. Consequently, all doses are administered over identical dosing intervals (e.g., every 24 h). The interval evaluated in these studies will be that which best describes the relationship between change in bacterial burden and the PK-PD index most associated with efficacy, as established by the results obtained from prior dose-fractionation studies. Common thresholds assessed in these studies include net stasis (i.e., no change in the density of bacteria or fungi from that observed at baseline) and 1- and 2-$\log_{10}$ reductions in the counts of CFUs relative to baseline observations.

Figure 22.4, which shows data from VanScoy et al. [15], illustrates the type of data that can be derived from an in vitro dose-ranging study. In these studies, a one-compartment in vitro infection model was used to simulate total-drug epithelial lining fluid (ELF) AUC values ranging from 33.3 to 7942 mg•h/L following administration of arbekacin, an investigational aminoglycoside. These drug exposures were evaluated against four *Pseudomonas aeruginosa* isolates, the MIC values for which ranged from 2 to 8 mg/L. The relationship between change in $\log_{10}$ CFU from baseline at 24 h and total-drug ELF AUC:MIC ratio,

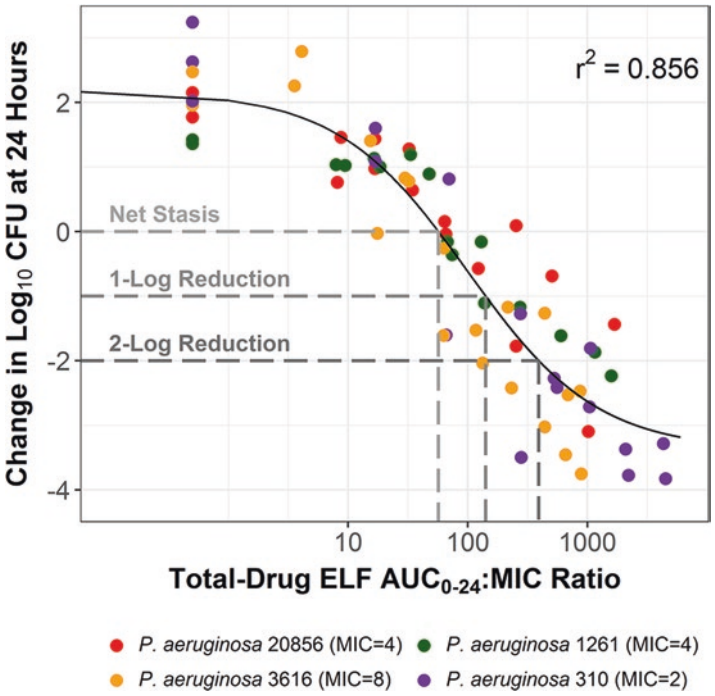

Fig. 22.4 Arbekacin total-drug ELF AUC:$MIC_{0–24}$ ratio targets associated with net bacterial stasis and 1- and 2-log_{10} CFU reductions from baseline for *P. aeruginosa* based on data from a one-compartment in vitro infection model [15]

the PK-PD index associated with arbekacin efficacy, was evaluated using a Hill model. Using this model, the magnitudes of total-drug ELF AUC:MIC ratio associated with net bacterial stasis and 1- and 2-log_{10} CFU reductions from baseline, which were 56.9, 142, and 393, respectively, were identified as shown in Fig. 22.4.

These data exhibit several characteristics which indicate the robustness of the above-described AUC:MIC ratio targets. We can be assured that the PK-PD relationship was well captured as evidenced by the nearly complete sigmoidal curve obtained by the fitted Hill model. This is a product of designing the dose-ranging study to obtain a wide range of AUC:MIC ratios by evaluating a large range of doses and various isolates with differing MIC values. Moreover, the coefficient of determination (r^2) of 0.856 for this relationship was high, which tells us that the relationship between change in log_{10} CFU and the AUC:MIC ratio is strong. Finally, the data pertaining to each of the various isolates evaluated are well dispersed along the fitted relationship with no apparent trends, indicating that no substantial differences in efficacy were observed across these isolates.

22.2.3 Accounting for PK-PD Variability

Devoting time and resources to the design of studies that account for variability in PK-PD relationships for efficacy is crucial to the development of a robust preclinical PK-PD package. Bacteria and fungi are extremely complex and adaptive organisms that can develop a myriad of antimicrobial resistance mechanisms and undergo changes in their inherent fitness. Consequently, the variability among isolates for a given pathogen needs to be considered when designing preclinical studies. The consideration of such variability provides an opportunity to better characterize the PK-PD of a given antimicrobial agent. The following will detail best practices for designing PK-PD studies in order to maximize the information that can be gained in light of the above-described variability.

To begin, let us review the design of dose-fractionation studies and consider how best to select an isolate for evaluation. Given that the primary objective when conducting these studies is to discriminate among the various PK-PD indices and determine which is most associated with efficacy for an antimicrobial, the intention should be to minimize the potential of generating variable and inexplicable results. Therefore, when evaluating an antimicrobial agent, it is best to select a well-defined isolate that is known to grow well in the in vitro or in vivo system intended for study and for which consistent and predictable PK-PD data have been generated previously (e.g., as observed in prior time-kill studies).

Regarding dose-ranging studies, the objective when selecting isolates should be to study a diverse collection of isolates such that inter-isolate variability can be adequately characterized. The challenge panel of isolates should have MIC values that encompass a clinically relevant range and that express applicable resistant determinants. Given that these studies are employed to derive PK-PD targets associated with efficacy which are then used to forecast dosing regimens for patients in the UDR setting or even the setting of MDR and XRD, it is important to account for the population of pathogens expected in either of these clinical settings. Examples of isolate collections used for PK-PD analyses that meet the above-described criteria are described below.

Figure 22.5 shows the relationship between change in $\log_{10}$ CFU from baseline and free-drug plasma $AUC_{0–24}$:MIC ratio for an investigational anti-staphylococcal agent, afabicin, against seven *Staphylococcus aureus* isolates with MIC values ranging from 0.004 to 0.06 mg/L, the data for which was obtained from studies utilizing a murine-thigh infection model [16]. When assessed relative to the range of MIC values (<0.001–0.25 mg/L, MIC_{90} = 0.008 mg/L) evaluated in a recent in vitro surveillance study of 660 *S. aureus* isolates collected from European, North American, Latin American, and Asian hospitals from 2013 to 2014 [17], the range of MIC values for the isolate collection studied was considered robust. In addition, the latter consideration is presented in Fig. 22.6 which shows data obtained from one-compartment in vitro infection model studies of eravacycline, an investigational tetracycline, against five *Escherichia coli* isolates stratified by those that were tetracycline susceptible and non-susceptible based on either the Clinical and Laboratory

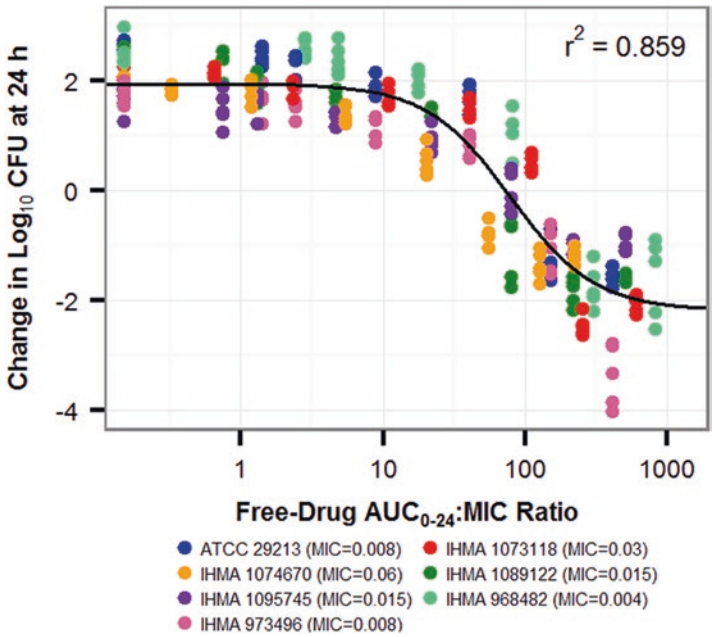

Fig. 22.5 Relationship between the change in $\log_{10}$ CFU from baseline at 24 h and free-drug plasma AUC_{0-24}:MIC ratio for afabicin based on data for seven *S. aureus* isolates studied in a neutropenic murine-thigh infection model [16]

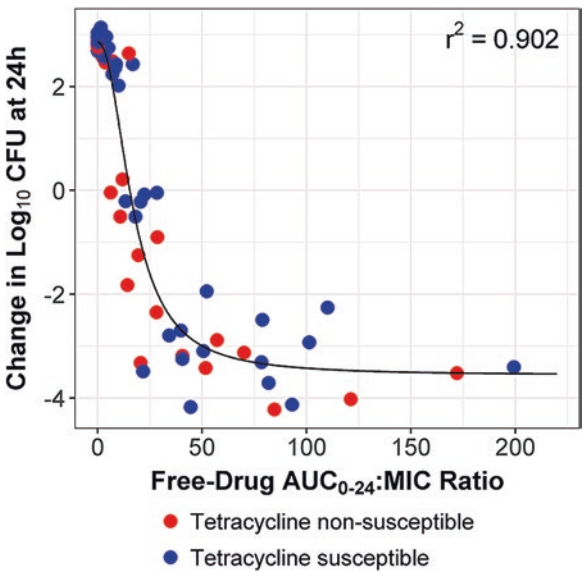

Fig. 22.6 Relationships between change in $\log_{10}$ CFU from baseline at 24 h and free-drug AUC_{0-24}:MIC ratio for eravacycline based on data for tetracycline susceptible and non-susceptible *E. coli* isolates studied in a one-compartment in vitro infection model [18]

Standards Institute or US FDA susceptibility breakpoints [18–20]. In both examples, the pooled data co-modeled well, producing robust sigmoidal relationships.

While there is no specific number of isolates from a family or genus of pathogens which must be utilized to effectively characterize variability in the relationship between the PK-PD index and reduction in bacterial burden, a sufficient number of isolates needs to be initially studied. Assuming the isolates selected account for all clinically relevant resistance determinants and embody a suitable range of MIC values, investigators should evaluate the mean and median values for the PK-PD targets to ensure they are relatively similar [21]. However, the ultimate decision regarding how many isolates should be evaluated must be made on a case-by-case basis and may best be determined iteratively after examination of results based on a reasonably robust number of isolates.

22.2.4 The Importance of Evaluating Pharmacokinetics

The importance of collecting samples to evaluate the PK within in vitro systems is often underestimated. Many investigators may choose to forgo collecting PK data when conducting studies, opting instead to simply assume the targeted PK profile was obtained within the system. However, this step is critical to ensuring accurate and reliable information is obtained when modeling PK-PD data. Despite the many variables which can be held constant (e.g., antimicrobial and media flow rates throughout the system), observed concentration-time profiles within in vitro systems can vary from those expected for a multitude of reasons such as drug degradation (e.g., hydrolysis or photodegradation), binding of drug to the infection model components, or even simple methodologic errors. Failing to account for these factors by neglecting to collect PK samples can result in errors of varying magnitude of impact when interpreting the study data. For instance, consider an in vitro dose-ranging study in which no PK samples are collected but rather for which it is assumed that the targeted concentration-time profile will be obtained. However, the concentrations actually achieved within the system are lower than those expected. Consequently, the exposures evaluated for PK-PD target determination will be falsely elevated, causing a rightward shift in the PK-PD relationship. Therefore, any PK-PD targets derived from these data will also be falsely elevated. Figure 22.7 provides an illustration of this concept, while Fig. 22.8 shows data from a study conducted by Louie et al. using a hollow-fiber in vitro infection model [22] for which lower concentration-time profiles for ceftaroline than expected were achieved.

In the above-described study [22], ceftaroline was administered with and without avibactam (also referred to as NXL104), a non-β-lactam-β-lactamase inhibitor, over a 10-day study period. The activity of this combination was evaluated against three *Klebsiella pneumoniae* isolates, one of which expressed several β-lactamase enzymes, including KPC-2, SHV-27, and TEM-1. As shown in Fig. 22.8, all study arms which included avibactam, achieved ceftaroline concentration-time profiles similar to those targeted. However, in the study arm which did not contain avibac-

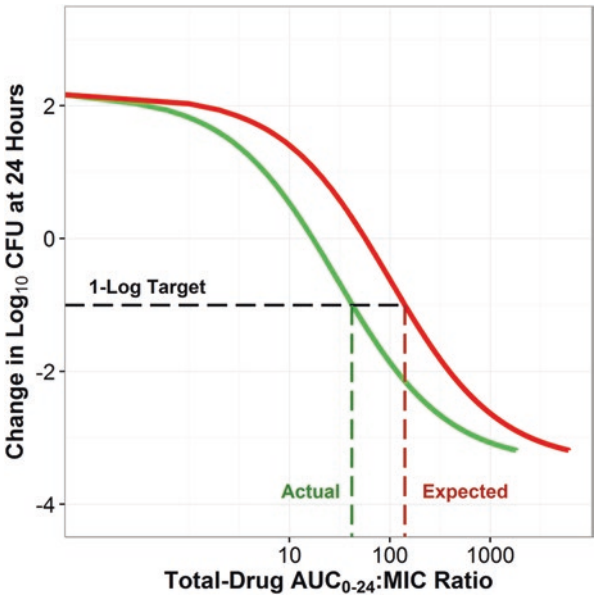

Fig. 22.7 Discrepancy between $AUC_{0–24}$:MIC ratio targets associated a 1-log_{10} CFU reduction from baseline based on evaluations performed using actual and expected PK

tam, ceftaroline concentrations were much lower than those expected. This is due to ceftaroline being hydrolyzed by the endogenous β-lactamase enzymes secreted by the above-described *K. pneumoniae* isolate. These data underscore the importance of collecting PK as part of all in vitro and in vivo infection model studies. Had these investigators not collected PK samples during their study, they would have greatly overestimated ceftaroline exposures achieved in the in vitro infection model.

The evaluation of in vivo PK is critical to conducting PK-PD analyses based on data derived from in vivo infection models. Thus, careful consideration needs to be given to the design of such studies to ensure that useful data are generated. To this end, measures can be taken to mitigate sources of variability in PK of the antimicrobial agent by controlling for factors such as route of administration, species, sex, and weight. The use of genetically modified animals of the same species, sex, and weight has allowed for control of the latter set of factors. Along these same lines, the route of drug administration is another important consideration. While intraperitoneal injections are routinely utilized due to the relatively large potential space for injection and the ease and rapidity with which drug administration can be carried out, training of research staff would be required to ensure proper technique in order to prevent drug administration into abdominal organs or adipose tissue. Intravenous (IV) injections are also desirable for the rapid delivery of drug directly into the bloodstream. However IV injections can be extremely difficult to administer to smaller species such as BalbC as compared to CD-1 mice. Subcutaneous injections are often the most preferred and relatively straightforward injection site, resulting in minimal variability among injections and technicians. Other factors to consider that can affect PK include the potential for drug interactions between the antimicrobial agent and analgesic or anesthetic agent.

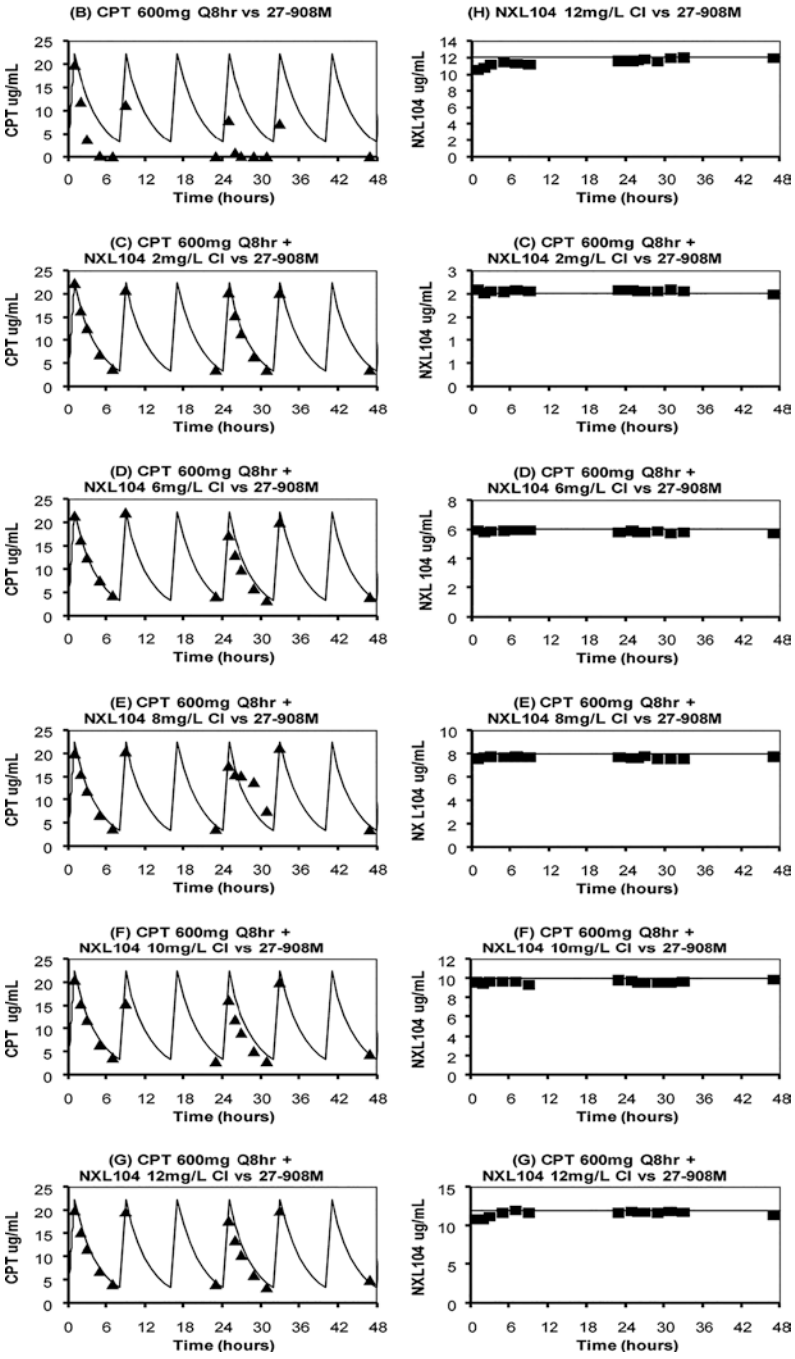

Fig. 22.8 Targeted (solid line) concentration-time profiles with observed concentrations (symbols) overlaid for ceftaroline (triangles) and avibactam (NXL104, squares) among in vitro treatment arms including both agents (C to G) or ceftaroline or avibactam alone (B and H, respectively). (Reproduced from Ref. [22] with permission from Antimicrob Agents Chemother. Copyright © American Society for Microbiology)

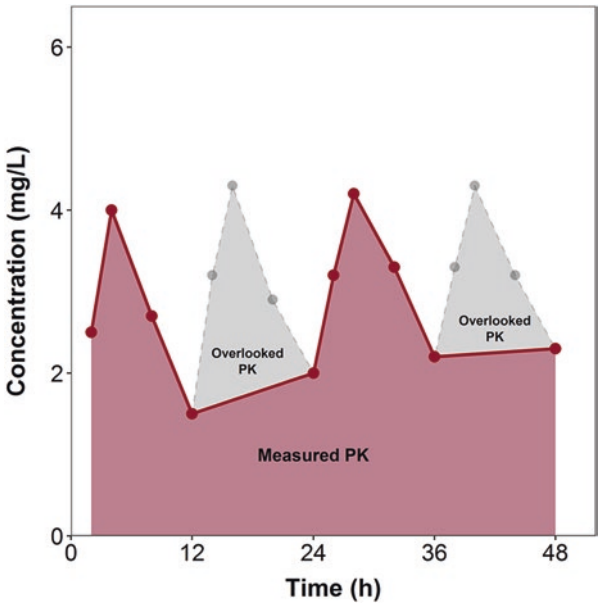

Fig. 22.9 Non-compartmental characterization of PK samples collected both intensively and sparsely following multiple dose drug administration

Other study design elements to consider include infection status and PK sampling. Although in vivo PK studies are often carried out in noninfected animals, it can be advantageous to evaluate PK in infected animals. Moreover, when applicable, PK samples from the effect site of interest should be collected in addition to blood samples (e.g., ELF in the case of murine-lung infection models). Intensive PK sampling enables non-compartmental analyses (commonly referred to as "SHAM analyses") of the PK data. Non-compartmental analyses allow for lines across the individual or pooled concentrations obtained from animals to be connected, thus allowing for the shape of the concentration-time profile(s) to be characterized. Based on these data, exposure measures such as the AUC and Cmax can be calculated. While PK sampling is typically intensive, it is also possible to design less intensive PK sampling strategies and use compartmental analyses to derive drug exposures. Compartmental analyses offer the benefit of allowing for more precise estimates of drug exposure with less reliance on intensively sampled PK from study animals. If non-compartmental analyses are used to evaluate PK data, intensive sampling around multiple doses may be required to ensure reliable estimates of drug exposure. Consider the case presented in Fig. 22.9 in which animals were dosed every 12 h over a 48-h period, but PK were only intensively sampled following the first and third doses. If a non-compartmental analysis were used to evaluate these data, exposures would be greatly underestimated given that the concentrations following the second and fourth doses would be largely ignored. Thus, it is important to ensure that the PK sampling strategy is adequate,

especially if non-compartmental analyses are planned. Given that PK sampling is often terminal for study animals, designing a study with less intensively sampled PK and using compartmental analyses is also beneficial from an ethical perspective. Compartmental analyses are also more desirable when greater variability in the PK of the antimicrobial agent is anticipated as would be the case when PK is studied in infected animals. Compartmental models and their use for evaluating clinical PK data in conjunction with Monte Carlo simulation to evaluate potential antimicrobial dosing regimens for clinical studies are discussed in Sects. 22.3 and 22.4, respectively.

