Thomas J. Dougherty
Michael J. Pucci *Editors*

Antibiotic Discovery and Development

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Editors

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Foreword

A strong case can be made that up to this point among the most important scientific achievements in history has been the discovery and development of antibiotics to treat bacterial infections. During most of human history, the number one cause of death was infection. The leading killer in the pre-antibiotic era was essentially conquered by the advent of antibiotics and average human lifespan increased dramatically. Most of us do not concern ourselves to a great extent with bacterial infections that would have terrified people less than one hundred years ago. Bacterial diseases have altered history from pneumococcal pneumonia to bubonic plague to tuberculosis all killing untold millions in their process. In the last century, an assortment of pills and injections has often turned the tide in the favor of the infected host and vanquished the pathogen. Sometimes it is difficult to recall just how grim infectious diseases were prior to introduction of antibiotics. As Lewis Thomas put it in his book The Youngest Science: Notes of a Medicine Watcher [1]: "For most of the infectious diseases on the wards of Boston City Hospital in 1937, there was nothing that could be done beyond bed rest and good nursing care." He notes that with the introduction of the first antibiotics "The phenomenon was almost beyond belief. Here were moribund patients, who would surely have died without treatment, improving within a matter of hours and feeling entirely well within the next day." The arrivals of these "wonder drugs" also signaled the rise of the pharmaceutical industry and have been lucrative products for these companies, remaining a 30 billion dollar business today. However, unlike most other therapeutic areas, antibiotics essentially have built-in obsolescence, as pathogens have become resistant both through mutations and through a number of often clever genetic exchange mechanisms. Drug resistant bacteria are on the rise, and in some cases, the options for effective treatment are very narrow. Healthcare-associated infections are at the forefront of resistance problems, with multiply resistant pathogens that are increasingly problematic to eradicate with current therapy. Further, resistant infections have escaped the hospital. In the US, MRSA infects >94,000 and kills >19,000/year., a toll that exceeds deaths due to AIDS. Antibiotics are also unusual because there is a societal aspect to their use. Unlike most other disease treatments, the use or misuse

of an antibiotic has a much broader impact on individuals beyond the immediate patient. The loss of effectiveness, due to the spread of resistance impacts all of us.

Thus far, in most cases, humans have been able to keep ahead of the rapidly evolving resistant microbes. However, the question remains very much open whether this will continue indefinitely or whether will we find ourselves heading back towards pre-antibiotic times. The IDSA issued their “Bad Bugs, No Drugs” report in 2004 [2], outlining the critical nature of the situation and the urgent need for new antibiotics to address multiply resistant pathogens. More recently, they have issued their ten new drugs by 2020 initiative [3], which spans across many key stakeholder groups. However, during the 1990s and 2000s, several large pharmaceutical companies either eliminated or downsized their antibacterial discovery efforts. At the same time and partly as a consequence, the number of newly marketed antibacterial drugs has also fallen. Some smaller companies have entered into this area, but the number of researchers trained in antibiotic discovery and development has greatly reduced as compared to the peak levels in the last century. At a time when the medical need for new antibiotics is increasing, there is less effort and fewer people trained and committed to the task.

While there are already many excellent texts that list the various antibiotic classes and their properties or explore mechanisms of action or mechanisms of resistance, the goals we set out to achieve in this book are different. Our aim was to provide the reader with a broad-based yet in depth perspective of the field of discovering and developing antibiotics. We asked ourselves the following question: what knowledge would be important for a newcomer to the field? What would a seasoned antibiotic drug hunter also find useful to have at hand? These were the questions we sought to address in assembling the overall book outline and recruiting expert chapter authors. In this volume, the intention is to attempt to capture the antibiotic discovery and development process and provide the reader with a sense of how it is done and where things stand in 2011.

The book begins with a solid historical review of the early years of antibiotic discovery & development (often referred to as “The Golden Years”). It is important to appreciate the early efforts and techniques employed to find new antibiotics in the mid-twentieth century. Many readers may find themselves surprised at the sophistication of screening methods employed 30 or 40 years ago. From that starting point, the book highlights the evolution of many of the individual classes of drugs in clinical use discovered during that time. In addition, there has been considerable effort recently to rejuvenate existing classes to address specific resistance problems, and these chapters also reflect that work. As a result, the individual drug chapters span examples of early compounds right up to the latest developments in each class. In some cases, separate chapters are presented on the prominent resistance mechanisms to individual drug classes as well as a review of the multi drug resistant efflux pumps, which are particularly problematic for Gram-negative bacteria. We also wanted to devote several chapters to the “worst offenders”; that is problem pathogens that are particularly challenging to current antimicrobial therapy. In this context, it is also important to appreciate the vast array of resistance mechanisms that different microbial pathogens have acquired and adopted.

Having set the stage with existing classes of compounds and problematic pathogens, the next aspect the book addresses is the drug discovery process and areas to be considered when identifying novel antibiotics. Two rather unique chapters address the issues of chemical and physical properties identified in current antibiotics and the challenge of antibiotic penetration through the several membrane barriers enroute to the target in the bacterial cell. There is also a chapter devoted to the important area of natural products, a major source of current antibiotic classes and the future of such efforts. Next, approaches to discovering novel antibiotics is covered, including genomic identification of targets, principles of enzymatic screening to identify potential leads, and the use of cell-based screens to identify inhibitors. The role of both NMR and X-ray structure techniques in identifying inhibitors, mechanism of action studies, and their utility in refining compounds are covered in two chapters. The chapter on a recent novel antibiotic program, the identification of an FtsZ cell division inhibitor, is presented as an excellent example of the process of modern antibiotic discovery.

Equally important to the refinement of lead interaction with the target and microbial inhibition is the issue of demonstrating efficacy in model animal infections. In this chapter, many of the standard animal infection models are described, along with the type of data generated and its interpretation, and the role of pharmacokinetics and pharmacodynamic models in infection research are addressed in their own chapter.

Finally, we round out the topics with a chapter on antibiotic resistance surveillance, an important area for anticipating what future resistance trends may be. There is also a chapter on the late stage development process for antibiotics; the types of studies necessary for the Regulatory authorities, and the process of submitting the documentation to place a new antibiotic on the market.

We were extraordinarily fortunate to have enlisted some of the leading scientists in the field from both industry and from academia to share their knowledge and experience. We are profoundly grateful for the encouraging responses we received from these individuals and their willingness to participate in this effort. The chapters we received were all extremely thoughtful and of high caliber. Without their contributions, this volume simply would not exist. Our hope is that the reader will learn and benefit from the information in this volume and that it will serve as a valuable reference source for antibiotic investigators, present and future.

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Part I
Introductory History of Antimicrobial Drugs

Chapter 1
The Early History of Antibiotic Discovery: Empiricism Ruled

Richard J. White

1.1 Introduction

For the last 60 or so years the chemotherapy of bacterial infections has been dominated by natural products and their semi-synthetic variants. Although the term antibiotic was initially used exclusively to describe those anti-bacterials of natural or semi-synthetic origin, it has become broadened in common usage to include antibacterial agents of purely synthetic origin as well. The emphasis of this chapter will be on the discovery of novel prototype structures that represent the different classes of antibiotic e.g. penicillins, cephalosporins, and macrolides a distinct from the multiple generations of improved analogues within a class that have typically followed an initial discovery. In some cases, the prototypical molecule discovered was developed and marketed without modification. In others, some modification proved necessary to make the drug clinically useful. An antibiotic class is defined by a characteristic core moiety, or pharmacophore, that is responsible for the observed antibacterial activity. Although dramatic and important improvements have been made through the chemical manipulation of the molecule that was first found, from the discovery standpoint, they represent variations on a pre-existing theme. An important consideration in this chapter will focus on the discovers' mindset and what led him/her to find a new antibiotic. Unfortunately such details are not always reported, and, at best, the information available is fragmentary. In some cases, comprehensive accounts of the events leading up to the discovery are well-documented, in others, however, only a minimal description is available. The extent of the information reported herein does not necessarily reflect the relative importance of individual discoveries, but simply what is readily accessible. In addition to the results published in scientific

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journals, several books have been written that touch on antibiotic discovery [8, 31, 35, 57, 62] and have proven to be an invaluable resource in compiling this chapter. The first publication documenting an antibiotic's *in vitro* and/or in vivo activity will usually be considered to be the year of its discovery. Only those antibiotics that were found to have clinical utility and were, for at least a time, marketed for the treatment of systemic bacterial infections will be considered. As will become clear, the gap in time between an antibiotic's discovery and its availability to prescribing physicians has increased dramatically over the years. This is largely a result of the burgeoning number of regulatory hurdles that have to be overcome. In turn, this is the outcome of an improved understanding of the different factors affecting safety and efficacy, coupled with the need to show that any novel antibiotic was at least as good as, if not better, than the standard of treatment available at the time. This chapter on the early history of discovery will only cover those classes of antibiotic found by the use of empirical screening methods, which in reality represents the majority. A subsequent chapter will cover those antibiotics that were discovered more recently by employing rational approaches. The transition from the historically successful empirical approach to that of a more rational screening approach was gradual with significant overlap occurring over a number of years starting in the late 1970s. In fact, the two most recent novel antibiotic classes to be launched in the first decade of the twentieth century (linezolid and daptomycin) were discovered empirically! The order in which the various antibiotic prototypes will be discussed is not strictly chronological because there were two tracks or approaches running in parallel: one a synthetic chemicals based approach and the other exploiting natural products. For convenience, there is some grouping of compounds on the same track. The structures of the antibiotics discussed in this chapter are shown in the accompanying Figs. 1.1–1.4, which are grouped by their provenance. In those cases where the molecule initially found needed chemical modification to achieve clinical utility, the marketed product is shown.

1.2 Birth of Chemotherapy

Luis Pasteur and Robert Koch are widely recognized as having played a critical role in formulating and developing the germ theory of disease; for this and related work, they were both awarded Nobel prizes. In 1892, Koch's postulates spelled out the criteria for proving that a particular bacterium was responsible for a specific disease. Meanwhile, a highly active German chemical dye industry had provided important reagents to histologists who had shown that some of these dyes could selectively stain tissues or pathogens. This set the stage for Paul Ehrlich to propose his concept of selective chemotherapy. He reasoned that it should be possible to create 'magic bullets' that would not just stain a pathogen, but selectively kill it as well.

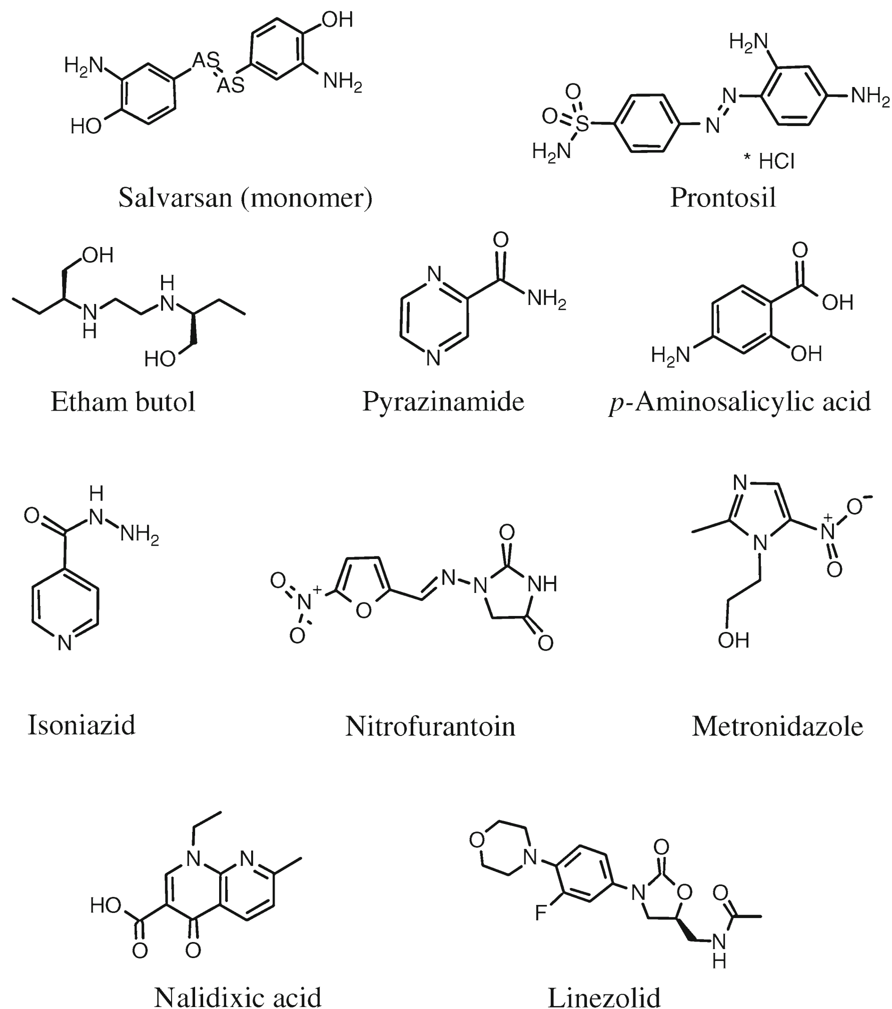


Fig. 1.1 Antibiotics produced through synthetic chemistry

1.2.1 Ehrlich's Discovery of Salvarsan

Ehrlich took a pathogen-targeted approach and systematically searched for a drug active against syphilis, caused by the bacterium *Treponema pallidum*. His starting point was atoxyl, an arsenic-containing compound that had some activity against African sleeping sickness, but had serious toxicity issues. Atoxyl was amenable to chemical modification and became the lead on which to base a chemical optimization

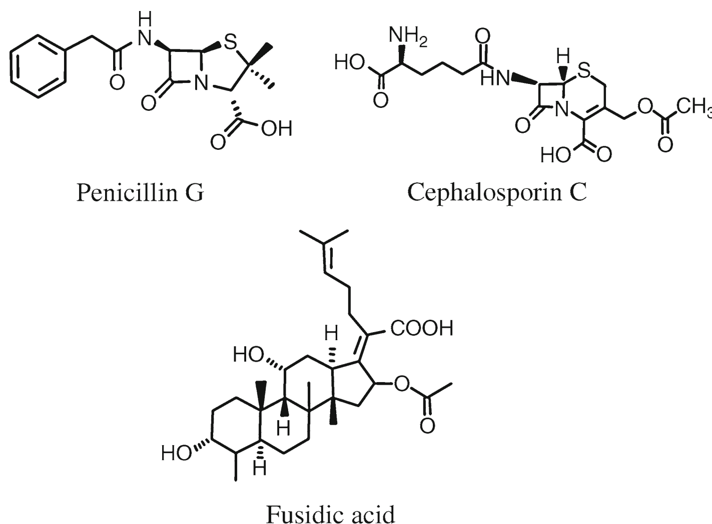
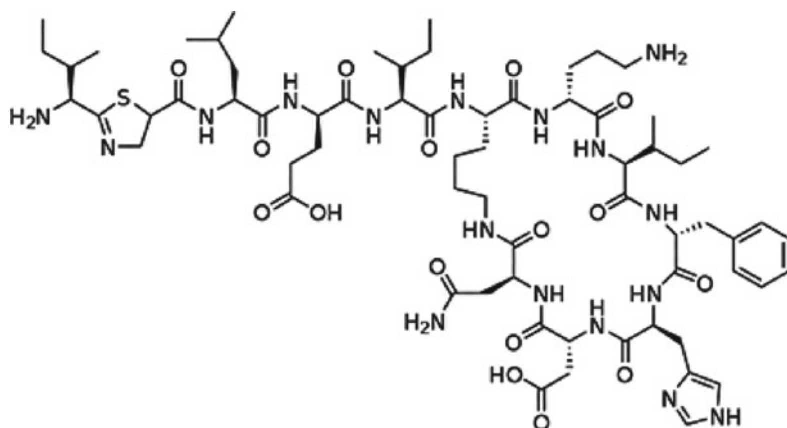


Fig. 1.2 Antibiotics produced by Fungi

program. He reasoned that although arsenic itself was known to be toxic, that an organo-arsenic derivative might become selectively toxic to a pathogen. This effort eventually led to the synthesis of analogue number 606, subsequently named Salvarsan, which cured syphilis infections in a rabbit model [66]. It is interesting to note that all the activity screening had out be carried out with an in vivo rabbit model since there was no *in vitro* test system available, which is still the case today. The drug was discovered in 1909 and amazingly (by today's standards) was in clinical use by the following year. It was extremely successful in treating syphilis, especially when compared with standard treatment of the day, Mercury salts. It provided Hoechst with the first blockbuster drug. Prior to the discovery of Salvarsan, Ehrlich was awarded the Nobel Prize for his work on immunology in 1908. Despite the important role that Salvarsan has played historically in the therapy of bacterial infections, its actual structure was controversial. In 2005, it was revealed that Salvarsan is actually a mixture of arsenic bonded species, which slowly gives rise to an oxidized species that is responsible for the activity against the pathogen [41].

1.2.2 Prontosil: Forerunner of the Sulfonamides

Continuing in the same vein as Ehrlich, Gerhard Domagjk of the Bayer Division of IG Farben (a consortium of German dye manufacturers) started testing azo related dyes for activity against bacteria. The program started in 1927, and in 1932 KI-730 (subsequently named Prontosil) was submitted for testing; it was devoid of activity *in vitro* but was still tested in vivo using a *Streptococcus pyogenes* infection in mice [62]. Surprisingly, it worked very well in vivo and its discovery was announced in 1935 [18]. As is often the case when news of a novel antibacterial leaked out,



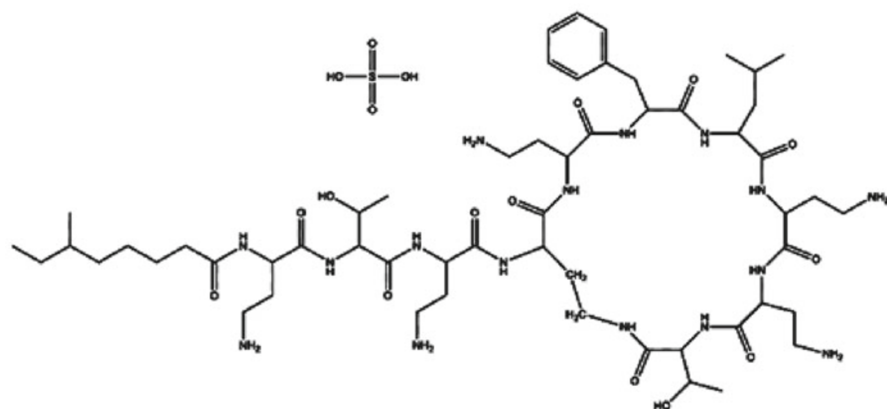
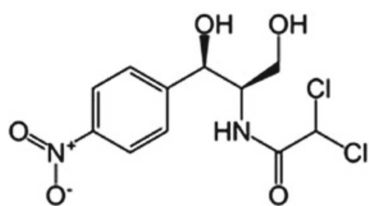
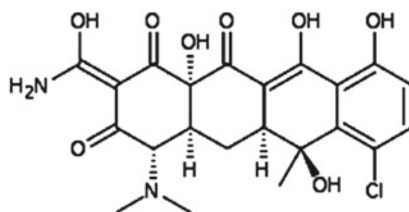


Fig. 1.3 Antibiotics produced by non-actinomycete bacteria

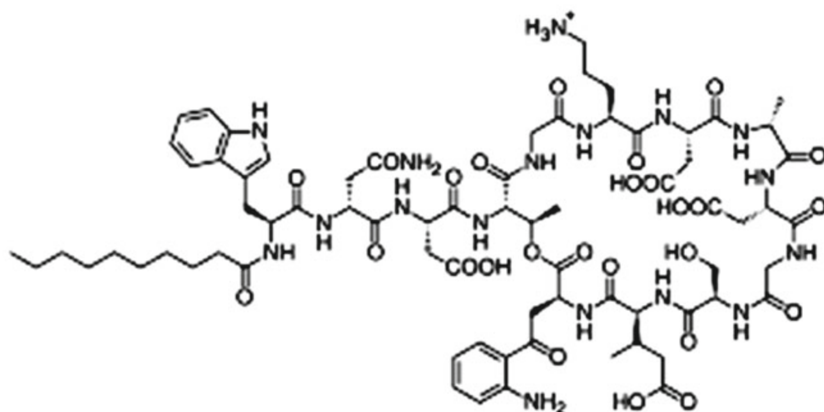
several other groups began work on analogues, and it was quickly recognized that Prontosil consisted of two moieties: a triamino benzene imparting the red color, plus a p-aminobenzene sulfonamide, which turned out to be the active part of the molecule. This knowledge led to the synthesis of a large number of active sulfonamides, and representatives of this class are still on the market today. Unlike Prontosil, they were active *in vitro* as well as *in vivo*. In 1940, it was recognized that the sulfonamides could be reversed by p-aminobenzoic acid; the sulfonamides are structural analogues of this natural metabolite [73]. For the first time, chemists had the benefit of starting with a molecule that was not intrinsically toxic, until this point, much of the efforts to discover novel antibacterials started off with a toxic molecule and tried to engineer out the toxicity (e.g., salvarsan).



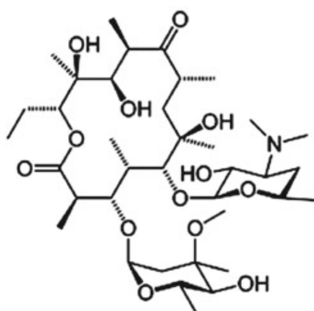
Chloramphenicol



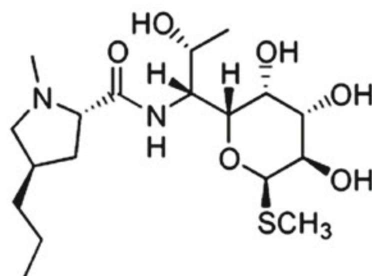
Chlortetracycline



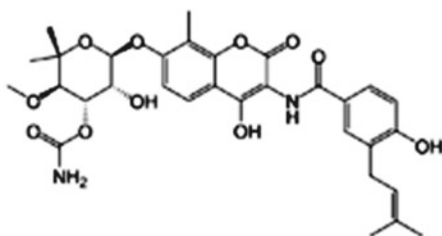
Daptomycin



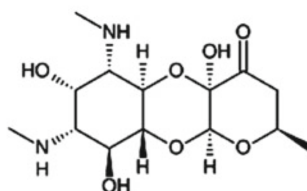
Erythromycin



Lincomycin

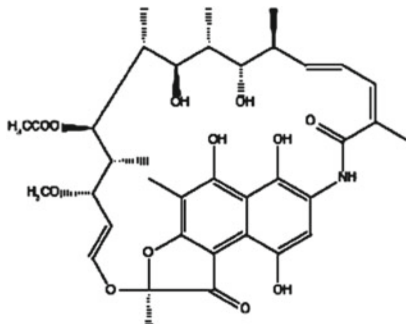


Novobiocin

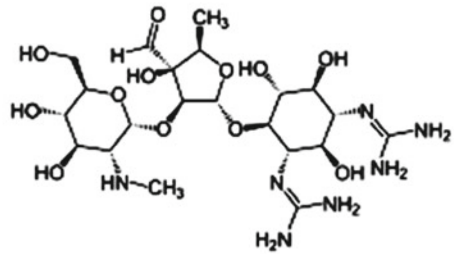


Spectinomycin

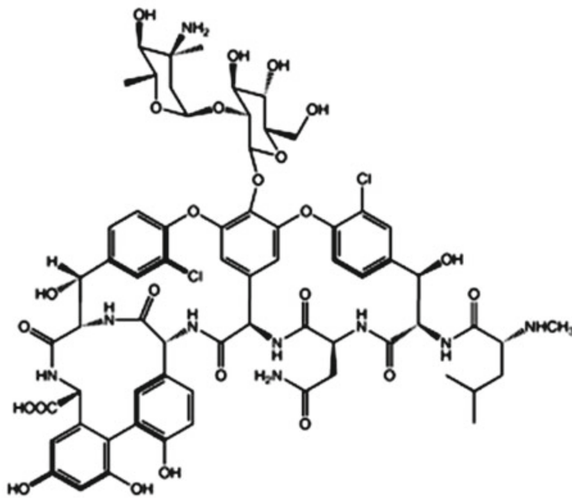
Fig. 1.4 Antibiotics produced by Actinomycetes



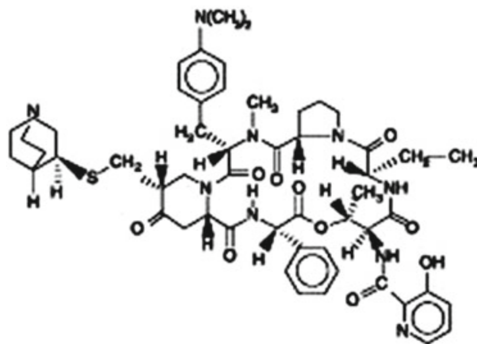
Rifamycin SV



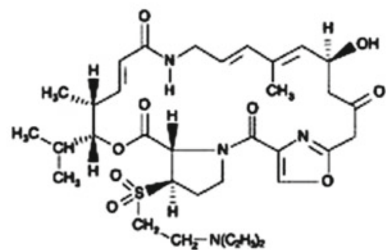
Streptomycin



Vancomycin



Synercid component 1 Quinupristin



Synercid component 2 Dalfopristin

Fig. 1.4 (continued)

1.3 Natural Products Enter the Scene

While the German chemical industry was actively pursuing synthetic chemicals as Ehrlich's magic bullets, Alexander Fleming was investigating the staphylococci and made the now famous, but fortuitous observation that a contaminating mold growing on one of his discarded Petri dishes inhibited growth of the surrounding staphylococci.

1.3.1 Discovery of Penicillin

Fleming's contaminating mold was identified as belonging to the genus *Penicillium*, which led to the name penicillin for the substance responsible for the antibacterial activity observed on the agar plate. Fleming published his work on penicillin in 1929 [25], reporting that extracts of the mold were able to kill a number of gram positive pathogens in addition to the staphylococci and even the gram negative pathogen responsible for gonorrhea. Over the next 10 years, Fleming tried to progress penicillin further but was hampered by an inability to isolate and purify it. Early attempts to use crude penicillin topically in patients were not very successful, and Fleming did little further work on its clinical potential, focusing instead on its utility as bacteriological reagent. He never tested it in a model infection in mice! Meanwhile, Ernst Chain, working as part of Howard Florey's team at Oxford, had taken on the task of isolating penicillin and solving its structure. The first results of this effort were published in 1940 [12], and by 1945, penicillin had demonstrated its amazing curative properties in the clinic and was being produced and distributed on a large scale. For their seminal work Florey, Chain, and Fleming were awarded the Nobel Prize in 1945. Over the ensuing years many generations of novel penicillins have been developed with improved spectrum, pharmaco-kinetics, and resistance to beta lactamase. Today, they remain a very important part of the antibiotic armamentarium.

1.3.2 The Actinomycetes Take Center Stage

Fleming's discovery of penicillin in 1928 coupled with Rene Dubos' discovery of tyrothricin in 1939 [19], led Selman Waksman to start investigating microbes found in the soil as a source of novel agents active against bacteria. Dubos' work that led to tyrothricin was very different from Fleming's fortuitous discovery of penicillin, as it resulted from the first deliberate search for compounds produced by soil microbes that were capable of killing pathogenic bacteria. He actually fed gram-positive bacteria at intervals to a large sample of mixed soils, hoping initially to find microbes that were capable of destroying the bacteria. In reality, he discovered a bacterium that produced an alcohol soluble compound capable of inhibiting the

growth of gram-positive bacteria that he called tyrothricin. The alcohol extract was actually a mixture of two compounds: tyrocidin and gramicidin. Although neither antibiotic proved to be of clinical utility, their discovery was a seminal event demonstrating the utility of screening soil microbes [51]. Tyrocidin proved very toxic, and, although gramicidin was able to cure experimental infections in mice, it also was too toxic for systemic use in humans. Gramicidin is a complex of six related compounds and still has utility today as a topical treatment for superficial infections; it is one of three constituents in Neosporin ointment. Natural products synthesized by soil microbes are frequently produced as a complex of related molecules. Waksman's group started testing all three of the known types of microbe found in the soil (bacteria, fungi, and actinomycetes) for their ability to produce antibiotic activity. It quickly became apparent that the actinomycetes were the most fruitful source of this activity. The subsequent systematic screening of soil actinomycetes led to actinomycin and streptothricin, which, like tyrocidin and gramicidin, were too toxic for clinical use as antibacterials. Nonetheless a clear direction had been set in the quest for novel antibiotics!