22.2.5 Resistance Prevention

In order to preserve the efficacy of an antimicrobial agent, we must determine not only the doses required for microbial killing but also those needed to slow or prevent the emergence of on-therapy resistance. Such dosing regimens hold the promise of durability. That is, antimicrobial dosing regimens which are selected on the basis of resistance prevention are likely to maintain their antimicrobial activity well after commercialization. Key to this effort is characterizing the time course and relationship between drug exposures and resistance emergence. The gold standard for obtaining such information is the hollow-fiber in vitro infection model, but many other less sophisticated in vitro tools such as mutation frequencies and one-compartment (chemostat) models may be utilized to obtain a basic understanding of this relationship.

In the case of one-compartment in vitro infection models, drug-free media and antimicrobial doses are pumped into a central compartment which contains a microorganism of interest. Waste and drug degradation by-products are pumped out of the system and enter a peripheral waste reservoir. This system allows investigators to precisely simulate targeted concentration-time profiles and is more cost-effective than a hollow-fiber model but is limited in its ability to determine exposures that prevent amplification of resistant subpopulations. This limitation is due to the inadvertent loss of microorganisms from the central compartment when waste is removed from the system, which ultimately leads to artificial decreases in susceptible and resistant organism density. Conversely, the hollow-fiber in vitro infection model is a two-compartment model which utilizes a cartridge comprised of thousands of small tubular filters (fibers). These filters allow the free diffusion of media, drug, and waste products while trapping the microorganisms within the cartridge. Consequently, the system enables the evaluation of resistance emergence over long periods of time (weeks to months) within the hollow-fiber cartridge.

Figure 22.10 presents data obtained from a hollow-fiber in vitro infection model evaluating the time course of resistance emergence across a wide range of doses for the cephalosporin-β-lactamase inhibitor combination, ceftolozane-tazobactam [23]. This system was used to simulate free-drug serum ceftolozane-tazobactam

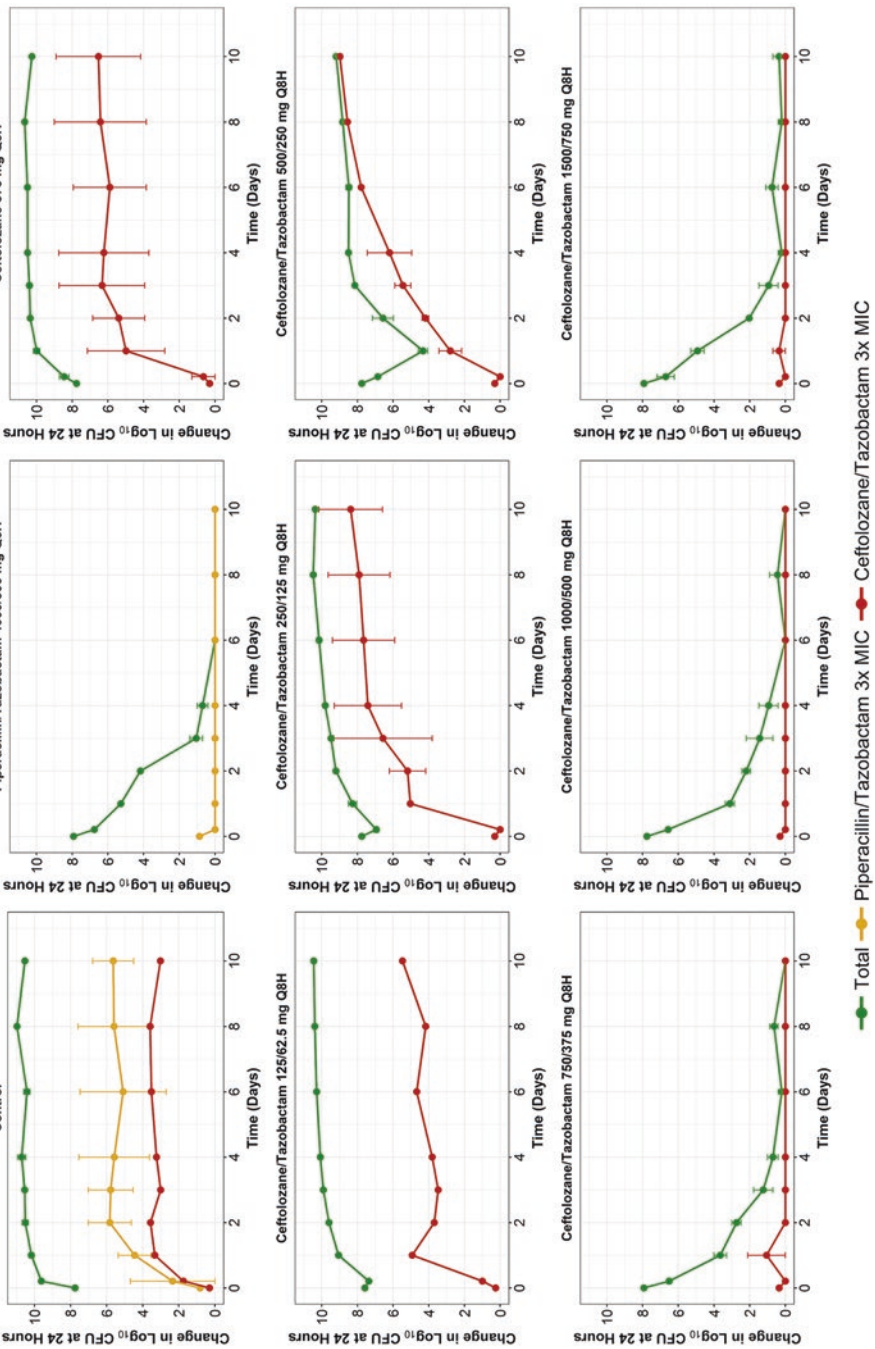

Fig. 22.10 Changes in the total and resistant bacterial populations over time for single ceftolozane, piperacillin-tazobactam, and control dosing regimens (**a**) and a range of ceftolozane-tazobactam dosing regimens (**b**). (Reproduced from Ref. [23] with permission from Antimicrob Agents Chemother. Copyright © American Society for Microbiology)

concentration-time profiles and evaluate the activity of these agents administered together against a laboratory-derived *Escherichia coli* strain in order to determine the combination of doses required to prevent the amplification of a resistant bacterial subpopulation. In order to evaluate this endpoint, changes in bacterial density for the total and resistant bacterial populations were determined. Samples for bacterial enumeration were collected at 0 and 5 h after the start of the experiment and on Days 1, 2, 3, 4, 6, 8, and 10 and plated on drug-free agar and agar supplemented with piperacillin-tazobactam or ceftolozane-tazobactam at concentrations that were three times the baseline MIC of the *E. coli* strain utilized. The total bacterial population and resistant subpopulation were represented by the CFUs which grew on drug-free agar plates and plates containing agar supplemented with drug concentrations that were three times the baseline MIC, respectively.

As shown in Fig. 22.10, the range of ceftolozane-tazobactam doses utilized yielded a wide range of effects. For the lowest ceftolozane-tazobactam regimen (125/62.5 mg), the growth of the resistant bacterial subpopulation was stabilized with no effect on the magnitude of the total bacterial population. The intermediate dosing regimens (250/125 and 500/250 mg) resulted in the amplification of resistance. Within these treatment arms, the resistant bacterial subpopulation was shown to steadily increase, representing a greater proportion of the total bacterial population over time. Finally, for the highest dosing regimens (750/375, 1000/500, and 1500/750 mg), resistance emergence was prevented as evidenced by the eradication of the resistant subpopulation and eventual sterilization of the in vitro system. Data such as these are highly informative and can aid in the selection of optimal dosing regimens which minimize the risk of on-therapy amplification of the growth of drug-resistant subpopulations.

The strength of the findings for the above-described example is that the *E. coli* strain evaluated was genetically constructed in order to isolate a specific mechanism of resistance for study, CTX-M-15 in this case. However, strains that are produced in this manner are often less biologically fit and less virulent than those encountered clinically. Thus, using clinical isolates that have been genotyped is a preferable approach when designing PK-PD studies to evaluate pathogens with selected resistance mechanisms.

Another important consideration for designing studies to evaluate the emergence of resistance is the target pathogens of interest and the likely resistance determinants that are expected to be observed. In this case, the evaluation of an *Enterobacter* isolate which can overexpress AmpC β-lactamases as a result of mutations in the AmpC transcription protein (AmpR) in response to β-lactam exposure [24, 25] would have provided the opportunity to pressure test the dosing regimen for ceftolozane-tazobactam (1000 and 500 mg) that was chosen for clinical study and ultimately approved for use for the treatment of patients with complicated intra-abdominal infections (cIAI) and complicated urinary tract infections (cUTI) [26]. The conduct of such studies in early-stage development provides the opportunity to evaluate decisions about both the choice of the partner β-lactam and β-lactamase inhibitor and the dose of each agent in combination.

22.3 Population Pharmacokinetic Analyses

Selection of a dosing regimen for Phase 2 and 3 trials is a critical decision in the development of new antimicrobials. The dose selected needs to be low enough to prevent severe drug-related toxicities, yet high enough to achieve efficacy in the majority of patients. Ideally, this dose should also be of sufficient magnitude to prevent the emergence of on-therapy resistance. The first step in selecting a clinical dosing regimen is to understand the PK of the drug in humans, including PK variability and factors contributing to this variability. Once this is known and thoroughly understood, simulations of various proposed dosing regimens can be conducted as will be discussed in Sect. 22.4.

Population PK modeling is the current gold standard for performing PK analyses in this context. Although simpler tools are available (such as non-compartmental, naïve pooling, and standard two-stage approaches), a population PK model offers a few key advantages. The first of these is that the population approach allows for the quantification of between-subject variability. This is particularly useful when simulating various dosing regimens as one can estimate the width and shape of the expected exposure distribution. The second advantage is the ability to quantify and explain variability in PK through factors such as body size, age, gender, or clearing organ function (i.e., patient-specific covariates). Again, this is useful when performing simulations as one can determine if the same dose can be administered to all patients or if dose should be adjusted based on patient characteristics (e.g., weight-based dosing or adjustments based on patient renal function). The final key advantage of population PK models is that they can be informed by sparsely collected PK data based on an optimized sampling scheme. This is especially important when utilizing data from Phase 2 and 3 trials as it is rarely feasible to collect rich PK data in all patients in these studies due to ethical and logistical constraints.

In essence, population PK models use differential equations to describe the time course of drug concentrations across a population of subjects. Traditionally, compartmental models are utilized, wherein the model compartments represent hypothetical spaces to which drug may distribute (e.g., vasculature, tissue, macrophages, etc.). The central compartment most often represents the vascular space and all areas of the body where drug rapidly equilibrates. Various peripheral compartments may be added to the base model to represent areas of the body with slower distribution characteristics. In addition, absorption compartments can be added to characterize the time course of drug disposition following various routes of administration such as absorption through the gastrointestinal tract after oral administration. Each compartment is represented with a differential equation using various PK parameters (e.g., clearance or volume of distribution) to describe the movement of drug into and out of the compartment. The values of these parameters can vary across subjects. For instance, take an oral antibiotic with a concentration-time profile which can be described with a one-compartment model. The model can be described with two differential equations – one for the gut/gastrointestinal tract and one for the plasma and other places in the body where the drug rapidly distrib-

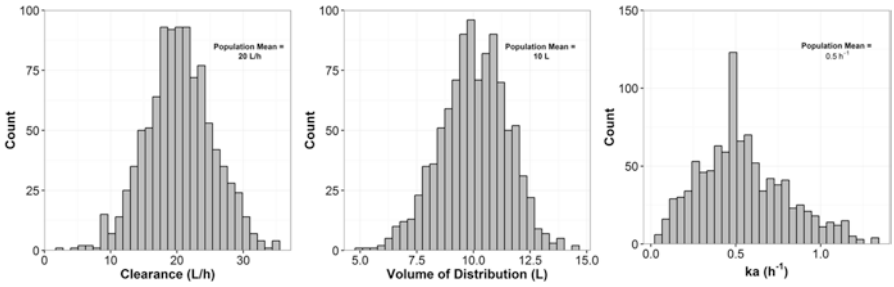

Fig. 22.11 Distributions of individual PK parameter estimates using a population PK model

utes. The differential equations often use a "ka" parameter to describe absorption from the gut/gastrointestinal tract, a volume of distribution term to relate drug amounts to measured drug concentrations, and a clearance term to describe removal of the drug from the body. Using a population PK model, one can determine a mean parameter value across all subjects (known as the "population mean value," as shown in Fig. 22.11). In addition, the model can also estimate an individual parameter estimate for each subject, as shown in Fig. 22.11. The distribution around the population mean value is termed "interindividual variability." Quantification of this variability is crucial for dose selection analyses, which are discussed further in Sect. 22.4.

The development of the population PK model should be an iterative process, beginning during Phase 1 development. If the studies are designed appropriately, analysis of Phase 1 data should allow for the identification of anomalies in PK, such as nonlinearity or nonstationary, early in drug development. Nonlinearity refers to a change in exposure which is not proportional to the change in dose. For example, a doubling of dose would typically be expected to cause a doubling of the AUC, but in the case of a drug which exhibits nonlinear PK, the AUC might only increase slightly following this doubling of dose. Oftentimes, this is due to saturation of an absorption process in the gastrointestinal tract following oral administration. More troubling are instances in which the dose doubles, but the resulting AUC is more than doubled. This is often due to the saturation of an elimination pathway. Nonstationary, on the other hand, refers to PK parameters that change with time irrespective of dose. For example, a drug with auto-induction of clearance can result in lower exposures on Day 5 of therapy relative to Day 1, even if the same dose is administered in both cases. Other common PK issues which can be observed in Phase 1 studies are food and diurnal effects. All of the abovementioned PK complexities can be built into the population PK model structure.

Throughout the iterative process of developing and refining a structural population model, covariate analyses can be conducted. Covariate analyses allow for identification and evaluation of factors that explain variability in the PK parameters. Typical covariates evaluated include body size measures [e.g., weight, height, body surface area, body mass index (BMI), lean body weight], sex, race, age, clearing

organ function (e.g., creatinine clearance or hepatic function tests), and genotypes. Trends between covariates and individual PK parameters are explored. Any covariate-parameter pairs with a considerable relationship are tested in the model, and those which are found to have statistically significant relationships remain in the model. Oftentimes, the value of these analyses may be limited during early-stage development when data stem largely from Phase 1 studies comprised of largely homogenous healthy volunteers. However, covariate analyses become more informative as they are enriched with data obtained from special populations such as patients with renal impairment or obesity and Phase 2 and 3 studies, which enroll large numbers of patients with greater covariate variability.

As evidenced by results of analyses that have demonstrated differences in clearance, volume of distribution, and tissue penetration in patients compared to healthy volunteers [27–29], differences in PK for patients relative to healthy subjects may be observed. This is not surprising given the physiologic changes observed in patients, particularly those who are critically ill. In infected and acutely ill patients, greater PK variability has been observed [30–32]. Regardless of the scenario, these differences can be quantified by refining the population PK model. Thus, after development of the Phase 1 population PK model, it is important that the model is refined using data collected from Phase 2 and/or Phase 3 studies. Phase 2 is typically the first time the investigational agent is administered to patients rather than healthy volunteers. However, in the case of the development of antimicrobial agents for MDR and XDR pathogens, the conduct of Phase 1b studies conducted in patients prior to small Phase 3 clinical studies is increasingly becoming commonplace.

Lastly, it is important to update the results of the covariate analysis after refining the population PK model as there is typically a wider range of covariates observed in Phase 2 and 3 studies. For example, the protocol for Phase 1 studies usually excludes patients with a BMI above 30 kg/m^2. However, in Phase 2 and 3, oftentimes there is no exclusion criteria based on BMI. Important relationships between BMI and PK parameters such as clearance may not be evident until a wider range of BMI data are included in the analysis datasets. In addition to the wider range of covariates for patients in Phase 2 and 3 studies relative to subjects enrolled in Phase 1 studies, additional covariates which could not be assessed in healthy volunteers (e.g., renal function, APACHE II score, and/or infection type) can be evaluated.

22.4 Monte Carlo Simulation and Dose Selection

Monte Carlo simulation is a mathematical technique that uses repeated random sampling to determine the impact of uncertainty when characterizing the probability of an event. Such an approach is useful to determine the probability of achieving a PK-PD target associated with efficacy among simulated patients with drug exposures that would be expected in the target patient population [33, 34]. These analyses, which are commonly referred to as PK-PD target attainment analyses, are widely used in both early- and late-stage drug development to support the selection

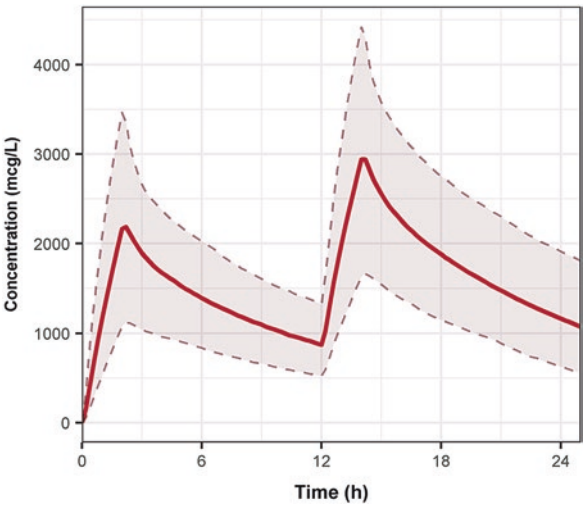

Fig. 22.12 Representative example of concentration-time profiles for a simulated patient population after administration of a given dosing regimen. The solid line represents the median profile and the lower and upper dotted lines represent profiles with concentrations at the 5th and 95th percentiles, respectively

of dosing regimens and develop in vitro susceptibility testing criteria (i.e., susceptibility breakpoints) [35]. In this section, we will briefly outline the use of Monte Carlo simulation to carry out PK-PD target attainment analyses and discuss how the results of these analyses may be used to evaluate dosing regimens and inform the selection of interpretative criteria for in vitro susceptibility testing.

The optimal application of Monte Carlo simulation for dose selection evaluations requires the use of compartmental models as previously discussed in Sect. 22.3. A population PK model for the antimicrobial under investigation can be used to generate drug exposures of interest for simulated patients. As discussed previously, interindividual variability can exist on many of the parameters within population PK models. As illustrated in Fig. 22.11, by randomly assigning parameter estimates for simulated patients based on the distributions for these parameters, interindividual variability can be considered. As shown in Fig. 22.12, PK parameter estimates assigned to simulated patients can be used to generate concentration-time profiles after administration of the dosing regimen of interest, which in this example was an intravenously administered antibiotic infused over 2 h twice daily. The distribution of these concentration-time profiles, as represented by the median and 5th and 95th percentiles, allows for an understanding of the variability expected in the actual patient population.

These above-described concentration-time profiles can be used in conjunction with fixed MIC values to calculate the PK-PD index associated with efficacy for a given pathogen by MIC for individual dosing regimens administered to each simulated patient. The percentage of simulated patients achieving a given PK-PD target by MIC is then determined. As shown by data assessing the percent probabilities of PK-PD target attainment by MIC for ceftaroline relative to nonclinical %T > MIC targets for *S. aureus* shown in Fig. 22.13 [35], these data are commonly interpreted in the context of observed MIC values for isolates based on

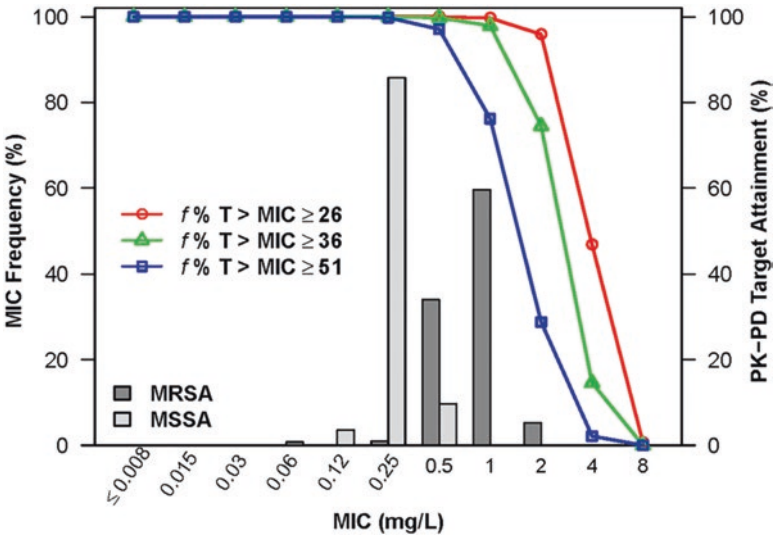

Fig. 22.13 Percentage of simulated patients with normal renal function (80≤ creatinine clearance ≤170 mL/min/1.73 m^2) achieving free-drug (*f*) %T > MIC targets by MIC following administration of ceftaroline fosamil 600 mg q12h, overlaid on a histogram showing the MIC distributions for MRSA and MSSA. (Reproduced from Ref. [35] with permission from Antimicrob Agents Chemother. Copyright © American Society for Microbiology)

in vitro surveillance data. In this example, a collection of 3965 *S. aureus* isolates collected from medical centers in the United States, stratified by the 2254 and 1711 isolates which were methicillin-resistant and methicillin-susceptible, respectively (MRSA and MSSA, respectively), were evaluated. Percent probabilities of PK-PD target attainment of ≥90% up to MIC values that represent the upper margins of the MIC distribution (i.e., the MIC_{90} which represents the MIC value at which ≥90% of isolates are inhibited) would be considered a favorable set of results for a given dosing regimen. While the evaluation of the probability of PK-PD target attainment by MIC is useful to support recommendations for dosing regimens, evaluations weighted over the MIC distribution also provide support for a given dosing regimen. The latter, which is commonly referred to as the overall probability of PK-PD target attainment, can be determined by multiplying the probability of PK-PD target attainment for a specific PK-PD target at a given MIC value with the probability of occurrence of that MIC value and then taking the sum of these percentages. When based on robust in vitro surveillance data for a given pathogen [36], the overall probability of PK-PD target attainment is a metric that provides an expectation of PK-PD target attainment in a simulated population based on the MIC distribution for that pathogen likely to be observed in clinical practice.

The choice of the PK-PD target used to support dose selection and susceptibility breakpoint recommendations is an important consideration for assessments of

PK-PD target attainment. Endpoints for such PK-PD targets range from net bacterial stasis to a 2-$\log_{10}$ CFU reduction from baseline. Typically, these data are derived from neutropenic murine-thigh or murine-lung infection models or when warranted, in vitro infection models. Net bacterial stasis has been suggested to be an appropriate endpoint for a PK-PD target when selecting dosing regimens to treat patients with infections associated with lower bacterial inoculums and/or for which source control, including surgical intervention, is an option. This endpoint may also be reasonable to assess for inferences about patient populations that are expected to be immunocompetent and for whom the response rate associated with no treatment is expected to be relatively high (e.g., ≥60%). Examples of indications that meet these criteria include acute bacterial skin and skin structure infections (ABSSSI), cIAI, and cUTI. Reduction of 1-$\log_{10}$ CFU from baseline has been suggested to be an appropriate endpoint for a PK-PD target when selecting dosing regimens to treat patients with infections associated with higher bacterial inoculums such as pneumonia, endocarditis or bacteremia, and/or for infected patients who are immunocompromised. In such populations, the response rate associated with no treatment may be low (e.g., ≤40%) [11, 37, 38].

Support for each of the above-described endpoints is based on successful translations between the results of previous PK-PD analyses based on nonclinical and clinical data [3, 39–42]. Results of analyses of these data have demonstrated that the same magnitude of the PK-PD target associated with net bacterial stasis from neutropenic murine-thigh infection models for a given antimicrobial agent was associated with a high percentage of successful outcomes among patients with cIAI or ABSSSI [3, 39–42]. The choice of a 1-$\log_{10}$ CFU reduction from baseline for the treatment of patients with infections with a higher no-treatment response is based on an assessment of PK-PD target attainment analysis results for antimicrobial agents that were evaluated for pneumonia. As the percent probability of achieving a PK-PD target associated with a 1-$\log_{10}$ CFU reduction from baseline increased, so too did the probability of a successful regulatory outcome [2]. The latter was considered an indicator of meeting non-inferiority in pivotal clinical trials.

While a 2-$\log_{10}$ CFU reduction from baseline has been suggested as an endpoint for indications such as HABP/VABP [11], attainment of a PK-PD target associated with such a level of bacterial reduction may not be possible for many antimicrobial agents, including those currently available and commonly used for these indications. As previously shown for a meropenem dosing regimen of 2 g q8h infused over 1 h [4], while it is possible to achieve the %T > MIC target associated with a 2-$\log_{10}$ CFU reduction from baseline, large interpatient variability can hinder the likelihood of achieving this PK-PD target in many patients. However, one strategy to overcome this is to administer the same dose as a prolonged infusion over 3 h [43]. Such a strategy was employed for development of meropenem-vaborbactam, a β-lactam/β--lactamase inhibitor combination recently approved by the US FDA [44]. From a drug development perspective, the margin of safety should be weighed against goals for efficacy when considering an endpoint of a 1- vs 2-$\log_{10}$ CFU reduction from baseline for indications such as HABP/VABP [38]. If an antimicrobial agent has a

wide safety margin, developers have a greater opportunity to utilize a 2-$\log_{10}$ CFU reduction endpoint.

In addition to the PK-PD target, it is important to consider exposures at the effect site when applicable. For example, if an antimicrobial agent is being developed to treat patients with pneumonia, it is important to evaluate the likelihood of achieving efficacious drug concentrations in ELF. To enable the consideration of ELF exposures, PK data from healthy volunteers and if available, from patients, should be considered when developing the population PK model. This model would then be used together with ELF PK-PD targets associated with efficacy derived from a murine-lung infection model and Monte Carlo simulation to assess PK-PD target attainment for dosing regimens.

As described above, the assessment of dosing regimens to evaluate in Phase 2 or 3 studies requires the use of a population PK model constructed using PK data from healthy volunteers enrolled in Phase 1 studies. As such, the interindividual variability in PK will be limited and may not be reflective of the target patient population. In such cases, inflating variance in PK parameters (e.g., increasing the interindividual variability terms on PK parameters such as clearance and volume) as a part of a sensitivity analysis may be a useful approach to further discriminate among candidate dosing regimens [38]. Another limitation of a population PK model developed using Phase 1 PK data is that covariate distributions are relatively narrow. Since studies for special populations, including subjects with renal or hepatic impairment, are typically not completed early in a clinical development program, the evaluation of covariates is not usually available until late-stage development. Thus, early-stage development decisions for dose selection should be confirmed after a population PK model has been refined using data from the target patient population and special populations. Additionally, an understanding of covariates that are highly influential on PK allows for the assessment of dosing regimens in simulated patients stratified by ranges of such covariates to support dosing recommendations for special populations. Data from simulated patients can be used to support dosing recommendations even if such dosing regimens were not assessed in clinical trials. This strategy was used for delafloxacin, a fluoroquinolone that was recently approved by the US FDA for the treatment of patients with ABSSSI. The delafloxacin dosing regimen approved for patients with severe renal impairment [45], 450 mg by mouth twice daily, was not studied in clinical trials [46, 47] but was supported by the results of population PK and PK-PD target attainment analyses [48].

The above-described strategy to use preclinical PK-PD data, population PK models, and Monte Carlo simulation both for early- and late-stage development decisions about dose selection allows developers to mitigate risk and increase the likelihood of regulatory success. The results of such analyses can also be used to inform recommendations for interpretative criteria for in vitro susceptibility testing criteria for the antimicrobial agent of interest against target pathogens. The data obtained from these simulations can be used in conjunction with clinical outcome data by MIC and pathogen susceptibility distributions to support susceptibility breakpoint decisions. Results of PK-PD target attainment analyses to support sus-

ceptibility breakpoint recommendations is critical information for drug developers not only when seeking regulatory approval but also early in clinical development. Given the lengthy process of incorporating an antimicrobial into automated susceptibility testing systems, it behooves developers to perform preliminary susceptibility breakpoint evaluations in order to ensure informed decisions are made.

22.5 Clinical Data for PK-PD Analyses

As described above, an understanding of the PK-PD characteristics of an antimicrobial agent early in drug development increases the likelihood of regulatory success. However, the evaluation of PK-PD relationships for both efficacy and safety based on clinical data collected in Phase 2 and 3 can be used to provide valuable information to confirm early-stage dose selection decisions and further improve the likelihood of regulatory success. Depending on the indication and whether the antimicrobial agent is being developed for a setting of UDR versus MDR or XDR pathogens, the robustness of the clinical data package required can vary. For indications involving relatively susceptible pathogens and for which a suitable comparator agent can be studied, the clinical data package includes data from clinical studies that are powered to demonstrate non-inferiority and that are large enough to detect safety signals. Such studies, especially when PK data are collected in all patients, provide a robust repository of data to use for evaluating PK-PD relationships for efficacy and/or safety endpoints. However, in the setting of highly resistant pathogens, large clinical studies are difficult to conduct in a reasonable time frame. An important challenge for conducting such studies is the lack of frequency of patients with such infections. Furthermore, when identified, study enrollment can be difficult to accomplish as these patients are often critically ill [1]. In order to develop a given antimicrobial agent for MDR or XDR pathogens in a reasonable time frame, clinical data for indications involving such pathogens will be less robust. Given that data from in vitro or in vivo infection models have demonstrated similar PK-PD relationships for efficacy among isolates with and without resistant determinants [18, 49], the most efficient development program for antimicrobial agents for MDR and XDR pathogens would be one that combines robust preclinical PK-PD data, the data package for which includes MDR and/or XDR pathogens, with data from clinical studies conducted in the UDR setting that are powered to demonstrate non-inferiority. While such programs, especially with even a limited number of clinical cases with MDR and/or XDR pathogens should be adequate to allow for labeling that includes indications for such pathogens, regulatory agencies to date have been less willing to formally establish drug development paths based on this premise. Instead, discussion has centered around a plan to encourage sponsors to assemble robust preclinical PK-PD and Phase 1 PK data packages together with a limited clinical data package to strengthen NDA submissions for indications due to MDR and XDR [1, 50]. Regardless of the path, there is a common requirement for both nonclinical and clinical PK-PD data to increase regulatory certainty.

22.5.1 Data Prerequisites

Irrespective of whether the clinical data package is robust or limited, the data derived from PK-PD analyses are valuable. However, as described below, objectives of such analyses will vary depending on the data package available. Important prerequisites for both types of clinical data packages include the collection of PK data from all patients and the evaluation of informative endpoints. As described in Sect. 22.3, the benefit of developing a population PK model based on Phase 1 data is that the model can be used to determine sparse PK sampling strategies for implementation in clinical trials. Such strategies are designed to ensure that optimal information to estimate drug exposure in each patient is obtained using a minimal number of blood samples for drug assay as possible. Using these sparse PK data, the goal of additional population PK analyses is to refine the existing model developed using Phase 1 data in order to enable precise and unbiased estimation of drug exposure in individual patients, including the applicable PK-PD index for efficacy (e.g., AUC:MIC ratio, Cmax:MIC ratio, or %T > MIC).

In addition to reliable estimates of drug exposure, well-defined and reproducible efficacy and safety endpoints are needed to evaluate PK-PD relationships for such endpoints. Objective criteria, determined by observations collected at informative time points, are required to assess drug effect. Clinical trial endpoints for efficacy are typically categorical variables, such as clinical response to therapy (success or failure) assessed at the test-of-cure visit (i.e., a window of time after the end of study drug; TOC) and/or at the end of therapy. However, recent US FDA guidance for a number of indications has described the assessment of efficacy endpoints evaluated earlier in therapy [5, 6]. For patients with ABSSSI and CABP, clinical response is assessed on Days 2 to 3 and 3 to 5, respectively. PK-PD relationships for efficacy have been largely described using dichotomous efficacy endpoints assessed at TOC [3, 31, 41, 51–54]. In contrast, there is comparatively less experience evaluating efficacy endpoints assessed earlier in therapy [55]. Despite the lack of experience with the latter, given the natural course of infection, which involves eradication of the pathogen followed by macrophage and inflammatory modulator activity, which is then followed by resolution of signs and symptoms, it may be difficult to identify PK-PD relationships for efficacy early after therapy has been initiated [56–58]. Consequently, the time at which efficacy is assessed can influence the likelihood of identifying PK-PD relationships for efficacy.

While dichotomous efficacy endpoints are typically evaluated in clinical trials for antimicrobial agents and serve as primary endpoints upon which sample size is determined, the evaluation of continuous or time-to-event efficacy endpoints can also be informative. Examples of continuous endpoints include change in bacterial density or lesion size, while examples of time-to-event endpoints include time to resolution of signs and symptoms, lesion size reduction, or bacteriologic eradication. Continuous or time-to-event endpoints have the benefit of being more sensitive than categorical endpoints for capturing drug effect. When measures of efficacy are assessed serially, this provides the opportunity to identify the time period during

which treatment effect is greatest [55, 59]. Evaluation of such endpoints for PK-PD analyses for efficacy has the potential to inform decisions about dose and duration using a relative smaller sample size than that for a dichotomous efficacy endpoint [59]. For example, while evaluations of clinical or microbiological response for 38 tigecycline-treated patients with CABP failed to reveal PK-PD relationships for efficacy, a relationship between free-drug AUC:MIC ratio and time to fever resolution was identified [60]. The median time to fever resolution was 12 and 24 h for patients with a free-drug AUC:MIC ratio >12.8 and ≤12.8, respectively. Thus, despite not representing the primary clinical trial endpoint for efficacy, relationships for such endpoints can be used to support dose selection and even provide potential insights about the duration of therapy.

For the assessment of PK-PD relationships for safety endpoints, the same principles as described above are applicable. While safety endpoints, such as the presence or absence of a given safety event or a dichotomous threshold for a continuous laboratory measure, are dichotomous in nature, the assessment of continuous endpoints including laboratory measures or physiologic measurements such as blood pressure collected serially provides the opportunity to develop informative multivariable models [61, 62]. Such models, which can be constructed to describe the effect of varying drug exposures on laboratory measures over the course of therapy in the context of other patient factors, can then be applied to simulated data to discriminate among potential dosing regimens to be studied in Phase 3 trials. For example, the percentage of simulated patients with laboratory measures above clinically relevant folds of the upper limit of normal (ULN) (e.g., 3, 5 or 10 × ULN) or in the case of systolic blood pressure, the percentage of patients with readings ≥160 mmHg can be determined for individual dosing regimens. This information, together with assessments of the percent probabilities of achieving each efficacy endpoints (based on clinical PK-PD relationships for efficacy) and/or nonclinical PK-PD targets, can be used to balance considerations for safety and efficacy. Or using multivariable models developed using Phase 2 and/or 3 data, percent probabilities of elevation of these safety endpoints can be evaluated among all simulated patients and subgroups at increased risk who receive intended dosing regimens. The identification of patient populations at increased risk and the characterization of the elevations for such safety endpoints can be used to inform use for labeling and/or clinical practice guidelines.

22.5.2 Analysis Objectives

As described above, the robustness of the clinical data package guides the objectives of the PK-PD analyses for efficacy. For antimicrobial agents for patients with infections arising from pathogens in the setting of UDR, the sample size of evaluable populations is expected to be sufficient to support the assessment of PK-PD relationships for efficacy. Thus, the objective is to determine if PK-PD relationships for efficacy endpoints can be identified. However, despite the robustness of the sample

size of analysis populations, other factors may influence the ability to characterize PK-PD relationships, including the duration of therapy. It is important to evaluate patients from the microbiologically evaluable population in order to consider patients who received a sufficient number of doses and who had pathogen(s) isolated at baseline. The former ensures that the lack of clinical response is not attributed to insufficient duration of drug exposure, and the latter allows for drug exposures to be indexed to pathogen MIC values in order to enable PK-PD indices to be determined. For infections for which there are baseline pathogens with anticipated or known (as determined by preclinical data) PK-PD characteristics that differ, evaluation of subpopulations may be necessary to characterize PK-PD relationships for individual pathogens. Or in the setting of infections with polymicrobial pathogens, careful consideration needs to be given to how the primary pathogen used for calculating the PK-PD index is identified. Additionally, consideration needs to be given to the definitions for clinical failure. If the reasons for declaring a clinical failure include those not related to study drug (e.g., an adverse events), data for patients failing for these reasons should be excluded given the potential for these data to impede the ability to identify PK-PD relationships.

Finally, and perhaps most importantly, while it is important to consider all of the above-described factors to ensure that every opportunity has been provided to allow for PK-PD relationships for efficacy based on a robust clinical data package to be identified, the lack of identification of PK-PD relationships for efficacy is a predictable outcome when patients have received PK-PD optimized dosing regimens. In such cases, it is still valuable to demonstrate that drug exposures from patients indexed to MIC values from pathogens identified at baseline exceed nonclinical PK-PD targets for efficacy to confirm the basis for dose selection. When PK-PD relationships for efficacy are identified based on clinical data from patients who received PK-PD-optimized dosing regimens, these relationships are usually based on a dichotomized variable for the PK-PD index of interest, the threshold for which is optimally determined using a number of statistical approaches. These approaches can include using the threshold of the PK-PD index representing the first split of a classification or regression tree, a receiver operating characteristic curve, or using a model fit to estimate a threshold for achieving a target efficacy outcome or probability. PK-PD relationships identified in this manner resemble step functions and allow for patients with both lower PK-PD indices and percentages of successful response to be contrasted from those with higher PK-PD indices and percentages of successful response [51, 59]. Table 22.1 summarizes the results of two separate PK-PD analyses of Phase 3 data for patients who received PK-PD optimized dalbavancin or oritavancin regimens. In both cases, PK-PD relationships identified were based on two-group variables for AUC:MIC ratio [63, 64]. The differences in the percentage of successful clinical responses between patients in the lower and higher exposure groups were 10.9 and 13.6%, respectively, with percentage of patients with successful clinical response in the lower AUC:MIC ratio groups of 89.1 and 82.6% for dalbavancin and oritavancin, respectively. Thus, when PK-PD-optimized dosing regimens are studied and PK-PD relationships based on two-group variables are identified, the differences between the lower and higher exposures groups are unlikely to be impressive.

Table 22.1 Summary of PK-PD relationships for efficacy for dalbavancin and oritavancin based on dichotomous two-group AUC:MIC ratio variables

Antimicrobial agent	Efficacy endpoint	PK-PD index	Threshold value of PK-PD index	Percentage of patients < or ≥ threshold achieving the efficacy endpoint (n/N)		*P*-value
				< threshold	≥ threshold	
Dalbavancin [63]	Clinical success at the test-of-cure visit[a]	AUC_{avg}:MIC ratio[b]	21,267	89.1 (98/110)	100 (52/52)	0.01
Oritavancin [64]	Clinical success at the post-therapy evaluation[c]	$AUC_{0–72}$:MIC ratio[d]	11,982	82.6 (19/23)	96.2 (126/131)	0.029

[a]The test-of-cure visit occurred 14 days [± 2 days] after the end of therapy
[b]AUC_{avg}:MIC ratio was calculated by dividing the average 24-h AUC from 0 to 120 h by the baseline MIC of the infecting pathogen
[c]The post-therapy evaluation occurred 7–14 days after the end of therapy
[d]$AUC_{0–72}$:MIC ratio was calculated by dividing the AUC from 0 to 72 h by the baseline MIC of the infecting pathogen

However, for limited clinical data packages in support of indications involving MDR or XDR pathogens, the sample size of evaluable patients will likely be insufficient to allow for formal analyses to be conducted. Thus, in such cases, the objective of the PK-PD analyses for efficacy will be to confirm that drug exposures indexed to MIC values from pathogens isolated at baseline exceeded nonclinical PK-PD targets for efficacy based on robust preclinical PK-PD data for all patients studied. Such information will thereby serve to support dosing regimens selected.

22.5.3 Historical Data and Bayesian Approaches for Clinical Trial Design

Given the current paradigm for obtaining robust preclinical PK-PD data and using these data with Phase 1 PK data and Monte Carlo simulation to predict doses for Phase 2 and 3 clinical trials, the likelihood for failed clinical trials has been reduced. Evaluation of data based on contemporary clinical trials that did not make full use of these approaches to select dose, together with innovative statistical approaches, provides the opportunity to answer questions about the no-treatment effect. Such data represent valuable inputs for power and sample size calculations for future clinical trials in the setting of UDR. As described below, data for tigecycline from 61 patients with HABP/VABP who were microbiologically evaluable and who had sufficient PK data, the clinical trial that failed to demonstrate non-inferiority

compared to imipenem/cilastatin in the clinically evaluable population [65], yielded a number of useful PK-PD findings [53].