1.3.2.1 The Discovery of Streptomycin

In 1943, Albert Schatz, a graduate in Waksman's lab found Streptomycin, which was active against gram negative bacteria and most importantly against *Mycobacterium tuberculosis*, the pathogen responsible for TB (tuberculosis). It was quickly shown to be active in animal models of TB and then to be capable of curing the disease in actual patients by 1946. Although the Merck company originally had rights to all the research in Waksman's lab, the dramatic need for large quantities of a life saving drug convinced Merck to allow other pharmaceutical companies to take out licenses to manufacture streptomycin; soon a 1,000 kg a month was being made. Ultimately the utility of streptomycin would be severely limited by ototoxicity, which is its principal side effect. This side effect unfortunately led to patients that were cured of TB but deaf as result of the treatment. Waksman was awarded the Nobel Prize in 1952 for his pioneering work with actinomycetes and for the discovery of streptomycin. His work, coupled with pioneering results of Dubos, provided the screening paradigm that would be applied so successfully for the next 30 or so years by the pharmaceutical industry in the quest for novel antibiotics. Sadly the story of streptomycin's discovery was clouded by a court case in which Dr Schatz claimed that he had not received the recognition he deserved for the early pivotal role that he had played. He had initially been excluded from royalty payments that Waksman had been receiving for streptomycin. Although this was subsequently rectified, Schatz still felt that Waksman had retrospectively manipulated the story [37, 57]. None of this drama, of course, should detract from the critical and broader role that Waksman played in pursuing and championing the actinomycetes as antibiotic producers. His work led to the golden era of antibiotic discovery.

1.4 The Pharmaceutical Industry Initiates Screening: Collaboration and Competition

Following from Waksman's work on streptomycin and the demonstration that soil microbes were capable of producing a variety of structures with antibacterial activity, the pharmaceutical industry instigated major screening programs. This led to an incredibly productive period from the late 1940s until the 1970s during which many of the major antibiotic classes were discovered. Initially all the major US pharmaceutical companies were heavily involved in making penicillin, and in some cases, streptomycin as well. Encouraged by the commercial success of these antibiotics, they were eager to discover their own [31]. In an unusual move, four US companies in the Midwest (Eli Lilly, Abbott Laboratories, Upjohn, and Parke Davis) shared information on attempts to chemically synthesize penicillin. Although these efforts failed to supplant the fermentation route of production, this unique collaboration continued with their antibiotic discovery programs. However, when Parke Davis discovered chloramphenicol in 1947, they left this partnership. The other three continued to collaborate until 1952 when erythromycin was discovered [31]. Not surprisingly, since then antibiotic discovery has been the subject of secrecy, intense rivalry, and competition between the many companies involved. In some cases, this secrecy has even led to accusations of industrial espionage and the stealing of key actinomycete producer cultures with criminal convictions resulting in some cases. Given the incredible productivity of these early antibiotic discovery programs, it's worth describing their key features and how they evolved over time and became increasingly sophisticated. Although the details of such programs varied, the general features of these efforts were remarkably similar and can be conveniently considered as consisting of three components:

1. Isolation and cultivation of novel producer organisms.
2. Screening of cultures for activity.
3. Purification and identification of the active metabolites.

1.4.1 Isolation of Novel Producer Organisms

Waksman's work on different soil microbes had shown that the actinomycetes were the most prolific producers of antibiotics. Usually the announcement of a novel antibiotic was coupled with description of a producer microbe that was itself a novel species. Thus efforts to isolate large numbers of novel actinomycete cultures were made, which required a systematic collection of soil samples. Pharmaceutical companies involved in the antibiotic screening enterprise rapidly put in place programs to insure that a wide variety of soil samples became available. There was a sense that the more rare and exotic the locale, the better the chances of coming up with something new. Employees vacationing or traveling abroad were encouraged to take special soil sample collection bags with them and to sample a wide range of

habitats worldwide. Ironically, it not infrequently turned out that the most interesting cultures came from their own back yard! Back in the laboratory, large numbers of pure cultures were isolated from this collection of soil samples. Each company had their own process for selecting which colonies they would pick off and culture from the agar plates on which diluted suspensions of the soils had been plated out. The practiced eye of a good soil microbiologist became invaluable as a means of recognizing unusual actinomycetes based on the morphology and color of the agar colonies. These soil isolates were then fermented in liquid media and the resultant fermentation broths were tested for *in vitro* activity against the pathogen of choice. The numbers involved were large, considering the limited availability of automated equipment at the time, a typical company perhaps processing in excess of 100,000 actinomycetes in a year. Not only was selection of novel actinomycetes required but it also became evident that the conditions under which they were grown influenced profoundly what the individual cultures produced. Antibiotics are examples of what are referred to as secondary metabolites. Unlike primary metabolites, like amino acids or nucleotides that are essential for the microbe's growth and survival, secondary metabolites are not essential (under laboratory conditions). It is now generally accepted that antibiotics do have a role in a sort of inter-microbial warfare that is waged amongst the inhabitants of the same ecological niche in competition for the limited nutrients available for growth and survival. The structural variation amongst the different antibiotic classes produced by the actinomycetes is astounding. The actinomycete group of bacteria is subdivided into several genera, the most productive of which (from the antibiotic standpoint) has been the *Streptomyces*. Although initially confused with the fungi because of the frequent presence of fungal-like mycelia rather than individual cells, the actinomycetes are true gram positive bacteria. Ironically, it turns out that some of the most notorious bacterial killers also belong to the actinomycete group: the pathogens responsible for TB and leprosy are both members of the genus Mycobacterium. As mentioned earlier, the novelty of producer organisms that were being screened played a critical part in improving a pharmaceutical company's chances of finding novel antibiotics. The emphasis shifted to finding what became known as 'rare actinos,' in the case of Schering Plough this meant acquiring a collection of the *Micromonospora*, an actinomycete genus that proved a rich source of antibiotics [43, 69] leading to the discovery of gentamicin. At Lepetit in Italy the focus was on another rare genus, the *Actinoplanes* [38] that also led to novel antibiotics. Special techniques were developed that allowed these companies to pick out these rare actinos amongst the background of commonly occurring ones. This frequently involved the use of selective media but also relied on the experienced eye of a soil microbiologist recognizing characteristic colony morphologies on the agar plates they were selected on. As a by-product of this intense focus of the pharmaceutical industry on the actinomycetes, large numbers were characterized and deposited with American Type Culture Collection and other national culture collections. Patenting the microbe producing the antibiotic was a key commercial strategy, since, in most cases, it was the only practical way to access the molecule. The structures were far too complicated to make by synthetic chemistry in a cost effective manner. Experience has shown that

antibiotics are typically produced late in the growth cycle and the fermentation media are very complex, frequently containing solids such as soybean meal [32]. Thus a complex media is inoculated with a suspension of an actinomycete and growth allowed to occur in specially made baffled conical flasks with shaking for several days. The length of time varied depending on the particular microbe involved, as also did the temperature (typically 5–7 days at 28°). The samples from these fermentation broths that were then screened, were either supernatants (after the removal of mycelial mass by centrifugation or filtration), or some form of organic solvent extract made of the whole culture, supernatant, or mycelium that was compatible with the screening test being used. As will be seen, although the focus and most of the success came from those programs based on the actinomycetes, some important antibiotics other than penicillin came from fungi (cephalosporins and fusidic acid) and other non-actinomycete bacteria (bacitracin and polymyxin).

1.4.2 Screening for Activity

The simplest and most straightforward test was to assay a fermentation broth sample for antibacterial activity by adding an aliquot to a growing bacterial culture in liquid or on agar and to look for inhibition of growth. The bacterial species chosen was usually the pathogen 'du jour,' for example *S. aureus*, or *E. coli* if one was interested in broad-spectrum activity. In practice, it turns out that activity against gram-positive bacteria is found much more commonly than activity against gram-negatives. The same is true of screening synthetic chemical libraries and is a reflection not so much related to different targets being present in gram positives and negatives, but rather access to the same targets resulting from the additional outer membrane present in gram-negatives, which severely restricts permeability. Many of the antibiotics discovered during this era progressed to clinical trials and were marketed with little or no knowledge of their mechanism of action. Antibiotics became important tools in sorting out the biosynthetic pathways of bacteria.

1.4.3 Isolating and Identifying the Active Metabolite

Identifying a novel soil isolate that produced a potentially novel *in vitro* antibiotic activity was only the start of a long and arduous path on the way to isolating the fermentation broth component responsible and solving its structure This required the isolation of increasingly large quantities of the fermentation derived molecule. As soon as enough material was in hand, the candidate antibiotic was subject to expanded *in vitro* testing to define its spectrum of antibacterial activity and simple physicochemical tests. In the early days, it was relatively facile to discover novel antibiotics through simple empirical screening of fermentation broths. However, the problem of re-discovering the same molecules became an increasingly difficult issue to deal with. The details of the way in which different pharmaceutical companies ran their

screening programs unwittingly biased them towards particular antibiotics and thus the same molecules would repeatedly turn up. Furthermore, the publication and patent filings on novel molecules by their competitors exacerbated the risk of re-discovery. There were many instances of the same molecule being discovered at about the same time and frequently there was a race to file a patent, which quite often was based on physico-chemical properties of a molecule in the absence of a defined structure. Various tricks were used to avoid the re-discovery problem and this whole process has become referred to as dereplication. A key issue was the ability to determine as soon as possible whether one had a novel molecule or not. Although the initial fermentation broth might show up as highly active in an *in vitro* antibacterial test, the molecule responsible was typically present at a few micrograms per milliliter, representing less than 0.01% of the total solids present. Attempts to identify the active molecule responsible became easier and more meaningful, as it was purified and concentrated, usually through extraction with organic solvents at various pH's and/or chromatography. The spectrum of antibacterial activity provided an early, rapid and cheap fingerprint for identification, which later became increasingly powerful with the addition of bacterial mutants that were resistant to specific antibiotic classes. Chemical dereplication frequently depended on UV visible and IR spectra, and required a higher degree of purity to become meaningful. The advent of HPLC and the ability to determine the UV/visible spectra of individual peaks was a major advance for dereplication. The gradual accumulation of samples over the years led to the building of comprehensive libraries of known compounds that facilitated identification enormously. Unfortunately, companies were not always willing to send out samples of newly discovered antibiotics to their competitors, which complicated this process. Data banks on the physico-chemical properties and biological activities of natural products became commercially available in hard copy and later in electronic form, and this helped the process of dereplication [4, 11, 55], especially those entering the field without the benefit of accumulated experience in the area and a library of samples of previously discovered antibiotics. Natural product screening programs had a tendency to mature and improve with time as the efficiency of dereplication improved. It increasingly became a numbers game as all 'the low hanging fruit' had already been picked and the law of diminishing returns was setting in.

1.5 Antibiotics Produced by Actinomycetes

1.5.1 Chlortetracycline

In 1944 Benjamin Duggar, a retired botany professor, joined Lederle Laboratories of Pearl River NY and took charge of a soil screening program. Amongst the hundreds of soil samples to be screened for antibacterial activity, it was hoped to find a safer alternative to streptomycin for the treatment of TB. This objective was achieved in 1945 with the isolation of *Streptomycin aureofaciens,* a gold colored actinomycete that produced an orally active broad-spectrum antibiotic initially called aureomycin [20]. It was subsequently re-named chlortetracycline when its structure was

resolved [64]. Chlortetracycline was first marketed in 1949 just before chloramphenicol. Disappointingly, it was of no use for TB. Other tetracyclines were quickly identified as the competition between the pharmaceutical companies heated up. Pfizer, concerned about the declining price of penicillin, put a team of over 50 scientists onto a soil screening program charged with looking at more than 100,000 soil samples from all over the world. The outcome of this effort was the identification of oxytetracycline that was produced by *Streptomyces rimosus* and turned out to have similar antibacterial properties to chlortetracycline [24]. The global diversity of their soil sample collection turned out to provide little or no advantage, since the key isolate, *S. rimosus*, came from a soil sample collected at their manufacturing site in Terre Haute IN! Tetracycline was found to be co-produced with chlortetracycline by *S. aureofaciens* at Lederle [6]. However, the proprietary situation became unclear when Bristol Labs found tetracycline produced by a different actinomycete, *Streptomyces viridifaciens,* and Pfizer succeeded in chemically converting chlortetracycline to tetracycline [14]. All three companies filed patents but were all initially rejected. Pfizer and Bristol persisted with their filings and were eventually awarded patents. An agreement was reached between the various tetracycline producers and their licensees, and this family of antibiotics became widely prescribed and came to rival penicillin as wonder drugs. The US Federal Trade Commission criticized this tetracycline 'cartel' for controlling prices and keeping competition at bay. The tetracycline story provides some idea of the intense competition that went on between the different pharmaceutical companies involved in this era of antibiotic discovery and is covered well by Sneader [62].

1.5.2 Chloramphenicol

In 1943 the Parke Davis Company set up a research collaboration with Paul Burckholder, a botanist at Yale, to screen potential antibiotic-producing microbes isolated from soil samples for activity against six different bacteria. Out of over 7,000 soil samples that were screened, one from near Caracas, Venezuela yielded a broad-spectrum orally active antibiotic: chloramphenicol (originally referred to as chloromycetin). It was the first broad-spectrum antibiotic to be marketed that could be used orally or systemically. The producing culture was given the name *Streptomyces venezuela* and sent to Park Davis in Detroit, where the active component was isolated in 1947 [21] and the structure solved rapidly thereafter [15]. By the end of 1947, it had already undergone preliminary clinical evaluation with impressive results. Chloramphenicol can be made on a large scale by synthetic chemistry obviating the need for fermentation, and by 1949, large amounts were being manufactured and sold. Sales of this drug catapulted Parke Davis into becoming the world's largest pharmaceutical company. Unfortunately, after testing in eight million or so patients, a rare but frequently lethal side effect was revealed. Although only as few as 1 in 100,000 patients treated, suffered from the aplastic anemia that it caused, this was enough to dramatically curtail its use.

1.5.3 Additional Aminoglycoside Antibiotics

Although the focus of this chapter is on the prototypical antibiotic class members that have been discovered, some mention of additional aminoglycosides is merited, since they have been marketed as novel natural products and were not the outcome of semi-synthetic chemistry aimed at generating a new improved generation of streptomycin.

1.5.3.1 Neomycin

In his continuing studies after streptomycin, Waksman found a complex mixture of related aminoglycoside antibiotics that was produced by the soil actinomycete, *Streptomyces fradiae* [70]. This complex of antibiotics was called the Neomycins, and Neomycin B was the component that became used clinically. Unacceptable systemic toxicity has limited neomycin B to topical use. As a group, the aminoglycosides generally have an ototoxicity and nephrotoxicity liability. They are typically broad-spectrum agents with excellent gram-negative activity and are rapidly bactericidal (a key property in treating serious systemic infections). They have a mechanism involving inhibition of bacterial protein synthesis, but interact with the 30 S subunit of the bacterial ribosome rather than the larger 50 S unit unlike other protein synthesis inhibitors (such as the macrolides, lincosamides, and tetracyclines) that are primarily bacteristatic in nature.

1.5.3.2 Kanamycin

Another aminoglycoside called Kanamycin was discovered in 1957 by one of the doyens of the antibiotic era, Hamao Umezawa [68]. It was produced by the soil isolate *Streptomyces kanamyceticus.* Although rarely used now, it is important as its chemical modification gave rise to several important derivatives including Amikacin.

1.5.3.3 Gentamycin

The story of gentamicin's discovery is unusually well-documented and is worth summarizing here as a useful illustration of the importance of working with novel actinomycetes and of industrial laboratories having external collaborations [42, 43, 69]. The Schering Corporation was late in joining the other pharmaceutical companies in the rush to discover novel antibiotics and had been focusing on steroids and their transformation. In 1957, G Luedemann joined Schering, after completing his doctoral research at Syracuse University NY. He was aware that Professor Carpenter, his mentor at Syracuse University, was retiring and that his collection of specimens of an unusual genus of actinomycetes, the Micromonospora, was going to be consigned to the autoclave. In 1958, another member of Carpenter's department,

A Woyciesjes, agreed to a small collaboration that would provide Schering with Micromonospora isolates and he set up a small laboratory in his own basement to do this work. These isolates were all collected locally. Cultures were screened at Schering as potential producers of antibiotic activity. Out of the more than 300 cultures sent to Schering over the next couple of years, 15 produced novel antibiotics, by far the most important of which was Gentamicin, a new complex of closely related aminoglycosides [71]. Unusually Gentamicin was and still is marketed as a complex mixture of at least five active components, presumably a result of the difficulty in obtaining a purified single component with a commercially viable process. It would be very difficult to get a mixture of this sort approved today with the significantly tightened regulatory requirements. Surprisingly, Woyciejes was not included as an inventor on the original Schering patent for gentamicin and, like Schatz did before him for streptomycin, successfully challenged the case in court.

1.5.4 Erythromycin A: The Macrolide Prototype

In 1952, James McGuire and coworkers at Eli Lilly isolated a strain of *Streptomyces erythreus* from a soil sample collected at Iloilo in the Phillipines [9]. This actinomycete produced a complex of at least six related molecules of which only one had useful potency against gram-positive bacteria: Ilotycin (now referred to as erythromycin A) [62]. The erythromycins were the prototypical members of the so-called macrolide class of antibiotics, characterized by a lactone ring that, in the case of the clinically useful members of this class, contained 14 or 16-membered rings. Erythromycin was active against the increasingly problematic penicillin resistant staphylococci. The structure of erythromycin was solved in 1956 [27]. The development program for erythromycin A involved dealing with a number of problems impinging on the ability to develop oral and parenteral formulations very bitter taste, poor aqueous solubility, and acid instability. At the time, Eli Lilly, UpJohn, and Abbott Laboratories were sharing the rights to antibiotic leads. Upjohn decided not to proceed considering it no more than a weak penicillin. However, the other two realized the full potential of erythromycin and Abbott, in particular, enjoyed a huge commercial success with it [31]. The azalides and ketolides were subsequent variations of this prototypical macrolide made by chemical modification.

1.5.5 Lincomycin

This protypical member of the lincosamide family of antibiotics was produced by a culture of *Streptomyces lincolnensis* isolated from a soil sample collected in Lincoln, Nebraska (hence the name of the producing organism and the antibiotic itself!) by researchers at the Upjohn Company in 1962 [47]. Lincomycin is active against

gram-positive bacteria and anaerobes; its clinical utility was limited and it was rapidly replaced on the market by the more efficacious clindamycin, a semi synthetic derivative of lincomycin containing chlorine. The lincosamides, like the macrolides, are primarily bacteristatic antibiotics that have a similar mechanism involving the inhibition of protein synthesis and also share a common resistance mechanism involving the methylation of ribosomal RNA.

1.5.6 Vancomycin: The Glycopeptide Prototype

This antibiotic came from the productive soil screening program at Eli Lilly in 1956 where McCormick et al. [48] isolated a gram positive active component from the fermentation broth of a novel actinomycete that was named *Streptomyces orientalis* (now renamed *Amycolatopsis orientalis*). Although the initial isolate came from a soil sample collected in Borneo by a missionary, it was subsequently found that two further strains of the same species from Indian soil samples produced the same antibiotic [40]. Several early pieces of information spurred interest in pursuing vancomycin; evidence of bactericidal activity, activity against penicillin, streptomycin, and erythromycin resistant staphylococci, and a low potential to develop resistance on repeated passage of pathogens in the presence of drug. Animal studies also indicated a low toxicity, but, for many years, the vancomycin used in the clinic contained significant impurities from the fermentation process and was prone to cause serious side effects in patients especially nephrotoxicity. These earlier brown colored preparations were dubbed 'Mississipi mud'. Later improvements in the commercial purification process led to a much cleaner and safer product. It was rapidly approved for the treatment penicillin resistant staphylococcal infections in 1958 but was quickly overshadowed by the introduction of methicillin in 1960. However, it was resurrected in the 1970s with the spread of methicillin resistant *Staphylococcus aureus* and is still recognised as an important antibiotic. The glycopeptides have a high molecular weight compared with most other antibiotics, lack oral activity, and their use is largely restricted to the intravenous treatment of serious systemic gram-positive infections.

1.5.7 Rifamycins: The First Ansamacrolides

In the mid 1950s, the Italian pharmaceutical company Lepetit, based in Milan, initiated a typical screening program examining soils collected from many different locations by traveling employees and business contacts worldwide. The Lepetit group, led by Piero Sensi, was looking for activity against several clinically important bacteria including *M. tuberculosis*. One particular soil sample came from the Cote d'azur, France, and had by chance been collected by the vacationing employee responsible for

isolating actinomycetes. Not surprisingly on returning to the laboratory, he gave his own reddish soil sample special attention! A novel actinomycete was isolated that produced a complex of related antibiotics highly active *in vitro* especially against the staphylococci and, most importantly, *M. tuberculosis* [58]. The producer organism was initially named *Streptomyces mediterranei*, but was subsequently reclassified as *Nocardia mediterranei* and even finally as *Amycopolis mediterranei*. The complex of antibiotics became called the rifamycins (the name deriving from the French movie thriller of the time “Rififi’). It proved difficult to isolate and elucidate the structure of the components of this complex. Two Swiss chemists, Prelog and Oppolzer [53], solved the unusual structure of this novel class of antibiotic. The first component to be isolated was rifamycin B, which itself turned out be inactive but underwent a slow spontaneous degradation, via the intermediate formation of rifamycin O, to yield the highly active rifamycin S (that became the first rifamycin to be marketed). The rifamycins were the first example of a new class of antibiotics referred to as the ansamycins, characterized by a ring system bridging a naphthalenic chromophore. Recognizing the unique chemical challenges that their limited chemistry resources would face in trying to further develop this novel class of antibiotic, Lepetit collaborated with the Swiss company Ciba-Geigy. The outcome of this effort was rifampicin [45], which is still today part of a first line treatment combination for TB. The rifamycins have a unique mechanism of action involving a highly selective inhibition of DNA dependent RNA polymerase. Concerns about a rather high frequency of resistance have mostly restricted rifampicin’s use to treating TB, in spite of its excellent *in vitro* activity against the staphylococci.

1.5.8 Novobiocin

This antibiotic was discovered almost simultaneously by several different groups and has had several different names assigned to it: Cathomycin, Streptonivicin, Albamycin, and Cardelmycin [30, 61]. Workers at the Upjohn Company announced the isolation of Streptonivicin from the culture broth of *Streptomyces niveus* (subsequently renamed *Streptomyces spheroides*) as a gram-positive antibiotic in 1956 [61]. Although marketed by Upjohn for several years in the 1960s as Albamycin alone, and in several fixed combinations with other antibiotics, it was subsequently withdrawn in the USA.

1.5.9 Spectinomycin

Another antibiotic discovered by the Upjohn group was Spectinomycin (initially referred to as actinospectacin). It was isolated in 1961 from the culture broth of *Streptomyces spectabilis* [46] and is an aminocyclitol with some structural resemblance to the aminoglycosides; interestingly, it also interacts with the 30 S

ribosomal particle inhibiting protein synthesis. It has rather weak *in vitro* activity *versus* gram positives, and its clinical use has been restricted to the treatment of uncomplicated gonorrhea.

1.5.10 Daptomycin: The First Lipopeptide to Be Marketed

In 1987, researchers at Eli Lilly published details of a novel complex of structurally related lipopeptide antibiotics referred to as A21978C, produced by *Streptomyces roseosporus* [17]. This complex mixture of at least six components could be resolved by incubation with cultures of *Actinoplanes utahensis* that yielded a single inactive peptide. The members of the complex carry different fatty acyl side chains that are selectively removed by an enzyme present in cultures of this actinoplanes species. This common core peptide became a critical intermediate for the synthesis of analogs in which the naturally occurring acyl side chains were replaced chemically with a range of new side chains. This led to the synthesis of the semisynthetic antibiotic Daptomycin (referred to earlier as LY 146032) [22] that was approved for marketing in 2003, almost 20 years after it was first described! Daptomycin is a gram-positive only antibiotic that is rapidly bactericidal, having a unique mechanism involving a lethal breakdown in membrane permeability.

1.5.11 Streptogramins: A Natural Synergy

The streptogramins represent a large class of antibiotics produced by the actinomycetes that have a restricted spectrum of activity against the gram-positives. The class is unique in that it comprises a mixture of two structurally distinct macrocyclic components that are co-produced by the same microbe and that act together synergistically [13]. Both components inhibit bacterial protein synthesis at the level of the ribosome, and the observed *in vitro* activity is primarily bacteriostatic. Cross-resistance is exhibited with certain other antibiotics that inhibit protein synthesis at the 50 S ribosomal sub unit (specifically the macrolides and lincosamides), referred to as MLSB type resistance. Pristinamycin is the most important member of this class, from the standpoint of human use, and was discovered at the laboratories of Rhone Poulenc (now part of Aventis) through an empirical fermentation screening program in the 1950s and eventually described in 1968 [54]. Although Pristinamycin has been marketed in some European countries under the trade name Pyostacine since the 1960s, it has never been approved in the USA. Pyostacine is orally active but was not available in a parenteral form, due to poor water solubility. In view of the increasing problem with gram-positive infections in the hospital setting, there was a need for new injectable anti-staphylococcal drugs. Rhone Poulenc initiated a semi synthetic program aimed at producing a pristinamycin analog that could respond to this requirement. The result of this effort was a combination of novel analogs of the two pristinamycin components, dalfopristin and quinupristin, which is marketed in the USA as Synercid.

1.6 Antibiotics Produced by Fungi

1.6.1 The Discovery of Cephalosporin C

Following on from the discovery of penicillin, Guiseppe Brotzu initiated a search for antibiotic producing organisms at the Instituto d'Igiene in Cagliari Sardinia. In a departure from the typical screening of soil isolates, he decided to examine cultures isolated from the seawater close to a local sewage outlet. He reasoned that the purification of the seawater that occurred could be due in some measure to microbial antagonism. In 1945, he isolated a fungus, *Cephalosporium acremonium,* which produced broad-spectrum antibacterial activity. After making many subcultures a variant, was identified that produced high levels of antibacterial activity detectable in filtrates of the fungal growth medium. In 1948, he published his results on crude extracts made from *C. acremonium* cultures that included data from patients who had their boils and abscesses caused by staphylococci and streptococci successfully treated by a topical application of this complex mixture [7]. It is a remarkable feature of early antibiotic screening that even very crude extracts frequently gave excellent *in vivo* activity in animal models and even patients (the same had been true for penicillin). The level of antibiotic present in these early preparations would more adequately be described as a contaminating impurity rather than a major component. At this stage, Brotzu did not have the resources to take the work further and identify the active constituent(s) and this project was undertaken at Florey's laboratories at Oxford University, where the crucial work on penicillin had been carried out. Examination of extracts of *C. acremonium* soon revealed that it contained multiple components possessing antibacterial activity. The first component to be identified was only active against gram-positive bacteria and was called cephalosporin P [10], which actually turned out to be a mixture of at least five related components itself. Next an unstable broad-spectrum component was identified, which had all the antibacterial activity originally described by Brotzu and was initially called Cephalosporin N. However, when Abraham and coworkers managed to isolate it in pure form and determine the structure, it turned out to be a new penicillin, and was renamed penicillin N [2]. Although the activity of penicillin N was much less active than benzyl penicillin, it had much better activity against gram-negatives. In comparing the structure of these two penicillins, it became apparent that the nature of the side chain was very important in determining the spectrum and potency of antibacterial activity. This was a key observation for the subsequent semi-synthetic programs leading to multiple generations of improved penicillins. The third component of the *C. acremonium* culture was noticed as an impurity isolated during degradation studies on penicillin N in attempts to determine its structure. It had weak antibiotic activity and was called cephalosporin C, and Abraham and Newton soon realized that it was related to the penicillins. What caught their interest was its greater resistance to bacterial beta-lactamases that were able to cleave the penicillin ring and inactivate the antibiotic. The Oxford group finally solved its structure in 1961 [1]. Cephalosporin C became the major starting point for the production of four very successful generations of semi-synthetic cephalosporins, although it was

never marketed as an antibiotic in its own right. It is of note that the proprietary situation for the cephalosporins was for many years dominated by a series of patents taken out by the National Research and Development Corporation in the UK, set up to exploit any discoveries that were made in British Universities and government laboratories. This was set up as a reaction to the fact that British companies had to pay large royalties to American companies in order to make penicillins, which had in reality been a British discovery. Several pharmaceutical companies (both British and foreign) took out licenses and the British taxpayer was richly rewarded for the government's investment! The key work here had been carried out in an academic laboratory; it is interesting to speculate whether work on the *C. acremonium* culture would have been allowed to proceed long enough in an industrial setting to allow for the identification of the all-important third, but weakly active antibiotic component, cephalosporin C; it took more than 12 years!