Panel A of Fig. 22.14 shows the fitted function and associated 95% pointwise confidence bounds for the relationship between clinical response and free-drug AUC:MIC ratio which was identified using univariable logistic regression [53, 66]. This function is overlaid on a histogram for the distribution of free-drug AUC:MIC ratio. Three important observations based on these data were the following: (1) As the free-drug AUC:MIC ratio increased, so too did the probability of clinical success; (2) the 95% pointwise confidence bounds around the logistic function were tight in the free-drug AUC:MIC ratio range in which the data density was high; and (3) a large proportion of patients (31%) had observed free-drug AUC:MIC ratios associated with a low probability of clinical success, an indicator that the chosen tigecycline dosing regimen, 100 mg IV followed by 50 mg IV every 12 h, was suboptimal for patients with HABP/VABP [53].

The above-described analyses were based on frequentist inference. In a follow-up analysis, Bayesian inference, which provides the benefit of considering prior information, was applied to reassess the PK-PD relationships for efficacy [66]. Specific objectives of the analyses were to determine and compare the magnitude of treatment effect and the ability of clinical trial endpoints to capture drug benefit using frequentist and Bayesian statistical approaches. Prior information that informed the Bayesian analyses were based on data from in vivo studies conducted using a neutropenic murine-thigh infection model. These data, which demonstrated that increasing AUC:MIC ratio was associated with improved response, served to

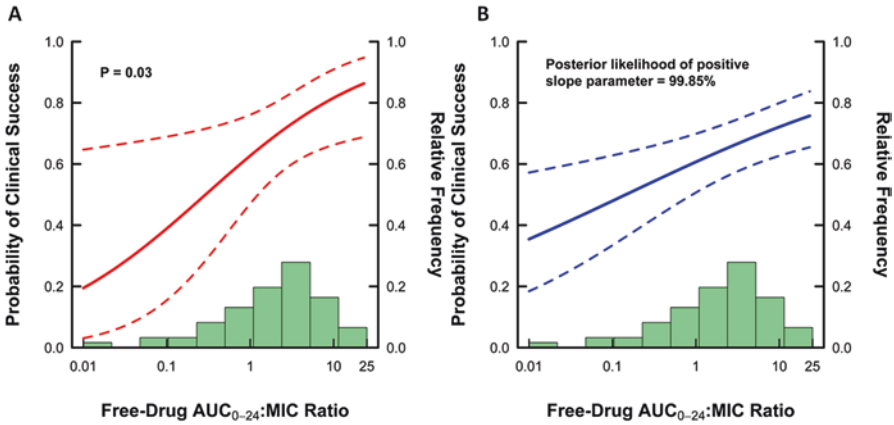

Fig. 22.14 Frequentist (**A**) and Bayesian (**B**) logistic regression-estimated relationships between clinical response and the tigecycline free-drug AUC:MIC ratio based on data from 61 patients with HABP/VABP. The solid lines represent the fitted functions based on frequentist and Bayesian logistic regression, while the dashed lines represent the upper and lower 95% pointwise confidence and credible bounds, respectively. The green histogram represents the distribution of observed values for free-drug AUC:MIC ratio. (Reproduced from Ref. [66] with permission from Antimicrob Agents Chemother. Copyright © American Society for Microbiology)

characterize the magnitude of the association between free-drug AUC:MIC ratio and efficacy. Input variables utilized were the slope and the dynamic range based on the nonclinical PK-PD relationship for *Staphylococcus aureus*, a major target pathogen. The slope was considered a useful parameter to inform the analysis since a positive sign for this parameter was an indicator that higher free-drug AUC:MIC ratios were associated with a greater magnitude of effect. Lower and upper limits of free-drug AUC:MIC ratio of 0.01 and 25, respectively, were employed. This range represented that over which the majority of drug effect in animals was observed and encompassed that observed in patients with HABP/VABP.

While Panel A of Fig. 22.14 shows the fitted function for the PK-PD relationships for clinical response based on frequentist logistic regression, panel B of Fig. 22.14 shows the fitted function for the PK-PD relationship for clinical response based on Bayesian logistic regression. In contrast to the 95% pointwise confidence bounds shown for the relationship based on frequentist logistic regression, tighter 95% pointwise credible bounds are shown for Bayesian logistic regression. As described below, treatment effect was estimated using these PK-PD relationships, both frequentist and Bayesian approaches, and three different methods based on the probability of a successful response at free-drug AUC:MIC ratios of 0.01 and 25.

For Method 1, treatment effect represented the difference in point estimates between the probability of clinical success at free-drug AUC:MIC ratios of 0.01 and 25. For Method 2, treatment effect represented the difference between the upper limit of a 95% interval for the probability of clinical success at a free-drug AUC:MIC ratio of 0.01 and the lower limit of a 95% interval for the probability of clinical success at a free-drug AUC:MIC ratio of 25. This approach is analogous to a fixed margin approach for estimating treatment effect for the design of non-inferiority clinical trials for antimicrobial agents [67]. Figure 22.15 shows a schematic for calculating treatment effect based on the relationship between the probability of clinical success and free-drug AUC:MIC ratio using Bayesian logistic regression and Methods 1 and 2. For Method 3, the 95% lower confidence and credible bounds for the treatment effect were obtained by using 1000 bootstrap samples and a bias-correcting acceleration method.

Treatment effect estimates for clinical response, which were determined using frequentist and Bayesian logistic regression and each of the above-described methods, are summarized in Table 22.2. Differences in point estimates of the treatment effect for clinical response between the frequentist and Bayesian approach were larger using Method 1. The comparatively tighter Bayesian credible intervals observed in panel B of Fig. 22.14 were, however, indicative of increased certainty with the latter approach. For Methods 2 and 3, treatment effect was greater based on using Bayesian logistic regression. These data demonstrate the utility of frequentist and Bayesian-based analyses to quantify treatment effect, a parameter which is important for powering clinical trials. These data also demonstrated that irrespective of the approach, use of bootstrapping to obtain lower bounds for the treatment effect allowed for improvements in the overly imprecise and arbitrary practice of taking the difference between the lower bound of the interval for the maximal effect and the upper bound of the interval for the minimal effect.

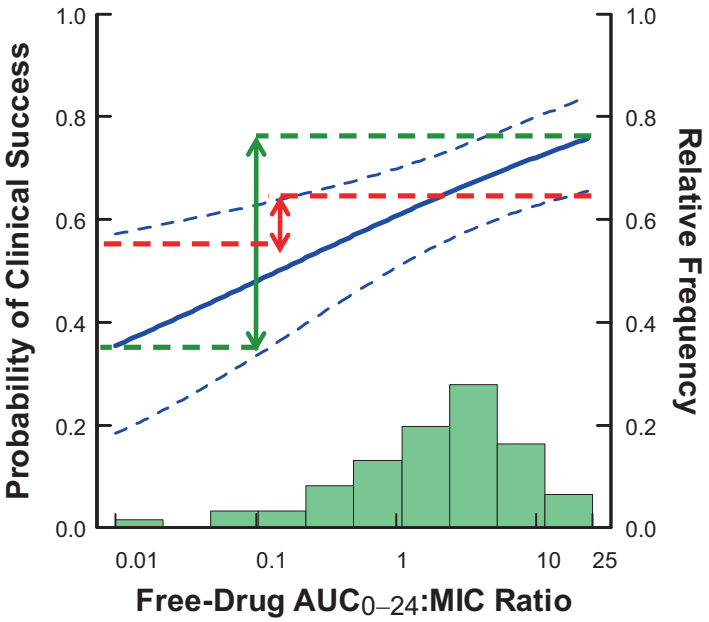

Fig. 22.15 Schematic showing the calculation of treatment effect based on an PK-PD relationship for efficacy using two methods. Antibiotic pharmacodynamics, evaluation of exposure-response relationships using clinical data: basic concepts and applications, 2016, page 143, Sujata M. Bhavnani, Christopher M. Rubino, and Paul G. Ambrose [59]. (This work is subject to copyright. All rights are reserved by the Publisher, whether the whole or part of the material is concerned, specifically the rights of translation, reprinting, reuse of illustrations, recitation, broadcasting, reproduction on microfilms or in any other physical way, and transmission or information storage and retrieval, electronic adaption, computer software, or by similar or dissimilar methodology now known or hereafter developed. With permission of Springer)

Table 22.2 Estimates of treatment effect for clinical response as determined using frequentist and Bayesian logistic regression and three different methods[a]

	Treatment effect estimated by method		
Approach	1	2	3
Frequentist logistic regression	0.672	0.043	0.211
Bayesian logistic regression	0.405	0.085	0.314

Data shown were reproduced from Ref. [66] with permission from Antimicrob Agents Chemother. Copyright © American Society for Microbiology

[a]Based on the probability of clinical success at free-drug AUC:MIC ratios of 0.01 and 25

Results of the above-described assessments of tigecycline PK-PD relationships for efficacy based on data from patients with HABP/VABP and Bayesian statistical approaches allowed for more precise estimates of the no-treatment effect. These data describing the no-treatment effect for this indication, which are from a contemporary clinical trial rather than historic sources, will be useful to inform clinical trial design in the setting of UDR. Although the above-described example was based on

an evaluation of a PK-PD relationship using a dichotomous efficacy endpoint and logistic regression, Bayesian approaches can be applied to the evaluation of PK-PD relationships using other types of efficacy endpoints. Regardless of the type of endpoints or statistical analyses undertaken, application of a Bayesian approach, which considers known a priori data, offers the benefit of increased certainty in the findings. The degree to which certainty can be increased will, however, depend on the quality and robustness of the prior information. With increasing robustness of preclinical data packages for new drug application submissions for antimicrobial agents, the use of the above-described approach to evaluate PK-PD relationships using clinical data is useful to consider.

22.6 Concluding Remarks

Whether it may be through a robust preclinical PK-PD package, population PK and dose selection analyses, or the evaluation of clinical data to establish PK-PD relationships for efficacy or safety, pharmacometrics can serve as a pillar of support for antimicrobial drug development programs. The use of PK and PK-PD analyses can help guide the selection of early- and late-stage clinical dosing regimens and ultimately be used to support final dosing recommendations for regulatory submissions and inform the selection of interpretative criteria for in vitro susceptibility testing. These tenets are even more applicable when developing antimicrobials for the treatment of patients with infections due to MDR and XDR pathogens. Such programs most often must rely on a limited pool of clinical data from which inferences regarding treatment effect may be derived. In these instances, pharmacometric analyses not only support decision-making and de-risk development programs for antimicrobial agents but also serve as the foundation for NDA submissions which strengthens the value of the often limited clinical data obtained.

Major Points

- The increasing prevalence of antimicrobial-resistant pathogens has begun to shrink our once plentiful antimicrobial armamentarium, creating a growing need for new agents to treat patients with infections due to multiple or extensively drug-resistant (MDR and XDR, respectively) pathogens.
- The use of pharmacokinetic-pharmacodynamic principles together with the broader science of pharmacometrics has enabled both early- and late-stage analyses supporting antimicrobial dose selection.
- When developing antimicrobial agents to treat patients infected with pathogens with usual drug resistance (UDR), pharmacometric analyses can serve as a pillar to provide decision support and greatly reduce program risks, greatly increasing the likelihood of regulatory success.
- When developing antimicrobial agents to treat patients infected with MDR and XDR pathogens, pharmacometric analyses can additionally serve as the foundation for new drug application submissions, strengthening the value of the often limited clinical data obtained in such programs.

- In addition to focusing on pharmacometric concepts for the development of antimicrobial agents in the setting of pathogens with UDR, this chapter discusses how pharmacometric analyses can be used to leverage robust preclinical PK-PD packages in conjunction with limited clinical data in order to support antimicrobial drug approval for the treatment of patients with infections due to specified MDR or XDR pathogens.

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Chapter 23
New Regulatory Pathways for Antibacterial Drugs

David Shlaes

23.1 Regulatory History of Antibacterial Drugs

Among the first drugs approved by the Food and Drug Administration in the United States were sulfonamides and penicillin in the 1930s and 1940s [1, 2]. Approval was based on very small clinical trials where control patients received a placebo or where historical data on untreated patients demonstrated the large treatment effect of the new therapy. With the exponential expansion of the number of antibiotics being tested, it became clear that one could not study these new products in the context of placebo-controlled trials, since depriving patients of lifesaving therapy would be unethical. Therefore, all new antibiotics were studied by comparing them to preexisting antibiotics for which efficacy had already been shown.

During the 1980s and 1990s, the US Food and Drug Administration (FDA), in concert with the Infectious Diseases Society of America (IDSA), formalized guidelines for the conduct of clinical trials that would lead to approval of new antimicrobial products [3]. Since it is statistically difficult to demonstrate equivalence, trial designs were based on the idea of non-inferiority in which a statistical margin for the error around the mean efficacy for the test compound, compared to the standard comparator used in the trial, was defined. This margin is an important number, as it defines the number of subjects that must be studied in any trial. The IDSA proposed, and the FDA accepted, margins that were based on trial feasibility rather than on any formal statistical consideration. Until the early 2000s, clinical trials were based on a non-inferiority margin of 15% such that in most cases enrollment of only a few hundred patients would be required. For example, two trials might require 700 patients in total, and two trials were required for each clinical indication, such as skin and skin-structure infection and community-acquired pneumonia. An exception

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I. W. Fong et al. (eds.), *Antimicrobial Resistance in the 21st Century*, Emerging Infectious Diseases of the 21st Century, https://doi.org/10.1007/978-3-319-78538-7_23

was hospital-acquired pneumonia: enrollment was much more difficult, and margins up to 20% were allowed.

The indications and endpoints also differed from those studied today. Prior to 2000, one could combine bacterial bronchitis patients with community-acquired pneumonia patients to receive approval for treatment of lower respiratory tract infection. The endpoints tended to be subjective assessments of cure or improvement at a defined point in time post-therapy. A typical example was 30 days.

Suddenly, at the turn of the century, statisticians at FDA became so concerned over these designs that they revolted [3]. Their concern was mainly based on the possibility of biocreep [4]. In this scenario, drug A is used as a comparator. The assumption is that drug A is superior to placebo by some treatment effect – say 30%. So if no therapy results in cure 40% of the time and therapy with drug A assures cure 70% of the time, there is a 30% treatment effect. But when drug B is compared with drug A and is effective within a 15% margin, that might mean (see below) that the drug is 15% less effective than drug A and that it has only a 25% treatment effect. They pointed out that by the time you get to drug E, you might be back to placebo efficacy without ever realizing it. Based on these considerations, the FDA statisticians insisted on narrower non-inferiority margins – usually closer to 10% – although initially they proposed margins of 5% for some trials. At a 10% margin, one needs to enroll two to three times the number of subjects as in a trial with a 15% margin. At 5%, enrollment must be as much as five times that number.

At that point, the pharmaceutical industry engaged in a counterrevolution. They began a rapid abandonment of antibacterial discovery and development. The new required trial numbers were so large that many companies were concerned that any return on investment would be annihilated by the increasing costs of the trials. With this turn of events, the IDSA tried to intervene, fearing that the discovery and development of new antibiotics needed to fight against ever-increasing bacterial resistance would cease altogether. Does this sound familiar?

One fact that everyone overlooked at the time is that two clinical trials, successfully conducted at a 15% non-inferiority margin, would falsely conclude non-inferiority less than 3% of the time even when calculated at the 10% level. Additionally, as long as the treatment effect of the comparator remains relatively constant, the risk of biocreep is low in any case [5]. And this is likely to be the case since subjects with infections resistant to the comparator are routinely eliminated from analysis. Many of us regarded the FDA statisticians' revolt as unscientific and unnecessary.

Then, in 2006, came telithromycin or Ketek, the drug that would doom us to 6 more years of FDA recalcitrance to the development and approval of new antibacterials [3]. Although safety was a key concern for telithromycin, it was approved in the United States based on the unusual process of examining data for safety in patients treated outside the United States, where the drug had been approved and was available. The reasons for this are complex and reviewed elsewhere [3, 6]. But in 2006, shortly after approval and entry into the US market, several cases of severe telithromycin-induced liver toxicity were reported [7]. This led to a condemnation of the entire non-inferiority trial approach to approval of new antibiotics and to a

withdrawal of the FDA from antibiotic approvals, with a few exceptions, for the next 8 years [3]. Examples of antibiotics whose development became impossible during the post-2006 era include omadacycline: the sponsor was forced to halt an ongoing clinical trial for skin infections while the FDA reconstructed its trial design requirements. The company ran out of funding and only recently was able to restart their late-stage trials. During these years, the FDA even reneged on previously negotiated trial designs. Replidyne and Advanced Life Sciences were caught in this web of changing FDA trial design requirements; both companies ultimately failed as a result. Theravance was forced to reanalyze their data in light of altered FDA trial requirements, thereby delaying the approval of telavancin for use in hospital-acquired pneumonia by several years. All this is well documented [3].

In May, 2012, FDA management, having realized that antibiotic development had slowed to a point where the late-stage pipeline was dangerously weak, announced a reset of the entire process [8]. Rachel Sherman and Janet Woodcock, in the Office of the FDA Commissioner, had decided that something drastic was required (Janet Woodcock, personal communication). They received some cover from Congress 2 years later with the passage of the GAIN (Generating Antibiotic Incentives Now) Act that required the FDA to provide feasible and speedy pathways for the development of new antibacterial drugs. The GAIN Act resulted in the FDA designation, qualified infectious disease product (QIDP), that provides for an expedited FDA review and, if appropriate, more rapid approval and entry into the marketplace. Since 2014, six new antibacterial drugs have been approved by the FDA. All were designated as QIDP.

23.2 Regulatory Pathways Today

Shortly after the FDA announcement of its reboot process, John Rex presented a proposal at a Gordon Conference on New Antibacterial Drug Discovery (summarized in Fig. 23.1 and subsequently published [9]). The problem that Rex and his colleagues confronted was that there was no intermediate pathway for antibacterial drug development other than the traditional (Tier A) – two large non-inferiority trials per indication on the one hand and the animal efficacy rule (Tier D; only efficacy in animal models is used to justify approval) at the other extreme. They proposed a solution with two intermediate pathways, Tiers B and C (Fig. 23.1). This approach, especially that embodied by Tier B, became the basis of the current regulatory approach to the development of antibacterial drugs for patients with unmet medical needs, which included resistant infections, drug allergies, and other problem issues [10, 11]. Tier C remains problematic and is the source of much discussion (see below).

The “new” approach by the FDA, as it has evolved since 2012, is summarized in Tables 23.1 and 23.2. As noted previously, both in Europe and in the United States, two clinical trials were required to obtain marketing approval for each clinical infectious disease indication. One exception was that one could carry out two trials for

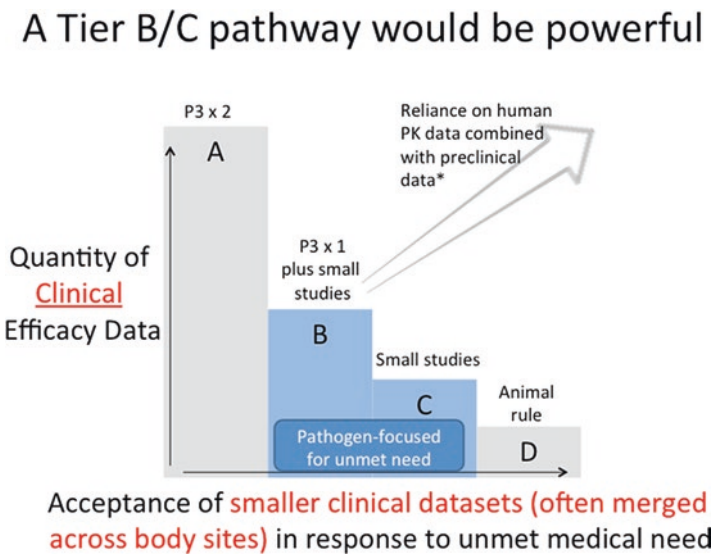

Fig. 23.1 A tiered set of strategies for the development of new antibiotics (from J. Rex – personal communication). Tiers A and D are preexisting pathways of antibacterial drug development. In Tier A, two non-inferiority trials are required to establish efficacy of a new antibiotic for any given infection (urinary tract, skin, etc.). In Tier D, the animal rule used to approve therapies for bioterror infections, efficacy, pharmacokinetics, and pharmacodynamics is studied in animals, safety and pharmacokinetic studies are carried out in humans, and a pharmacodynamic argument supplants the need for clinical trials in humans showing efficacy since these are not possible. To support trial designs in the middle-tier strategies, Tiers B and C, strong pharmacodynamic data from preclinical models is absolutely required, as is solid in vitro antibacterial data. Based on this, in tier B, a single non-inferiority trial could be paired with, for example, an open-label trial of the new antibiotic demonstrating efficacy in infections that would not have been expected to respond to standard therapy – such as those caused by essentially pan-resistant pathogens. In Tier C, the only trials might be those where the antibiotic is targeting highly resistant infections

community-acquired pneumonia and a single trial for hospital-acquired or ventilator-associated pneumonia and obtain approval for both indications. Table 23.3 shows a typical clinical development plan (this one for tigecycline) around the turn of the last century. In this plan, two trials were envisioned for each of several indications including skin and skin-structure infections, complicated intra-abdominal infections, and community-acquired pneumonia, plus a single trial for hospital-acquired pneumonia. Table 23.3 shows the effect of reducing the non-inferiority margin from 15% to 10%. Today, both in Europe and the United States, for antibiotics addressing an important unmet need, such as those active against resistant pathogens, the number of non-inferiority trials required has been reduced considerably (up to 50%), as shown in Table 23.1. The regulatory authorities reasoned that since an antibacterial targets the bacterial cause of infection, data from one clinical indication, such as intra-abdominal infection, could be used to support data for a second indication, such as complicated urinary tract infection. For both of these examples, the causative pathogens are similar (Gram-negative bacteria). In order to justify this

Table 23.1 The evolution of FDA policy

FDA Pre 2012	FDA post 2012 reboot
In general, two independent NI trials	Single NI trial in ABSSSI plus a single NI trial in CABP – Allows for approval in both indications
Required for each indication	Single NI trial in cUTI plus single NI trial in cIAI – Allows for approval in both indications
Exception – 2 trials in CABP +1 in HABP	Small, pathogen-specific trials may be allowed. controls and other parameters for such trials
NI margins generally 10%	Remain to be established for individual products
Exception – HABP – 15–20%	Placebo controls no longer required for AOM
AOM, ABS, ABECOPD – Placebo controls required	

NI Non-inferiority, *CABP* community-acquired bacterial pneumonia, *HABP* hospital-acquired bacterial pneumonia, *ABSSSI* acute bacterial skin and skin-structure infection, *cUTI* complicated urinary tract infection, *cIAI* complicated intra-abdominal infection, *AOM* acute otitis media, *ABS* acute bacterial sinusitis, *ABECOPD* acute bacterial exacerbations
Adapted from Shlaes [opal chapter]

Table 23.2 Clinical endpoints for trials of antibacterial drugs

Indication	Primary FDA endpoint	Non-inferiority margin	Primary EMA endpoint	Non-inferiority margin
Skin and skin structure infection	20% reduction in erythema/swelling day 2–3	10%	Cure at test of cure	10%
Community-acquired pneumonia	Improvement in signs/symptoms day 3–5	12.5%	Cure at test of cure	10%
Ventilator associated and hospital acquired pneumonia	All cause mortality at fixed time day 14–28	10%	Cure at test of cure	12.5%
Complicated urinary tract infection	Clinical and Mircobiological success	10%	Similar	10%
Complicated intra-abdominal infection	Clinical success	10%	Similar	12.5%

reduction in trial subjects needed for each indication, the regulatory authorities emphasized the importance of a strong set of pharmacokinetic and pharmacodynamic data, both preclinically and in volunteers and patients. These data assume increasing importance as we approach situations in which clinical data become more and more difficult to obtain. For further details on pharmacodynamic approaches, see Chaps. 21 and 22 in this volume.

Table 23.3 Clinical development plan for tigecycline (circa 1999)

Study population (based on presumed cure rates)			
Indication	Cure rate	10% NI margin	15% NI margin
CAP	85%	1532	688
Skin	80%	2248	1000
IAI	70%	2948	1316
HAP/VAP	65%	1598	710
Total		**8326**	**3714**

CAP community-acquired pneumonia, *Skin* acute bacterial skin and skin-structure infection, *IAI* complicated intra-abdominal infection, *HAP/VAP* Hospital acquired and ventilator-associated pneumonia, *NI* non-inferiority

23.3 Evolution of Primary Endpoints at FDA for Clinical Trials of Antibacterial Drugs

For many in the clinical community, the adverse events following the FDA approval of Ketek resulted from the use of the non-inferiority clinical trial design, which had been the foundation of antibacterial drug development for decades. Immediately after the Ketek scandal, under pressure from Congress and from organizations such as Public Citizen [3], the FDA issued guidance on how to justify the non-inferiority margins for proposed clinical trials (FDA Guidance on Use of Non-Inferiority Trials [12] – this final version came after its first draft in 2006). The guidance noted that the underlying assumption validating the non-inferiority design is that the comparator being used provides a treatment effect superior to that of a placebo. Thus, sponsors were obligated to provide such evidence to justify their proposed margins. As noted above, this type of approach by the agency stopped antibiotic development in its tracks.

Through the work of the Biomarkers Consortium of the Foundation for the National Institutes of Health and the diligence of the FDA itself, data from the pre-antibiotic era were used to establish treatment-effect levels for placebo with key indications. But the endpoints utilized in the 1900s were often quite different from those that have been used in modern times. The most notorious example concerns the treatment of skin and skin-structure infections, as defined by FDA guidance [13]. The only clinical trial data that could be identified in which a placebo was utilized came from two studies of sulfonamide antibiotics in the treatment of erysipelas in the 1930s [14, 15]. The endpoints used were the decrease in the extent of the skin lesion (redness and swelling) during the first 48 h following initiation of therapy. There are a number of problems with these data. For example, the placebo was actually UV light therapy that some argue has a treatment effect beyond any real placebo. Second, in those days, most cases of erysipelas or cellulitis were caused by *Streptococcus pyogenes*. Today the most common cause is *Staphylococcus aureus*. This change in pathogen brings the entire approach into question. Third, the early response endpoint (48 h) is considered to be clinically unimportant by most clinicians, even though they all follow skin erythema and swelling to determine whether

the patient is responding to therapy. The primary endpoint preferred by clinicians, and the one still used in Europe, is cure as assessed at some point after completion of therapy. Cure remains an "important" secondary endpoint for the FDA. The treatment effect for the early endpoint, defined by the FDA, is around 20% for the sulfonamides compared to UV light, and the non-inferiority margin specified by the FDA for such trials is 10% – about half of the treatment effect. This is entirely appropriate if, in fact, the placebo treatment estimate is valid. But the endpoint itself remains controversial.

The story for community-acquired pneumonia is similar. Here the pre-antibiotic literature is full of well-documented studies of pneumonia prior to the availability of antibiotics and after the introduction of sulfonamides and other antimicrobial therapies. Again, though, the endpoints used in the early studies were mostly resolution of fever, which was felt to be a good surrogate for ultimate clinical cure. The other endpoint that was studied throughout the pre-antibiotic era is mortality. In fact, the FDA considered requiring that mortality be used as the only primary endpoint for trials for community-acquired pneumonia, and this endpoint still appears in their guidance as an option [16]. But because the mortality of patients treated in the context of a modern clinical trial is so low, the number of subjects required to achieve a valid result is so high as to be unattainable under almost any circumstance. Again based on the pre-antibiotic literature, the FDA chose, as an alternative to mortality, an early endpoint of improvement for at least two key symptoms between days three and five after initiation of therapy. The guidance states: "The primary efficacy endpoint of clinical success is defined as improvement at day 3 to day 5 in at least two of the following symptoms: chest pain, frequency or severity of cough, amount of productive sputum, and difficulty breathing. Symptoms should be evaluated on a four-point scale (absent, mild, moderate, severe), with improvement defined as at least a one-point improvement from baseline to the assessment at day 3 to day 5 (e.g., from severe to moderate, from moderate to absent, or from mild to absent)."

The FDA estimated that the treatment effect (subjects attaining the endpoint when treated with antibiotic vs. those attaining the endpoint without antibiotic therapy) ranged from 30 to 77%, as determined from pre-antibiotic era data [16]. Theoretically, if the non-inferiority margin should be about 50% of the treatment effect, one could justify a margin of 15–38%. The FDA conservatively chose a margin of 12.5%. Europe uses an endpoint of cure and requires a margin of 10% (EMA addendum 2012). Most global trials today, therefore, are powered to detect a non-inferiority margin of 10% using cure as a test.

The most complex and controversial primary endpoint discussions concern hospital-acquired and ventilator-associated pneumonia. Both European regulators and the FDA in the United States treat these diseases as a single complex that can be studied with a single trial. However, the diseases can be very different clinically. If an episode of hospital-acquired pneumonia requires ventilator support, the disease does in fact resemble ventilator-associated pneumonia. If not, it more closely resembles community-acquired pneumonia, a very different condition [18].

For the FDA, numerous factors have led it to choose a very different primary endpoint than used by colleagues in Europe – all-cause mortality at days 14–28. The European Medicines Agency still uses cure at some point after completion of therapy. The main problem for the FDA, given its commitment to provide a justification for the non-inferiority margin in trials of that design, was that it was unable to establish a clear placebo effect level for any endpoint other than mortality. This feature is clearly and extensively reviewed in their guidance document for clinical trials in these indications [17]. In addition, for the FDA and its advisers, the determination of cure or even clinical improvement for this serious and complex disease was too subjective. The FDA, and many clinicians and statisticians, preferred a "hard and clean" endpoint of mortality. Yet this, too, is not without controversy. It seems clear that up to 50% of the mortality at day 28 among patients with ventilator-associated pneumonia is related to comorbidities rather than to the infection under therapy [19]. Therefore, in a non-inferiority trial, this feature tends to push the result to the null. Of interest, recent trials using the mortality endpoint have all used the 28-day time point rather than the 14-day endpoint. This may be to take advantage of the insensitivity of the assay, since that would favor a finding of non-inferiority. Alternatively, it may be because mortality at 14 days is lower than at 28 days, making it more difficult to reach the FDA's preferred level of 15% mortality in the control arm of the trial.

The FDA also allows a "mortality-plus" endpoint – all-cause mortality plus no disease-related complications. The Foundation for the National Institutes of Health in the United States recently posted a comment to the FDA's draft guidance for these trials (FNIH Comment to FDA Guidance 2017 - https://www.regulations.gov/document?D=FDA-2010-D-0589-0027) in which they show that using the adverse events from sepsis in the MedDRA listing is a valid and efficient way of looking at "mortality plus."

A separate issue for all indications studied, but especially for ventilator-associated pneumonia, is that of prior antimicrobial use. Regulatory agencies in the United States and Europe discourage use of antibiotics prior to enrollment of patients in a trial of a new antibiotic for obvious reasons. But this is not so easy. It takes precious time to enroll a patient, and physicians are loath to withhold therapy while waiting. Antibiotic use is generally high in hospitals, especially in intensive care units [20, 21] such that finding patients who have not been treated or are not currently being treated can be difficult. The new data provided by the Foundation of the National Institutes of Health suggests that, at least for ventilator-associated pneumonia, there is no effect of prior antibiotic treatment on 28-day, all-cause mortality across several recent trials. This may be reassuring to regulatory agencies and may allow more flexibility in patient enrollment. Other indications, including complicated intra-abdominal infection and complicated urinary tract infection, share roughly similar endpoints in Europe and in the United States.

23.4 Clinical Trials for Pathogen-Specific Antibacterial Drugs

In the FDA reboot of their approach to clinical development of new antibacterials and in the recent addendum provided by the EMA, various approaches were suggested to allow studies of drugs that targeted only specific pathogens [10, 11]. In spite of this, the desire of the regulatory agencies for controlled clinical data has led companies to embark on trials that were ultimately shown to be infeasible [22]. One example is the study of Carbavance compared to best available therapy for a variety of infections. The study is projected to take 4 years to enroll 150 evaluable subjects (https://clinicaltrials.gov/ct2/show/NCT02168946?term=meropenem+medicines+company&rank=4). Achaogen's superiority trial of plazomicin took 3 years to enroll 69 patients (https://clinicaltrials.gov/ct2/show/NCT01970371?term=achaogen&draw=1&rank=4). Until the present, the drugs that have been studied were all broad-spectrum agents where the sponsors wanted to show activity against specific resistant pathogens, such as Gram-negative bacteria resistant to carbapenems [23].

This desire on the part of antimicrobial sponsors is understandable. They are trying in good faith to address the specific unmet need of drug-resistant infections with direct clinical data. They also believe that such data will be more persuasive to physicians when it comes to marketing their new antibiotic. But the superiority trials upon which they have embarked have been difficult, if not impossible, to enroll suitable numbers of subjects [23]. Some experts have recommended a non-inferiority approach that does not target resistant pathogens per se [24]. These experts reasonably argue that a few patients with perhaps not so highly resistant infections (so-called usual resistance), plus strong pharmacokinetic and pharmacodynamics data, will provide a compelling argument for the regulatory authorities and render trials more feasible. Whether the clinicians who will ultimately use these new antibiotics are suitably conversant with such data remains to be seen.

More recently, several companies have developed compounds that are truly pathogen-specific. That is, they have no activity or only poor activity against non-target bacteria. The best example of this is POL-7080, a peptide active only against *Pseudomonas aeruginosa* that is being developed by Polyphor [25]. In response to the clear need for compounds like this and the absence of a clear and feasible pathway by which development can proceed, the FDA has organized a number of workshops and advisory committee meetings to address the problem. We can expect additional regulatory guidance in the near future. The basis of the approach likely to be undertaken by the regulatory agencies has recently been published by the IDSA [26] and is enshrined in the twenty-first Century Cures Act that was signed in December 2016. The legislation established a pathway to approval, LPAD (Limited Population Antimicrobial Drug), that provides for studies of small populations and a limited label upon approval [27]. The program proposed by Boucher et al. calls for a very strong package of preclinical and clinical pharmacokinetic and pharmacodynamics data to support a clinical dosing regime, especially in the types of patients likely to be treated with the new agent. The IDSA notes that it is not necessary to

study only pathogens having a very high MIC, since, given a lack of cross-resistance between the new agent and previous antibacterials, as shown in vitro, strong pharmacodynamic and pharmacokinetic data should provide support for use of the new agent. This will make these trials somewhat easier to enroll compared to recent attempts [23]. Further, it is suggested that clinical indications, such as pneumonia, bacteremia, urinary tract infection, and others, should be pooled within the context of a single trial. This will make endpoint determination challenging. The IDSA notes that superiority trials, defined, I believe, as randomized controlled trials to demonstrate superiority of the test agent compared to control treatment, are not feasible and are not desirable, since we hope that control therapy will still be effective at the time the trials are conducted. Nevertheless, even the IDSA has previously considered the use of superiority trials [27], and some of us believe that they will be feasible under certain circumstances [28]. Boucher et al. and the IDSA now seem to favor a non-inferiority design. They suggest that even small trials be designed as randomized controlled trials, but they recognize that enrolling sufficient patients to achieve a statistically significant result may not be feasible. The IDSA recommends consideration of external controls – but they caution that if such controls are used, the treatment effect of the new therapy should be large compared to the control group and that the controls used should be validated. To validate such external or historical controls, prospectively identified patients who could have been enrolled in the trial could be used. Additionally a small, randomized control set from the prospective trial could be used to validate the external control set. Nevertheless, it seems clear that some clinical efficacy data to support approval of these pathogen-specific antibacterials will be required.

23.5 The Next Frontier

Our greatest challenge will not be the regulatory environment or the scientific difficulties of discovering drugs active against Gram-negative bacteria. It is the problem of economics [29]. For a complete discussion of this problem and potential solutions, see the chapter by Larsen in this volume (Chap. 24). But without a strategy that makes antibacterial drugs economically viable, all of the regulatory reform and scientific advances in the world will be for naught.

23.6 Conclusions

Bacterial resistance is and will always be with us. Every new antibacterial drug, if used, will ultimately lead to resistance. How quickly this occurs and how much it spreads will depend on a variety of variables. But there is no avoiding resistance, at least insofar as history tells us. We may be able to delay the onset of resistance by using various strategies such as limiting use, using dosing regimes designed to avoid

resistance, and choosing drugs for which the potential for emerging resistance appears to be low. We may be able to delay the spread of resistance through strict measures of hygiene in the hospital and the community. But resistance is inevitable. To address this, we will still need a robust pipeline of antibacterial drugs active against resistant pathogens.

The history of regulation of antibacterial drug development is one of tragedy followed by inspired progress. The tragedy was a decade in which antibacterial drug development slowed to a crawl. Inspired progress has led us to a place where antibacterial drugs that we could not have imagined developing just a few years ago can now be developed and approved. Today, streamlined pathways for approval of antibacterial drugs that address key unmet medical needs, such as serious infections caused by resistant pathogens, are readily available. These streamlined paths provide for rapid and cost-efficient entry into the global marketplace.

The last and most important hurdle to overcome in the near term is market failure ([29], Larsen, Chap. 24, this volume). If we do not solve this problem, all the regulatory progress in the world will not provide the robust pipeline of new antimicrobials that we desperately need but do not have.

Major Points

- We underwent a long period of regulatory uncertainty after the turn of the last century.
- 2012 saw a reboot of the US Food and Drug Administration approach to the development of antibacterial drugs.
- While regulation of antibacterial drug development is still a work in progress, there now exist streamlined pathways to approval for agents that meet key unmet clinical needs.
- The most anticipated upcoming guidance is that focusing on the development of antibacterial drugs that target specific species or genera of bacteria. It is likely that the clinical data required to support approval will be limited and that regulatory action will rely even more heavily on both animal and human pharmacokinetic and pharmacodynamic data.
- Regulatory clarity and the feasibility of clinical trials to achieve approval welcome changes compared to those in years between 2000 and 2012. But none of this important progress solves the problem of market failure for antibacterial drugs. Unless this problem is addressed, many of our other efforts may come to naught.

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Chapter 24
Economic Incentives for Antibacterial Drug Development: Alternative Market Structures to Promote Innovation

Marina L. Kozak and Joseph C. Larsen

Abbreviations

ADAPT	Antibiotic Development to Advance Patient Treatment
AMR	Antimicrobial resistance
BARDA	Biomedical Advanced Research and Development Authority
CDC	US Centers for Disease Control and Prevention
CMS	US Centers for Medicare & Medicaid Services
DISARM	Developing an Innovative Strategy for Antimicrobial Resistant Microorganisms Act
DNDi	Drugs for Neglected Diseases Initiative
DRG	Diagnosis-Related Group
DRIVE-AB	Driving Re-investment in R&D and Responsible Antibiotic Use
ENPV	Expected Net Present Value
FDA	US Food and Drug Administration
GAIN	Generating Antibiotic Incentives Now
GARD	Global Antibiotic Research and Development Partnership
GUARD	Global Union for Antibiotics Research and Development
HHS	Health and Human Services
IDSA	Infectious Diseases Society of America
IMI	Innovative Medicines Initiative
IP	Intellectual Property

Disclaimer: The views expressed are those of the authors and not necessarily those of the Biomedical Advanced Research and Development Authority, the Assistant Secretary for Preparedness and Response, or the United States Department of Health and Human Services.

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I. W. Fong et al. (eds.), *Antimicrobial Resistance in the 21st Century*, Emerging Infectious Diseases of the 21st Century, https://doi.org/10.1007/978-3-319-78538-7_24

JPIAMR	Joint Programming Initiative on Antimicrobial Resistance
MER	Market Entry Reward
ND4BB	New Drugs for Bad Bugs
NIAID	National Institute for Allergy and Infectious Diseases
NIH	US National Institutes of Health
NPV	Net Present Value
NTAP	New Technology Add-on Payment
OTA	Other Transactional Authority
PACCARB	President Advisory Committee on Combating Antibiotic-Resistant Bacteria
PCAST	President's Council of Advisors for Science and Technology
PRV	Priority Review Voucher
QIDP	FDA qualified infectious disease products
R&D	Research and Development
READI	Reinvigorating Antibiotic and Diagnostic Innovation Act
ROI	Return on Investment
TATFAR	TransAtlantic Task Force on Antimicrobial Resistance
TIPR	Transferable Intellectual Property Rights
WHO	World Health Organization

24.1 Introduction

Over the past several decades, there has been a steady decline in companies developing new antibiotics. Generally, this relates to the limited commercial returns and lower profitability of antibiotics compared to other therapeutics areas. Treatment periods are often short and curative; antibiotics have wide availability, are easy to use, and are generally low cost compared to chronic conditions, such as cancer. This is further compounded with a physician's reservation to use the newest antibiotics only as a last resort therapy when other treatment options fail, resulting in today's antibiotic market, where new drugs are underused and undervalued. In fact, antibiotics are one of the only classes of drugs whose use limits their life span of utility. As the current model links profit to the number of new drugs sold, these factors do not lend themselves to a robust business model for companies to pursue and have resulted in a significant innovation gap for new antibiotics. From 2007 to 2012, the number of patents filed for new antibiotics decreased by 34.8% [35]. There has not been a new class of antibiotics to treat hospital-acquired Gram-negative infections in over 45 years. There are very few other technology sectors where no major innovation has occurred in that period of time. For example, the current oncology pipeline has over 800 candidate therapies in clinical development [33]. In contrast, the antibiotic pipeline has 26 in Phase 2/3 development [31].