1.6.2 Fusidic Acid

In the early 1960s, a team at Leo Pharmaceuticals headed by Godtfredsen was searching for an amidase enzyme that would cleave penicillin to yield 6-aminopenicillanic acid, a critical intermediate for the synthesis of novel semisynthetic penicillins. As part of this quest, they screened a number of *Fusarium* species, and, by chance, came across one that produced activity against staphylococci. This particular species, *Fusarium coccineum*, was cultured from monkey fecal matter, not from a soil sample! The weakly acidic active component was isolated and its structure determined. It was shown to be quite different from any other antibiotic having an unusual steroid-related structure and was named fusidic acid [29]. Cephalosporin P, isolated by Abraham et al. [2] during their work leading to cephalosporin C, had a similar structure and was subsequently shown to be cross resistant with fusidic acid. In 1962, positive clinical trial data was generated for fusidic acid [28] and the antibiotic was subsequently marketed in oral, intravenous and topical formulations in several major markets, but not in the USA. Fusidic acid is a gram-positive only antibiotic that inhibits protein synthesis and is bacteriostatic. No further generations of this interesting molecule have been registered for use. The penicillins, cephalosporins, and fusidic acid represent the only antibiotics in clinical use that are of fungal origin.

1.7 Antibiotics Produced by Bacteria that are not Actinomycetes

Although the actinomycetes have been the predominant group of bacteria found to produce useful antibiotics, there are two examples of antibiotics produced by other gram-positive bacteria.

1.7.1 Bacitracin: A Gram-Positive Only Peptide Antibiotic

In 1945, a group working at Columbia University College of Physicians and Surgeons announced the discovery of a novel peptide antibiotic that they called bacitracin [33]. Their research was unusual because it was not part of a commercially driven screening program, but rather an attempt to understand a discrepancy between the bacteria isolated from infected wounds, which were cultured directly in broth and on blood agar plates. On occasions, organisms present on the blood agar plates were absent from the broth cultures. This happened most often when the broth cultures contained a large number of gram-positive sporulating rods. Many of these gram-positive cocci were shown to antagonize the growth of other gram-positive cultures when they were plated together. One culture in particular, produced a very potent antibiotic effect and was identified as belonging to the *Bacillus subtilis* group of organisms (later it was shown to be a strain of *Bacillus licheniformis* [34]). This particular bacterium was isolated from a patient called Tracy, leading to the name assigned to the antibiotic produced. Bacitracin has a gram-positive only spectrum with a mechanism involving the inhibition of cell wall synthesis, and, although, its main use is as a topical, due to a serious nephrotoxic liability, it is available in an intramuscular form for the treatment of serious staphylococcal infections.

1.7.2 Polymyxins: Gram-Negative Only Peptide Antibiotics

The polymyxins (A through E) are a group of closely related antibiotics produced by the gram-positive *Bacillus polymyxa*. They were first discovered in 1947 at the Stamford CT laboratories of American Cyanamid during a screening program for producers of antibiotics with gram-negative activity [63]. Candidate cultures on agar plates were overlaid with a suspension of *Salmonella schottmuelleri* and subsequently examined for zones of inhibition. The colonies of *B. polymyxa* were very unusual in that they gave very large zones of inhibition of the salmonella species used but virtually no zone if *Staphylococcus aureus* was used as the indicator organism instead. At that stage, no antibiotics were known that were more active against gram-negatives than gram-positives, giving an early indication of presumptive novelty. In fact, purified polymyxin B, the most interesting component from the selectivity standpoint, is devoid of gram-positive activity. The polymyxins have a cyclic peptide structure with a lipophilic tail attached, their unusual spectrum of activity results from a selective interaction with lipopolysaccharide, a polymer found exclusively in gram-negative bacteria. In 1949, Colistin was discovered in Japan and introduced to clinical use in 1959 as a novel antibiotic. However, in 1963 [72] it was shown to be identical to Polymyxin E. In the case of colistin, a methane sulfonate derivative, it had been made to reduce the nephrotoxic liability. Colistin and Polymyxin B remain on the market with a limited use (due to safety concerns) for the intravenous treatment of serious gram-negative infections; however, they are most frequently used as important components of topical formulations.

1.8 Antibiotics Produced Through Synthetic Chemistry

Although natural products have played a predominant role in antibiotic discovery, there have been a number of significant antibacterial agents that have had their origins in synthetic chemistry. As we shall, see a lot of the early effort in this approach was directed towards finding better antibiotics for TB.

1.8.1 Para Amino Salicylic Acid

At about the same time that Waksman was working on Streptomycin as a TB antibiotic, Jorgen Lehmann made a very important prediction: para amino salicylic acid would be active against the TB pathogen and was urging a Swedish pharmaceutical company to make it. This prophecy derived from Lehmann's reading in 1940 of a very brief publication that was sent to him by a friend, Frederick Bernheim from Duke University. The title of this note was 'The effect of salicylate on the oxygen uptake of the tubercle bacillus' [5]. The effect described was quite dramatic with small quantities of salicylic acid (aspirin) causing a major stimulation of oxygen consumption. Lehmann reasoned that this meant: (1) Salicylic acid was able to penetrate the bacteria (a major issue for TB since it has very waxy outer covering that rendered it impermeable to most substances). (2) Analogues of the molecule could potentially interfere with bacterial respiration via competitive inhibition and should be able to penetrate in the same way as the parent molecule. At this stage it was known that the sulfonamides worked as analogues of the natural metabolite para aminobenzoic acid. This was also the reason why Lehmann was suggesting the para analogue rather than the meta or ortho versions. Thus the proposal to synthesize para aminosalicylic acid was made without Lehmann having conducted a single experiment! In reality, the chemical proved exceedingly difficult to synthesize, and it was not until the end of 1943 that Karl Gustav Rosdahl succeeded in cracking the synthetic problem and delivered the first supplies of PAS for testing. The drug was very potent against the TB pathogen in vitro and worked very well in a guinea pig model of TB. The first patient was treated in early 1944, only 3 months after the research began. It proved a safer drug than streptomycin, it could be given orally, and it had less of a resistance problem. Later it came to be used in combination with other TB antibiotics. A detailed description of the PAS story and other antibiotics to treat TB can be found in a book by Ryan [57].

1.8.2 Isoniazid

Although isoniazid was first synthesized in 1912, it was almost another 40 years before its activity against TB was discovered. In 1951, three pharmaceutical companies (Bayer, Hoffman la Roche, and Squibb) independently recognized the remarkable activity of isoniazid. These research programs had their origins in the earlier

work of Domagk (the discoverer of prontosil) who had been studying a series of thiosemicarbazones as potential TB antibiotics [26]. One such analog was actually marketed in Germany as Contaben. A large number of analogs were synthesized in what would seem a largely empirical effort. At Squibb, it was the fortuitous testing of a synthetic intermediate en route to a thiosemicarbazone that led to their discovery. These efforts culminated with the finding that substituting a benzene ring in the thiosemicarbazone with a pyridine led to isoniazid; a highly potent, orally active, and well tolerated drug. Furthermore, isoniazid was very cheap to synthesize and remains today part of primary therapy for TB as part of a triple combination.

1.8.3 Pyrazinamide

The discovery of pyrazinamide was announced by Kushner and coworkers of Lederle Laboratories in 1952 [36] and came from a synthetic chemistry program in which analogs of Nicotinamide were being made (nicotinamide was already known to have activity against TB). Later evidence showed that it was in fact a prodrug that was converted into pyrazinoic acid by a mycobacterial enzyme. The mechanism only became understood much later in 2000 [74] when it was shown to involve the inhibition of fatty acid biosynthesis by pyrazinoic acid.

1.8.4 Ethambutol

Continuing research at Lederle Laboratories led to the discovery of ethambutol in 1961 and had its origins in an empirical screen of synthetic chemicals for *in vitro* activity against the TB pathogen [67]. Once again, knowledge of the mechanism would not be established until much later in 1995, when it was shown to inhibit the biosynthesis of the arabinam component of the mycobacterial cell wall arabinogalactan [50].

1.8.5 Nitrofurans

In the 1940s, many furans were synthesized in the quest for novel anti-bacterials; it was noted that introduction of a nitro group at position 5 of a 2 substituted furan ring resulted in a marked increase in antibacterial activity [8]. Subsequently, further synthesis led to the derivative, Nitrofurantoin, which was first used in the mid 1950s for the oral treatment of urinary tract infections. After oral administration the concentration of the drug was only high enough in the urine to achieve a therapeutic effect. At these concentrations, it was bactericidal. Mechanistically Nitrofurantoin is a prodrug, requiring reduction of the nitro group by a bacterial enzyme. The reduced drug then inhibits the Krebs cycle at multiple points preventing the generation of essential ATP [49].

1.8.6 Metronidazole

Although metronidazole is a product of synthetic chemistry, its origins go back to the discovery of azomycin in 1953 by the prolific laboratory of Hamao Umezawa in Tokyo, Japan [44], its structure was solved in 1955 [52]. Azomycin was produced by an unidentified species of streptomyces and had antibacterial activity. Noting the similarity of the azomycin structure to agents active against trichomonas such as aminitrozole, researchers at Rhone Poulenc found that it also had trichomonacidal activity. Azomycin itself turned out to be too toxic to be of clinical utility, but it inspired the synthesis of a series of analogs by the French team that led to Metronidazole, an important drug for treating trichomonas infections [16]. Serendipitously, it was found to be active against ulcerative gingivitis in 1962, a bacterial infection of the gums [59], and this led to a realization of its broader antibacterial activity. It is especially active against anaerobic bacteria such as *Bacteroides fragilis* and is approved for a number of indications that involve this pathogen.

1.8.7 Nalidixic Acid: The Prototype Quinolone

As part of a study to devise a novel chemical synthesis of chloroquine in 1946, researchers at the Sterling Winthrop Research Institute in Rensselear, NY noted the occurrence of a by-product (7-chloro-1,4-dihydro-1-ethyl-4-oxyquinoline-3-carboxylic acid) [65]. Nothing much happened with this fortuitous by-product until it was included in a screening program and found to be active against fowl coccidiosis. George Lesher and co-workers then began synthesizing analogs of this synthetic by-product and in 1962 announced the discovery of nalidixic acid, the first quinolone antibacterial that would reach the clinic [39]. It had quite good gram-negative activity, it was rapidly bactericidal, but it lacked activity against the gram-positives. Importantly, there was no cross-resistance with other marketed antibiotics, but resistance to the drug itself could be obtained quite easily. Early work on the mechanism of this prototypical quinolone proposed that it was an inhibitor of DNA gyrase, but in 1990, it was shown that there is in fact a dual mechanism with a second enzyme Topoisomerase IV also being inhibited [23]. Both target enzymes are involved in maintaining the integrity of the DNA helix. When Nalidixic acid was eventually approved by the FDA in 1967, the indication was for the treatment of urinary tract infections; a reflection of the fact that the drug was rapidly cleared from the systemic circulation and concentrated in the urine. Subsequent work on the quinolone class led to the identification of flumequine by researchers at the Riker Laboratories in 1977, the first fluoroquinolone [56]. Unlike nalidixic acid, flumequine had some gram-positive activity. Continued chemical manipulation of the flouroquinolone pharmacophore was able to further broaden the spectrum, reduce the frequency of resistance, and improve the pharmacokinetics. This has led to multiple generations of broad-spectrum antibiotics, useful in the treatment of a range of systemic infections by both oral and parenteral routes.

1.8.8 Linezolid: The First Oxazolidinone to Be Approved

This novel class of synthetic antibiotic had its origins in the late 1970s in a program at Dupont that had started with a search for chemicals active against fungal and bacterial plant pathogens at their agricultural division [3]. The chemicals tested were also chosen for antibacterial screening by the pharmaceutical division. One of the oxazolidinones tested was reported as having potent *in vitro* activity, and this spurred an interest in this group of compounds leading to *in vivo* testing. Ironically, the initial *in vitro* observation turned out to be false, fortunately clear evidence of *in vivo* activity was found. Further testing of purer samples confirmed the poor *in vitro* activity, and this turned out to be a general property of the series. An encouraging finding at this stage was an absence of natural resistance to the oxazolidinones. Walter Gregory and coworkers at Dupont continued their work with this new chemical class of antibacterials, and, by 1987, their efforts had led to the identification of two analogs: DuP-721 and DuP-105. These analogs were active *in vitro* against staphylococci (including MRSA), streptococci and *Bacteroides fragilis*, and importantly were shown to be active *in vivo* by both parenteral and oral routes [60]. However, shortly thereafter the program at Dupont was terminated based on liver toxicity seen in rats. There the oxazolidinone story might have ended were it not for a young Upjohn chemist, Steve Brickner, who had seen the Dupont data on posters at the 1987 ICAAC meeting. He initiated his own small program at Upjohn (now Pfizer) in an attempt to make improved oxazolidinones. It's important to note that the culture at Upjohn encouraged scientists to invest up to 10% of their time on their own, 'self-initiated' projects. Other colleagues at Upjohn also recognized the potential of this new class, and a small but growing group of scientists quickly became involved in what became a mainstream project. What was different about the Upjohn project was that rather than basing the SAR solely on *in vitro* MIC's, early estimation of toxicity was a key driver. Cachexia (severe weight loss) in treated animals was one unusual side effect that had been seen. This emphasis on early toxicological studies required the synthesis of much larger amounts of the individual analogs than is typical for a program at this stage. By 1995 this led to the identification of two significantly improved analogs (subsequently named Eperezolid and Linezolid) that proceeded to phase 1 testing in human volunteers. Linezolid was selected for phase 2 evalution on the basis of its superior pharmacokinetics in humans. In 2002 the FDA approved Linezolid. It had taken about 25 years starting from the original work at Dupont for this novel class of antibiotic to yield a clinically useful drug.

1.9 Concluding Comments

The use of intact bacteria as a simple test for antibiotic activity has served the pharmaceutical industry well, having been responsible for the discovery of most of the drugs currently used to treat bacterial infections. This was largely the result of brute

force screening efforts with big resource commitments. However, with the benefit of our current understanding of mechanisms of action/resistance, coupled with genomics, robotics and improvements in analytical chemistry, the whole process could have been much quicker and less labor intensive. In comparing the antibiotics from nature and synthetic chemistry, one cannot help but be impressed by the marked difference in the level of complexity of the structures. It is difficult to imagine how we could ever have arrived at the complex, exquisitely specific structures produced by the soil microbes. Our understanding is still quite limited when it comes to designing an antibiotic *de novo*.

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Chapter 2
Rational Approaches to Antibacterial Discovery: Pre-Genomic Directed and Phenotypic Screening

Lynn L. Silver

2.1 Introduction

Early antibacterial discovery might be divided into the synthetic and antibiotic traditions. The salvarsan-prontosil strand led to exploration of antimetabolite and other chemotherapeutic approaches, while penicillin's discovery led to the fruitful exploitation of natural products produced by microorganisms. With the investigation of the mechanisms of action of synthetic and antibiotic agents and the principles of selective toxicity and specificity of action, the separation becomes less clear. Both modes of discovery yield small molecule inhibitors of essential bacterial functions; both started with empirical discoveries but eventually the search evolved to favor more directed methods of compound selection and design. Over time, the yield of novel antibacterial classes via both synthetic and natural product routes has declined, paralleling the increase in more rational screening methods. Is this a causal relationship or merely a correlation?

The history of these directed attempts, especially in the natural products area of antibacterial antibiotic discovery, has been obfuscated due to the conscious effort by pharmaceutical companies to hide their methodology from competitors. Although commercially relevant discoveries were revealed through patent and publication with reasonable speed, it is often only through retrospective reviews written much later by the discoverers – or their informants – that the methods used were uncovered. Often, we do not know when such screens were first used. Luckily, much of the screening methodology used to detect inhibitors of peptidoglycan synthesis has been published over time and forms the bulk of the material in this chapter. In the 1980s through 1990s, when there was actually a good deal of innovative screening

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being carried out, the screens in use and their general output were rarely published and this has led to the misperception in much recent literature that little such directed screening was done. Unfortunately, this chapter will not be full of revelation of those methods, though an effort has been made to discuss the types of phenotypic screens that were based on or reflected screens for mutants, early reporter screens, and the mindset that was involved in developing these screens. The question of low output, compared to the riches of the earlier empirical efforts, will be more subjectively dealt with at the end of the chapter.

2.2 Antibacterial Chemotherapeutics: Antimetabolites Versus Enzyme Inhibitors

2.2.1 Prontosil

The "magic bullet" of Ehrlich (Nobel Prize in 1908) was the idealized agent that kills the infecting organism while preserving the host. Ehrlich's work on syphilis involved the systematic synthesis of chemical variants of dyes that selectively stained spirochetes but not host cells and their testing in an animal model; this eventually yielded a (relatively) selective agent, salvarsan, that cured mice and men of syphilis (Fig. 2.1). Working with other dyes, Domagk (Nobel Prize 1939) discovered prontosil rubrum (Fig. 2.1), an azo-dye manufactured by IG Farben, that was active in curing mice of streptococcal infection in vivo but had no in vitro activity [41]. Prontosil provided the first truly selective antibacterial therapeutic with broad

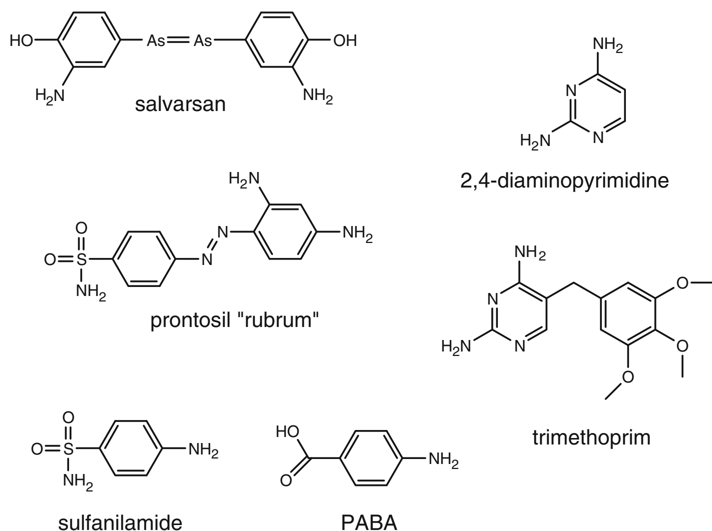

Fig. 2.1 Salvarsan and inhibitors of the Folate pathway

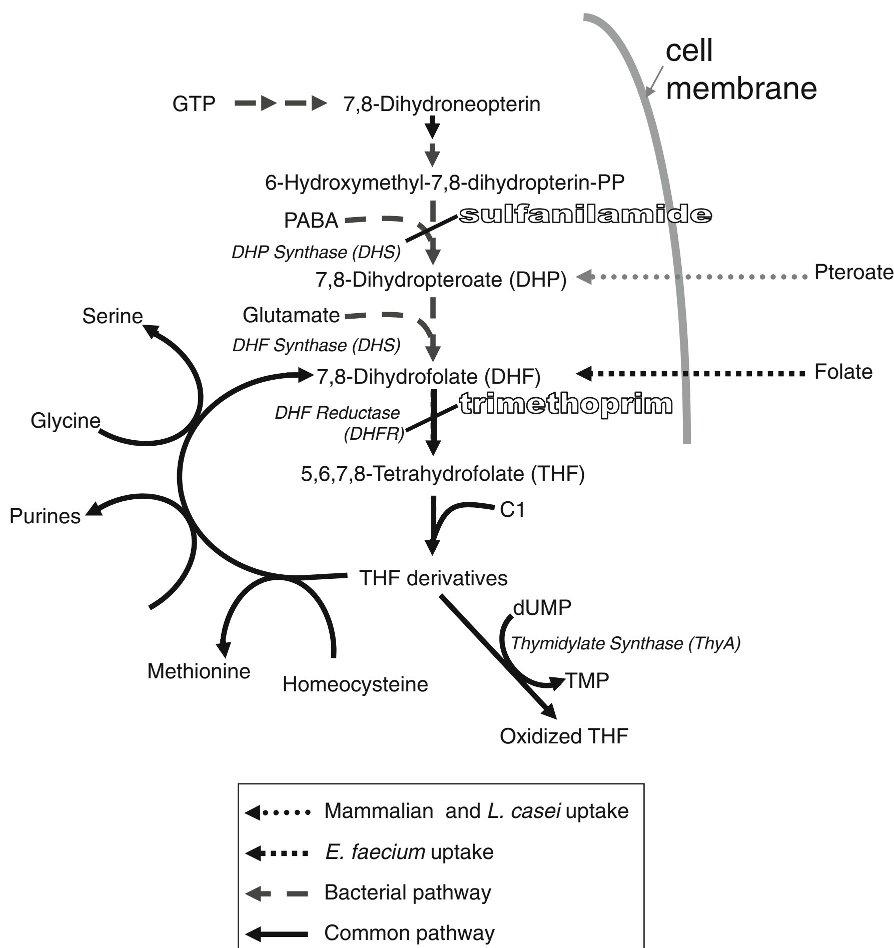

Fig. 2.2 *Folate pathway*. The general de novo pathway of folate synthesis is shown. While most bacteria cannot take up pteroate or folate, mammals and *L. casei* can take up and incorporate pteroate into 7, 8-dihydropterate; E. *faecium* can take up and incorporate folate into 7, 8-dihydrofolate

usage. Later work showed that the activity was due to a metabolite, "prontosil album," shorn of its red chromophore, identified as para-aminobenzenesulphonamide [33], known as sulfanilamide (Fig. 2.1). Fildes had hypothesized that certain disinfectant and other agents inhibited bacterial growth by interfering with substances essential for the growth of the organism [52] and at his suggestion, Woods investigated the interference by an unknown factor in yeast extract with the activity of sulfanilamide, finding that the substance was likely to be para-aminobenzoic acid (PABA, Fig. 2.1) [196]. This led Woods to postulate that sulfanilamide was active due to its resemblance to PABA, which enabled its competition with PABA for an essential anabolic enzyme. At the time, the role of PABA in bacterial (or any) metabolism was unknown. It was soon shown to be critical in the folate pathway (Fig. 2.2), which provides intermediates to a number of metabolic pathways. This is,

essentially, the basis for rational chemotherapy hypothesized by Fildes and Woods, the design and selection of compounds that resemble true metabolites. But how does the competition of sulfanilamide with PABA explain the selective toxicity of sulfanilamide? Would other such antimetabolites show species selectivity?

The idea of selective toxicity was addressed by Work whose main thesis was that selective toxicity could only be approached rationally if the basis for selectivity between host and infecting organism was understood [197]. He argued that even when antibacterials showed excellent selective toxicity and also appeared to inhibit formation of a specific cellular molecule, it could not be said that the selectivity is due to species specificity in the synthesis of that molecule without an understanding of the characteristics of the synthesis that define species specificity.

Indeed, in the case of sulfanilamide, selectivity is based on the fact that most bacteria cannot take up folates but must endogenously synthesize dihydrofolate via several steps including the synthesis of dihydropteroate from PABA and 6-hydroxymethyl-7, 8-dihydropterin-PP via dihydropteroate synthase (DHS), while humans cannot synthesize folate but must take it up from exogenous sources (Fig. 2.2). Thus the antimetabolite concept is not any insurance of selectivity.

2.2.2 *Trimethoprim*

The approach to chemotherapy via rational design and screening for antimetabolites was explored at Wellcome by Hitchings, Elion (Nobel Prize for their efforts in 1988), and their coworkers, who made great strides in anticancer, antiparasite, and antibacterial chemotherapy in part through investigation of folate antagonists. Initially working with *Lactobacillus casei*, a bacterium that *can* utilize exogenous folate (Fig. 2.2), Hitchings and coworkers recognized that folate utilization was competitively inhibited by nearly all 2, 4-diaminopyrimidines (Fig. 2.1) [72]. However, they recognized that the competitors showed more tissue and species specificity in this competition than did 4-amino analogs of folate and so might not be acting as simple antimetabolites. The target of the inhibitors was not recognized until the later steps in the pathway were elucidated (Fig. 2.2), and it was shown that the 2, 4-diaminopyrimidines blocked the reduction of dihydrofolate to tetrahydrofolate by the enzyme dihydrofolate reductase (DHFR), an enzyme present in bacteria *and* humans [73, 74]. Once the basis for specificity and selectivity was understood to reside at the level of enzyme interaction and cellular uptake mechanism, a more rational approach to design and testing of analogs could be undertaken.

Trimethoprim (2, 4-diamino-5-[3,4,5-trimethoxybenzyl]pyrimidine, Fig. 2.1) was the result of this effort, its final choice based on its tolerability in monkeys and excellent antibacterial potency [27]. Additionally, it was shown that trimethoprim and sulfanilamide were synergistic in their action by the use of in vitro methods [27, 48] by dint of their double blockade of the folate pathway.

As will be discussed below (Sect. 6.1), antifolate screening among natural products was undertaken at Fujisawa [136] using a phenotypic whole cell screen.

2.3 Natural Product Screening

The earliest antibacterial discovery – salvarsan, sulfas, trimethoprim – focused on synthetic chemicals, but the "golden age" was one of natural product screening. The work of Fleming (Nobel Prize 1945), Waksman (Nobel Prize 1952), and others has been much reviewed and is covered in the previous chapter. The screening of fermentation broths of Actinomycetes yielded a variety of antibiotics that were relatively quickly developed for clinical use starting in the 1940s. The general screening method was an agar diffusion assay in which fermentation samples were usually applied to filter paper discs that were placed on an agar plate inoculated with a bacterial culture (Fig. 2.3). By the 1950s, the rate of detection of novel compounds had declined. As reviewed by Baltz [10], work at Merck and Lilly in the 1950s showed that between 12.5% and 25% of randomly isolated Actinomycetes produced antibacterial antibiotics and that between 10,000 and 20,000 cultures had to be screened to yield about10 novel compounds. That is, novel products were found in 0.1% of cultures and any specific novel compound might be found at a frequency of ~10^{-4} per culture screened. Clinical candidates were found among novel compounds at a frequency of 2–10%. As more cultures were screened and more "knowns" accumulated, the frequency of any specific novel compound being found decreased to between 10^{-6} and 10^{-7} per culture by 1976. This rarity is exacerbated by the high prevalence of very common compounds (such as streptothricin, actinomycin, streptomycin, tetracycline), in addition to the accretion of the relatively lesser known compounds over time. Clearly, random screening for inhibitors of bacterial growth followed by unprioritized isolation of activities had become impossible early on in

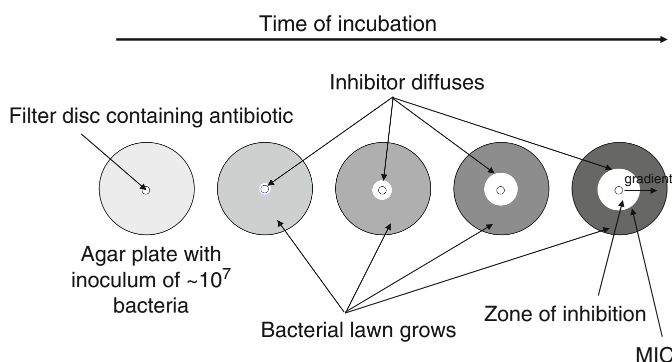

Fig. 2.3 *Agar diffusion assay for antibiotic activity*. Petri dishes are filled with a thin layer of agar containing growth medium. Bacteria (~10^7) may be inoculated directly in the molten agar (when sufficiently cooled) or spread on top of the hardened agar. Samples are applied to filter paper discs on the agar or into wells cut in the agar. As the culture grows and any antibiotic on the disc diffuses into the agar, a zone of inhibition (ZOI) is formed around discs containing antibiotic. The size of the ZOI is a function of the concentration of antibiotic on the disc, the diffusion rate of the antibiotic compound at the incubation temperature in the given medium, and the growth rate of the bacterial strain. The outer edge of the ZOI lies at the effective minimal inhibitory concentration (MIC)

the history of antibiotic discovery. Indeed, prioritization was required. The methods used to distinguish novel compounds from known compounds at the earliest stage possible in the discovery process are known collectively as "dereplication."