Concomitant with the decline in the development pipeline is the rise in antibiotic resistance owing to misuse of existing drugs and a lack of adequate tools to diagnose and appropriately treat infections. While statistics vary, the Centers for

Disease Control and Prevention (CDC) has estimated that at least 2 million people acquire serious infections with bacteria that are resistant to one or more of antibacterial drugs designed to treat those infections in the USA alone. Approximately 23,000 of these individuals will die as a result of the drug-resistant infection and amount to as much as $35 billion a year in excess direct healthcare costs [8]. Given an unaltered current trend, the rise and spread of antibiotic resistance will have a crippling economic and human impact as our ability to treat even simple infection will disappear [27].

The decline in innovation appears to be directly proportional to the number of companies who are researching and developing new antibacterial drugs. In 1990 there were 18 large pharmaceutical companies developing antibiotics [7]. Today, there are only four with ongoing clinical development. Much of the innovation in antibacterial drug development is occurring with small- and medium-sized biotechnology companies. Many of these companies develop products to late stages of clinical development and are either acquired or sell the candidate antibiotic to a larger company that is capable of commercializing the product. In fact, among the last eight antibiotics that were approved in the USA from 2010 to 2015, only one, SIRTURO® (bedaquiline), did not change ownership over the course of its lifecycle. All other antibiotics approved in that time period changed ownership at an average of 2.86 times during development [11]. This turnover of ownership only adds to the fragility of the antibiotic market and the need to establish a dedicated cadre of scientists that understand the entire antibiotic development pipeline.

It is clear that the current era of antibacterial drug development is driven less by research and innovation and more by commercialization, where a limited number of large pharmaceutical companies purchase late-stage molecules developed by smaller biotechnology companies or other large pharmaceutical companies looking to exit the sector. In August 2016, Pfizer acquired a substantial proportion of AstraZeneca's antibiotic portfolio. Pfizer purchased the portfolio for up to $1.575B USD plus royalties. This move followed Pfizer's decision, in 2011, to relocate its antibacterial research and development program from the USA to China. In late 2014, Cubist Pharmaceuticals was acquired by Merck for $9.5B USD, based largely upon the commercial success of Cubicin® (daptomycin). Other assets that were purchased were all late-stage molecules including Sivextro® (tedizolid phosphate) and Zerbaxa® (ceftolozane-tazobactam). Soon after the merger, Merck decided to eliminate the approximately 120 research and development positons that were associated with Cubist [17]. While both the Pfizer and Merck decisions are likely based upon valid business factors, they nevertheless impact the overall brain trust of people conducting research and development for new antibacterial drugs and therapies. Combined with scaling back of academic research due to broader funding constraints, the number of antibiotics has steadily decreased over the last three decades.

Concerns over the lack of innovation and activity in antibacterial drug discovery have led to the development of a scientific roadmap for antibiotic discovery developed by Pew Charitable Trusts [32]. The roadmap makes several key recommendations to improve the pace of new antibiotic development. These include increasing our understanding of the inherent scientific barriers to antibiotic development, such

as the ability to get molecules across the Gram-negative outer membrane; developing tools to enable both conventional antibacterial drugs and non-traditional approaches, such as bacteriophage and virulence inhibitors; and increasing focus on greater coordination of information sharing, expertise, and reagents across the research and development community to hasten the pace of discovery. To engender success, Pew's roadmap will require a significant investment in human capital and training to allow for sustained innovation and redevelopment of the scientific expertise that has been slowly degraded over the last 30 years.

In addition to a well-developed scientific base, the price of antibiotics should relate to their value. Historically, the sales of new antibiotics have not been robust, particularly when compared to other therapeutics classes. Sales or projected sales for years 1–2 postlaunch of the last six marketed antibiotics (Avycaz®, Teflaro®, Zerbaxa®, Sivextro®, Dalvance®, and Orbactiv®) were $20 M–$80 M USD [22]. This is in contrast to sales of more widely prescribed medications, Januvia®, Lyrica®, and Spirivia®, used to treat type 2 diabetes, fibromyalgia, or asthma, respectively, whose 1–2 year projected sales were between $800 M and $1.5B USD. Given the vast difference in immediate returns for investors, it is evident why companies are not robustly pursuing research and development programs for new antibacterial drugs.

To reinvigorate innovation in antibacterial drug development, while promoting appropriate use, and ensuring patient access to these critically important medicines, several policy proposals have been put forward. The following sections describe the advantages and disadvantages to fixing the broken economics of antibiotics development and provide context for ongoing policy discussions around which type of economic incentives should be considered for implementation.

24.2 Characteristics of a Strong Incentive

There are several characteristics economic incentives for antibacterial drug development need to possess in order to be effective. In general, they should balance promoting innovation, ensuring access for patients that need them, and promoting conservation and stewardship.

24.2.1 Stability

While any package of incentives will have secondary effects in the market, incentives need to minimize disruptive effects to the greatest extent. For example, proposals involving the creation of vouchers that extend patent life or expedite regulatory review timelines may generate a secondary market, where these vouchers could be bought and sold or may create a situation, where more widely used medications in other disease areas are priced higher for longer periods, thereby effecting patient

care in larger populations. A recent study found that without fixing the price of priority review vouchers, the commercial value of vouchers depended on the number of vouchers available on the market thereby de-linking the private value of the voucher from the drug in development and potentially lowering the overall incentive value to below the cost of the development program [38].

24.2.2 Sustainability

Incentives require sustainability. If markets or companies cannot rely on the incentive, then it will not make the long-term research and development investments to obtain them. Incentives, and specifically the funding for them, should be minimally affected by political whim.

24.2.3 Stewardship

Increasing developer returns alone do not address the problems of antibiotic overuse. Thus, incentives should also be utilized to accomplish public health objectives. Constructing incentive packages to have mandatory requirements, such as restricted or eliminated marketing of the antibiotic, development of educational programs for clinicians and pharmacists to teach appropriate use, and even imposing limits on annual production could be effective means of rewarding innovation, while ensuring public health measures are achieved.

24.2.4 Innovation

Ultimately, to achieve their goal of promoting innovation, incentives need to improve the net present value (NPV) calculation and improve it to a level that is sufficient to spur private sector investment in this area and entice companies to initiate research and development programs for antibacterial drug development. The NPV metric governs the risk/benefit and profitability of pursuing development in the pharmaceutical industry. NPV is the sum of all investment costs in development and the expected value of future revenues, while taking into account the value of time of money of a given development program. In other words, it is the amount of profit one could anticipate, factoring in failures along the way and recognizing that a dollar invested today is discounted over the development time of the product. It is estimated that for antibacterial drugs, converted to USD, the NPV is approximately $42.61 M USD [42]. In contrast for neurological or musculoskeletal drugs, NPVs are estimated to be between $720 M and $1.1B USD.

One analysis of private and societal NPVs for antibiotics examined the estimated expected net present value (ENPV) for developers considering initiating preclinical research for antibiotics to treat various infections [41]. Across six indications that cover the major areas of antibiotic use (including acute bacterial otitis media, acute bacterial skin and skin structure infections, community-acquired bacterial pneumonia, complicated intra-abdominal infection, complicated urinary tract infection, and hospital-acquired and ventilator-associated bacterial pneumonia), the group found wide distribution of both the private value to a developer and the societal importance of the drugs. This variability was due to the total market size, the real opportunity cost of capital, and the total time to market. In spite of this variability, the private values fell far short of the estimated threshold needed to initiate preclinical research. Meanwhile, these antibiotics ascribed a significant societal value based upon the estimated value of the new antibacterial drug to the individual, the estimated societal burden, monetized societal burden of the illness, and calculated NPV of the total societal burden for the projected useful life of the new antibacterial (i.e., 20 years), and the estimated reduction in total societal burden of the disease attributed to the new antibacterial drug. These results describe the significant discrepancy of how society values these products versus what the current market will tolerate in terms of setting a sustainable price.

If the NPV for new antibacterial drugs remains far below the societal value, few companies will invest in research and development further perpetuating the current crisis. However, focusing on increased drug sales alone and therefore increased antibiotic use will lead to further antimicrobial resistance; thus the value of antimicrobials is highest when the drugs are used as little as possible. Alternative approaches and incentives are therefore needed to increase the NPV, drive drug development, and fill this gap between private and public value of antibiotics.

24.3 Incentive Types

There are two primary types of economic incentives, push and pull. Push incentives subsidize up-front development cost, while pull incentives provide some guaranteed return on investment (ROI) only if the research is successful (Table 24.1).

24.3.1 Push Incentives

Push incentives may include grants, contracts, public-private partnerships, tax credits, and clinical trials networks. Generally, push incentives can promote basic research that builds a knowledge base for applied and commercially exploitable research, requiring less funding to implement, and allow public health priorities to guide the product development agenda. A disadvantage of push incentives is the risk of projects failing in development leading to financial pressures that result in

Table 24.1 Selected incentive types and examples used throughout the chapter. Push incentives are intended to fund or reward research and development effort irrespective of outcome, while pull incentives are intended to fund or reward research and development effort if outcome successful

R&D Incentives	**Push**	Government Grant	
		Tax Incentive	
		Clinical Trial Network	
		Portfolio Approach	E.g., Megafund
		Public: Private Partnership	E.g., OTA/Portfolio Partnership, CARB-X
	Pull	Prize/Voucher	E.g., PRV
		Market Exclusivity	E.g., TIPR
		Advanced Market Commitment	E.g., Project Bioshield
		Tradable Intellectual Property	
		De-linkage	
		Lego-regulatory	E.g., GAIN act

developers misrepresenting the project progress in order to receive funding. Alone, push incentives are insufficient to ensure profitability for the developer or equitable access for patients who need medicines.

24.3.2 Pull Incentives

Conversely, pull incentives may include regulatory incentives like priority review vouchers, tradable patent extensions, additional market exclusivity, tax credits (if structured to pay off at a predefined point), advanced or milestone payments, advanced market commitments or volume guarantee, and value-based or higher reimbursement. Pull incentives only reward successful research programs, thereby ensuring a remarkable sense of efficiency with this incentive type. They provide for the ability to target specific outcomes of research and development, though the developer assumes the majority of the risks with this approach with the incentive payoff late in development (i.e., regulatory approval or market entry) making it challenging to incentivize early-stage research. Product developers may not gravitate toward pull incentives if the reward is not significant enough, particularly among small- to medium-sized enterprises that may lack the resources to transition candidate products to late-stage development or regulatory approval. Some of the challenges of pull incentives are defining the criteria or milestone where a developer would receive the reward and determining the appropriate size of the reward (i.e., ascribing value to the candidate antibiotic).

Pull incentives that affect the ROI through revisions to government policy or higher reimbursement are known as lego-regulatory pull incentives. Similarly, these incentives only reward successful research and development, but the reward is instead linked to changes in the current regulatory framework. These could include

changes in market exclusivity, patent protection, or pricing/reimbursement. The primary advantage of this incentive type is that they don't require funding to establish or sustain. Without a financial payout, the uncertainty around the level of payment is subsequently removed thereby also easing political hurdles. Generally, the challenges with these incentives relate to reduced levels of innovation in markets where substantial levels of market exclusivity already exist. These will be discussed in greater detail in the sections below.

24.4 Current Incentive Landscape

The current incentives used in the United States (US) and the European Union (EU) are generally push incentives that subsidize development costs. However, given the dearth of novel antibiotics in development, these strategies require re-evaluation.

24.4.1 US Approaches

In the US, the National Institutes of Health, National Institute of Allergy and Infectious Diseases (NIH/NIAID) supports early development projects through a number of push incentives, related to antibiotic resistance, including funding for basic research, targeted research areas, and translational research to prepare programs for advance research and development. NIAID also supports companies developing new antibiotics by providing a suite of preclinical services to help progress molecules in development.

In addition to early development support, the Biomedical Advanced Research and Development Authority (BARDA), a component within the US Department of Health Human Services (HHS), conducts advanced research and development of medical countermeasures for public health emergencies. BARDA makes available medical countermeasures (vaccines, therapeutics, and diagnostics) that address bioterrorism and naturally emerging threats by utilizing a mixture of push and pull incentives to create a pipeline of product candidates and ensures an appropriate return on investment for developers through product procurement. Under the Project Bioshield Act of 2004, BARDA was provided $5.6B over 10 years to purchase medical countermeasures for use in public health emergencies [46]. In essence, the program provides an advanced market commitment. Companies receive funding to support research and development and then transition to procurements as a means of incentivizing industry to develop a pipeline of medical countermeasures. Since 2010, BARDA has supported the development of several antibiotics through its antibacterial program and has advanced several candidates to Phase 3 clinical development.

Another funding approach utilized by BARDA is through partnership development. One such instrument is called the Other Transactional Authority (OTA),

which allows BARDA to form strategic alliances with antibiotic developers and take a portfolio approach managing the programs. Instead of focusing on a single antibiotic candidate, BARDA supports a portfolio of candidates and distributes the risk of development across several molecules, such that this mechanism allows for technical attrition of candidates and the reallocation of resources to account for and mitigate unforeseen risk. BARDA has awarded four OTAs for antibiotic development and intends to form additional partnerships in the future.

More recently, BARDA established another push approach that supports early-stage preclinical development of new antibiotic candidates. In July 2016, BARDA established CARB-X, a novel public-private partnership that will identify, select, and manage a portfolio of approximately 20 high-quality antibacterial drug candidates and develop them to first-in-human testing. CARB-X is a collaboration between NIAID and BARDA and four life science accelerators, the Wellcome Trust, AMR Centre, California Life Science Institute, and MassBio [30]. The tools provided by CARB-X include non-dilutive research funding, research support services, and business mentoring services to companies in the portfolio. The goal of CARB-X is to develop two antibiotic candidates into Phase 1 clinical testing over the 5 years of the program. CARB-X's remit is global and is focused on promoting innovation in antibacterial product development.

While more limited, a lego-regulatory pull incentive also exists in the US, provided under the Generating Antibiotic Incentives Now (GAIN) Act. The GAIN Act of 2012 grants an additional 5 years of market exclusivity for new antibiotics that are designated "qualified infectious disease products" or QIDP [47]. This 5-year exclusivity limits the approval of similar drugs during the period and is in addition to any existing exclusivity. The QIDP status enables the drug to receive priority review and also the fast-track designation. Coupling the drug with a companion diagnostic test provides an additional 6 months of exclusivity. However, the GAIN Act is limited in that it includes no provisions to practice stewardship, or appropriate use, and the increased exclusivity will increase the cost of healthcare potentially limiting patient access. One could argue, in fact, that the period of exclusivity could be a driver to sell as much as possible during that period, thereby potentially promoting inappropriate use and contributing to rising resistance. Overall, the limited financial returns from this incentive are unlikely to be sufficient to entice industry to robustly engage in the research and development of new antibacterial drugs. The profits obtained during the exclusivity period will offset some, but not all of the research and development costs, weakening its utility as an incentive.

24.4.2 International Approaches

Since 1999, antibacterial resistance research in the EU has been funded via the EU framework programs for research and innovation, including the current program called Horizon 2020. Additional support for early discovery and development comes from targeted projects like ENABLE, or the European Gram-negative

Antibacterial Engine, that provides a 6-year €100 M program dedicated to accelerating the discovery and development of new antibiotics for infections caused by Gram-negative bacteria [21]. The goals for the ENABLE project are to identify three antibacterial lead molecules, two clinical stage candidates, and one molecule that will advance into Phase 1 clinical development. Programs that focus on advanced development in the EU include the Innovative Medicines Initiative (IMI), which launched the New Drugs for Bad Bugs (ND4BB) program to support antibacterial drug discovery and development programs, and the InnovFin Infectious Diseases, which was launched more recently in 2015. As a joint initiative by the European Investment Bank and the European Commission, this program was designed to provide a wide array of finance tools ranging from standard debt instruments to risk-sharing instruments. Under the program, companies who are investing in vaccines, diagnostics, or treatments for infectious diseases are eligible for loans between €7.5 M and €75 M. Projects that have passed the preclinical stage of development are eligible for support that finances clinical development. Under the program, loan recipients must fund at least 25% of the project costs. The loan will cover 50% of the development cost, and the recipients must identify a third party to cover the remainder. A critical analysis of the impact of this program was conducted citing challenges to companies with this approach in terms of securing the additional financing to qualify them for the loan [6]. Further, there is no mechanism to ensure sustainability that is tied to commercial success. As designed, the best outcome for the program is the repayment of the loan; the worst circumstance is where money was given to a failed commercial enterprise with no prospect of repayment. Regardless, the availability of an additional incentive to subsidize development costs is helpful in the current antibiotic market.

Beyond Europe, the Joint Programming Initiative on Antimicrobial resistance (JPIAMR) is also focused on providing push incentives. This initiative was established to coordinate fragmented national research efforts in order to make better use of resources and to address the common challenges posed by antimicrobial resistance (AMR) more effectively. Engaging with international stakeholders, including the World Health Organization (WHO), industry, and the JPIAMR member states, members voluntarily agree on a common Strategic Research Agenda that is jointly implemented and includes translational research [23].

The Drugs for Neglected Diseases Initiative (DNDi) has established a program in May of 2016 called GARDP or the Global Antibiotic Research and Development Partnership [18]. Borrowing from their experience in financing drugs for neglected diseases, GARDP hopes to test new incentives and contribute to the access and conservation of new antibiotic treatments. GARDP collaborates closely with the WHO to ensure disease, and pathogen priorities are adequately addressed. Their goal is to support three to four projects that will address urgent global health needs and be ready for implementation by the end of 2017.

24.5 Alternative Incentive Structures and Considerations

As biomedical innovation, as a whole, has become riskier, more costly, and more difficult to finance, there is an increased need to examine alternative models to structure public or private sector investments. Several alternatives are explored below.

24.5.1 Portfolio Approach

One proposal, based upon portfolio theory, is to create a financial structure, where a large number of programs at various stages of development are funded by a single entity as a means of reducing the collective risk of the investment [14]. A portfolio, or megafund model, would be able to finance companies by issuing debt. The megafund would issue "research-backed obligations," and the intellectual property of the portfolio of products would serve as collateral of the debt. This would create dynamic leverage that would be based upon the principle that as a portfolio of biomedical products progresses, the level of risk should decrease. By proxy, the amount of debt that could be supported should increase as a function of the percentage of the total capital required, effectively decreasing the amount of equity required [25]. Dynamic leverage would allow for time-varying amounts of debt, which could aid in building a portfolio of early-stage preclinical or discovery-based programs. The amount of debt could be adjusted related to factors like the probability of default. It is estimated that a fund of $5–15B would yield an average investment return of 8.9–11.4%. This rate of return is lower than typical venture capital rates but could potentially be attractive for larger institutional investors. The advantage of this concept is that risk is distributed across multiple development programs, thereby increasing the probability of a return on the investment. This model has been proposed for orphan drugs targeting rare diseases, because these companies may be limited in their ability to raise funding through traditional means [13]. Due to the unique nature of drug development for orphan drugs, including lower development times, lower attrition, and more rapid regulatory approvals, it is believed that less capital is needed to de-risk a portfolio of these programs. The authors suggest that a megafund of $575 M could yield double-digit returns with only 10–20 projects in the portfolio. For antibiotics specifically, it is unclear if this would be a viable model. Unlike orphan diseases, where the development costs may be lower, the costs to develop a new antibacterial drug are more commensurate with traditional drug development. Further, whether the antibacterial drugs that were marketed under this approach would make enough profit to ensure an appropriate return on investment for those who invest in the portfolio is unclear. Lastly, this approach does not incorporate attempts to address public health objectives to encourage appropriate use and stewardship. Nevertheless, the megafund and other dynamic

leverage approaches represent novel financial models that should be assessed for their suitability in supporting a portfolio of antibacterial drugs.

24.5.2 Rethinking Clinical Trials

One major component of antibacterial drug development is the planning and execution of pivotal Phase 3 clinical trials. These studies are typically large, expensive, and technically challenging to execute for certain disease indications (e.g., hospital-acquired/ventilator-associated pneumonia). At present, the planning and establishment of the complex infrastructure to conduct a Phase 3 clinical trial for an antibiotic candidate are done de novo, with each sponsor investing time and resources to set up the trial each time one is to be conducted. One way antibacterial drug development could be incentivized is by introducing efficiencies in the execution of these clinical trials. The development of a clinical trial network that focuses on a subset of infections caused by Gram-negative bacteria and utilizes a master clinical protocol may represent a means of improving the efficiency of clinical trial execution and potentially saving patient resources.

The 2014 US National Strategy on Combating Antibiotic-Resistant Bacteria calls out this specific approach to reducing obstacles faced by drug companies developing new antibiotics and states that the US government will examine the feasibility of generating and applying master clinical protocols to multiple test groups of patients while sharing a common control group [44].

The characteristics of a master clinical protocol generally include some combination of the following: (1) allow for the study of multiple drugs using the same master protocol, (2) may include multiple arms (or sub-protocols) that allow for studying different types of disease, (3) utilize a shared control arm, (4) may overlap the study periods for different investigational drugs, (5) may include adaptive design elements, and (6) provide a shared standing infrastructure for testing multiple drugs.

The development of a clinical trial network can be used to implement several of these concepts [24]. In this network, a common control arm would be utilized and would be continually enrolling the standard of care for a specific indication (e.g., complicated urinary tract infection). Investigational products would be evaluated but would share the control arm. McDonnell et al. estimate that this could reduce the trial cost by anywhere from 33 to 43%. Given the substantial costs of conducting Phase 3 clinical trials, the clinical trial network could serve as an effective push incentive to subsidize development costs. The current antibiotic pipeline, with its limited number of candidates, potentially at different developmental stages, may not warrant the substantial investment of resources required to establish a network of this complexity. There may, however, be mechanisms for industry to begin incorporating adaptive trial design elements, such as hierarchical borrowing, that may allow for the gradual introduction of certain efficiencies without the substantial investment needed for a trial network [3].

Table 24.2 Characteristics of different pull incentives for antibacterial drug development

Incentives	Promotes innovation	Allows for patient access	Promotes stewardship	Sustainability
Higher reimbursement	+++	+	+	++
Diagnosis confirmation	++	+	++	++
Patent extension voucher	+++	+ (to patent-extended medicine)	+	+++
Priority review voucher	+	+	+	+++
Options market	++	+++	+	++
Market entry rewards – Full de-linkage	+++	+++	+++	+
Market entry rewards – Partial de-linkage	+++	+++	+++	++

+++ strong effect, ++ moderate effect, + weak effect

Further complicating the ability to evaluate potential new drugs are the still relatively rare pathogens, which harbor multi- and pan-resistance, including *Pseudomonas aeruginosa* and *Acinetobacter* spp. While randomized controlled trials are still the gold standard for reducing uncertainty about the safety and efficacy of a new therapeutic, their conduct for rare pathogens can be altogether impractical. In recognition of this problem, the Infectious Diseases Society of America (IDSA) has put forth a set of recommendations that address the challenges associated with developing narrow spectrum drugs [5]. The white paper considers alternative mechanisms to address the uncertainty of conducting trials where patient resources are limited. These include PK-PD dosing optimization, pharmacokinetic dose justification in relevant patient populations, efficacy confirmation using multiple animal models, validated external controls, and small clinical datasets that pool data from multiple body sites. Taken together the proposed approaches can help supplement data packages where patient resources are limited.

24.6 Additional Pull Incentives and Considerations

There are several pull incentives that are currently under discussion with governments and the broader antimicrobial resistance community. Table 24.2 provides their various strengths and weaknesses as it relates to their ability to (1) promote innovation, (2) allow for patient access, (3) promote stewardship, and (4) support overall sustainability. Detailed description of the incentives and their pros and cons are provided in the sections below.

24.6.1 Voucher Programs

Some, particularly in the pharmaceutical industry, have recommended awarding a tradable voucher that extends the patent life of a product to companies that develop new antibiotics. This voucher, which would be given upon approval of the new antibiotic, would give the owner the ability to extend patent exclusivity for a given number of years to any one drug patent the company owns. In most instances, the patent would be purchased by the company that owns the most valuable patent nearing expiration. The price that would be paid for such a voucher could easily be in the billions of dollars. Tradable patent vouchers offer a powerful incentive tool and would likely facilitate reinvestment in antibacterial drug development. Further, as a sustainable incentive, it does not require government funding to ensure its continued existence. However, some believe that it is a blunt and inefficient mechanism for promoting innovation. In fact, creation of this voucher program would be an unprecedented step in US intellectual property law, where protections related to exclusivity would be granted due to innovation in a completely separate area. The incentive to increase antibacterial drug development would be funded by the purchasers of the drug whose patent is extended. One would be subsidizing one area of healthcare at the expense of another. There is also concern that this mechanism of patent extension would have a negative impact on patient care, by keeping more widely used medications on patent longer and delayed development of generic drugs. From both a societal and healthcare perspective, the overall cost of this incentive may be disproportional to the effect of the incentive. However, there has been some economic modeling of this incentive. One study examined the societal impact of the patent extension voucher for a *Pseudomonas aeruginosa* narrow-spectrum antibiotic [43]. They estimate that the cost to society for the first 2 years of the patent-extended drug would be approximately $7.7B over the first 2 years and $3.9B over the next 18 years. If the new antibiotic eliminated the costs of treating 50% of drug-resistant *P. aeruginosa* infections, it would save society $2.7B in cost over the first 2 years. It is estimated that the costs would be neutral by year 10 and would save society approximately $4.6B by year 20. These data suggest that the patent extension voucher could be an effective means of incentivizing antibiotic development without having to raise substantial funding. However, it is unclear if this incentive would be as effective in other resistant infections that occur with far less frequency. In those cases the incentive may be disproportionate. Lastly, this incentive does not ensure appropriate use, as profit of the antibiotic is still a direct proportion of its volume sold and/or used.

Modifications to this policy have been proposed that may aid in limiting its disruptive effects [29]. A proposed area of improvement would be to ensure the social value of the antibiotic is directly tied to the innovation. A potential mechanism for this would be to tie the value of the voucher to public health needs. For example, drugs that treated the most highly prioritized threats on the CDC list would be valued greater than those that provided incremental value over existing drugs. Vouchers

could also be capped, both in terms of time and revenue to control the impact of the incentive on healthcare costs. For example, the voucher could be given to extend the patent for 12 months or $1B in sales, whichever came first. The value or duration could be modulated to account for public health need. A completely new class of antibiotic that targeted Gram-negative bacteria would be given larger caps on revenue or duration than one that possessed more limited public health utility. Further, additional measures that aligned to public health objectives should be considered for incorporation into this incentive type.

Alternatively, and perhaps more directly, the government could simply auction off a set of vouchers every year to generate revenue. The funding generated could be placed into an antibiotic innovation fund to support full or partial de-linkage payments. A few $1B capped vouchers could easily provide the necessary funding for an incentive fund and would allow public health officials a role in the incentive prioritization. A complexity to this incentive is how global agreements on intellectual property or trade would factor into the decision to extend the patents in the US.

One example of this type of voucher program is the priority review voucher (PRV), particularly for neglected and tropical diseases and rare pediatric diseases. PRVs became law in 2007 and were designed to accelerate the review time for a selected product, forcing the US Food and Drug Administration (FDA) to render a decision on that product in 6 months saving an average of 7–8 months of review time [19]. PRVs can be used for a company's candidate product or can be sold to another developer. If PRVs were to be used to incentivize antibacterial drug development, there would be two distinct advantages. First, PRVs would accelerate the approval and availability of new products in a number of different therapeutic areas, and second, they would theoretically motivate industry to engage more greatly in antibacterial drug development. Historically, however, PRVs have been sold for anywhere between $67 M and $350 M (www.priorityreviewvoucher.org), an amount unlikely to be sufficient to ensure adequate ROI.

There are several disadvantages to PRVs as an incentive. First, the price and value of the PRV will depend on the current supply in the market. In other words, the more PRVs that get issued as a result of successful antibacterial drug development, the less value they will possess. A recent analysis suggests that if four PRVs were available at any given time, their value would decrease to approximately $100 M and cautioned Congress from further expansion of the program [38]. Second, PRVs do not guarantee FDA approval, potentially impacting the value of the voucher. Third, PRVs do nothing to ensure access and appropriate use and still ensure a model exists where profits are still intrinsically linked to volumes sold. To date, many awarded PRVs remain unused making their impact on drug development difficult to gauge. Taken together, PRVs would seem to provide a limited ROI and would be limited in their ability to encourage innovation, access, and appropriate use.

24.6.2 Reimbursement Strategies

Higher prices would be an effective means of incentivizing antibacterial drug development. For example, oncology drugs routinely demand high prices, and their pipeline of candidates reflects this, with over 800 candidates in Phase 2/3 clinical development [33]. Despite providing life-saving benefits, antibiotics have historically been priced much lower. Further, antibiotics in the US are typically approved on the basis of non-inferiority data, suggesting that there is no strong basis to price them higher than the clinical trial comparator. While higher prices might impact conservation or stewardship but from a financial standpoint versus a public health goal, more expensive drugs may not readily be prescribed to patients out of concerns over their affordability. Higher prices could instead incentivize companies to market their drug more strongly to ensure a greater return on investment. Thus, simply increasing the price of antibacterial drugs may drive greater innovation and stewardship but also limit patient access and further increase healthcare costs.

One model that utilizes higher prices as a pull incentive is referred to as the diagnosis confirmation model [22]. Under this model, antibiotics are priced at two different levels, a lower empiric treatment cost and a premium cost that would be levied if the diagnosis confirmed the pathogen of interest; such that initially, clinicians would use the drug empirically, based upon clinical response therapy, which would be de-escalated. If the patient remains on the therapy and the organism is identified and confirmed to be sensitive to the treatment, then the hospital would charge the higher premium price. There are a number of disadvantages to this approach. First, this model possesses no built-in function to ensure equitable availability to patients. Second, it does nothing to de-link the profitability of an antibiotic from the volume sold. Third, it is relatively complex and relies entirely upon the diagnostic capabilities within the healthcare setting administering the antibiotic and does not address a false or inaccurate test result. There are some advantages, however, as it encourages de-escalation of therapy if infection with a multidrug-resistant pathogen is not confirmed or strongly suspected. It would also ensure the use of diagnostics, as their results would be critical in determining if the patient remained or was taken off of the new antibiotic. The data collected in the healthcare setting could allow for hospitals to better maintain stewardship. Ultimately the model would discourage the empiric use of the novel therapy as long as cheaper effective options are available. However, the decision to use or de-escalate would be with the treating physician, and decision related to this would be contingent on a robust diagnostic capability.

Another proposed model for higher reimbursement is to fundamentally alter the way that antibiotics are reimbursed in the hospital care setting. Currently, inpatient healthcare expenses are classified using a system called the diagnosis-related group (DRG). This system divides possible diagnoses into more than 20 major body systems and subdivides them into nearly 750 groups for the purposes of Medicare reimbursement [2]. For example, there is a DRG code for appendicitis that reimburses based upon the average cost of care for patients with that condition, capturing

the spectrum of patients with no complications, resulting in the lowest costs, to severe complications, with highest costs.

Each DRG has an associated payment rate. The payment rates are updated annually to reflect the relative cost hospitals incur for the various DRGs 3 years prior. DRGs for which costs are rising over time at an above-average rate tend to be underpaid relative to actual costs because payment rates lagged compared to DRG cost increases. DRG's costs may increase over time if the need for expensive antimicrobials is rising over time due to increasing pathogen resistance to cheaper antimicrobials. However, because drug costs are only one component of total hospital costs, this effect is mitigated. One approach to update this system is to modify the DRG code to include a code for drug-resistant bacterial infections. For example, there could be a subcategory for each typical condition (e.g., abdominal infection, urinary tract infection, nosocomial pneumonia) that would classify these infections as resistant and would therefore reimburse at a higher level. Higher payment would presumably be predicated on being able to confirm or detect the resistant pathogen and not necessarily on a lack of a positive clinical response. While this incentive has the advantage of not relying on obtaining significant public sector funding and the political will to maintain this modification would be minimal, it does little to address stewardship of new antibiotics and may in fact result in driving the use of new antibiotics as hospitals seek higher reimbursement rates and would perpetuate a model where the US subsidizes the global pharmaceutical market.

To the extent that DRG's costs are rising because new treatments are introduced, a new technology add-on payment (NTAP) can be awarded for a period of 3 years if the new technology is demonstrated to result in improved clinical outcomes. Implemented in 2000, NTAP reimburses hospitals at the standard DRG plus an extra payment of up to 50 percent of the cost of the new technology. However, the payment has annual caps, and the US Centers for Medicare & Medicaid Services (CMS) is selective about the drugs and devices that qualify for the add-on program. To date, one antibiotic product has been approved for NTAP. Dificid® – a targeted therapy for treatment of *Clostridium difficile*-associated diarrhea – was awarded a NTAP because the drug demonstrated substantial clinical improvement over existing therapies [34]. Specifically, in its Phase 3 clinical trial, Dificid achieved comparable initial clinical response when compared to vancomycin but achieved superior sustained clinical response, with patients experiencing fewer recurrences following treatment. Incentives in the form of altering the NTAP program either by awarding NTAP payments for a period longer than 3 years or by making all antibacterial treatments eligible for the program could be utilized. Such an approach would not require significant public sector funding and have minimal maintenance needs. Broadening of NTAP would allow CMS to support public health goals by targeting the use of NTAP to antibiotics that provided the greatest public health benefit. However, this incentive would do little to ensure access and appropriate use as the program is US centric, while the problem of antibacterial resistance is global; therefore a substantially higher US payment could exacerbate the drug pricing imbalance between the US and other countries.

24.6.3 Options Markets

Advance market commitments or purchase options have been proposed as additional pull incentives [6]. Under this model, the company begins development of a new antibiotic with the intent of targeting that drug for use in developing nations against, for example, pathogens causing cholera or typhoid fever, and may also be able to be applied in developing countries for pathogens that are still rare but frequently drug resistant (e.g., *Acinetobacter*). The purchaser would buy options for the antibiotics that could be redeemed once the drug is approved and on the market. If purchased early in development, the price of the options would be low. If purchased late in development, when the risk is lower, the price would be higher, likely closer to what the price at which the product would be sold once in the commercial market place. Upon approval by regulatory agencies, the options holder could purchase the drug for use or could sell the drug or options to governments or patients for a profit. If the antibiotic failed to make it to market, the purchaser would lose the original investment, the size of which would depend on the stage of development. A key benefit of this approach is allowing investors to modulate their level of risk based on the drug's phase of development. If an investment is made early but accompanied by substantial risk, significant savings could be achieved. The option model, however, is limited in its ability to promote stewardship and its dependence on the free and open exchange of scientific data between developers and the option purchaser.

24.6.4 De-Linkage Programs

The current economic model for antibiotics intimately links the amount of profit of an antibiotic to the volume sold. Companies strive for high sale volumes to improve their ROI, which can increase overuse and drive resistance. This is often counter to public health objectives of minimizing overuse and ensuring that use is limited to only those patients that need the drug. As a result, alternative business models have been proposed. De-linkage is a model where companies are not paid on sales volumes but are given market entry or milestone payments to provide a definitive return on investment for introducing a new antibiotic into the commercial market. Payments under a de-linkage approach could also be effectively tethered to public health objectives, like conservation and stewardship.

Full de-linkage is a type of de-linkage model where the payments are made to fully buy out the use of the product once it is ready for market. This payment would have to be sufficient to ensure adequate ROI and ensure that a minimum supply of production could be maintained. As a condition of accepting this payment, the company would agree to not market or sell their antibiotic. The size of these payments would be substantial and would be estimated to be between $2 and 3B, depending upon the public health value of the antibiotic. The purchaser would then retain the

full right to market and supply the product. Some reports have suggested that this could occur via a global purchaser who would coordinate countries' investments, ensure stewardship of the antibiotics purchased, and allow access in low- and middle-income countries [28]. A primary advantage of a global purchaser is that a single organization would be capable of implementing public health measures, such as ensuring countries are held accountable for how they utilize the antibiotic. This would allow for use to be dictated by clinical need versus commercial interests. A challenge, however, with this approach is determining the appropriate price for the buyout. There is a need to reward the innovator and ensure an appropriate return on investment but also avoid paying too much. Additionally, this system would require broad international agreement and oversight. If countries could not come to an agreement under a global buyer model, it is unlikely that a global purchaser and other markets could effectively run in parallel.

Alternatively, partial de-linkage or market entry reward is a financial model where the drug developer supplements its ROI though milestone payments that work to de-link profits from volumes sold. Such that under this model, the drug developer retains intellectual property and is responsible for approval, manufacturing, and sales of the antibiotic while ensuring payments are attached to conditional requirements for stewardship or level of sales. For example, to receive milestone payments, a company would have to agree to cease additional annual sales of the product in any given year once a certain sales volume threshold was reached for that year or ensure a specific price in low- or middle-income countries. One advantage of the partial de-linkage model is the predicted protection against market disruptions, thereby possessing minimal secondary disruptive effects, particularly when compared to other pull incentives. In considering various economic incentives, the secondary disruptive effects of the incentive are a critical consideration, such that, if the price of antibiotics were to increase by ten-fold, there would be consequences, both predictable and unforeseen. Additional advantages include the capacity to provide a known ROI for developers, the ability to target antibiotics for high unmet medical need, and the ability to be designed with provisions that could ensure global access and proper use. Conversely, partial de-linkage models are disadvantaged by their expense and their sustainability. It is predicted that payments under a partial de-linkage model would be approximately $1–1.3B per antibiotic [28]. In the absence of a tax or alternative revenue-generating mechanism, governments will have to sustainably finance a fund that would administer these de-linkage payments. Questions also remain related to implementation of this incentive, particularly related to the size of the payments, when payments are provided, how products are prioritized for the incentive payments, and how stewardship conditions can be incorporated, particularly on a global scale.

Considering the ability of the partial de-linkage model to balance the key strengths, including promoting innovation while maintaining sustainability, allowing patient access, and promoting stewardship, this approach may be best suited for adaptation. In the US, for example, BARDA's role and experience in administering push and pull incentives to mobilize the pharmaceutical and biotech industries to produce medical products for public health emergencies place BARDA in a position

to readily implement such a model to provide a known ROI for companies developing new antibacterial drugs.

24.7 Policy Initiatives and Reports

There have been several policy documents, peer-reviewed publications, and sponsored studies that have made recommendations on incentives for antibacterial drug development. An analysis conducted by the Trans-Atlantic Task Force on Antimicrobial Resistance (TATFAR) made three primary recommendations [39]. First, a global AMR threat assessment should be conducted to guide prioritization of pathogens and the antibacterial drugs that would receive a particular set of incentives. Second, a combination of both push and pull incentives should be used that span all phases of antibacterial drug development. Lastly, de-linkage models designed to address public health objectives, such as stewardship, are recommended. Above all, these models should be coordinated internationally over time. Several policy initiatives addressing these recommendations are summarized below.

24.7.1 PCAST Report

In 2014, the US President's Council of Advisors for Science and Technology issued a report that provided several key recommendations for economic incentives for antibiotic development [44]. The report estimates that sales of approximately $500 M per year over a 10-year period would be needed to ensure an adequate ROI for industry. The report recommended increased push incentives across all phases of development and three main pull incentives for consideration. First, consider increasing pricing and reimbursement as a potential incentive. As previously noted, this recommendation carries with it challenges associated with the uncertainty of whether the market-tolerated increases in price would be sufficient to provide an adequate ROI and with increased inappropriate use tied to a volume-based model. Second, they recommended examining the use of patent vouchers that would extend the patent of an already approved, likely more profitable drug. While vouchers are anticipated to be highly valued, they delay the transition to a generic market and have a higher total social cost compared to other incentives. It could be viewed as a hidden tax on the already-approved drug. On the other hand, vouchers do not require an additional appropriation of funding to implement and would still adhere to free-market principles. Third, de-linkage was also recommended as a potential incentive.

Two de-linkage forms were discussed: complete de-linkage, where developers would receive a one-time $1B USD payment for the registration of a new antibiotic, and partial de-linkage, where milestone payments of approximately $400 M USD were given as market entry rewards based upon concurrently implementing required

stewardship objectives. Under complete de-linkage, the government would be responsible for the control, access, and distribution of the antibiotic. This could allow focus of the development of new antibiotics for areas of highest unmet medical need. The PCAST report discussed the possibility of establishing an antibiotic incentives fund to provide advance market commitments and milestone payments to reward bringing a new antibiotic to market and recommended BARDA, as the government entity administers this fund [44]. Given their experience in awarding and managing complex public-private partnerships with industry, BARDA could support a level of $4B USD over 10 years, which they contended could result in one new approved antibiotic every 2 years.

In 2015, a systematic analysis of 47 different economic incentives to stimulate antibacterial drug development recommended that two separate push mechanisms and one substantial pull incentive would be effective toward ensuring companies had an adequate return on investment [36]. They favored de-linkage models as an approach because it provides developers a known ROI, decreases the motivation for developers to market or over sell their product, and does not impact access of drugs to patients. They also cautioned that a package of incentives should first be developed that addressed the market challenges of developing a new antibiotic prior to public health objectives while considering incorporating international coordination. This would reduce the potential of stagnation while trying to develop an economic policy capable to addressing the complex issues related to AMR globally.