2.3.1 Dereplication

Various dereplication methods of classifying fermentation broths as containing compounds "already seen" have been employed in order to reduce the number of "hits" from a screening system requiring time consuming chemical isolation. Importantly, it is necessary to track the biological (antibiotic) activity as well as the chemical entity during dereplication [162]. For chemical classification or identification sufficient to recognize whether an activity is novel or previously seen, it is often necessary to follow the activity through several, usually orthogonal, fractionation steps, in order to correlate the biological activity with the chemical signature. Potent activities present in small quantities may be practically chemically invisible in the face of more major components.

The chemical fractionation and isolation techniques used to track biological activity to a specific chemical entity have evolved over time, as have methods for identification of chemical structure. Biological methods for identifying, or at least classifying activities as common or potentially novel with minimal need for fractionation have also evolved. One such method is the use of a panel of bacterial isolates that have been selected as resistant to specific common compounds. This method was proposed by Stansly [167] and put into practice by Stapley [168]. Selected fermentation broths are tested for their antibacterial activity on the panel of resistant isolates; if the broth contains a compound to which one of the test panel is resistant, then the broth may be classified as containing a known or at least a compound cross-resistant with a known. This type of panel for dereplication and identification has been employed and refined over time with the use of a broad spectrum of bacteria, often under varying media or growth conditions or in the presence of specific β-lactamases, giving patterns of sensitivity characteristic of specific compounds. In large part, the differences among "wild type" bacteria in their innate sensitivity to antibiotics is based on their permeability to the compound much more so than to the presence or absence of a given target. In any case, by creating a large "deck" of results from a panel of organisms, patterns can be recognized that indicate the presence of a previously seen compound. But can that find novelty?

Since many, if not most, Actinomycete broths contain multiple antibiotics (or at least, the isolates have the capacity to make multiple antibiotics), the patterns seen in biological dereplication panels may reflect mixtures. Such mixtures may give seemingly unique novel patterns that turn out, upon fractionation, to resolve into combinations of knowns. Even with mixtures, if there are enough key organisms (resistant to specific compounds) and specific signatures, most broths will be classified as

non-unique (i.e. as containing mixtures of knowns). However, as noted above, one must dereplicate both chemical and biological activity. The biological dereplication tool may identify major knowns in a mixture, but it may miss novel minor components. Thus, while chemical and biological dereplication tools are available, they are inefficient for finding novelty among empirically screened antibiotic activities selected from tens of thousands of fermentations of randomly isolated organisms. Underlining the need to avoid common randomly selected organisms for screening, Baltz [10] has reasonably posited that the rare compounds sought are those for which the producing machinery has most recently evolved while the commonly seen antibiotics are older and their producers more widespread. Thus, groups engaged in natural product screening have made great efforts over the years to include uncommon producing organisms, exploit remote ecological niches, and use novel isolation methods and selective media to raise the likelihood of finding the rare producers of novel compounds. While those rare producers may be more prevalent among screened organisms, the likelihood is still great that the bulk of antibiotic activities seen will be common and still require dereplication. As a focus on rare organisms alone did not solve the problem, the primary screening process itself evolved from empirical "kill-the-bug" screens to more directed, "rational" screening.

2.3.2 Screening as a Means of Dereplication

An intrinsic problem of empirical screening is the ease with which gram-positive organisms such as *S. aureus* and *B. subtilis* are killed by common natural products and members of synthetic chemical libraries. Gram negatives are more resistant to large and hydrophobic compounds due to the inherent selectivity of their two bounding membranes and potentiated efflux mechanisms [160]. Thus, one could choose to use gram negatives for high throughput empirical screening such as a multiply resistant *E. coli* strain as recommended by Baltz [11, 12]. It is true that this will eliminate the few gram positive-specific molecular targets and the novel compounds to which the screening organism is impermeable (but which might provide a lead for chemical modification to improve spectrum), but this approach provides both selectivity and a degree of dereplication or elimination of the common knowns (to which resistance can be obtained). In fact, much of the early screening (after Waksman's initial screening for antimycobacterials) was directed toward broad spectrum or gram-negative activities. When novel compounds with solely gram-positive spectra were discovered, they were often directed toward animal health and animal growth promotion.

While retrospective accounts of early target directed screening may emphasize the choice of target [54, 56, 134], it is clear that choosing to evaluate a subset of antibiotics, selected by some practical or rational criterion, has the benefit of reducing the amount of dereplication to be done. One need only ask if this new thing is

like the finite number of other things acting in a similar way. Thus, screening for inhibitors of a specific pathway or with a particular selective screen is a method, in itself, of dereplication. This has been termed targeted dereplication and is illustrated in screening for HIV inhibitors [161].

The β-lactam and glycopeptide antibiotics found in empirical screening had proved to be efficacious and display high selectivity and low toxicity, because they inhibit the bacterial specific peptidoglycan synthesis pathway. The other major classes of antibiotics found early on targeted protein synthesis and the basis for selective toxicity of those was not as clear at the time. Indeed, many, if not most, antibacterial protein synthesis inhibitors also inhibit mitochondrial protein synthesis but maintain selectivity most likely on the basis of selective permeability, or short-term use. Furthermore, as will be seen in the following section, the study of cell wall inhibitors had revealed a number of characteristic phenomena that could be exploited in screening. Thus, a large number of screens for cell wall inhibitors were devised and run starting in the 1960s, combining the rational choice of a desirable target pathway and the opportunity for dereplicating natural products by narrowing the range of active compounds to which an unknown must be compared in order to predict novelty at an early stage after detection.

2.4 Rational Screening for Inhibitors of Cell Wall Synthesis

The recognition of mechanism of action of antibiotics and antibacterial chemotherapeutic agents began with the concept of antimetabolites and continued with the discoveries of antibiotics. For penicillin, the mechanism of action was delineated via both biochemical and morphological means [106, 141, 170]. Park's discovery of the nucleotide-linked peptide intermediate of cell wall synthesis (UDP-GlcNAc-L-ala-D-glu-L-lys-D-ala-D-ala, called "Park nucleotide") that accumulated during penicillin treatment of *S. aureus* was a key finding in the understanding of the peptidoglycan synthesis pathway [141]. Thus penicillin, and later other antibiotics, notably protein synthesis inhibitors, proved useful tools for studying bacterial physiology in dissecting the basic pathways of macromolecular synthesis in bacteria. The pathway of cell wall synthesis (in *E. coli*) is shown in Fig. 2.4. The first committed step is catalyzed by MurA, starting the *mur* cascade of cytoplasmically located steps that are sequentially required for the synthesis of Park nucleotide which is translocated to the undecaprenol-P carrier lipid by MraY to form Lipid I on the inner surface of the cytoplasmic membrane. GlcNAc is transferred to Lipid I by MurG to form Lipid II, which is transported to the outer surface of the cytoplasmic membrane. The disaccharide-pentapeptide of Lipid II is joined to existing chains of disaccharides by transglycosylase (a function mainly of the dual function large penicillin binding protein, PBP1b) and the peptide chains are cross-linked by the transpeptidase activity of several different PBPs. The undecaprenyl-P is recycled to the inner face of the cytoplasmic membrane.

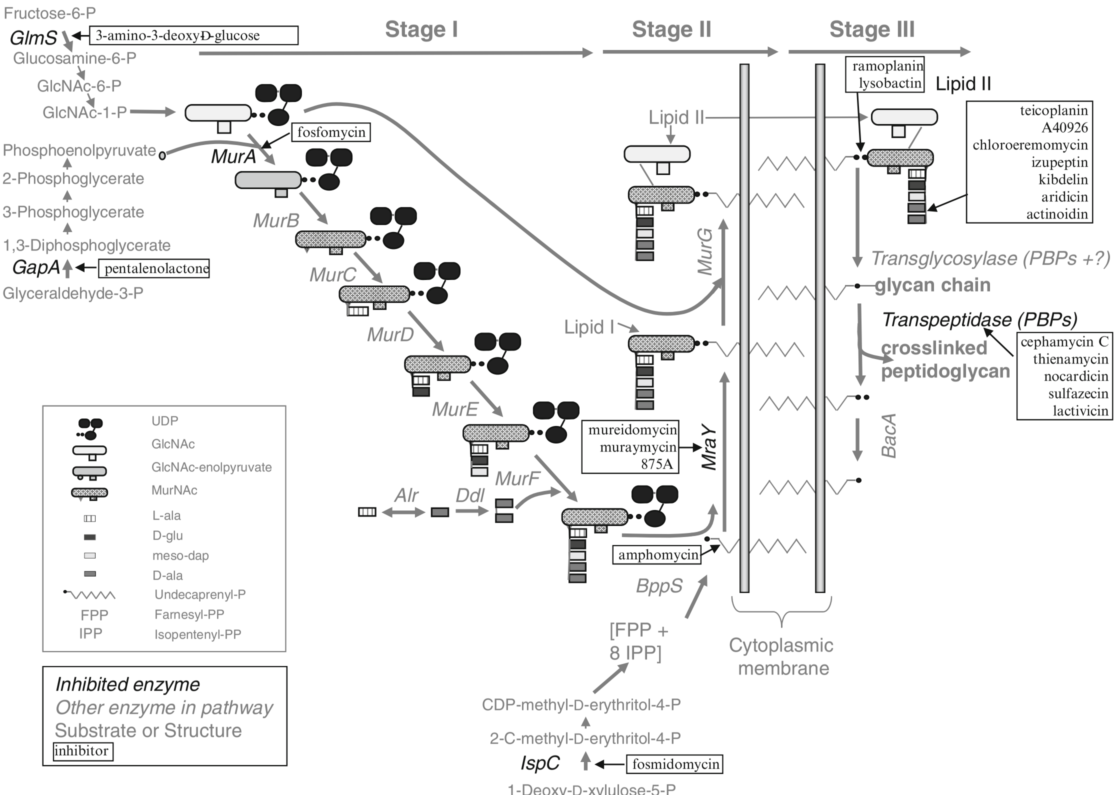

Fig. 2.4 *Cell wall pathway and inhibitors*. The committed steps of the peptidoglycan synthesis pathway of *E. coli* are shown, from MurA through transpeptidation by PBPs. Additionally, parts of pathways providing substrates for the cell wall pathway, N-acetyl-glucosamine (GlcNAc), undecaprenyl-P, and phosphoenolpyruvate are indicated. Inhibitors discovered through cell wall pathway-directed screening are noted in *boxes* (This figure is adapted with permission [158])

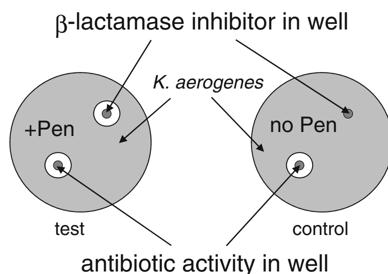

Fig. 2.5 *Beecham "KAG" assay for β-lactamase inhibitors*. As described in the text, blood agar plates are prepared with one of each pair containing benzylpenicillin. The plates are seeded with *Klebsiella aerogenes* producing a β-lactamase that renders it insensitive to the penicillin; samples containing β-lactamase inhibitor candidates are deposited in wells in the paired plates (i.e., the same sample is applied to both of each pair of plates). During incubation, the bacterial lawn grows and any β-lactamase inhibitor in the wells diffuses and inhibits the β-lactamase produced by the bacterial inoculum, thus rendering the cells sensitive to the penicillin in the plate. Hence, a zone of inhibition (ZOI) will be formed on the penicillin-containing plate around wells containing such β-lactamase inhibitors. Wells containing antibiotic activity will yield ZOI on plates both with and without penicillin

2.4.1 β-Lactamase Inhibitors

Soon after the introduction of penicillin into the clinic, resistance, due to degradative enzymes, the β-lactamases, became evident [125]. Interestingly, these enzymes were found to have been present in pathogens, dating from long before the introduction of penicillin [14, 15]. Accordingly, some of the earliest rational approaches to antibacterial therapeutic discovery among natural products concerned the search for inhibitors of β-lactamases. The methods by which the β-lactamase inhibitors were discovered have been noted and reviewed by various authors [23, 75], including those involved in the discoveries, but, since the screening procedures were often published after disclosure of compounds, the timing of the discoveries is not always clear.

First published in 1976 [25], although reported to have been used as early as 1967 [23], the KAG screen of Beecham (Fig. 2.5) employed a blood agar plate containing benzylpenicillin seeded with a lawn of *Klebsiella aerogenes* resistant to penicillin by dint of its production of a Class A β-lactamase. Fermentation samples were inoculated into wells cut in the agar and plates incubated overnight. Diffusible β-lactamase inhibitors would produce a zone of inhibition due to protection of penicillin from degradation by the lactamase. A control plate without penicillin in the agar served to counterscreen against samples producing antibiotics. A differential between the control and test plate was taken to indicate the presence of a β-lactamase inhibitor. Clavulanic acid, an oxapenam (Fig. 2.6), was discovered at Beecham [147] using the KAG assay [25] in an investigation of secondary metabolites produced by *S. clavulagirus*, which had already been described as producing several cephalosporin C related compounds, including cephamycin C, and penicillin N. Clavulanic acid was very successfully developed and introduced in 1981 by Beecham for use in combination with amoxicillin (Augmentin, Co-amoxiclav). Augmentin is generally used orally, although there is a parenteral formulation.

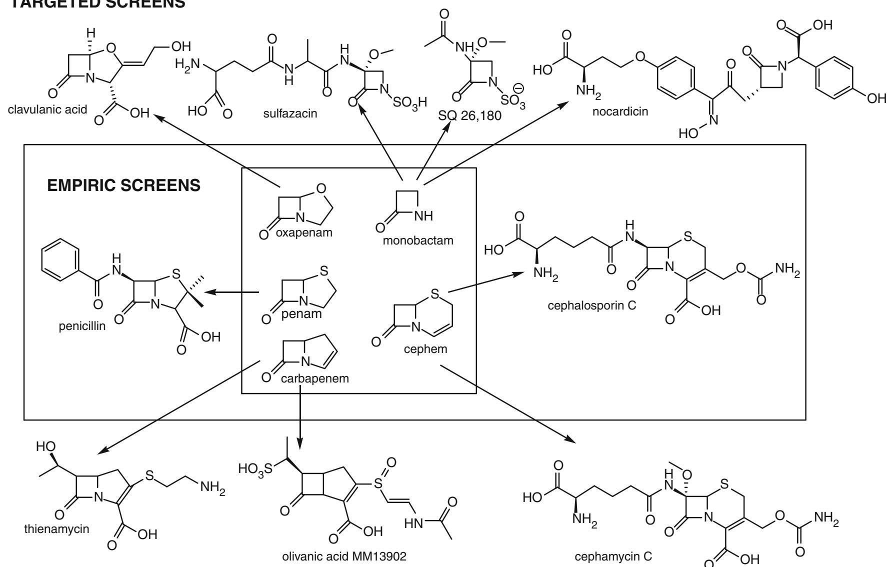

Fig. 2.6 *β-lactams from nature*. Structures the inner box illustrate the basic β-lactam plaforms. *Middle-box* structures were found through empiric screening. Structures in the *outer box* were found through directed screening

Of the commercially available β-lactamase inhibitors, clavulanic acid is the only one that is orally bioavailable.

Olivanic acids (Fig. 2.6), sulfated carbapenem derivatives, were also discovered by Beecham [24, 28] from *S. olivaceus* apparently by use of the KAG screen [23]. The discovery of two β-lactamase-inhibitory compounds from *S. fulvoviridis* had been reported earlier by Umezawa and coworkers [187], one of which, MC696-SY2-A, was later shown [112] to be an olivanic acid (equivalent in structure to MM 4450 of Beecham). It is likely that an agar diffusion assay described in the discovery paper [187] was used for screening. *S. aureus* was used as an indicator strain and agar plates were prepared containing appropriate amounts of penicillinase, penicillin and the test organism. Paper discs containing an inhibitor were applied and the plates incubated overnight. Since the penicillinase to penicillin ratio was adjusted so as to leave no undigested penicillin, the diffusion of inhibitor from the paper discs would thus lead to the formation of a zone of inhibition on the test strain due to protection of the penicillin.

A screen for β-lactamase inhibitors was published by Squibb in their disclosure of a novel, potent non-β-lactam inhibitor of β-lactamase, izumenolide (EM4615, Fig. 2.7) [26, 110]. In this screen (Fig. 2.8), fermentation samples were added to 11 mm cellulose discs, the discs dried and applied to agar plates containing the Type A β-lactamase TEM-2. Plates were incubated at 37°C for 3 h, the discs removed and the plates flooded with a solution of a chromogenic cephalosporin (presumably nitrocefin [173]) that turns red upon hydrolysis of the β-lactam ring. Thus, a positive response in the assay is a light yellow zone (of non-hydrolyzed nitrocefin) around the site of sample application against a lawn of red (hydrolyzed nitrocefin).

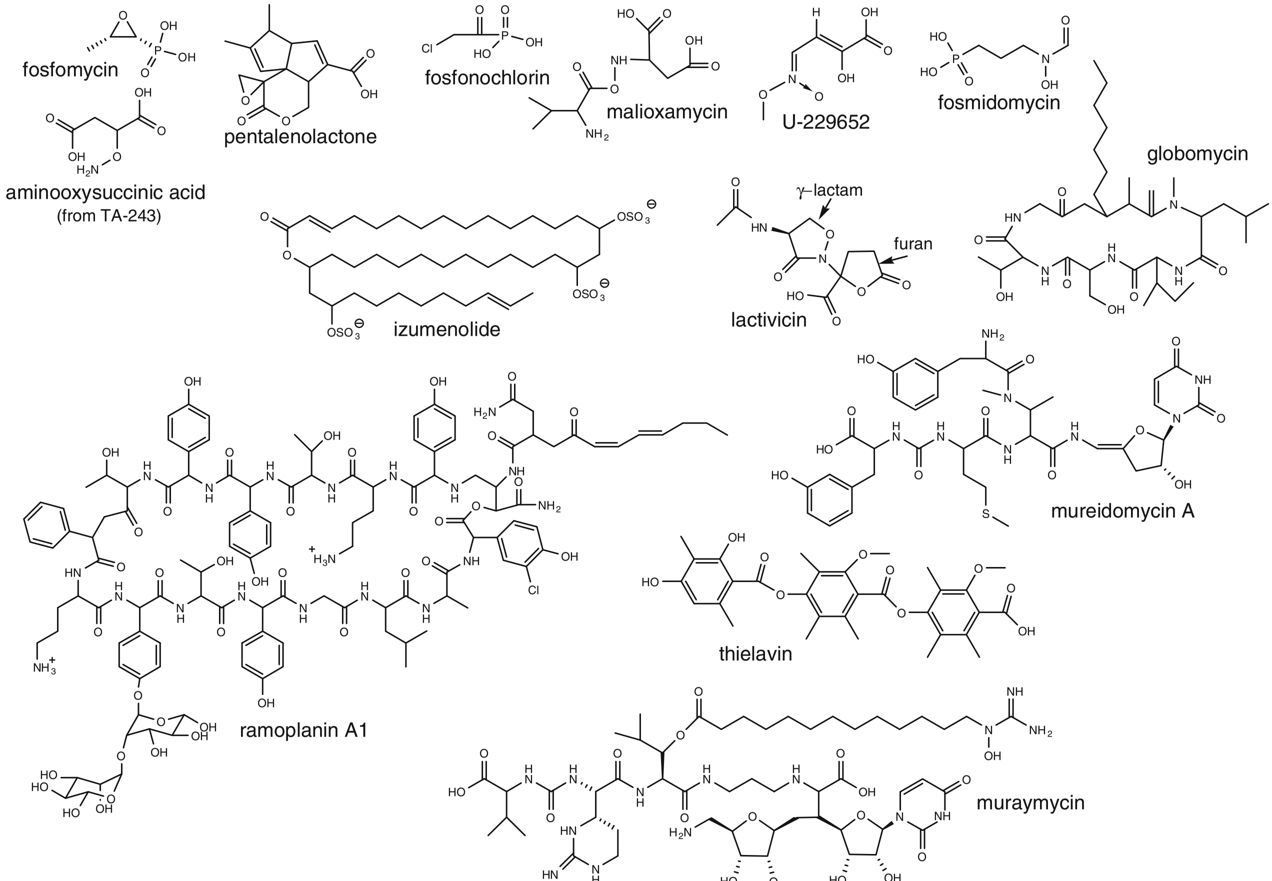

Fig. 2.7 Some non-β-lactams found through directed cell wall screening

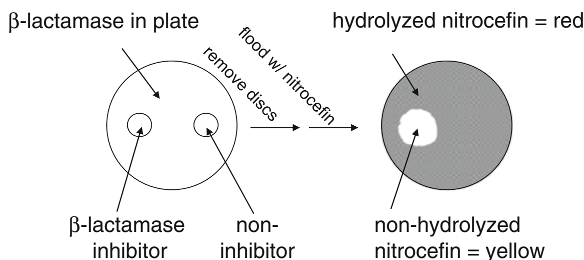

Fig. 2.8 *Squibb assay for β-lactamase inhibitors*. Plates containing β-lactamase in agar are prepared and 11 mm filter discs containing β-lactamase-candidate samples are distributed on the agar. Plates are incubated for 3 h at 37°C, then discs removed and the plates flooded with a solutionof nitrocefin. In the absence of a β-lactamase inhibitor, the β-lactamase in the plate digests the nitrocefin, yielding the red hydrolytic product. If a β-lactamase inhibitor diffuses from the disc, hydrolysis is prevented, and the nitrocefin retains its native yellow color

The Smith Kline group, also using nitrocefin as a substrate, published their screen for β-lactamase inhibitors [188]. A culture of *K. pneumoniae* 1200 (an isolate containing a β-lactamase, chosen after testing of a variety of strains for their behavior with the positive and negative controls) was grown overnight in a relatively colorless medium and distributed into small test tubes followed by addition of 0.05 ml aliquots of test inhibitors and mixing. No inhibitor was added to negative control tubes. After 5 min incubation at room temperature, one drop of a 0.05% nitrocefin solution was added. Red color (due to hydrolysis of nitrocefin) developed rapidly in negative control tubes but not in tubes containing the positive control clavulanic acid. No screening results were indicated; it is likely that this assay was used for optimization of synthetic or semi-synthetic β-lactamase inhibitors.

In addition to clavulanic acid, the successfully developed β-lactamase inhibitors, sulbactam and tazobactam, are semi-synthetic penam sulfones designed and synthesized at Pfizer [49] and Taiho [7], respectively. Sulbactam is used parenterally most often in combination with ampicillin (Pfizer's Unasyn), and tazobactam was developed by Wyeth in combination with piperacillin (Zosyn).

2.4.2 Screens for Spheroplast Formation

In 1992, two reviews appeared that summarized some of the screening strategies for cell wall and other antibiotics that had been used during the 1960s through 1990s, principally at Fujisawa [134] and at Merck [56]. While the Fujisawa screens had been noted previously (see Sect. 2.4.3.), the Merck review was the first disclosure that a spheroplasting method had been used as a primary screen (SPHERO) at Merck for 30 years. As noted in the Gadebusch review, "By the early 1960s it was apparent that a deliberate search for cell wall antibiotics was warranted based on considerable evidence of their efficacy and unusually low toxicity" As early as 1962, Dr. Eugene Dulaney at Merck "set about performing detection assays using the

physical effects produced by cell wall-active agents as the clue for the detection of such microbial products" [56]. It is both fitting and frustrating to note Dr. Dulaney's contribution here, as he was this author's greatly esteemed mentor in the art and science of antibacterial screening but, throughout his long career, he never published on any of the screens, including SPHERO, that he devised.

Details of the relative robustness (false positives, reproducibility, throughput) of the SPHERO screen have not been disclosed but it is known [56, 169] that conditions for spheroplasting were based on the findings of Lederberg [106]. In an effort to find conditions for studying protoplasts of gram negative bacteria, Lederberg observed that protoplasts (later called spheroplasts in gram negatives) were formed upon treatment of *S. typhimurium* and *E. coli* with penicillin in the presence of sucrose and Mg++ under conditions which supported growth. At Merck, samples, generally clarified natural product broths or extracts, were added to bacteria in osmotically stabilized medium and cell morphology was observed by direct microscopy after a period of several hours. Spheroplasts appear as large round refractile bodies. As inhibition of mass increase will prevent the spheroplasting action of penicillin, such a screen would be interfered with by the presence of agents inhibiting certain other cellular functions such as RNA or protein synthesis. Similarly, the presence of membrane active agents could lead to lysis of any spheroplasts formed. As natural product broths often contain a mixture of antibacterial substances, this can be problematic. One way of overcoming it is to run several dilutions or to grow isolates in several media and conditions in the hopes that mixtures will be made in or diluted to varying ratios so that a positive response may be revealed. Conversely, the fact that the production of spheroplasts requires that the cell's mass increase is not compromised, the positives that are found in such screens are less likely to be toxic or have multiple mechanisms of action. Once activities were detected in the SPHERO primary screen, dereplication could be undertaken. This included determination of the bacterial spectrum and relative potency of spheroplasting activity of the unknown active, its sensitivity to various β-lactamases and other tests of antibacterial spectra to help distinguish old true positives from novel activities, as discussed above (Sect. 2.3.1).

The SPHERO screen discovered fosfomycin, cephamycin C, and thienamycin as well as other carbapenems (epithienamycins) and the uracil containing antibiotics, A859A and 875A, likely inhibitors of MraY. Ensanchomycin and prenomycin, compounds related to the transglycosylase inhibitor, moenomycin were also discovered in SPHERO. Pentalenolactone (Fig. 2.7) and many previously seen compounds, including cycloserine (inhibitor of alanine racemase (Alr) and D-ala-D-ala ligase (Ddl) (see Fig. 2.4) were also detected as spheroplast-formers [56, 169].

Fosfomycin (Fig. 2.7), a broad-spectrum phosphonate antibiotic produced by several streptomycetes [71], targets MurA [88], the first committed step in the peptidoglycan pathway (see Fig. 2.4). It was developed in Europe by CEPA, Madrid, which had collaborated with Merck in its discovery.

Cephamycin C and thienamycin, both β-lactams (Fig. 2.6), became the subject of extensive chemical programs. Cephamycin C [169], also discovered by Lilly in the course of examining known Streptomyces producers of penicillin [124], was the first cephem discovered that is produced by bacterial sources, including *S. lactamdurans*. It is highly resistant to many β-lactamases and has an almost exclusive

gram-negative spectrum that was broadened through chemical modification, yielding the semi-synthetic cefoxitin [94]. Cefoxitin (Mefoxin) has been a highly successful parenteral compound.