24.7.2 O'Neill Report

The UK government commissioned a review on antimicrobial resistance chaired by Lord Jim O'Neill, former Chief Economist of Goldman Sachs and now Secretary at the UK Ministry of Treasury. In addition to multiple published reports on establishing or improving economic incentives for antibacterial drug development and preventing spread of antimicrobial resistance, the May 2015 report (Securing New Drugs for Future Generations: The Pipeline of Antibiotics) proposed a series of interventions to balance commercial profitability with antimicrobial access and conservation, considering the balance with new drugs at the expense of off-patent drugs that could still be effective [28]. For example, new drugs could be reserved for treatment until existing drugs have failed. A Global AMR Innovation Fund was recommended as a "push" incentive. De-linkage models were recommended as a means to commercially sustain antibiotic development and encourage earlier investments. A proposed global buyer, representing a multitude of coordinated nation states, could purchase the global sales rights (estimated at $2–3B USD) to new antibiotics and manage the supply and distribution internationally, controlling stewardship, and use and provide access in developing nations. The developer could not market the new drug but would reimburse an adequate ROI. This model is potentially risky related to the uncertainty of establishing the buy-out price (with potential to overpay for rights) or projecting resistance to existing drugs. A coalition of

countries will need to be willing to contribute to a global buyer methodology and accept the risks with controlling supply. Some of these risks could be addressed with a hybrid de-linkage model due to less coordination and funding, as it would rely on a single global funding body ($1–1.3B USD/product), but companies would retain the ability to sell the drug in the market and receive payments to ensure an adequate ROI. Payments could be linked to stewardship and global access goals (i.e., price setting in specific countries) to address market-based rewards and meeting public health.

The report also recommends the establishment of a short-term multi-targeted global innovation fund for antibiotic research and development (estimated $2B USD over 5 years), acknowledging that funding for push incentives is needed to effectively populate the pipeline of novel antibacterial clinical candidates. With these fixed market incentives and private capital flow back, the innovation fund is proposed to be sufficient to reinvigorate research for the long term. The global innovation fund should address: (1) reevaluating old libraries of antibiotics and novel combinations that may be efficacious as "resistance breakers," (2) pursuing a bold approach to AMR (directed funding for novel approaches) that looks across and beyond established avenues of research, (3) improving and promoting scientific understanding of drug resistance, and (4) developing diagnostic tools for AMR.

24.7.3 Chatham House Report

The report, Towards a New Global Business Model for Antibiotics De-linking Revenues from Sales, made several key recommendations for new business models that considered funding, intellectual property (IP), stewardship, and regional and global implementation: (1) de-linkage models that guarantee an adequate ROI independent of sales volume, prioritizing access to new antibiotics and encouraging conservation, (2) increased public financing of incentives (tax credits, contracts, and prizes) across the entire antibiotic life cycle to target antibiotic development against microbes identified by a global threat assessment, and (3) a global threat assessment based on infection incidence, transmissibility, available treatments, and societal impact, to identify threats arising from resistance and prioritize the classes/types of products needed [9]. Global prioritization of antibiotics was recommended to be a fully transparent, independent process where the effectiveness of proposed incentives will need to be determined. Lastly, the report called for appointment of a secretariat to foster global coordination and the development of a global incentive fund.

24.7.4 DRIVE-AB Consortium

DRIVE-AB, which stands for Driving Re-investment in R&D and Responsible Antibiotic Use, is a public-private consortium supported by the European Union's Innovative Medicines Initiative (IMI) that was created to study different economic models for antibacterial drug development [20, 22]. It consists of 16 public and 7 private partners from 12 different countries and has received €9.4 M EUR in funding. The goal of DRIVE-AB is to quantify the value of a new antibiotic and create, test, and validate new economic models that will incentivize the development of new antibiotics. Initially, they intend to develop a definition of responsible antibiotic use. Data from surveillance systems and published literature will be used to determine the current impact of antibiotic resistance in both clinical and economic terms. Models are projected to be created that estimate the value of existing and new antibiotics to physicians, patients, and ultimately society. These determinations will aid in the creation of new economic models that will enhance and perpetuate the development of new antibiotics while ensuring the appropriate stewardship and conservation measures. Their recommendations are projected to be released in late 2017.

24.7.5 GUARD Initiative

Early in 2017, the report, entitled "Breaking Through the Wall, A Call for Concerted Action on Antibiotics Research and Development," was published by the German Ministry of Health and outlines the development of the Global Union for Antibiotics Research and Development (GUARD) initiative to facilitate the launch of badly needed antibiotics [4]. Specifically, four proposals are offered to reinvigorate the antibiotic value chain and ensure clinical needs are met. These include (1) identifying the target product profiles to ensure research funding is appropriately directed to the greatest clinical need, (2) building an infrastructure to fund promising research through the establishment of a Global Research Fund, (3) funding development projects through a forgivable loan instrument (only repaid if the drug is launched successfully) in order to steer development toward clinical and public health urgency, and (4) rewarding product commercialization through the Global Launch Reward acting as a pull incentive. While implementation of all four proposals would require significant scientific, organizational, and international support as well as funding and implementation plans, the successful execution of the initiative would likely advance the antibiotic pipeline from basic research to commercialization.

24.7.6 B20 Health Initiative Report

Recognizing the integral nature of health in economic development, the B20 Health Initiative, providing a platform between the global healthcare industry, governments, international organizations, and society to jointly drive change toward innovative health systems, published a set of recommendations and policy actions related to healthcare [1]. Their third recommendation focused on combating antimicrobial resistance with three policy actions: (1) scaling up R&D, (2) setting guidelines for antibiotics in food production, and (3) capacity and infrastructure building in low- and middle-income countries. Specifically, in order to incentivize product development, appropriate push and pull mechanisms, such as development funds and launch rewards, are called out as possible mechanisms.

24.7.7 Office of Health Economics Report

A briefing released in the Office of Health Economics, similarly to the B20 report, concluded push incentives alone will not generate new medicines [15]. The report evaluated market-based incentives that could be put in place in Europe to stimulate R&D for new antibiotics. The assessment found that the priority review voucher (PRV) was unlikely to be widely applicable and that Transferable Intellectual Property Rights (TIPR) risk overpayment compared to market entry rewards (MER), which carry political and credibility risk. Despite the risks, however, both TIPR and MER should be further explored for use in the EU as a regional "pull" incentive. Along the same lines, a US market survey evaluating the power of an incentive, the potential for administrative burden or unintended consequences, and the ease of implementation concluded that a hybrid approach of market entry coupled to exclusivity extension warrants further exploration in the US as well [40].

24.8 Framework for Prioritization

A significant question that remains in the discussion around incentives is related to decisions on which antibiotics will qualify for what set of incentives. One goal would be to align these incentives to areas of greatest unmet medical need. The CDC issued a report in 2013 that classified public health pathogens into three categories, urgent, serious, or concerning public health threats [8]. While extremely helpful in setting priorities from an epidemiological perspective, the CDC report was not intended to guide research and development priority setting. More recently, the WHO released the Global Priority List of Antibiotic-Resistant Bacteria to Guide Research, Discovery, and Development of New Antibiotics [53]. The WHO's objective is intended to incentivize funding for the research and development of novel

Table 24.3 A side-by-side comparison of priority lists from the CDC and WHO. Bolded pathogens highlight distinct entries in each list

2013 CDC priority pathogens	2017 WHO priority pathogens
Urgent	Critical
Clostridium difficile	*Acinetobacter baumannii*, carbapenem-resistant
Carbapenem-resistant *Enterobacteriaceae* (CRE)	*Pseudomonas aeruginosa*, carbapenem-resistant
Drug-resistant *Neisseria gonorrhoeae*	*Enterobacteriaceae*, carbapenem-resistant, ESBL-producing
Serious	High
Multidrug-resistant *Acinetobacter*	*Enterococcus faecium*, vancomycin-resistant
Drug-resistant *Campylobacter*	*Staphylococcus aureus*, methicillin-resistant, vancomycin-intermediate and vancomycin-resistant
Fluconazole-resistant *Candida* (a fungus)	
Extended spectrum beta-lactamase-producing Enterobacteriaceae (ESBLs)	*Helicobacter pylori*, clarithromycin-resistant
Vancomycin-resistant enterococci (VRE)	*Campylobacter* spp., fluoroquinolone-resistant
Multidrug-resistant *Pseudomonas aeruginosa*	*Salmonella*, fluoroquinolone-resistant
Drug-resistant nontyphoidal *Salmonella*	*Neisseria gonorrhoeae*, cephalosporin-resistant, fluoroquinolone-resistant
Drug-resistant *Salmonella* Typhimurium	
Drug-resistant *Shigella*	
Methicillin-resistant *Staphylococcus aureus* (MRSA)	
Drug-resistant *Streptococcus pneumoniae*	
Drug-resistant tuberculosis	
Concerning	Medium
Vancomycin-resistant *Staphylococcus aureus* (VRSA)	*Streptococcus pneumoniae*, penicillin-non-susceptible
Erythromycin-resistant group A *Streptococcus*	***Haemophilus influenzae*, ampicillin-resistant**
Clindamycin-resistant group B *Streptococcus*	*Shigella* spp., fluoroquinolone-resistant

antibiotics; therefore, infections that have multiple therapeutic options, such as *Neisseria gonorrhoeae*, are ranked lower compared to the CDC's list. Despite the differences in objectives, the two lists captured in Table 24.3 are highly overlapped.

There may be an opportunity to use the prioritized lists in conjunction with a payment model. One such framework for providing payment in the context of a de-linkage model has been described [37]. Under this proposal, payments would be made for 5 years beginning at initial registration. All base payments would be made via a single global purchaser under this model. Based upon the characteristics of the antibiotic, this base payment could be enhanced. Characteristics include novelty of

mechanism of action or class, whether it is the second, third, or fourth member of that class, and the alignment to the prioritized pathogens, whether there is a commitment to conduct pediatric studies and whether there is an oral formulation of the antibiotic. These characteristics add multiples of value to the base payment, thereby aligning public health objectives with the incentive structure. In the absence of a defined research and development agency, the use of economic incentives to shape research and development priorities may be an effective mechanism to produce the antibiotics that may not possess great market share but directly address significant public health objectives.

24.9 International Coordination

There is a growing consensus around the need for economic incentives for antibacterial drug development. Increasingly these discussions are gaining traction both domestically and internationally. The reports and policy documents, described above, call for increased coordination not only around programs that address AMR but also the economic incentives to be structured and administered globally.

One example of international cooperation was the establishment of the TransAtlantic Task Force on Antimicrobial Resistance (TATFAR). Created in 2009, TATFAR has the goal of improving cooperation between the US and EU in three key areas: (1) the appropriate therapeutic use of antimicrobial drug in medical and veterinary communities, (2) the prevention of healthcare and community-associated drug-resistant infections, and (3) the strategies for improving the pipeline of new antimicrobial drugs. In 2015, as part of its annual work plan, TATFAR elected to focus some of its attention toward making recommendations for economic incentives to improve antibacterial drug development. TATFAR assessed the current literature and published a preliminary set of recommendations [39]. TATFAR recommended:

1. A global AMR threat assessment process to coordinate data on resistant pathogens, the public health threat, and effectiveness of existing antibiotics should be developed. This process should consider additional criteria to guide prioritization for which new antibacterial drugs receive a particular set of incentives.
2. A constellation of economic incentives comprised of both push and pull mechanisms that address all phases of antibacterial drug development is needed to effectively incentivize industry.
3. Models that fully or partially de-link profit from volume sold should be developed, implemented, and evaluated. Initially, these should be initiated by a core group of countries capable of obtaining funding. Over time, models that account for conservation and access should be developed and could be governed by a collective mechanism.

In January 2016, more than 80 companies from the pharmaceutical, biotechnology, and diagnostic industries representing 16 countries published a declaration on

combating antimicrobial resistance [10]. As of April of 2016, there is now a total of 98 companies; 11 industry associations in 21 countries have added their names to the declaration. They called upon government to work with them to develop alternative market models that provide more reliable and sustainable market models for antibiotics and to commit to implement those models in the near term. Both delinkage models and value-based pricing were recommended as potential economic incentives. The declaration called upon policymakers and payers to recognize and account for the societal value these drugs provide and allow that to factor into the rewards they provide industry.

24.10 Future Directions

Successful implementation of an economic incentive model has precedence. The US Orphan Drug Act is an example of a package of economic incentives that was successfully able to stimulate the pharmaceutical industry to develop products for orphan diseases [16]. The Act was passed to create incentives to entice pharmaceutical companies to want to develop therapies for diseases where there is a limited market potential. The Orphan Drug Act provides additional 7 years of market exclusivity, tax credits that cover 50% of the Phase 2/3 clinical development costs, and grant funding. Since the passage of the Act, there have been over 400 drugs approved by the FDA to treat orphan diseases. Expansion of this Act to include antibacterial drugs may be a simple initial approach with minimal disruptive effects.

Additionally, there are a number of incentives that are currently under consideration by the US Congress. The Developing an Innovative Strategy for Antimicrobial Resistant Microorganisms Act of 2014 (DISARM Act), introduced in 2014, would provide additional payments under Medicare's New Technology Add-on Payment (NTAP) program for certain antibiotics [48]. These antibiotics would have to be qualified infectious disease products, described under the GAIN Act, and in general would have to address an unmet medical need or treat a pathogen with high rates of mortality or morbidity.

In 2013, the Antibiotic Development to Advance Patient Treatment (ADAPT) Act was introduced [49]. The ADAPT Act would direct FDA to approve new antibiotics that address unmet medical need in more limited populations of patients. It would allow FDA to consider a variety of evidence to determine whether to approve an antibiotic for a limited population. This limited population approval pathway could serve as an incentive, as it would theoretically lower the cost of clinical trials by requiring fewer patients. However, there are a number of significant challenges in conducting pathogen-specific clinical trials for antibacterial drugs under current FDA guidance, which may limit the true scale of this proposed incentive.

In 2015, the Reinvigorating Antibiotic and Diagnostic Innovation (READI) Act was introduced [50]. The READI Act would provide a tax credit to cover clinical trial costs for qualified infectious disease drug and rapid diagnostic tests. The tax credit would be transferable and would cover 50% of the clinical trial cost annually

and would function as a push incentive. At the time of drafting this chapter, none of these proposed legislative initiatives have passed either house or senate.

In 2016, the Duke-Margolis Center for Health Policy initiated the Antimicrobial Payment Reform Project [12]. This project seeks to utilize stakeholder engagement to evaluate several economic incentives, including de-linkage models and other reimbursement reforms that would support increased development of new antibacterial drugs and promote stewardship. The project will outline a path toward implementation of incentives and reimbursement reforms specifically within the US healthcare system. This work represents one of the first major policy discussions in the USA that brings together government officials, industry representatives, and academic experts to discuss economic incentives for antibacterial drug development.

The President Advisory Committee on Combating Antibiotic-Resistant Bacteria (PACCARB) was created in 2015 to provide advice, information, and recommendations to the Secretary of the US Department of Health and Human Services regarding programs and policies related to the National Strategy and Action Plan for Combating Antibiotic-Resistant Bacteria [44, 45]. In March 2016, the Secretary of HHS directed the PACCARB to provide her recommendations on economic mechanisms to incentivize development of therapeutics, rapid diagnostic, and vaccines for both humans and animals that maximized return on investment and encouraged appropriate stewardship and patient access. The PACCARB has commenced deliberation on this topic and will provide recommendations to the Secretary. Ideally, this work will aid in advancing policy discussions and allowing the US government to take a position on economic incentives for antibacterial drug development.

In December of 2016, the senate passed and the president signed into law the twenty-first Century Cures Bill [51], which among its many provisions establishes a new FDA "limited population approval pathway" for antimicrobials that treat serious or life-threatening infections for which there are unmet medical needs, explores novel statistical approaches to facilitate implementation of the limited population antibiotic development pathway, establishes a new pathway for rapid development and approval of new diagnostic devices called Breakthrough Diagnostics, and enables faster updating of antimicrobial susceptibility interpretive criteria, referred to as breakpoints, which are used for the development of the antimicrobial susceptibility tests that help doctors figure out the best antibiotic treatment for their patients. While this bill provides a much needed first step in rethinking the conduct of clinical trials for antimicrobials alone, it will be insufficient in filling the research and development gap and adequately stimulating the pipeline.

In 2017, Congress introduced the Improving Access To Affordable Prescription Drugs Act [52]. The bill intends to accomplish numerous goals, including requiring greater transparency with respect to R&D costs for new drugs and intending to stimulate research and development for new antibiotics to treat drug-resistant infections through the establishment of the "Antibiotics Prize Fund." If enacted, the bill would authorize $2 billion USD to be used to award "up to three prizes for qualifying products that provide added benefit for patients over existing therapies in the treatment of serious and life-threatening bacterial infections" as demonstrated in

superiority trials. While the prize criteria would be set by the NIH director, the bill does add a provision waiving all exclusivity rights by the developer thereby making the new therapy effectively a generic as soon as it is marketed. For consumers, this may be an attractive option to ensure low prices; for pharmaceutical companies, however, the value of the prize would need to greatly outweigh the future value generated through the sale of a truly beneficial therapies.

Other prize models are already being tested, including the Antimicrobial Resistance Rapid, Point-of-Need Diagnostic Test' Challenge; a collaborative effort between NIH and BARDA is intended to develop prototypes of diagnostics to improve detection of drug-resistant bacteria [26]. The prize, complementing existing BARDA and NIH portfolios, will award equal to or greater than $18,000,000 to be divided among a maximum of three awardees at its completion. These prize approaches may be an attractive mechanism to supplement the incentive landscape.

Other attempts to pass legislation intended to stimulate antibacterial drug development have been limited. In fact, there is no formal US government position on whether or what types of economic incentives are needed. The absence of a formal position is a significant obstacle toward the development of a legislative strategy that would lead to implementation of an incentive package. Additional efforts will be needed to bring together relevant stakeholders in the US, build consensus, and get policymakers to agree on an incentive package.

24.11 Conclusion

Evidenced by the minimal research and development investments, which has translated into a substantial innovation gap with no new classes of antibiotics invented to treat the most severe infections in 45 years, the current market incentives for antibacterial drug development are insufficient. Additional economic incentives are needed to not only keep the remaining companies engaged in antibacterial drug development but to entice companies to reenter this therapeutic area and make the necessary investments toward a sustainable research and development infrastructure for the discovery of new antibacterial drugs. A mixture of incentives comprised of both push incentives that subsidize research and development costs and a strong pull incentive that provides a known ROI is needed to generate positive NPV values for many antibiotic development programs. These programs should be capable of incentivizing small, medium, and large enterprises and should be present across all phases of development. The creation and implementation of an incentive package also present governments with a remarkable opportunity to shape the incentives to achieving broader public health objectives. Stewardship, educational campaigns, limits on marketing, and even limits on annual production are all possible measures that could be incorporated into incentives. By using a significant pull incentive as a known return on investment, the fundamental deficiencies in the market could be rectified, specifically that profit is tied to volume, which limits the utility of an

antibiotic. Failure to incorporate and maintain these public health measures into any incentive package would represent a significant missed opportunity for public health and public policy. If industry can't rely on the incentive and have it factor into their NPV calculations, it will do little to stimulate private sector investment. The structure and support of the incentive therefore cannot be subjected to political whim. One feasible approach may be strong lego-regulatory pull incentives, such as a patent extension voucher, with public health measures incorporated as condition of acceptance of the extension as well as caps on the duration of extension and total amount of revenue collected. While still possessing secondary disruptive effects, this incentive could be structured with limits that concurrently promote conservation and stewardship.

There is significant discussion in the antimicrobial resistance community on economic incentives for antibacterial drug development. There are several groups across the US and Europe actively discussing what types of incentives would be most effective in stimulating antibacterial drug development. Time is of the essence. With significant attention focused on the issue of antimicrobial resistance, economic incentives for antibacterial drug development have received added attention. Recognizing that no strategy will be perfect, consensus on a package and type of incentives is needed soon to translate the political momentum around antimicrobial resistance into legislative action. The window for implementation may close if time elapses and other more pressing issues of the day materialize. With all the political momentum around antimicrobial resistance and economic incentives, failure to achieve substantive policy change in this area would be profoundly damaging to the field and would likely lead to further reductions in the number of companies interested in the research and development of new antibiotics.

Major Points

- Alternative market models to support innovation in antibacterial drug development are needed.
- Current business models, where profit of an antibiotic is directly proportional to the volume sold, are at odds with public health objectives of stewardship and conservation.
- A mixed approach of push and pull economic incentives is needed to create an ecosystem of economic incentives that promote re-investment in research and development.
- Models that de-link the profit of an antibiotic from the volume sold could be used to reward innovation, while concurrently promoting stewardship and conservation of new antibiotics.
- There is general consensus within the research and development community that a mixture of push/pull incentives and de-linkage models are favored over other market incentives that possess greater secondary disruptive effects.

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Correction to: Transmissible Antibiotic Resistance

George A. Jacoby

Correction to:
Chapter 11 in: I. W. Fong et al. (eds.), *Antimicrobial Resistance in the 21st Century*, Emerging Infectious Diseases of the 21st Century, https://doi.org/10.1007/978-3-319-78538-7_11

Owing to an oversight on the part of Springer Fig. 11.2 and Fig. 11.3 of this chapter were initially published with errors. The error has been corrected and is reflected in both print and electronic versions.

The updated online version of this chapter can be found at
https://doi.org/10.1007/978-3-319-78538-7_11

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I. W. Fong et al. (eds.), *Antimicrobial Resistance in the 21st Century*, Emerging Infectious Diseases of the 21st Century, https://doi.org/10.1007/978-3-319-78538-7_25

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