Thienamycin (Fig. 2.6), the first carbapenem discovered, has an extremely potent broad antibacterial spectrum including *P. aeruginosa* and *S. aureus*, and was isolated from *S. cattleya* (which also produces Cephamycin C) [1, 90, 91]. The compound proved unstable and, although the subject of extensive fermentation improvement and chemical isolations studies [183], difficult to ferment in commercial amounts. Stability was much improved by use of the amidine derivative, N-formimidoyl-thienamycin, or imipenem, and a multi-step synthetic route worked out for its commercial production [120, 150]. Imipenem's lability to a human renal degradative enzyme, dehydropeptidase I, was countered by discovery of an inhibitor, cilastatin [62], that could be given in combination with imipenem to preserve its activity [89]. The combination drug, Primaxin (Tienam) has been a staple in the ICU since its introduction in the late 1980s.

It is clear that the ability of compounds to form spheroplasts was a part of the screening and characterization system at Sankyo, as there is a series of papers noting discovery of a number of compounds with spheroplast forming activity. But it is not clear that spheroplasting was run as a primary screen (as it was at Merck); this is not an empty difference. The nature of the primary screen run, its hit rate, false positive rate, reproducibility, and sensitivity are all critical to the success of any screening program, especially a natural products screening program depending upon fresh fermentation broths or extracts (as most of these were) where downstream sample availability, handling, purification and identification are all labor and resource intensive. Spheroplast-forming antibiotics discovered by workers at Sankyo include mureidomycin [83], globomycin [79], malioxamycin, pentalenolactone and iso-U-22956 [176], and fosfonochlorin (a fungal product) [177] (Fig. 2.7). Mureidomycin has been demonstrated to be an inhibitor of peptidoglycan synthesis, specifically an inhibitor of MraY [82]. Fosfonochlorin selectively inhibits incorporation of diaminopimelic acid (DAP) into cell wall of *E coli* over arginine into protein but its mechanism is unknown. Malioxamycin is a weak gram-negative antibiotic that preferentially inhibits DAP incorporation into cell wall but also inhibits protein synthesis to some extent. No mechanism for iso-U-22956 or its previously discovered isomer, U-22956 [116], have been proposed. The mechanisms of action of pentalenolactone and globomycin and their relation to spheroplast formation are discussed below (Sect. 2.4.2.1).

2.4.2.1 Mechanisms of Action of Spheroplasting Compounds Acting Outside the Committed Steps of the Cell Wall Pathway

While the spheroplasting screen and other cell wall screens, certainly detected inhibitors of peptidoglycan synthesis, they also detected activities that were unexpected. They are mentioned here to emphasize the place of serendipity in the discovery process and to underline the useful "fuzziness" of such assays. For natural product screening, where the goal is to find novelty and selectivity, it is not critical that the hits be "on target" but that the screen is robust and turns up interesting compounds.

Fosmidomycin and Other Phosphonates

Workers at Fujisawa, discovered a number of phosphonate compounds containing an N-acylhydroxamino function produced by several Streptomycetes by screening for activity against a nocardicin supersensitive *P. aeruginosa* strain. All of these, including fosmidomycin (FR-31564, Fig. 2.7), FR-900098, FR-32863, FR-33289 [103, 135], yielded spheroplasts in hypertonic medium and were thus thought to inhibit cell wall synthesis. They were later found to inhibit synthesis of menaquinones via the non-mevalonate (MEP) isopentenyl-diphosphate biosynthetic pathway present in many bacteria, plants and parasites but not mammals [153, 182]. Fosmidomycin and FR-900098 are specific inhibitors of 2-*C*-methyl-D-erythritol 4-phosphate synthase (IspC) the first committed step in the non-mevalonate pathway [104]. Fosmidomycin is synergistic with cell wall and some other inhibitors [126]. It is likely that spheroplasts are formed by fosmidomycin because in bacteria using the non-mevalonate pathway, undecaprenyl-P, the cell wall carrier lipid, is a product of the MEP pathway (Fig. 2.4). The MEP pathway appears to be a reasonable antibacterial target and a whole cell phenotypic screen for such inhibitors has been described [182]. Both fosmidomycin and FR-90098 have shown oral efficacy in mouse *Plasmodium vinckei* (a rodent malaria parasite) infection [84]. In humans, rapid reduction in *P. falciparum* parasitemia was seen in many subjects but recrudescence was common [107]. Studies of clindamycin-fosmidomycin combinations appear more promising but more trials are needed [22].

Pentalenolactone

Pentalenolactone (Fig. 2.7), also called arenaemycin, was reported to form spheroplasts in the Sankyo screen [176], was active in many of the cell wall screens run at Merck and was shown to synergize fosfomycin [43], as do many inhibitors of peptidoglycan synthesis. It is a specific inhibitor of glyceraldehyde 3-phosphate-dehydrogenase (GapA), an enzyme in the glycolytic pathway, and required for the synthesis of phosphoenolpyruvate, a substrate of MurA (Fig. 2.4). Bacterial resistance to pentalenolactone is mediated by altered glyceraldehyde-3-phosphate dehydrogenase, as demonstrated in the producing organism*, S. arenae* [55], but the target is not selective for bacteria. A fungal product, heptedelic acid (also known as koningic acid), has the same target.

Globomycin

Globomycin (Fig. 2.7), a cyclic peptide antibiotic with a gram-negative spectrum, inhibits signal peptidase II that is specific for the processing of the major lipoprotein of gram negatives, Lpp [76, 81]. Globomycin prevents the release of the prolipoprotein form of Lpp from the cytoplasmic membrane, but it does not prevent the covalent attachment of unprocessed prolipoprotein to the diaminopimelic acid of peptidoglycan; it is this linkage to peptidoglycan that is lethal for globomycin treated cells [198].

Cells lacking Lpp are not killed or spheroplasted by globomycin and incorporation of radiolabeled DAP into cell walls is preferentially inhibited by globomycin at concentrations above its MIC [80]. Thus, it appears that spheroplasting by globomycin is due to the covalent attachment of cytoplasmic membrane and peptidoglycan via unprocessed Lpp, which may ultimately prevent peptidoglycan synthesis.

2.4.3 Strains Supersensitive to Cell Wall Active Agents

An approach to screening for cell wall active agents, especially β-lactams, was undertaken independently at several Japanese companies. At Fujisawa, a mutant of *E. coli* derived through N-methyl-N'-nitro-nitrosoguanidine (NTG) mutagenesis was described with greatly increased sensitivity to certain, but not all, β-lactams [4]. Sensitivity was increased 30 to >200 fold to penicillin, cephalosporin C and cephamycin C, but not to cycloserine or fosfomycin. Sensitivity to nocardicin, a Nocardia product discovered in this screen, was 400× enhanced. Interestingly, mecillinam, which is specific for PBP2, was not more active on the supersensitive strain. It seems likely that the screening strain was, at least in part, a *ponB* (PBP1b) mutant, which could render the strain hypersensitive to β-lactams [172, 178]. Nocardicin [5, 70] was one of (if not the) first natural product monocyclic β-lactams (later called monobactams) discovered in screening (Fig. 2.6).

Takeda scientists [99] isolated a β-lactam supersensitive strain of *P. aeruginosa* through three rounds of NTG mutagenesis and selection. Again, the increased sensitivity appeared to be relatively specific for β-lactams. At least one facet of the increased sensitivity was the loss of the type C β-lactamase, but an additional PBP mutation, most likely PBP1b, is likely to be present. For screening, a four plate agar diffusion assay was used: the parent strain (PS) the supersensitive strain ($PSC^{ss)}$, PSC^{ss} in the presence of cephalosporinase from *E. cloacae*, and PSC^{ss} in the presence of penicillinase from *B. cereus*. The first two plates provided a primary screen for β-lactams while the second two were useful for classification. Thirty thousand strains of fungi, yeasts, bacteria, and actinomycetes were tested with filamentous fungi and actinomycetes yielding the only positives. Thirty-six actinomycetes and 90 fungi produced cephalosporin and/or penicillin N while 25 fungi produced penicillinG type compounds. A number of previously unknown cephalosporins and one penicillin strain were identified in this system. New carbapenems were found [78] in a Streptomyces strain. Screening of gram-negative bacteria for activity against β-lactam hypersensitive strains yielded the monocyclic β-lactams sulfazecin and isosulfazecin from a new species of Pseudomonas [77]. An interesting non-β-lactam compound, lactivicin (Fig. 2.7), was isolated from *Empedobacter lactamgenus* and *Lysobacter albus* and found to bind to essential PBPs [129]. Lactivicin contains N-acetyl-L-cycloserine (interestingly, itself an isooxazolidinone and a γ-lactam) connected via a C-N bond to a furan moiety (see Fig. 2.7). It is sensitive to and induces β-lactamases and, against gram positives such as *B. subtilis*, it behaves solely like a β-lactam. Against *E. coli*, where it behaves as a β-lactam at the MIC, other mechanisms likely to involve inhibition of sulfhydryl-containing membrane

proteins are apparent at fivefold the MIC [128]. It is relatively toxic to mice upon parenteral dosing [67].

A β-lactam hypersensitive mutant of *S. aureus* was derived at Otsuka and used in screening, with the reasoning that use of hypersensitive gram negative strains in screening had perhaps biased selection for the more gram negative directed cephalosporins. From among 10,000 actinomycetes and 1,000 eubacteria, 51 carbapenems, 3 cephamycin-C, 5 penicillin-N, 5 fosfomycin and 8 tunicamycin producing strains were found [92]. The high detection rate of carbapenems may justify the initial reasoning.

The Otsuka group also reported a screen using a mutant of *S. aureus* selected for hypersensitivity to D-cycloserine, an inhibitor of alanine racemase (Alr) and D-ala-D-ala ligase (Ddl) [93]. The defect is unclear; however, and testing showed good specificity for specific hypersensitivity to D-cycloserine over all other antibiotics tested. In the screen, besides many instances of D-cycloserine discovered, often from novel producers, O-carbamyl-D-serine, FR-900148 and a novel compound, T-243, were discovered. TA-243 was taken up via peptide transport and subsequently hydrolyzed to aminooxysuccinic acid (Fig. 2.7), an inhibitor of Alr. In fact, aminooxyacetic acid and aminooxysuccinic acid are general inhibitors of pyridoxal enzymes, which include Alr. Accordingly, the antibacterial activity of aminooxyacetate and aminooxysuccinate are reversed by pyridoxal or pyridoxine [176].

The use of strain hypersensitive to other classes of antibiotics have been reported as well (see Sect. 2.5.1)

2.4.4 *Acholeplasma* Screen

In 1979, scientists at the Kitasato Institute published their screening procedure for cell wall inhibitors [137]. The screen was based on the observation that Mycoplasma (now considered a genus of the class Mollicutes) such as *Acholeplasma laidlawii* are insensitive to penicillin [6], since they lack a cell wall [115]. The primary screen involved running a sample under two conditions. Samples were selected that inhibited a sensitive wall-containing organism, *B. subtilis*, but not *A. laidlawii*. As the lack of activity against *A. laidlawii* also indicates that the sample is less likely to be membrane-active or generally toxic;this type of screen provides a surrogate for the selective toxicity desired of an antibacterial drug. The Kitasato procedure involved other secondary assays as well. In testing a variety of compounds with known mechanisms of action, it was found that while cell wall active agents did not affect Acholeplasma, there were some false positives (i.e., certain compounds with other mechanisms of action also did not inhibit). Thus *B. subtilis* positive, *A. laidlawii* negative samples were tested for their specificity in inhibiting incorporation of radiolabeled precursors into cell wall but not into protein. Finally, activities passing through a membrane filter with a putative size cut-off of 1,000 molecular weight were prioritized for further isolation.

Around 10,000 broth filtrates of fungi, bacteria and actinomycetes were tested in this screening system. One new antibiotic, azureomycin, and six previously discovered compounds were identified. Azureomycin is a glycopeptide of as yet unknown

structure, but is likely to act similarly to vancomycin. Of the six previously seen compounds, which included penicillin, cycloserine, ristocetin A and B, amphomycin, and 3-amino-3-deoxy-D-glucose, the mechanisms of action of the latter two had not been previously known.

Amphomycin was shown to inhibit the activity of pentapeptide translocase (MraY; Fig. 2.4) [179, 180], by Ca++ dependent binding to and sequestration of its substrate, undecaprenyl-P [13]. Amphomycin analogs (friulimicin, MX-2401) have recently been under study as drug candidates [189]; 3-amino-3-deoxy-D-glucose was found to inhibit the formation of glucosamine-6-phosphate from fructose-6-phosphate catalyzed by glucosamine synthetase (GlmS) [181], later recognized as a potential antibacterial target [9, 16] as it is a precursor of the MurA substrate, UDP-GlcNAc (Fig. 2.4). The fact that amphomycin and the ristocetins have molecular weights higher than 1,000 was not entirely unexpected as the MW cutoff of the filter used is not clean. In a later paper, 15 higher molecular weight inhibitors selected in this screen were further examined and the izupeptins, previously unknown glycopeptides, found [164].

Interestingly, a Japanese academic group described a polyether compound laidlomycin, (similar to the ionophore monensin), selected from a screen precisely for its activity against *A. laidlawii*, noting that they were successful in finding novel compounds by this method [98]; this is likely due to exquisite sensitivity of Mycoplasma to certain membrane active agents since they lack the diffusion barrier of the cell wall. Although generally active against mammalian cells, some ionophores have a sufficient therapeutic window for use as coccidiostats in animal health, explaining the interest in screening for such compounds.

2.4.5 L-Form Assay

L-forms of bacteria grossly lack cell walls and are bounded simply by the cytoplasmic membrane (although recent work shows that gram negative L-forms do retain a small, essential amount of cell wall material which is required for growth [85]). They can arise "naturally" or through selection in hypertonic medium after treatment with various cell-wall disruptive (e.g., lysozyme) or inhibitory compounds (e.g., penicillin, cycloserine). On solid media they give "fried-egg" colonies resembling those of Mycoplasma. Early workers noted that L-forms were insensitive to inhibitors of cell wall synthesis and sensitive or hypersensitive to other types of antibiotics [38, 59, 87, 195]. Thus, once methodology for reproducible production of relatively stable L-forms was available, their use in screening for cell wall active agents is not surprising. Comparison of L-forms to their parental cell types, rather than Mycoplasma compared to a gram positive wall-bearing bacterium, would likely give a more robust screen, since false positives could arise in the Mycoplasma screen due to differences in non-cell wall targets between the unrelated pairs. Workers at Lepetit used various L-form screens for a number of years in concert with a culture isolation program designed to enrich for Actinoplanes isolates [60, 140]. Teicoplanin (teichomycin A2, Fig. 2.9) [139] and ramoplanin (A −16686, Fig. 2.7) [30] were

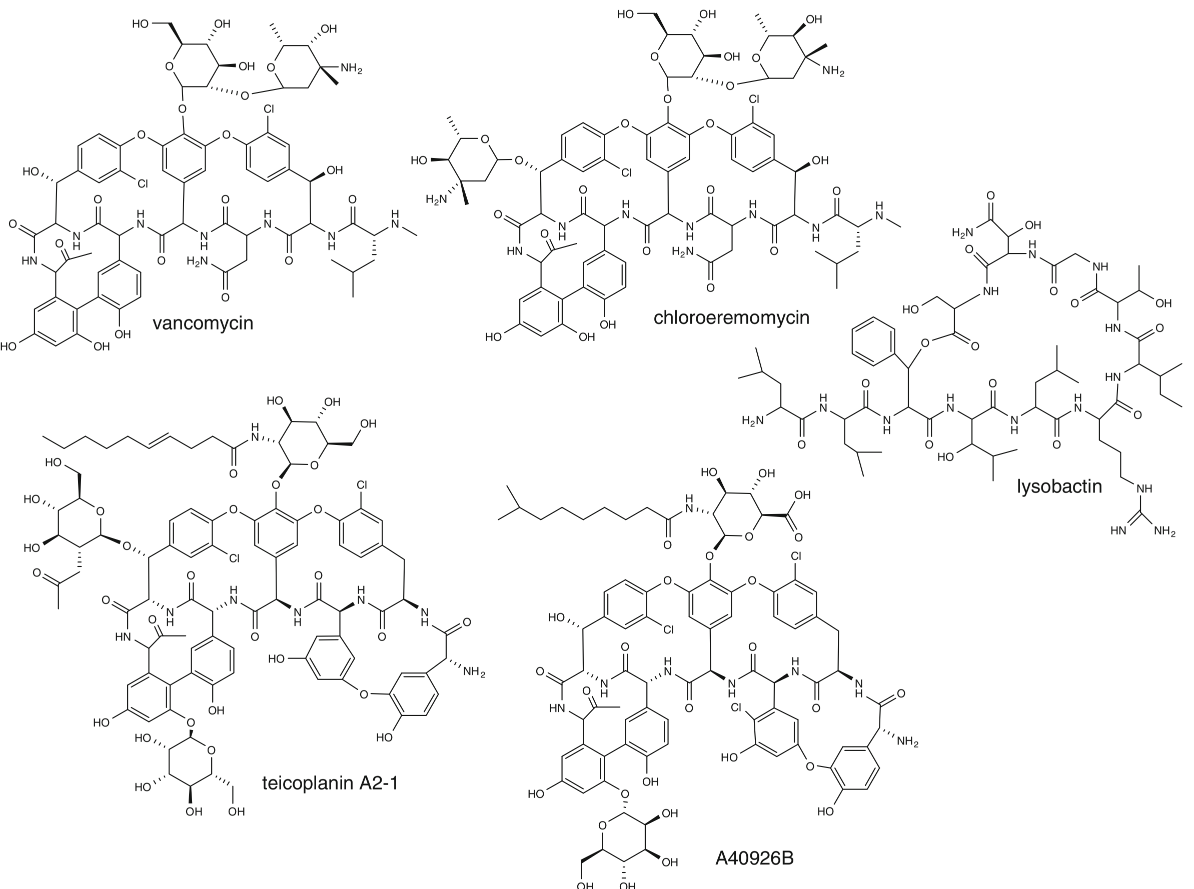

Fig. 2.9 Glycopeptides and Lysobactin

discovered by these methods. In the case of ramoplanin, the screen utilized a methicillin resistant *S. aureus* parent strain to prevent detection of β-lactams [117, 140].

Teicoplanin is a commercially successful parenteral antibiotic used outside of the US. Although the mechanism of action of teicoplanin is the same as that of vancomycin (see below), it is chemically distinct from vancomycin (Fig. 2.9). It has a longer serum half-life and much higher serum binding than does vancomycin and can be given by bolus or IM injection while vancomycin requires slower infusion and multiple daily dosing [44, 123]. Teicoplanin's spectrum is similar to that of vancomycin. It is active against inducible VanB strains of *E. faecalis* where vancomycin is variably inactive, due to failure of teicoplanin to induce VanB expression. As would be expected, constitutive VanB strains are resistant to teicoplanin [50].

Ramoplanin (Fig. 2.7) is a cyclic glycolipodepsipeptide that binds to both lipid I and lipid II and inhibits enzymes utilizing it as substrate including MurG and transglycosylase (see Fig. 2.4). As it is not likely to enter the cytoplasm, its target in whole cells is most probably the transglycosylase reaction [51, 111]. It has broad gram-positive activity, no oral absorption, and poor parenteral pharmacokinetics. Ramoplanin has been under clinical investigation for use in eliminating intestinal vancomycin resistant enterococci in at risk patients and for *C. difficile* associated diarrhea.

The L-form screen was used at Vicuron (which had absorbed Biosearch Italia, the latter form of Lepetit after its sojourn with Merrel Dow) for the detection of novel lantibiotics [29]

2.4.6 Specific Screens for Novel Glycopeptides Based on Mechanism of Action

The discovery of the glycopeptide vancomycin was reported by Lilly in 1955 [118] and that of ristocetin by Abbott in 1957 [63]. While their structures were not fully elucidated until much later [68, 69, 192], they were rapidly introduced into the clinic as anti-staphylococcal agents. The mechanism of action of these compounds was initially recognized as inhibition of peptidoglycan synthesis [148, 190] and treatment of sensitive cells with vancomycin was shown to lead to the accumulation of UDP-MurNAc-pentapeptide (Park nucleotide) in *S. aureus*, as does penicillin [3]. Vancomycin and ristocetin were found to bind to cell walls [18, 19] and to various intermediates of peptidoglycan synthesis terminating in D-ala-D-ala [143]. A synthetic peptide, diacetyl-αλ-L-diaminobutyryl-D-ala-D-ala, was shown to compete with UDP-MurNAc-L-ala-D-isoglu-m-dap-D-ala-D-ala for vancomycin binding and could reverse growth inhibition by vancomycin of *B. megaterium*, *M. lysodeikticus* and *S. aureus* [127]. Those competition assays formed the basis of later screens for novel glycopeptides. Thus, a number of screening systems for glycopeptides based on their preferential binding to cell wall precursors terminating in D-ala-D-ala were run by several companies.

At Smith Kline, French, a screening system designed to find compounds with the same target-binding site as vancomycin was designed and used in tandem with a

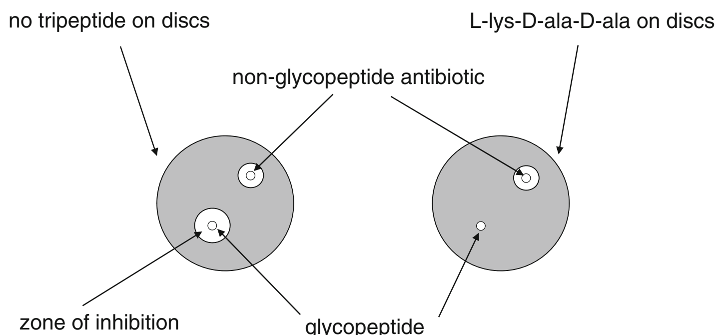

Fig. 2.10 *Tripeptide antagonism screen for glycopeptides*. Plates are prepared as for standard agar diffusion (Fig 2.3). Samples are applied in duplicate to filter paper discs, one disc also containing 100 μg of L-lys-D-ala-D-ala. In this illustration, discs on the right plate contain the tripeptide. Diffusion of glycopeptides that bind to the tripeptide is severely retarded, preventing the formation of a ZOI

culture selection and medium development program [146]. Two different strategies were used in screening. In the first, a prescreen for differential activity on wild type versus a vancomycin resistant isolate of *S. aureus* was followed by a tripeptide reversal screen. Later, the two screens were run in parallel. The selection of a highly vancomycin resistant derivative (vancomycin MIC >100 μg/ml) of a sensitive *S. aureus* strain (vancomycin MIC 1.6 μ/ml) consisted of plating 0.1 ml of an overnight culture on Mueller-Hinton agar containing 100 μg/ml vancomycin, subjecting the plate to UV irradiation (sufficient to give 99% killing) and incubation of the plate for 40 h at 37°C. Resistant isolates were obtained, which seems somewhat surprising. Use of the resistant organism paired with its parent detected 7 of 11 glycopeptides but also detected 30% of 165 other known antibiotics; additionally, this test was not very sensitive. The motivation to use this singularly non-robust, poorly selective and insensitive test as a primary screen was apparently the initial limited availability of the tripeptide used in the secondary screen.

The tripeptide antagonism screen (Fig. 2.10) consisted of measuring the zone size differential between identical samples on two 6.5-mm paper discs, one of them impregnated with 100 μg of diacetyl-L-lys-D-ala-D-ala, applied to a lawn of *B. subtilis*. Control studies showed that 100 μg of tripeptide reversed the activity of 3.1 μg of vancomycin on the disc. Hence, the zone of inhibition of antibiotic samples containing glycopeptides is strongly reduced around discs containing the tripeptide. The tripeptide reversal screen appeared to be completely selective for glycopeptides. With the vancomycin resistance prescreen, 344 initial hits (14%) out of 2,457 cultures screened ultimately yielded 5 glycopeptide producers (0.2%). With the tripeptide reversal primary screen, 2.9% (57 of 1,936 cultures) were positive of which 41 (2.1% of input cultures) were identified as glycopeptide leads. The 2.9% hit rate is remarkably high and reflects the screening of samples which were prepared from cultures isolated on and fermented in media selected to favor the capacity to produce and the production of glycopeptides. A number of glycopeptide classes were found in this campaign, including the aridicins [163] and the similar kibdelins [152], Actinoidin A2 [40] parvodicin [32] and A42867 [149].

At Merrell Dow (which had subsumed Lepetit), the glycopeptide A40926 (Fig. 2.9) was selected in a screen that involved passage of fermentation samples over agarose-ε-amino-caproyl-D-ala-D-ala [35] and elution of adsorbed glycopeptides with 1% aqueous ammonia at pH11 [61]. A40926 had been selected for further work on the basis of its activity against *N. gonorrhea*. It is the precursor of dalbavancin, which is currently in development by Pfizer (by way of the re-emergence of the Lepetit unit, then called Biosearch Italia, from Merrell Dow and its the merger with Versicor to form Vicuron which was then acquired by Pfizer). Dalbavancin has a long half-life and an alkyl side-chain like teicoplanin, aridicins, and kibdelins. This resin-dipeptide screen has the advantage over the tripeptide reversal screen run on agar plates of being able to detect glycopeptides in the presence of interfering activities or other antibiotics, as they would have the potential of masking the differential.

Lilly developed a solid state ELISA assay for screening broths [113] using a BSA-linked pentapeptide [ending in D-ala-D-ala] adsorbed to a microplate. Binding of vancomycin covalently linked to alkaline phosphatase could be measured by alkaline phosphatase activity and unknown samples tested for competition with vancomycin for that binding. Lilly discovered eremomycin and chloroeremomycin (Fig. 2.9, part of the A82846 complex), compounds produced by *Nocardia orientalis*, with this screen [64]. Chloroeremomycin was the progenitor of the semi-synthetic lipophilic compound, LY333328, later named oritavancin [34]. Oritavancin has good activity against staphylococci and vancomycin resistant enterococci, due to chloroeremomycin's potential for dimerization that increases its intrinsic potency and to additional mechanisms of action mediated by the synthetic alkyl side chain [189]. Initially a Lilly development candidate, oritavancin was licensed to Intermune and then Targanta which has recently been acquired by The Medicines Company, which appears poised to continue its development. It should be noted that concurrently with Lilly's discovery of A82846, the equivalent compounds, orienticin and deschloroorienticin were discovered at Shionogi with no directed lead screen identified [184], as was eremomycin by scientists at the USSR Academy of Medical Sciences [57].

2.4.7 Competition with Unfractionated Cell Wall Material

In order to find compounds binding to cell wall components, but with specificities different from those of the glycopeptides, a screen was run at Squibb that used competition with unfractionated wall material, rather than the purified tripeptide used in the glycopeptide screens [133]. A preparation of *S. aureus* cell wall material (boiled in 20%TCA, precipitated and treated with trypsin) was used to prepare agar plates and samples were run on plates with and without this murein addition. Cell wall binding agents are expected to give smaller zones on the murein containing plates. Lysobactin (Fig. 2.9), a dibasic depsipeptide antibiotic, produced by Lysobacter sp, was discovered in this way [133], as were the janthinocins [132], cyclic depsipeptide lactones which also possess moderate activity against gram-negatives.

Lysobactin specifically inhibits trace radiolabel into cell wall and not RNA, DNA, or protein at the MIC [21]. Its spectrum is similar to that of vancomycin but with 2–4-fold increased potency and some activity against gram negatives. When the cell wall material was lysozyme digested (thus liberating acyl-D-ala-D-ala), lysobactin was still inactivated but vancomycin was not. Lysobactin thus appears to bind to a different portion of Lipid II than does vancomycin (Fig. 2.4) [21]. Lysobactin treatment does not lead to accumulation of Park nucleotide and causes membrane leakage (of bacteria) above 10-fold the MIC. While the authors suggested that it might be similar to LY146032 (daptomycin), which has some cell wall activity [2] and also depolarizes membranes, lysobactin is structurally more similar to the katanosins and plusbacins discovered at Shionogi [154, 155], which also appear to bind to Lipid II [114]. All of these are quite potent in vivo with apparently reasonable therapeutic indices, but they have not been developed.

2.4.8 β-Lactamase Inducers

SQ 26,180 (Fig. 2.6), isolated from *Chromobacterium violaceum*, was the natural product monobactam progenitor of aztreonam [174, 191]. Although the discovery of SQ 26,180 and other eubacterially produced monobactams were initially reported to be based on a supersensitive screen using *Bacillus licheniformis* said to be specific for β-lactams, the screen actually involved sensitive detection of induction of β-lactamase by novel β-lactams [175]. A strain of *B. licheniformis* producing a low titer of β-lactamase was inoculated into agar, plates poured and, after drying, paper discs containing samples were placed on the agar surface and the plates incubated 2–3 h at 37°C. The plates were then overlayed with a solution of nitrocefin that turns red upon hydrolysis with the rapidity of color production dependent upon amount of enzyme. This assay was shown to be 15 times as sensitive as the supersensitive *E. coli* mutant screen of Aoki [4], 200 times more sensitive than microscopic detection of elongated cells of *P. mirabilis* and 3,000 times more sensitive than spheroplast formation in *P. mirabilis*. The only false positives detected in screening eubacteria with the *B. licheniformis* assay were some β-lactones. Squibb discovered a number of monobactams, a carbapenem and cephalosporins produced by bacteria using this screen.

A later screen employing β-lactamase induction was developed by workers at Millennium [171] who discovered that a chromosomal (AmpC type) β-lactamase from *Citrobacter freundii* when present (with its regulator, AmpD) on a plasmid in a permeable *envA* (*lpxC*) strain of *E. coli*, was induced by inhibitors of many steps of peptidoglycan synthesis, not only β-lactams inhibiting transpeptidase. The relatively high throughput screen was run in microplate format and used nitrocefin as an indicator of β-lactamase induction. Fosfomycin, cycloserine, cefoxitin, vancomycin, moenomycin and ramoplanin were active in this screen, as was a temperature sensitive *murG* mutant at the non-permissive temperature. The permeable strain was

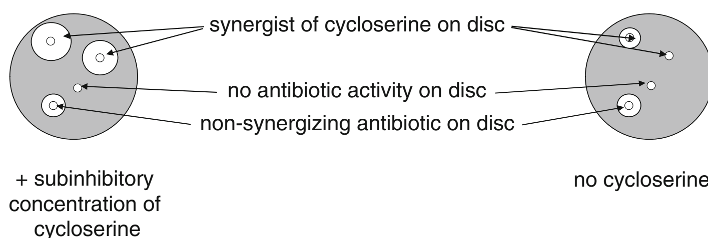

Fig. 2.11 *Synergy screen*. Plates are prepared as for standard agar diffusion (Fig 2.3). A subinhibitory concentration (generally 1/4–1/8 MIC) of cycloserine (or any antibiotic to be tested) is added to the molten agar of half the plates. Samples are applied to plates with and without cycloserine. After incubation, zone sizes on plates with and without cycloserine are compared and initial hits with a differential in zone diameter of 5 mm or greater are selected for retesting

sensitive to some membrane perturbants that gave false positives but allowed the detection of the larger, usually gram-positive specific peptidoglycan synthesis inhibitors like vancomycin and ramoplanin. It may well be that this β-lactamase induction screen was used for the detection of inhibitors of the cytoplasmic steps of the *mur* pathway that were disclosed by Wyeth (which had been collaborating with Millennium), without revealing the screening procedure; [53, 108]. The induction screen was also used in tandem with a secondary specific assay for inhibitors of late steps in cell wall synthesis (stages II and III; see Fig. 2.4) [36]. Inhibitors of transglycosylase and of MraY (Fig. 2.4), including muraymycin (Fig. 2.5) [119] were found in this screen.

2.4.9 *Synergy Screens*

Based on the idea that a sequential blockade of the cell wall pathway will lead to synergy of inhibitors within the pathway [42], Kuroda screened for substances whose activity was increased on a plate containing D-cycloserine (Fig. 2.11) [102]. A compound, FR-900130 (L-2-amino-3-butynoic acid) was isolated, which was a spheroplast former found to be an inhibitor of *S. aureus* alanine racemase.

In the 1990s, when the incidence of MRSA was strongly increasing, a number of groups reported on synergists of β-lactams (such as methicillin), against MRSA. A Microcide patent [20] described and claimed synergy screens for potentiators of antibacterial agents against strains resistant to those agents; the antibacterials included glycopeptides, macrolides, quinolones, tetracyclines, aminoglycosides and an exhaustive list of β-lactams. Examples were given of a number of sterols that potentiated the activity of methicillin against MRSA. Other programs did not explicitly mention screening programs, but it is highly likely that synergy screening was involved. Among synergists of methicillin against MRSA were components of tea extracts [199], carbobenzoxy-diphenyl-ala-pro-phe [45], and TritonX-100 [101].

2.5 Screens for New Members of Previously Described Classes of Antibiotics

2.5.1 Aminoglycoside Screens

Recognizing the success of screening programs using strains specifically hypersensitive to β-lactams and other cell wall agents, scientists at Bristol-Myers Tokyo extended the approach to aminocyclitols and aminoglycosides [130], For the screening strain, they started with a *Klebsiella pneumoniae i*solate, Kp-8, carrying an R-factor rendering the strain highly resistant to a variety of antibiotics including gentamicin and subjected it to serial mutagenesis with NTG followed by replica-plate screening for isolates with increased sensitivity to sorbistin, an aminocyclitol antibiotic produced by a eubacterium (rather than a Streptomycete). The screening strain, Kp-126 was highly sensitized to all aminoglycosides tested (MICs < 0.2 μg/ml vs ≥ 25 μg/ml on the parental Kp-8 strain for all but 4'-deoxybutirosin and amikacin which had MICs of 3.1μg/ml). MICs for other classes of antibiotics were much less affected. Cephalosporin C was the most affected, its MIC decreasing 32-fold. Fermentation samples of about 20,000 soil isolates (presumably eubacteria) were screened using paired strains Kp-8 and Kp-126 in an agar diffusion assay with paper discs. Ten antibiotics showed increased activity on Kp-126. Seven previously detected compounds were detected (sorbistins, butirosins, capreomycin, BMY-28160 [a peptide antibiotic], and streptothricin); two were unidentified antibiotics of "uninteresting activity" and one was a novel amino sugar antibiotic, BMY-28521 produced by *Bacillus pumilis*, identified as 3, 3'-neotrehalosadiamine (Fig. 2.12) [185].

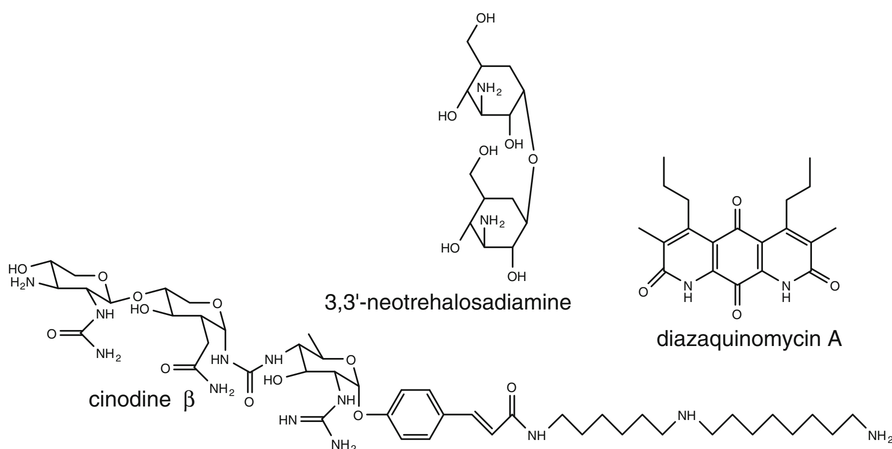

Fig. 2.12 Actives from non-cell wall directed screening

The basis for the apparent selective hypersensitivity of Kpn-126 for aminoglycosides is unknown and might certainly have led to biases in the types of compounds selected depending upon whether the differential was based on permeability, efflux, active transport, target alteration or a combination of these. However, the validity of such a directed natural product screen is based on its sensitivity combined with relatively low hit rate, reasonably low percentage of false positives and identification of novelty. There will always be false negatives in screening, but the enterprise should be pragmatic and results oriented.

A more specific and very sensitive ELISA-based screen for aminoglycosides was developed at Lilly [200]. ELISA plates were coated with anti-gentamicin polyclonal antibody and incubated with gentamicin-alkaline phosphatase conjugate. The amount of conjugate bound after washing was quantitated by measuring alkaline phosphatase activity after addition of the substrate p-nitrophenyl-phosphate. Aminoglycosides, except for neomycin B and C, competed with the conjugate for binding to the gentamicin antibody and no competition was seen with non-aminoglycosides. The ELISA assay was very sensitive, detecting gentamicin at 10 pg/ml, and insensitive to interference by substances in fermentation broths. While screening results were not shown, tests with known producers of aminoglycosides demonstrated the feasibility of the approach. The authors note that this type of methodology could also be used for the elimination of known classes in a screening cascade.

2.5.2 Reporter-Based Screening Platforms

A classical method is the use of indicator plates for the expression of β-galactosidase [121], an enzyme that can be used as a reporter of gene expression when fused to a suitable promoter. The standard "zone of inhibition" (ZOI) assays employed for empirical screening of fermentation broths and chemicals (Fig. 2.3) were adapted starting in the 1970s for use with various dyes and chromogenic or fluorogenic substrates so that reporter genes could be used to monitor the inhibition or induction of genes caused by interaction of a compound with a molecular target (Fig. 2.13). Eventually, these assays were transferred to liquid format, first in 96-well microplates and later much higher order vessels. But one beauty of the agar plate method is lost in liquid, the formation of a concentration gradient of the test compound, such that the effect of a broad range of concentrations (below and above the "minimal inhibitory concentration") can be observed with a single sample (Fig. 2.3); this is important when a desired phenotypic readout is only seen at a specific or narrow range of concentrations, often below the MIC. Thus, on an indicator plate using samples on filter discs, a ZOI may be seen with a surrounding zone of color indicating expression of a specific enzyme (such as β-galactosidase) (Fig. 2.13).

The use of β-galactosidase, as a reporter in screening for several classes of antibiotics, was demonstrated by Kirsch and coworkers at Squibb [97]. For tetracycline, chloramphenicol, and macrolides, the reporter gene was fused to promoters of drug-specific inducible resistance promoters: tetA/tetR from Tn10 in *E. coli*, *B. subtilis*

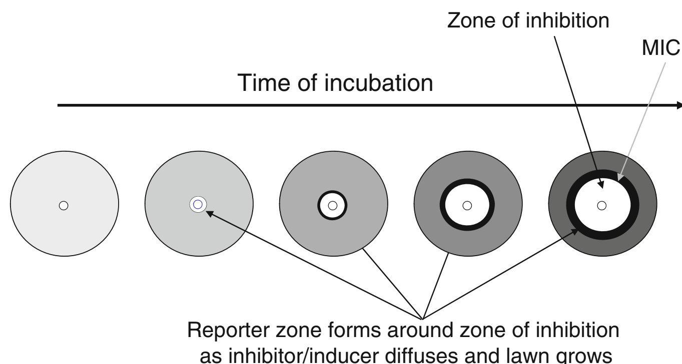

Fig. 2.13 *Reporter screen using agar diffusion format*. Reporter screens are run in the same way as standard agar diffusion screens (Fig 2.3), except that (1) the bacterial strain used contains a reporter gene (β-galactosidase, for example) under the transcriptional or translational control of a regulatory element that leads to turn-on of the reporter gene in response to the presence of a specific type of antibacterial inhibitor, and (2) the agar contains an indicator substrate or dye that will signal the expression of the reporter (for example, Xgal, a chromogenic substrate of β-galactosidase). In a strain in which β-galactosidase is under control of an SOS promoter, such as the *sfiA* or *recA* promoter, a DNA damaging agent or certain inhibitors of DNA replication will lead to upregulation of the β-galactosidase gene and a zone of blue (indicating Xgal hydrolysis) will be formed around the ZOI, at a concentration of inhibitor below the MIC

carrying the *S. aureus cat86* (chloramphenicol acetyl transferase) promoter, and *B. subtilis* carrying *S. aureus ermC* promoter, respectively. At the time this work began (although sadly not true by the time of its publication), no vancomycin resistance mechanisms were known, so a promoterless-*lacZ*-containing transposon library in *B. subtilis* was screened for strains containing promoters inducible by vancomycin. In each system, induction of β-galactosidase was monitored by an agar diffusion method using a specific pH indicator (2,3,5-triphenyltetrazolium chloride), chromogenic (6-bromo-2-naphthyl-β-D-galactopyranoside) or fluorogenic (methylumbelliferyl-β-D-galactoside) substrate. For each screen, suitable control constructs were used to counterscreen against non-specific inducers and artifacts. This type of screen is designed to be more sensitive than a simple ZOI readout, since the reporter expression is seen outside the ZOI, at sub-MIC levels. The tetracycline screen was eightfold more sensitive than a ZOI readout. Workers at Lederle published a similar screen for tetracyclines in the same time period [31].

The vancomycin construct was tested for inducibility by other glycopeptides which were, as hoped, shown to be active [97]. Sensitivity of the screen was not markedly better than for the ZOI readout, most likely because glycopeptides are large, generally slightly basic and tend to diffuse slowly in agar, thus limiting the size of the indicator zone. When used in screening several thousand broths, the screen yielded a number of actives that were secondarily tested for decrease in zone size in the presence of diacetyl-L-lys-D-ala-D-ala. A variety of glycopeptides were detected by the assay and no other known actives were detected, indicating the validity of the assay.

Early studies of the regulation of vancomycin resistance in *E. faecalis* led Handwerger to the interesting finding that inhibitors of transglycosylase, moenomycin as well as vancomycin, induced the VanB operon [66] whereas it was already known that teicoplanin did not induce VanB. It was later shown that the VanA and VanB operons of *E. faecium* and *E. faecalis*, respectively, are both under the control of a two component system, VanR (intracellular response regulator) and VanS (extracellular sensor) [8, 50]. A number of groups used various reporter fusions to determine what types of compounds were recognized by VanS and hence could induce these two operons, both as an academic pursuit and in order to screen for novel antibiotics. Depending upon the strain background, reporter and screening format used, varying results were obtained. While it is clear that vancomycin and teicoplanin induce VanA while vancomycin but not teicoplanin induce VanB, other results were contradictory. Ulijasz found that a variety of inhibitors of cell wall synthesis induced the VanA operon, monitored by a *lacZ* reporter fusion, when cloned into *B. subtilis* [186], while Lai found that glycopeptides, bacitracin, moenomycin and some membrane active agents induced VanA in an *E. faecium* indicator strain using a *cat* fusion [105]. Scientists at Millennium used an *E. faecalis* VanB strain with a *lacZ* reporter for screening and reported on discovery of a transglycosylase inhibitor from a fungal source, thielavin (Fig. 2.7). No breakthrough compounds came from these screens, but the methodologies presaged later screening modes.

As should be obvious, the survey for promoters induced by specific known drugs could be extended in many ways. While the Squibb screens noted in this section were designed to find new members of old classes, the screens and assays based on vancomycin resistance operons appeared, at least in some forms, to find a variety of types of inhibitor. The concept arises naturally of looking for promoters inducible by different inhibitors of several steps of a pathway, or by stress such as the SOS response discussed below (Sect. 2.6.2). Indeed, many groups recently using modern genomic era methodologies, a subject covered in a later chapter, have carried this out.

2.6 The Concept of Phenotypic Screening

The cell wall screens in Sect. 2.4 were generally based on phenomena caused by known inhibitors of peptidoglycan synthesis. The microbial genetic advances of the 1960s through the 1980s led to a general understanding of the essential functions of bacteria via conditional mutants and to great facility in devising selections for mutations, which would produce a desired phenotype. As the mechanism of action of the first wave of antibiotics was understood, it became evident that many of them had specific targets, and that inhibition of them might be mimicked by the effect of conditional mutations. Conversely, the phenotypes of conditional mutants when grown under non-permissive or semi-permissive conditions might be exploited for screening of inhibitors mimicking these effects. Indeed, the essential genes revealed by

microbial genetics were the potential targets for future antibacterial agents: (It should be noted that all the work of the genomics era starting in 1995, has turned up few if any targets that had been unrecognized previously.) Thus, starting in the 1970s, screens were devised that are analogous to the methods used to select for bacterial mutants, that is, by looking for the appearance of a specific phenotype that reflects the desired changes to the genotype. In drug screening, one can look for changes in appearance, behavior or biochemistry of bacteria that reflect inhibition of desired bacterial targets. Thus, screening for antibiotics took on the character of screening for mutants.

Like mutant hunts, screens could be of the "brute force" type, as might be used when screening a population for an auxotroph by replica plating. In drug screening mode, this would involve comparison of two (or more) conditions, for example, a pair of sensitive and resistant strains to find cross resistant compounds. This could be contrasted with a more selective screen, analogous to selecting for a revertant of an auxotroph to prototrophy (or a for resistance to an antibiotic), where the only colonies that appear are the desired mutants (at least in theory!). The screens of Squibb and Lederle for tetracyclines by use of *lacZ* under *tet* promoter control are the selective counterpart of a screen for differential between a sensitive and tet-resistant pair. Using two conditions as opposed to one for a primary screen is not especially "brute force," but use of a single-condition primary screen can preserve limited sample sources.

2.6.1 Folate Pathway Revisited: Interplay of Genetics, Biochemistry, and Screening Strategies

Since there are few examples in the literature of phenotypic screens, the following ramble is presented as a means of illustrating the way genetic and biochemical information can be integrated in various was to establish phenotypic screens.

In *E coli*, growth in minimal medium in the presence of trimethoprim, an inhibitor of dihydrofolate reductase (DHFR) at 5–10 μg/ml and thymine at 50 μg/ml leads to selection of thymidylate synthetase (*thyA*) mutants [165] because tetrahydrofolate (THF) is required catalytically for other synthetic steps (serine, methionine, purines), but THF is oxidized during synthesis of TMP by ThyA, [17]. Thus, loss of ThyA (as long as thymine or thymidine is supplied) spares THF and allows growth in the presence of a DHFR inhibitor like trimethoprim. Hence, in the presence of thymine, *thyA* mutants are more resistant to trimethoprim than *thy*+isogenic strains. It was also found that in media supplying amino acids and purines, sensitivity to trimethoprim is dependent upon thymidine concentration; under these conditions, thymidine raises the MIC of trimethoprim [100].

Taking these findings into account, Omura described a screen for natural product inhibitors of folate synthesis [136]. The screen employed *E. faecium*, which (like humans) lacks the dihydropterate synthase enzyme (FolP, target of sulfanilamide) and therefore requires exogenous pteroate (or other folate). Omura reasoned that in a

medium providing amino acids, purines and a limiting amount of pteroate, inhibitors of DHFR would not allow growth of *E. faecium* in the absence of added thymine but should allow growth in its presence (much as was seen by Koch in *E. coli*). Such a screen was run on 8,000 soil isolates. Four known and three unknown antibiotics were found. The known antibiotics showdomycin and oxazinomycin were reversed by other nucleosides as well as thymidine; tirandamycins A and B, known inhibitors of RNA polymerase were slightly reversed by thymidine but not other nucleosides. The novel compounds were AM-8402, an inhibitor of bacterial DHFR, and diazaquinomycins A and B (Fig. 2.12) which are ThyA inhibitors [122]. Indeed, detection of ThyA inhibitors would have been predicted based on the simple analogy to a mutant screen for a thymine auxotroph.

Based on the folate system, one might envision other screens: (1) a screen for DHFR inhibitors using a pair of *thyA* + and *thyA* isogenic *E. coli* strains grown in the presence of thymine; the *thyA* strain should be more resistant to DHFR inhibitors but that resistance would be function specific, not compound specific. (2) A selective screen for ThyA inhibitors, quite analogous to the original selection for *thyA* mutants. A *thy* + strain of *E. coli* would be used to inoculate a plate containing 5 μg/ml trimethoprim (the amount of trimethoprim would be selected to just prevent growth of a lawn after incubation) and 50 μg/ml of thymine. Samples would be applied on discs or in wells and overnight incubation allowed. As thymine is present, ThyA inhibitors should not give a ZOI but instead should promote growth in the presence of trimethoprim (i.e., give a zone of exhibition (ZOE)). This screen (and all antibacterial screens, for that matter), must be piloted with a large set of known antibiotics and, if natural products are to be tested, with a large number of fermentation samples to ascertain that there is a very low hit rate and that the screen is not intrinsically sensitive to common false positives. In this case, there might be common medium components or other compounds that promote growth of the lawn for other, unforeseen reasons. As a rule of thumb, the hit rate for natural products should be <0.1% since a higher hit rate would not be expected for an inhibitor of a specific enzyme for which natural product inhibitors are rare or unknown.

2.6.2 Phenotypic Screening for DNA Replication Inhibitors

When the fluoroquinolones ciprofloxacin and norfloxacin, which were known to target DNA gyrase, entered clinical trials in the early 1980s, it was quickly realized that other DNA replication enzymes were potential antibacterial targets as well. (DNA topoisomerase IV was not recognized as a second target of the fluoroquinolones until 1994 [95, 96].) In fact, this author began her career in antibacterial discovery in 1982 based on her academic research interests in bacterial DNA replication. Although, as noted repeatedly here, the ongoing screening programs were not published upon until much later or not at all, it should be stated that phenotypic screening for inhibitors of DNA gyrase as well as other inhibitors of DNA replication was ongoing during the 1980s and beyond. A 1990 review proposed DNA replication

proteins as suitable antibacterial targets, but mentioned no screens [156]. As most of these screens are not in the public domain, the academic work that was ongoing and which provided fodder for development of one type of DNA screen will be reviewed here briefly, as will some of the eventually published screens.

The SOS hypothesis first enunciated by Evelyn Witkin in the mid 1970s [193, 194] proposed that UV-induced mutations are produced by an error-prone repair system whose induction is coordinated with other *lexA+/recA+* dependent processes, such as prophage λ induction and filamentous growth, in response to UV irradiation, thymine starvation, DNA damaging agents such as mitomycin C or other inhibitors of DNA synthesis such as nalidixic acid. Schuster had already shown that prophage λ is induced under non-permissive conditions by strains carrying temperature sensitive mutations in the DNA replication genes *dnaB*, *dnaE*, and *dnaG* but not *dnaA* [151]. Taken together, this implies that screens devised to monitor SOS induction should detect DNA damaging agents and DNA synthesis inhibitors.

Colorimetric tests for inducers of prophage λ were described that could be used to screen chemicals and natural products for DNA damaging agents acting as carcinogens or with potential use as anticancer agents [46, 47]. These screens employed a permeable *envA* (= *lpxC*) *E. coli* strain containing a λ-lacZ fusion prophage in which induction could be monitored by measurement of β-galactosidase. In 1982, a reporter strain (the SOS chromotest strain) for inducers of the SOS response that used the *lacZ* gene fused to the *sfiA* (= *sulA*) promoter was described [145]. Thus by the mid 1980s, it was clear to industrial scientists involved in antibacterial screening that such indicator strains, which were readily available at the time, should respond to fluoroquinolones (as they were the descendants of nalidixic acid, a known inhibitor of DNA gyrase [58]) and inhibitors of a subset of DNA replication functions. It was later shown directly, through use of a strain in which the *recA* promoter was fused to *lacZ*, that fluoroquinolones did, indeed, induce the SOS response [144]. Of course, such strains also responded to DNA damaging agents of certain types. So any antibacterial screen using such strains would have had to be used in conjunction with counter screens against frank DNA damaging agents and secondary screens for specific inhibition of DNA replication proteins and/or DNA gyrase.

The first published use of an SOS-type screen for gyrase inhibitors is that of Osburne [138] using *B. subtilis* (since it is much more permeable than *E. coli*) with *lacZ* under control of a damage-inducible *din23* promoter. Actually, two strains were used, one containing in addition to the *lacZ-din23* fusion, a *recM13* mutation and the other a *recM+* allele. As certain DNA damaging agents induced *lacZ* expression in either the *recM+* or *recM13* backgrounds, but true DNA gyrase inhibitors (nalidixic acid, norfloxacin and novobiocin) induced *lacZ* on both strains, compounds that induced in both strains were selected as hits. Cinodine, a natural product inhibitor of DNA gyrase (Fig. 2.12), was identified by the screen.

Another illustration of the application of academic reports of interesting phenotypes of mutants in potential antibacterial target genes is a screen for inhibitors of Gyrase B supercoiling [65]. The screen relied upon the growth-dependence of a Topoisomerase I (*topA*) deletion mutant of *E. coli* upon inhibitors of supercoiling. This was based on the initial observations of DiNardo [39] that, in *E. coli*, *topA*

deletions selected rapidly for compensatory mutations in *gyrB* or *gyrA* and of Stankiewicz [166] that an amber mutant of *topA* in a *ts*-amber suppressor background was dependent upon novobiocin for growth. Thus, the screen [65] used a strain of *E. coli* transduced with a *topA* deletion that required novobiocin, or another suitable inhibitor, for rapid growth at 28°. When plated on agar in the absence of such a compensating inhibitor, the strain gave a very pale lawn. However, when filter discs containing *gyrB* inhibitors were placed on the plate before incubation, luxuriant growth was seen after incubation in the area of the plate where the compound had diffused from the filter, giving a zone of exhibition (around a zone of inhibition). No results of the assay were disclosed, but novobiocin and coumermycin were used as positive controls, and could be found upon screening.

2.7 Why Rational Screening Has Had a Low Yield: Implications for Current Programs

Aside from the successes in the cell wall area (the discoveries of the carbapenems, monobactams, β-lactamase inhibitors, fosfomycin, ramoplanin, new glycopeptides) and despite programs designed to find new antibacterial classes by target-directed methods, rational screening for antibacterials has been remarkably unsuccessful; this applies not only to the largely shrouded whole-cell phenotypic screens of the 1970s through 1990s, but to the more openly described genomics-based and biochemical screens that have followed. Several factors could be implicated for this low output, but a major problem has been the mismatch between the sample sources used and the screening strategies.

The early mechanism-based screens (at least those that were disclosed!) were mostly cell wall directed. They were used to screen natural product sources and served in part as dereplication tools. The concept, born in the early chemotherapeutic era, of choosing candidate targets for their likelihood of selectivity is valid and deserves weight. The choice of the bacterial-specific peptidoglycan synthesis pathway over, say, membrane lytic or DNA damage as mechanisms to be exploited for directed antibacterial screening is logical. But inhibition of cell wall synthesis may have been a singularly rich source of morphological and phenotypic screening possibilities – where inhibition of any point in a long pathway could give rise to similar phenotypes or phenomena. With the revelation by microbial genetics and later genomics, of essential bacterial genes that could be considered targets for new antibacterials, screening became directed more and more toward specific single targets. But the narrowing of target choice was counterproductive for natural product screening. Such highly rational (and hence politically appealing) targeted screens competed successfully with broader pathway (and other) screens for limited screening slots in natural product programs. The beauty of the natural products source is the likelihood that these compounds have evolved, at least in part, to inhibit growth of bacteria [37] (although this has been argued against as the main raison d'etre of microbial secondary metabolites [109, 201]), and while the molecular targets (or, let

us say, receptors) are unknown, they are preselected. If a natural product inhibitor has not already been found for a newly selected target, it is likely that such an inhibitor is rare, since all essential targets have been screened for empirically for decades. To make the most of natural products requires screens that can detect the widest breadth of chemical novelty.

The lure of target-based screening was a reasonable development from the chemotherapeutic concepts developed in early anti-infective and cancer therapies. Certainly, in the 1980s, success in cardiac pharmacology, with angiotensin converting enzyme (ACE) inhibitors and the HMG Co-A reductase inhibiting statins, laid the groundwork for such screens. Furthermore, the idea that chemical inhibitors could be designed for enzyme targets was also rational. Indeed, for chemical collections, target-based screening made theoretical and logical sense. The innate problems of natural product screening and the need to dereplicate before chemical isolation is obviated when the structure of the hit is known. However, the quality of chemical libraries used for antibacterial screening during the 1980s and even now, has been poor, in the sense that most antibacterial hits are surfactants or otherwise generally toxic and, for some reason, it is hard to find hits even for biochemically screened enzyme targets [142]. It is likely that the nature of the libraries has been skewed toward non-anti-bacterial structures. But a critical problem is that bacterial entry, especially into the gram-negative cytoplasm, requires characteristics not prevalent in standard libraries [131, 142]. Gram-negative entry favors polar, charged molecules for outer membrane entry and an uncharged species for cytoplasmic membrane passage [131, 160]. Along with the mismatch of screens and sources, the concentration on preselected targets has led naturally to a focus on single enzyme targets and this emphasis leads to a high potential for rapid resistance development [157, 159]

Among natural products, there has been a long-standing push for use of organisms from novel niches, and more recently, for culturing or cloning unculturable organisms [12, 86]. Furthermore, screens of natural product sources should be hypersensitive (exploiting the likelihood that previous searches might have missed compounds in low concentration) and, above all, aimed at finding novelty.

How can this be done? As later chapters will note, a variety of whole cell screening modes using under-expression of specific genes have been used. For efficient use in natural product screening, these should be deployed in groups, in banks of screens. In this way, a specific hit in a single screening organism (among others in a bank) would indicate selectivity, and likely novelty. These banks should be used in primary screening, to make use of the sensitivity of under-expression. For chemical screens, specific phenotypic assays should be used either as primary screens or to immediately validate that an in vitro hit with antibacterial activity actually targets the desired enzyme. In many, if not most, cases, the antibacterial activity of a hit in an enzyme assay is due to non-specific, often surfactant activity (especially if selection is for anti-gram positive activity). Similarly, optimization of inhibitors via medicinal chemistry is often found to make a quantum leap from lack of antibacterial activity to possession of such activity. It is critical to monitor such optimization with a specific phenotypic screen or assay to avoid chasing non-specific effects.

Correlation of antibacterial activity and anti-enzyme activity is insufficient to support the assumption of causality. While it is perfectly reasonable to develop an antibacterial with multiple modes of action, it is more likely that toxicity will be a problem if the "second" mode is non-specific, surfactant, and alkylating, or otherwise toxic. Thus whole cell phenotypic assays that tie antibacterial activity to action against a desired cellular target are still highly desirable. They may have failed to provide us with novel antibacterial classes over the last 30 years, but there are ways in which such phenotypic screens can be better designed and deployed against improved sample sources to find new entries into the rapidly obsolescing antibacterial armamentarium.

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Part II
Marketed Major Classes of Compounds

Chapter 3
Beta-Lactam Antibiotics

Malcolm G.P. Page

3.1 Introduction

The beta-lactam antibiotics constitute one of the oldest and most popular classes of anti-bacterial agents. The beta-lactams first used for human therapy, were isolated from molds, particularly *Penicillium chrysogenum*. The story of the discovery of the antibacterial properties of molds goes back to the earliest recorded history [50, 135]: in 3000 BC, Chinese scribes documented the use of moldy soya beans to treat infected wounds [22]; in the sixteenth century BC, a Greek peasant woman reputedly cured wounded soldiers using mold scraped from cheese [82]; the Ebers papyrus from Egypt, dated around 1550 BC, gives a prescription for treating infected wounds with "spoiled barley bread" [52]; in the second century BC, soldiers in Sri Lanka applied poultices made from moldy oilcakes to wounds. The therapeutic usage of molds continued in such ways through to the nineteenth century without much consideration of how the molds might be exerting their influence. A number of studies carried out in the late nineteenth century mark the beginning of more scientific approaches and, in particular, drew attention to the antibacterial properties of *Penicillium* species [125]. John Burdon Sanderson [123] reported that bacteria did not grow in culture medium covered with mold, which prompted further investigation of this phenomenon by Lister, who showed that urine samples infected with a mold he identified as *Penicillium glaucum* Link (1809) did not develop bacterial contamination [88]. Lister wrote to his brother "should a suitable case present I shall endeavor to employ Penicillium glaucum and observe if the growth of the organisms be inhibited in the human tissues." Although he did not report it himself, such a case did indeed present itself in the person of Ellen Jones, who had developed a deep gluteal abscess after being injured by a horse-drawn cart. Lister was able to

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cure the infection using local administration of an extract of *P. glaucum* [57]. Also inspired by Sanderson, John Tyndall performed a series of experiments with infusions derived from turnips and mutton in which motile bacteria had multiplied and found that the bacteria died in most of those that became contaminated with *Penicillium* [148]. His friend Thomas Huxley made similar observations, although they differed in their interpretation of the effect: Tyndall believed that it was deprivation of oxygen that killed the bacteria, whereas Huxley suspected it was due to a direct effect of the mold. In France, Louis Pasteur and Jules Joubert [113] reported the inhibitory effect of molds on *Bacillus anthracis* and Ernest Duschene [51] reported the healing property of *P. glaucum*. Vicenzo Tiberio in Italy made extracts of various molds and injected them into animals infected with virulent bacteria [144]. Andre Gratia and Sara Dath in Belgium reported that a *Penicillium* strain exerted a highly bacteriolytic activity against *Bacillus anthracis* [56] and Clodomiro Picado Twight, working in Costa Rica, studied the antibacterial and curative properties of *Penicillium* extracts between 1915 and 1927 (Enrique Tovar, "El legado de Clorito" La Nacion Digital, accessed August 2009). In none of these instances is it really possible to say whether it was penicillin or some other active metabolite produced by fungi that was exerting the antibacterial effect (for example, Duschene and Twight both mention activity against typhoid fever, which the penicillins obtained from *P. chrysogenum* do not have). It was the detailed report by Alexander Fleming (1929) on the inhibitory effect of *P. chrysogenum* Thom 1910 (known then as *P*. *notatum* Westling 1911) on staphylococci, including his description of the inhibitory effect of crude "penicillin" on pathogenic bacteria and the suggestion that it might find a use in medicine, that paved the way to the isolation and widespread use of the natural penicillins.

3.2 Development of Penicillins

The early development of the pencillins as therapeutic agents has many interesting facets that reflect on drug discovery today, and the story is worth considering once more, especially in the light of the looming crisis in antibiotic chemotherapy fuelled by decreasing numbers of new chemical entities and rising bacterial resistance to those drugs on the market [25]. Fleming somewhat lost his interest in penicillin in the 1930s, frustrated by the instability of his preparations and several failed attempts to purify the active principle, but he did make his strains available to anybody who was interested in them. Among these was Cecil George Paine at the Sheffield Royal Infirmary, who used Fleming's "droplets," isolated from cultures of *Penicillium* he had obtained from Fleming, to successfully treat babies suffering from *opthalmia neonatum*, a gonococcal infection, and a coal miner with a streptococcal infection of a corneal laceration. Paine did not report what was, in fact, the first successful clinical use of penicillin and when he was asked why, after his notes were rediscovered in 1983, he replied: "I was a poor fool who didn't see the obvious when placed in front of me." [150]. Howard Florey was the Joseph Hunter Professor of Pathology

at Sheffield from 1932 until his move to Oxford in 1935. Florey knew of Fleming's work and was probably reminded of the therapeutic effect of penicillin by Paine, but he did nothing about it until the late 1930s, when he and Ernst Chain received a grant to make a survey of antimicrobial substances produced by microorganisms, more as an academic exercise than as a search for medically useful substances [2]. Serendipitously, penicillin was one of the first three substances chosen for investigation [6]. When he had moved to Oxford, Florey had assembled a multidisciplinary team, including chemists, biochemists, and a bacteriologist as well as pathologists in Oxford. By bringing together this group of scientists representing the critical areas of expertise, Florey enabled the rapid characterization of the systemic activity of penicillin, producing observations that Florey described as "so gratifying as to be at times almost unbelievable." The first human trials, conducted in 1941, were hampered by the very small amounts of penicillin that could be isolated from Fleming's original isolate of *P. chrysogenum*. The material was very impure, containing only 0.3–5% of penicillin. It is very lucky that none of great excess of impurities was toxic, for otherwise, progress would have been considerably delayed. Nevertheless, the first two volunteers to receive crude penicillin experienced a rise in temperature, shivering and rigor, and the pyrogenic component had to be removed by an additional chromatography step. Several of the early patients were children, chosen in the hope that they would need less penicillin for effective treatment than would adults. Five of the patients had life-threatening streptococcal and staphylococcal infections that had not responded to other treatments. The results were highly favorable despite the limitations imposed by insufficient amounts, which led the team even to re-isolate the drug from the patient's urine.

An unusual aspect of the development of penicillin was that Britain was by that time at war with Germany. The preliminary findings on the isolation and demonstration of *in-vivo* activity were published in Lancet [6] and fuller description, including details of the critical extraction step, in a second article a year later [5]. The Lancet articles drew attention to the remarkable properties of penicillin and stimulated interest both in the USA and in Germany (where the Lancet was obtained *via* neutral Sweden). Work started at universities and companies in both countries: in the USA at Columbia University, working with Chas., Pfizer and Co., and also at Merck and Co., and Squibb and Sons, and in Germany at most of the constituent companies of *IG Farbenindustrie* (Bayer, Hoechst, *IG* Elberfeld, E. Merck, Schering *AG*, Schott and Genossen, *inter alia*). However, the drug development followed very different courses on the two sides [105, 113].

The Anglo-American efforts were conducted in a spirit of open collaboration between the allies, with private–public partnerships and governmental support and guidance [152]. The US government immediately started to direct research, production and clinical trials of penicillin through the US War Production Board (the USA was not yet at war) and the US Office of Scientific Research and Development. These agencies provided resources to stimulate penicillin research, for example, through a grant to the Northern Regional Laboratory in Peoria in the summer of 1941. Florey and Norman Heatley visited the USA and Canada in July 1941 hoping to gain support for large-scale production of penicillin in order to obtain enough

material to carry out a more comprehensive clinical study involving up to 100 patients. Penicillin research was taken up at the Northern Regional Laboratory directly after Florey and Heatley's visit and the first penicillin meeting, including representatives from Lederle, Merck, Pfizer, and Squibb took place in October 1941. Heatley stayed in the USA for 6 months to transfer the knowledge gathered in Oxford to the consortium of universities, national research institutes and pharmaceutical companies interested in penicillin. He was also involved in the first use of penicillin in the USA. John Bumstead, a physician at the Yale-New Haven hospital had a patient dying of β-haemolytic streptococcal sepsis. He managed to obtain a sample of 5.5 g of penicillin through tortuous routes, involving among others another patient, John Fulton, who was a member of an influential National Research Council committee and personal intervention by Heatley at Merck. Bumstead and Charles Grossman administered the first dose on 14th March 1942. A second lot of 5.54 g was shipped on 27th March, but nevertheless, the team collected the patient's urine for Heatley to take back to Merck for re-isolation of the excreted penicillin [136]. In war-torn Britain, the uptake of penicillin production was hampered by lack of personnel, apparatus, and materials. Nevertheless, Imperial Chemical Industries Ltd (ICI) sent representatives to Oxford to learn the necessary techniques, and, by early 1942, a pilot plant was running 100 L fermentations that supplied Florey with 5 g of penicillin for clinical trials. Glaxo Laboratories Ltd., started independently in 1940 with the help of Raistrick, who had worked with Fleming in his attempts to isolate penicillin, and, by 1942, was also producing penicillin on a pilot scale. Glaxo came together with Boots, British Drug Houses Ltd., Burroughs Wellcome and Co., and May and Baker Ltd., to form the Therapeutic Research Cooperation of Great Britain Ltd (TRC), which was later joined by ICI. One of the TRC's goals was to improve penicillin research through exchange of information between its members, which was coordinated by a subcommittee later named the Penicillin Producer's Conference. The British government recognized the importance of penicillin in 1942, when the Ministry of Supply installed the General Penicillin Committee to enhance penicillin production. The British institutions collaborated very closely with those set up in the USA, exchanging strains and technical information, while publication on penicillin was stopped in 1942 for strategic reasons. Several contributions combined to dramatically improve the production of penicillin. It was found that the addition of corn-steep liquor, a by-product of starch production that was available in large amounts in Peoria, increased the yield tenfold and led to production of penicillin G rather than penicillin F (see below). Replacement of glucose by lactose further improved yield. Researchers from Peoria screened many strains of *Pencillium* and eventually isolated a highly efficient strain of *Penicillium chrysogenum* from a moldy cantaloupe melon found in a local market. By 1945, the USA average penicillin production was 570,000 mega units of penicillin per month, and, in Britain, it was 26,000 mega units.

In contrast to the coordinated activities in Britain and the USA, research into penicillin production in Germany was much less successful and the amounts eventually produced much less (about 50 mega units per month in 1945). There were several reasons why research did not advance as rapidly as it did in the Anglo-American consortium [105, 119]. One problem stemmed from the kind of strategic

consideration that affects natural product research even today. The first sulfonamide antibiotic (Prontosil rubrum®) had been discovered by Gerhard Domagk at *IG* Elberfeld, and the class had become accepted worldwide as antibacterial agents. They were readily synthesized and had become highly profitable to the German pharmaceutical industry. In comparison, the production of the new penicillin was tedious and complicated and its activity relatively unproven. The second, major problem was the lack of a good producer strain. Although Fleming had provided his strain to *IG* Marburg some years before the war had started, by the time interest in penicillin had been awakened by the publications of Florey's group, they were unable to culture it. German scientists attempted to obtain producer strains from the collections of institutes in occupied countries, including the Pasteur Institute (Paris), the University of Copenhagen and the *Centraal Bureau voor Schimmelcultures* (CBS, in Baarn, Holland) but eventually resorted to screening numerous isolates for themselves. Hans Oeppinger at Hoechst started systematic screening in May 1942 and eventually obtained three effective strains that were then used for investigation of fermentation conditions. Oeppinger produced the first German penicillin preparation for injection in late 1944, but production was stopped in early 1945 by American occupation of the factory. Domagk also directed research into penicillin at Bayer using strains obtained from the CBS as well as self-collected isolates, but the primary objective appears to have been comparison with sulfonamides rather than isolation in larger quantities. Merck, Knoll, and Schering also started working on penicillin production, but the activities were either terminated or severely hampered by Anglo-American bombing raids. Outside of the pharmaceutical industry, Hans Knöll, working the bacteriology laboratory at the Schott and Genossen glass factory, isolated his own set of producer strains and set up a surface-culture method that produced sufficient quantities of penicillin wound-powder to treat up to 20 patients a day. Konrad Bernhauer at the *Vierjahresplan-Institut* in Prague started screening isolates in 1943 and provided producer strains to the labs at Bayer, Hoechst and Merck. There was, however, no central reference laboratory and many activities were carried out independently, duplicating effort and lacking government co-ordination or support. Eventually the government did pull the various groups together but, as Oeppinger said, "by the time of that meeting, we could get no yeast, no acids, no supplies or materials. It was all over."

3.2.1 The Natural Penicillins

Five compounds (Fig. 3.1) were isolated in pure form from the fermentation broths of *P. chrysogenum* strains. The aliphatic compounds (e.g., penicillin F) were predominant in the extracts produced in Britain, whereas penicillin G was predominant in the American fermentations to which corn steep liquor was added. The antimicrobial activities of the early compounds were rather similar, with a spectrum that covers Gram-positive bacteria but little or no activity against most Gram-negative bacteria. Penicillin G (benzyl penicillin) became the most widely used clinical product, because of its greater convenience of production. The difference between the end products

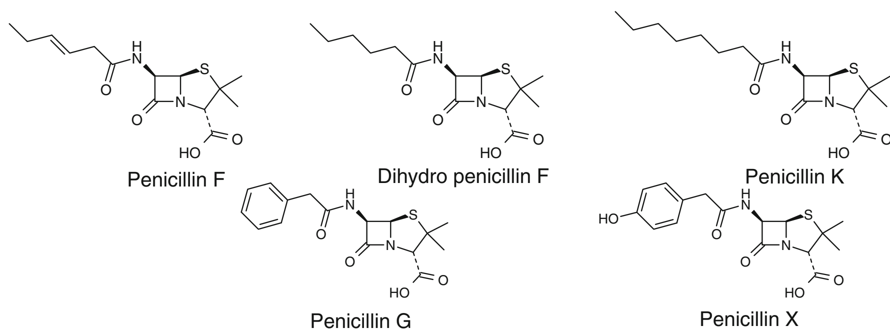

Fig. 3.1 Structures of the first natural penicillins to be isolated

obtained by the two fermentation methods was attributed to the presence phenyl acetate in the corn steep liquor the Americans used, and it was subsequently shown that adding it to the growth medium would indeed greatly enhance the yield of benzyl penicillin at the expense of the other products [96]. This observation encouraged attempts to produce new pencillins by feeding the fermentations with other carboxylic acid derivatives. *Pencillium* turned out to be moderately receptive to mono-substituted acetic acid derivatives and over 100 "biosynthetic precursor" penicillin derivatives were described in the patent literature of the late 1940s, but none of them appeared to present an advantage over penicillin G. Chemical modification of penicillin X at positions *ortho* to the hydroxyl group was also achieved, but again did not bring significant changes in the spectrum of action [40].

3.2.2 The Hunt for Penicillinase-Stable Penicillins

John Tyndall, in his experiments with turnip infusions, noticed that one of the tubes maintained a healthy population of motile bacteria, with no trace of mold, and developed a bright yellow-green color. He commented, "It cannot be doubted that the mould-spores fell into this tube also, but in the fight for existence the colour-producing bacteria has the upper hand" [148]. This must be the first report of resistance of a bacterium (almost certainly a species of *Pseudomonas*) to the inhibitory action of *Penicillium*. Fleming described the resistance of Gram-negative bacteria to penicillin in detail [55] and Chain and Abraham went on to discover the first penicillinase (or beta-lactamase) in *E. coli* [3]. In the early 1950s, strains of staphylococci that produced beta-lactamase appeared in hospitals, and it looked as though the possibility to combat life-threatening staphylococcal infections using penicillin would be lost. This possibility stimulated the search for new derivatives of penicillin that could withstand the attack of beta-lactamase.

Ernst Brandl, working on penicillin production at Biochemie Kundl in Austria in the late 1940s, was dogged by frequent contamination of the fermentations by beta-lactamase-producing bacteria that penetrated the tanks and destroyed the

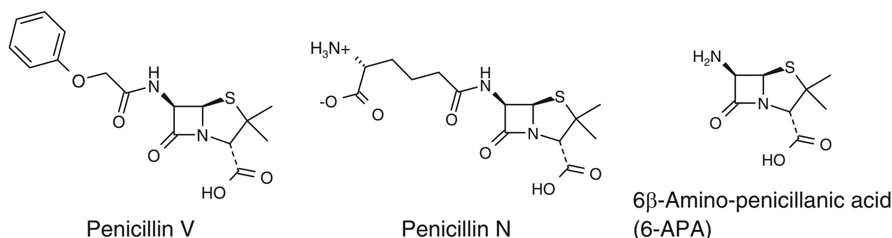

Fig. 3.2 Structures of influential early penicillin derivatives obtained by fermentation

precious penicillin [127]. He started a systematic search for substances that would prevent the bacterial contamination but not interfere with penicillin production. He eventually found that addition of 2-phenoxyethanol to the fermentation not only controlled the contamination but also appeared to produce a penicillin with greater biological activity. Brandl and Hans Margreiter isolated the novel penicillin and found that it was penicillin V (Fig. 3.2), which had already been described as a biosynthetic-precursor penicillin. [23]. However, Brandl and Margreiter realized that the apparently superior activity was due to its better stability and that penicillin V was particularly acid–stable and therefore much more suitable for administration by mouth than penicillin G. Penicillin V became the second penicillin to be widely used in clinical practice, especially for oral application.

Inspired by the reports of the activity of penicillin, Giuseppi Brotzu, at the Hygiene Institute in Cagliari, Italy, set out to look for new antibiotics in 1945. He decided to collect samples from the sea near the outlet of a sewage pipe, based on his hypothesis that self-purification of sewage might be due to antibiosis. He isolated a strain of *Acremonium strictum* Gams 1971 (formerly *Cephalosporium acremonium* Corda (1839)) that exerted antibacterial effects and used fermentation extracts to treat patients suffering from a variety of infections, finding beneficial effects in the case of typhoid fever, against which penicillin was not effective [31]. Brotzu sent his strain to Oxford, where Abraham and Guy Newton eventually purified two beta-lactams from the culture broths. One was penicillin N (Fig. 3.2) and the other was cephalosporin C (see below). Although penicillin N was still labile to beta-lactamase, its enhanced activity against *Salmonella* spp. demonstrated very clearly that changes in activity could be achieved by modifications of the side chain [101]. They chemically modified the amino group of the adipic acid side chain without achieving major change in the antibacterial spectrum. Parallel experience was made with modification of 4-aminobenylpenicillin, obtained by feeding fermentation broths with 4-nitrobenzylacetic acid [145] or 4-aminophenylacetic acid [86]. The side-chains of all of these early semi-synthetic penicillins were monosubstituted carboxylic acids and the penicillins were therefore good substrates for penicillinase. The possibility to produce more fundamental changes in the penicillin side-chain came when chemists working at Beecham realized that 6β-amino-penicillanic acid (6-APA) was also produced in fermentation broths and that this could be readily derivatized by chemical acylation [100].

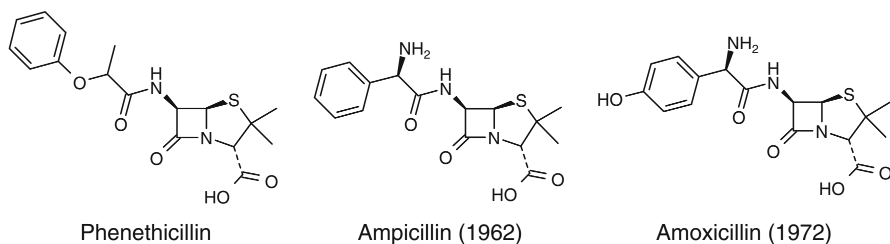

Fig. 3.3 Structures of semi-synthetic penicillin derivatives improved for oral administration. The dates in parentheses indicate the year of publication as a U.S. adopted name

3.2.3 Penicillins with Improved Oral Bioavailability

Some of the first semi-synthetic penicillins prepared from 6-APA were in fact prepared in order to obtain compounds that would be suitable for oral administration [25]. Bristol Myers produced a series of analogues of penicillin V which were substituted at the α-carbon atom, including phenethicillin (Fig. 3.3) and propicillin, while Beecham produced a series of penicillins derived from α-amino acids, including ampicillin and amoxicillin (Fig. 3.3) that have been the most widely used oral semi-synthetic penicillins. Not only do they have good oral bioavailability, but they offer the advantage of distinctly better activity against some Gram- negative bacteria such as *Haemophilus influenzae*, *Proteus mirabilis* and *E. coli* (provided they do not produce beta-lactamase). The improved oral availability comes through better acid stability. An electron-withdrawing substituent in the α-position of the side chains hinders intramolecular rearrangements in which the oxygen atom of the amide group attacks the beta-lactam under acid conditions [1]. The enhanced Gram-negative activity of the α-amino penicillins can be attributed to the positive charge formed by protonation of this group at physiological pH, as noted for penicillin N by Abraham and Newton.

3.2.4 Penicillins with Improved Stability to Staphylococcal Penicillinase

The penicillinase-producing staphylococci had become a major clinical problem by the late 1950s. All of the natural penicillins and the first semi-synthetic penicillins were susceptible to hydrolysis by the staphylococcal beta-lactamase and finding a stable compound took on a high priority at Beecham. The first compound to show enhanced stability was triphenylmethyl penicillin, but it was not active *in vivo*, probably because of very high protein binding. Since other tri-substituted methyl penicillins had similar stability towards beta-lactamase, it was reasoned that steric hindrance around the amide group decreased the affinity for the enzyme. This line of thought led to the synthesis of methicillin in 1959, nafcillin in 1960, and of a series of 4-isoxazoylpenicillins up to 1962 (Fig. 3.4).

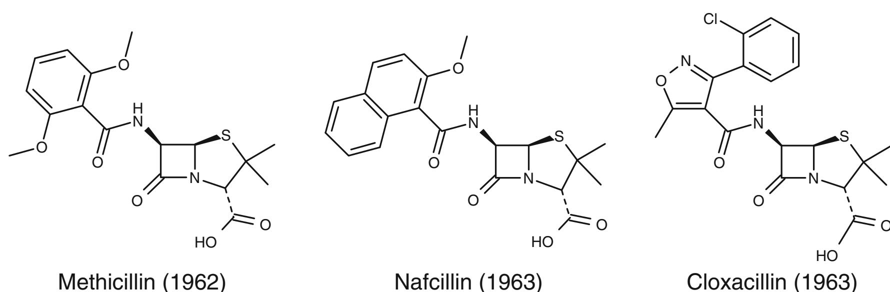

Fig. 3.4 Structures of semi-synthetic penicillin derivatives improved for beta-lactamase stability

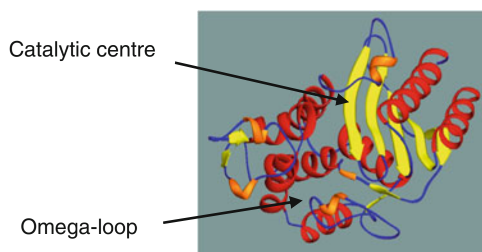

Fig. 3.5 Structure of staphylococcal beta-lactamase

All had similar *in-vitro* activity against staphylococci, including penicillinase-producing strains, but differed in their stability under acid conditions, and therefore suitability for oral administration. Methicillin has very poor acid-stability and had to be administered by injection, whereas cloxacillin, selected on the basis of blood levels following oral administration to volunteers, could be administered orally. The analogues flucloxacillin and dicloxacillin achieve particularly high blood levels but are strongly protein-bound because of their hydrophobicity.

The availability of the crystal structure of the staphylococcal beta-lactamase obtained by Osnat Herzberg and John Moult [70], now allows one to understand the molecular basis of the improved stability towards this enzyme. The catalytic centre of the enzyme has a rather shallow side chain-binding site, delimited by the omega-loop (Fig. 3.5). The bulky, sterically hindered side-chains found in this group of semi-synthetic penicillins prevent the beta-lactam ring from penetrating deeply enough into the catalytic centre for attack to occur.

3.2.5 Broad-Spectrum and Beta-Lactamase Stable Penicillins

In the 1960s, infections due to *Pseudomonas aeruginosa*, particularly in debilitated or immuno-suppressed patients, were becoming a significant clinical problem. Although ampicillin had demonstrated improved activity against Gram-negative

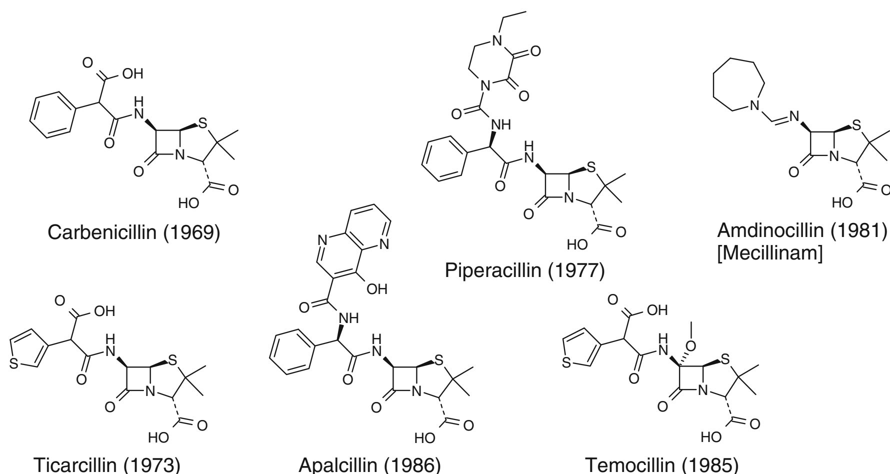

Fig. 3.6 Broad-spectrum and beta-lactamase-stable penicillins

bacteria, there was very little activity against this organism. The first penicillin with useful, albeit rather moderate, activity against *P. aeruginosa* was carbenicillin (Fig. 3.6), introduced by Beecham in 1967 and followed a few years later by the slightly improved ticarcillin. Carbenicillin and ticarcillin are stable towards the chromosomal class C beta-lactamase found in Gram-negative bacteria. Unlike the penicillins stable to the class A beta-lactamase of staphylococci, carbenicillin and ticarcillin react with the class C beta-lactamase to form a relatively stable acyl-enzyme intermediate that is only slowly hydrolyzed to regenerate free enzyme. Thus, they act as inhibitors protecting themselves from further action of the enzyme. In contrast, carbenicillin and ticarcillin are relatively readily hydrolyzed by the class A beta-lactamases, such as the staphylococcal penicillinase or the plasmid-encoded, extended spectrum beta-lactamases now prevalent among the Enterobacteriaceae.

Later developments included a series of derivatives of ampicillin that had modifications on the primary amino group. These included the ureido-penicillins such as piperacillin (Fig. 3.6) from Toyama, azlocillin and mezlocillin from Bayer, and apalcillin (Fig. 3.6) from Sumitomo. These compounds are not as stable towards beta-lactamases, but their side chains bring enhanced penetration into *P. aeruginosa.* Piperacillin, which is distinctly more active than ticarcillin against *P. aeruginosa* and Gram-positive bacteria, has been the most widely used of these.

Two unusual penicillins, mecillinam (amdinocillin), and temocillin (Fig. 3.6), which are basically only active against Enterobacteriaceae, will be discussed here, as they have received interest because of their relative stability towards beta-lactamases. Mecillinam derives from 6β-dimethylformamidinopenicillanic acid, which was prepared at Leo Laboratories as an intermediate in the synthesis of penicillin analogues with novel ring systems. Although the latter were not successful, the intermediate proved to be active in the routine screens and many analogues, culminating in mecillinam, were synthesized [91]. Mecillinam lacks the typical 6β-acylamino side chain,

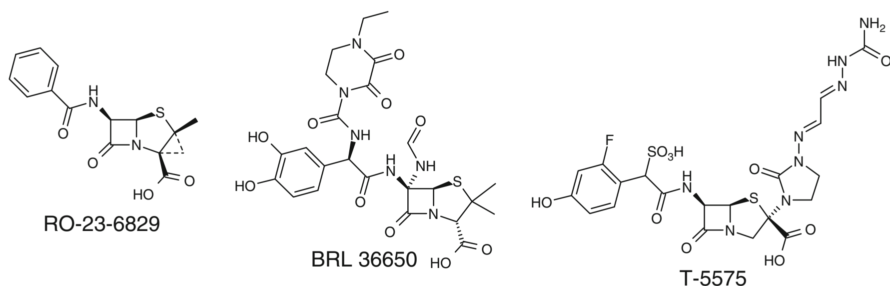

Fig. 3.7 Experimental penams

having instead an amidino function. This affects its recognition by beta-lactamase and may make it an inhibitor of class C beta-lactamase. It is active against a number of Gram-negative bacteria, including *E. coli, Enterobacter* spp., *Proteus mirabilis, Salmonella* spp. but not *P. aeruginosa, H. influenzae,* indole-positive Proteae, most anaerobes or Gram-positive bacteria. It retains good activity against strains of susceptible organisms that have express elevated levels of class C beta-lactamase, but elevated MICs may be observed with strains expressing extended-spectrum class A beta-lactamases [30]. Temocillin has a conventional 6β-acylamino side chain, derived from ticarcillin, but it also has a 6α-methoxy substituent. The α-methoxy substituent was first discovered in the natural cephamycins (see below), where it was found to confer significant beta-lactamase stability, which prompted chemists at Beecham to synthesize the corresponding 6α-methoxy penicillins. The early analogues did not show useful activity [20], but eventually temocillin (6α-methoxy ticarcillin) was synthesized [130]. The 6α-methoxy substituent interferes with the hydrolytic mechanism of the class A beta-lactamase and makes temocillin refractory to hydrolysis by these enzymes. The ticarcillin side chain of temocillin confers stability towards the class C beta-lactamases, which are able to hydrolyze most beta-lactams with a 6α-substituent.

Temocillin is active against *H. influenzae, E. coli, Proteus mirabilis, Salmonella* spp., and some strains of *Enterobacter* spp., but not *P. aeruginosa*, anaerobes, or Gram-positive bacteria. It retains good activity against strains of susceptible organisms that express the classical class A and class C beta-lactamases, against which it is very stable [89].

3.2.6 Experimental Penams

A number of experimental compounds with the same nucleus as the penicillins have been investigated for their antibacterial properties. Among these, tricyclic 2,3 methylene penams (Fig. 3.7) synthesized at Hoffmann-La Roche proved to have good activity against Gram-positive organisms (similar to that of penicillin G) and to penetrate somewhat more readily into Gram-negative bacteria [47]. The interaction of RO-23-6829 with beta-lactamases was more like that of a cephalosporin than

penicillin G: it was not readily hydrolyzed by staphylococcal penicillinase but was rapidly destroyed by the class C cephalosporinases. BRL 36650 was discovered at Beecham, during the investigations of 6α-substituted penicillins that lead to temocillin. It was found to have potent activity against Gram-negative bacteria but poor activity against Gram-positive organisms; therefore, having a spectrum rather similar to ceftazidime or aztreonam [18]. T-5575 (Fig. 3.7) and T-5578 are novel 2-carboxypenams synthesized at Toyama. They had an antimicrobial spectrum similar to aztreonam with good activity against Gram-negative bacilli, including *P. aeruginosa*, but little activity against Gram-positive organisms [154].

3.3 Cephalosporins, Cephamycins and Other Cephems

The *Acremonium* strain isolated by Brotzu in 1948 produced two active beta-lactams: one was penicillin N, which was influential in the development of broad-spectrum penicillins, and the other was identified as cephalosporin C [4, 91]. The antibacterial activity of cephalosporin C (Fig. 3.8) was so low that it was only detected after the molecule had been obtained in pure form. It showed low, but rather broad activity, and, importantly, was resistant to hydrolysis by penicillinase. Florey immediately became interested in the new molecule and demonstrated that it could protect mice even against penicillinase-producing staphylococci.

Although Brotzu had used crude extracts that probably contained cephalosporin C, and Florey thought that it could be used for treatment given in large amounts by intravenous infusion, it was the semi-synthetic cephalosporins that went into clinical use. During their investigation of its structure, Abraham and Newton found that ring system of cephalosporin C was more stable than that of penicillin and that it was thus possible to obtain 7-aminocephalosporanic acid under controlled exposure to dilute acid. Further, they showed that the acetoxy moiety could be replaced by nucleophiles such as pyridine or removed enzymatically to yield deacetylcephalosporin C. These findings, which came at a time when the Beecham group were

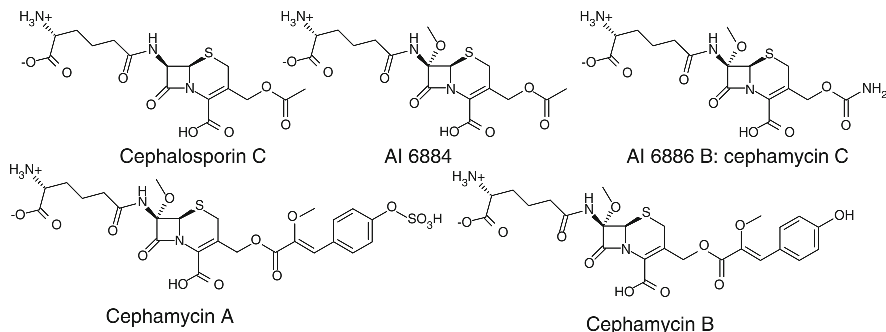

Fig. 3.8 Natural cephems: cephalosporin C and the first four cephamycins

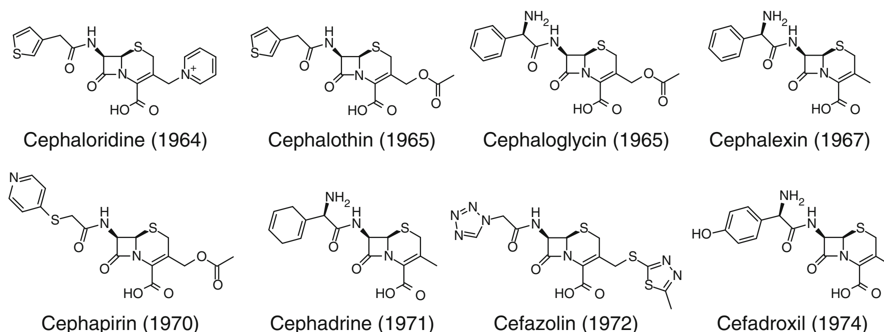

Fig. 3.9 First generation cephalosporins

having marked success with semi-synthetic penicillins such as methicillin, paved the way for extensive chemical modification that has made cephalosporins one of the most important classes of antibiotics today. The semi-synthetic cephalosporins are commonly grouped into four generations [60], based on their anti-microbial activity. The recent anti-MRSA cephalosporins (see Sect. 3.5), which possess a novel spectrum of activity, can be added to these groups.

3.3.1 First-Generation Cephalosporins

The first semi-synthetic cephalosporins to appear on the market were cephaloridine and cephalothin (Fig. 3.9), launched by Eli Lilly Ltd in the mid-1960s. The first-generation cephalosporins have simple 7β-acylamino side chains, in some instances derived from the oral semi-synthetic penicillins. The 3' substituents of the early congeners were derived from the parent 7-ACA (e.g., cephalothin), or by simple chemical modification (e.g., cephaloridine). The members of this group typically have activity against staphylococci, including penicillinase-producing *S. aureus* and *S. epidermidis*, and streptococci, including *S. pneumoniae*, group B *Streptococcus* and group A beta haemolytic *Streptococcus*. The activity against Gram-negative bacteria is relatively modest, but organisms that are covered include strains of *Klebsiella pneumoniae, E. coli, P. mirabilis* and *Shigella* species that do not produce β-lactamases or only produce penicillinases.

Commonly prescribed first-generation cephalosporins include the following: cefadroxil (Duricef®, Bristol Myers Squibb), cefazolin (Ancef®, Smith Kline Beecham), cephalexin (Keflex®, Lilly), cephapirin (Cefadyl®, Apothecon), and cephradine (Velosef®, Apothecon). Oral first-generation agents are well tolerated and have been widely used, particularly in respiratory tract infections, urinary tract infections, and peri-operatively: cefazolin has been an agent of choice in surgical prophylaxis**.** There have, however, been calls to restrict the use of first-generation cephalosporins in hospital practice now that better alternatives are available [43, 118].

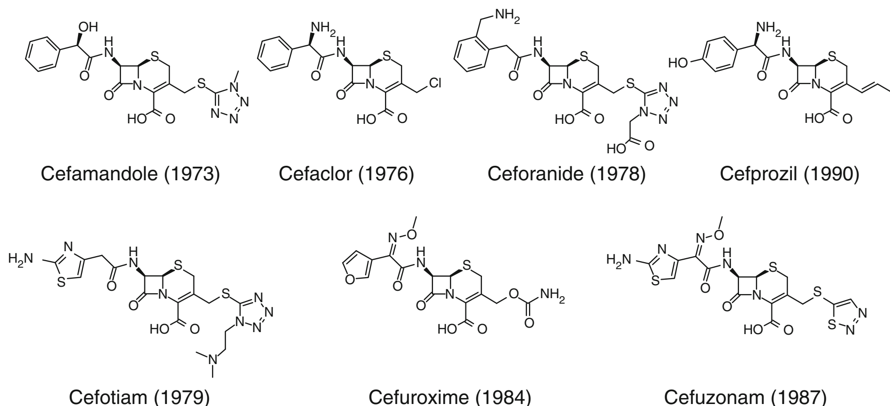

Fig. 3.10 Second-generation cephalosporins

3.3.2 Second-Generation Cephalosporins

The side chains found effective in the semi-synthetic penicillins continued to be used through the 1970s. The cephalosporin nucleus offered an advantage over that of penicillin because the 3' substituents can be varied to modulate the antimicrobial activity or pharmacokinetic properties, and numerous variants with improved activity against Gram-negative pathogens were synthesized. The modifications in some compounds, for example, cefuroxime (Fig. 3.10), decreased the oral absorption of the drugs, and a prodrug (cefuroxime axtel) was made by masking the carboxylic acid of the cephalosporin nucleus with as unstable acetyloxyethyl ester. The prodrug was rapidly converted to the active species by esterases in the blood stream, releasing acetate and formate. The first compounds with significantly modified 7β-aminoyacyl side chains appeared towards the end of the 1970s and at the beginning of the 1980s (e.g., cefotium from Abbott and cefuroxime from Glaxo).

The typical spectrum of this group includes organisms that are susceptible to first-generation cephalosporins, but with less activity against Gram-positive organisms, except for penicillinase-producing organisms where the improved beta-lactamase stability of the second-generation cephalosporins may give better efficacy. In addition, they exhibit activity against *Moraxella catarrhalis, H. influenzae, Enterobacter, Citrobacter, Acinetobacter, Serratia, Neisseria,* and *Providencia*.

Examples of second-generation cephalosporins include the following: cefaclor (Cefaclor®, Lilly), cefamandole nafate (Mandol®, Lilly), cefuroxime axtel (Ceftin®, Zinacef®, Glaxo Wellcome), cefprozil (Cefzil®, Bristol Labs). Parenteral cefuroxime is the only second-generation cephalosporin that penetrates the CSF sufficiently well to be effective in meningitis caused by *S. aureus,* pneumococci, meningococci, and *H. influenzae*.

Several of the second-generation cephalosporins with a 1-methyltetrazole-5-thio side chain have been associated with an unpleasant flushing reaction when taken

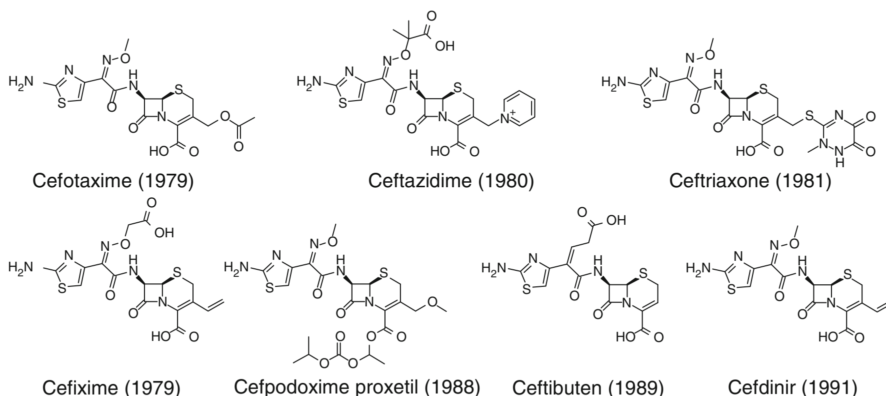

Fig. 3.11 Third-generation cephalosporins

some time before the drinking of alcohol. It appears that the side chain is liberated in vivo during a chemical rearrangement triggered by opening of the beta-lactam ring. The thiol is oxidized to 5, 5-dithiobis(1-methyltetrazole) or to a mixed disulfide analogue that can inactivate aldehyde dehydrogenase.

3.3.3 Third-Generation Cephalosporins

Third-generation agents have acquired a good reputation for efficacy and tolerability. Generally, they were expensive, but considered appropriate for hospitalized patients with severe infections. Since nearly all are now available as generics, the price is considerably lower. Most are administered parenterally, although there are a few with good oral bioavailability (e.g., cefixime, ceftibuten, cefdinir, Fig. 3.11). There has been considerable interest in the pro-drug approach (e.g., cefpodoxime proxetil, Fig. 3.11) to obtain oral activity.

The members of this class have less activity against Gram-positive bacteria than first-generation cephalosporins, but greater *in vitro* activity than first- and second-generation cephalosporins against Gram-negative organisms, especially those with beta-lactamases. The typical spectrum includes *E. coli, Klebsiella, Enterobacter, Acinetobacter, Serratia, Providencia, Proteus, Morganella*, and *Neisseria*. Some drugs are also active against *B. fragilis* and *Pseudomonas* species, for example ceftazidime. Cefotaxime, ceftriaxone, ceftazidime and ceftizoxime are useful in Gram-negative bacillary meningitis but other cephalosporins have poor penetration into the central nervous system. Commonly prescribed third-generation cephalosporins include the following: cefixime (Suprax®, Lederle), cefpodoxime proxetil (Vantin®, Pharmacia and Upjohn), ceftazidime (Fortaz®, Glaxo-Wellcome), cefdinir (Omnicef®, Abbott), cefotaxime (Claforan®, Hoechst-Roussel), ceftibuten (Cedax®, Schering), and ceftriaxone (Rocephin®, Hoffmann-La Roche).

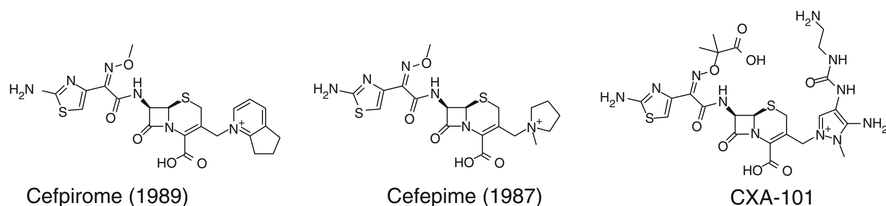

Fig. 3.12 Fourth-generation cephalosporins

The 7β-(aminothiazoyl)oxyiminoacetamido side chain of the third-generation cephalosporins plays an important role in their stability towards beta-lactamases. It is a bulky, rigid structure that does not fit into the active site of class A beta-lactamases such as the staphylococcal penicillinase, or the TEM-1 beta-lactamase found in Gram-negative bacteria. Thus, the third-generation cephalosporins often exhibit low affinity for the class A beta-lactamases except for those that have acquired point mutations that increase flexibility in the active site region and widen the side-chain binding pocket [111]. The situation is different in the class C beta-lactamases: here the side chain actually fits quite well into the active site, where it aligns with a beta-strand that forms one edge of the side-chain binding pocket. Thus, the third generation cephalosporins often exhibit high affinity for the class C beta-lactamases. However, the extra interactions, involving additional hydrogen bonds, help to stabilize the acyl-enzyme intermediate and slow down the later steps in the catalytic cycle [106].

The widespread use of third-generation cephalosporins has been associated with increases in beta-lactamase-mediated resistance amongst Gram-negative pathogens, super-infection with resistant staphylococci, and enterococci as well as antibiotic-associated diarrhea and onset of pseudomembranous colitis due to *Clostridium difficile*, and there have been calls to restrict the use of these agents [72, 103, 107, 111, 114]. Adverse events associated with the use of third-generation cephalosporins are rare, and mostly compound-specific. Ceftriaxone has been associated with fatal immune-mediated haemolytic aenemia in patients with HIV infection, sickle cell disease, and leukemia. Seizures, encephalopathy, coma, asterixis, neuromuscular excitability, and myoclonia have been reported from renally impaired patients treated with unadjusted dosing regimens of have been reported with several cephalosporins, including ceftazidime.

3.3.4 Fourth-Generation Cephalosporins

The fourth-generation cephalosporins (Fig. 3.12) are considered to possess extended Gram-negative coverage as compared to third-generation cephalosporins, due to increased stability towards hydrolysis by β-lactamases and, perhaps, less induction of β-lactamase-mediated resistance. They derive from compounds such as ceftazidime (third generation), which has better beta-lactamase stability than some of its

congeners. The positive charge in the 3' side chain appears to increase the ability of the cephalosporins to inhibit class C beta-lactamases as well as to promote uptake into the periplasm of Gram-negative bacteria. Although the fourth-generation cephalosporins are quite stable to the class C beta-lactamase, they are still hydrolyzed by the extended-spectrum beta-lactamases that evolved to hydrolyze the third-generation cephalsporins. Thus, there is a propensity to select for resistant strains, especially those with ESBLs and it has been shown that cefepime restriction improves decreases the overall resistance among Gram-negative organisms [44, 48].

The spectrum of activity of fourth-generation cephalosporins includes Gram-negative organisms with multiple drug-resistance patterns (e.g., *Enterobacter* and *Klebsiella*). Cefepime (Maxipime®, Squibb) and cefpirome (Cefrom®, Hoechst-Roussel) are the most important fourth generation cephalosporins. Cefepime penetrates into the CNS and therefore may be also useful for treatment of bacillary meningitis. CXA-101, formerly FR264205 discovered at Astellas [67], is an experimental compound in clinical development for treatment of infections caused by Gram-negative bacteria. CXA-101 exploits both these features to achieve improved resistance to hydrolysis by the *P. aeruginosa* class C β-lactamases. Like ceftazidime, CXA-101 penetrates well into *P. aeruginosa* and is not greatly affected by the expression of efflux pumps [137], but it is more stable towards the class C beta-lactamases of this organism [67]. The MICs reported for *P. aeruginosa* with resistance mediated by class C beta-lactamase were ≤4 mg/L [125]. Unfortunately, it remains sensitive to class B metallo-beta-lactamase (MIC for one strain of *P. aeruginosa* reported as >128 mg/L) as well many class A and class D extended-spectrum β-lactamases. Its utility against a broad range of Enterobacteriaceae is therefore more uncertain.

3.3.5 Anti-MRSA Cephalosporins

The first reports of *S. aureus* that was resistant to methicillin came in 1961, shortly after it was launched. These were, however, a series of isolated outbreaks, and methicillin-resistant *Staphylococcus aureus* (MRSA) did not become a serious clinical problem until the 1980s. After that time, the incidence of MRSA has steadily risen in most parts of the world, such that the clinical efficacy of beta-lactams against staphylococci is threatened once again. Resistance is due to the expression of an alternative penicillin-binding protein, PBP 2a or PBP 2', which is immune to inhibition by all currently available beta-lactams but can still carry out the essential step in cell wall biosynthesis. During the late 1980s and early 1990s, a number of companies initiated chemical modification programs to restore the activity of cephalosporins against MRSA.

The anti-MRSA cephalosporins exhibit extended Gram-positive coverage compared to first- and second-generation cephalosporins, including for the first time useful activity against methicillin-resistant staphylococci. There are two compounds from this class that are in late stage clinical development (Fig. 3.13). Ceftaroline,

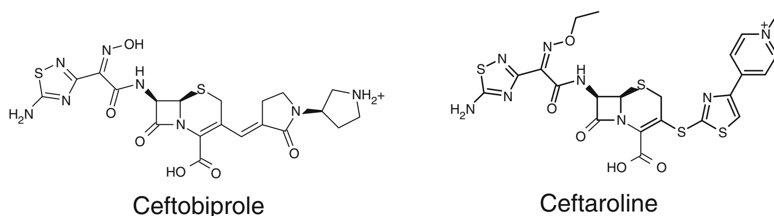

Fig. 3.13 Anti-MRSA cephalosporins

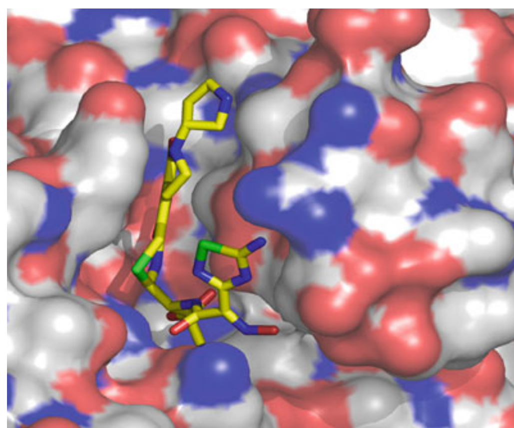

Fig. 3.14 Structure of the active site in the acyl-enzyme complex formed between ceftobiprole and *S. aureus* PBP2a (Redrawn from [88])

discovered at Takeda [77], has been approved for use against complicated skin and structure infections as well as community-acquired pneumonia. Its coverage of Gram-negative pathogens has been compared to that of cefotaxime or ceftriaxone, with no useful activity against *P. aeruginosa* or beta-lactamase-producing Enterobacteriaceae. Ceftobiprole, discovered at Hoffmann-La Roche [68], has been investigated in clinical trials against complicated skin and structure infections as well as community-acquired and nosocomial pneumonias. Its coverage of Gram-negative pathogens is comparable to that of ceftazidime or cefepime.

A common feature of the anti-MRSA cephalosporins is the long, rather hydrophobic 3'-side chain that terminates in a basic residue [97]. This is a requirement imposed by the structure of the active site of PBP 2a [87], where the side chain has to bind in a narrow, apolar cleft ending in a cluster of polar, mostly negative charged residues (Fig. 3.14). The aminothiadiazoyl, rather than aminothiazoyl, group in the 7β-acyl side chain allows an additional hydrogen bonding interaction that stabilizes the acyl-enzyme complex and therefore enhances the inhibition [82, 98].

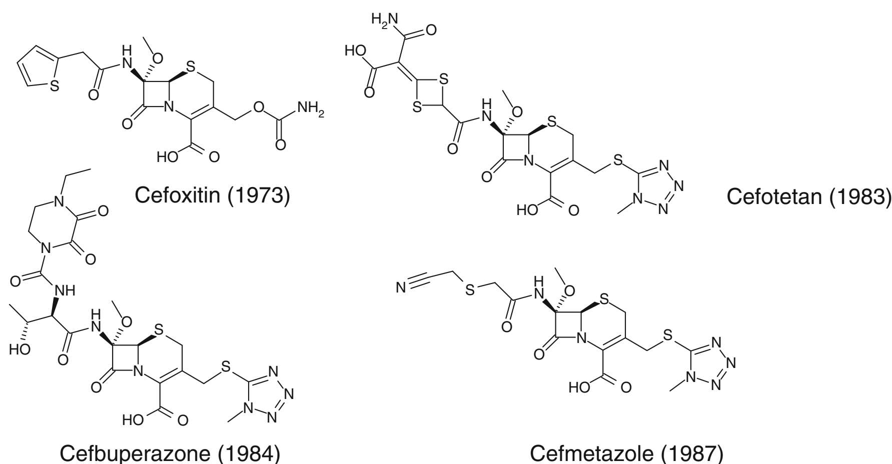

Fig. 3.15 Semi-synthetic cephamycins

3.3.6 Cephamycins

The cephamycins were discovered in screening programs conducted at Merck and Lilly around 1970 [98, 128]. The first four compounds discovered (Fig. 3.8) had the same α-aminoadipoyl in the 7β position as does cephalosporin C from which they are differentiated by the 7α methoxy substituent. The cephamycins were the first beta-lactams to be found in fermentation broths of actinomycetes and, as such, heralded the discovery of many new classes of beta-lactam. Cephamycins A and B have a broad spectrum of low potency activity, including both Gram-positive (except enterococci) and Gram-negative organisms (except *P. aeruginosa* and *S. marcescens*), but are both chemically unstable. Cephamycin C has a similar spectrum of activity, but is more potent against Gram-negative bacteria. A very important characteristic of the cephamycins is their stability towards beta-lactamases. All of the natural cephamycins are much more stable than cephalosporin C towards the serine beta-lactamases, which sparked off an intense effort on the chemistry of penicillins (see temocillin in Sect. 2. 4, above) and cephalosporins. Advances in semi-synthetic cephamycins (Fig. 3.15) came through the development of methods for exchange of the side chain [80] and of stereospecific introduction of the 7-methoxyl group into the cephem molecule [39].

The antimicrobial spectrum of the semi-synthetic cephamycins is generally similar to that of second-generation cephalosporins and these molecules are frequently classified with this group of cephems. The important difference is their greater stability towards the class A extended-spectrum beta-lactamases. Cephamycins are not so stable to the class C beta-lactamases, and a number of the plasmid-encoded class C enzymes (e.g., FOX-1 enzyme) were identified by their ability to hydrolyze these compounds [60]. Introduction of the 7α-methoxy substituent into third and fourth

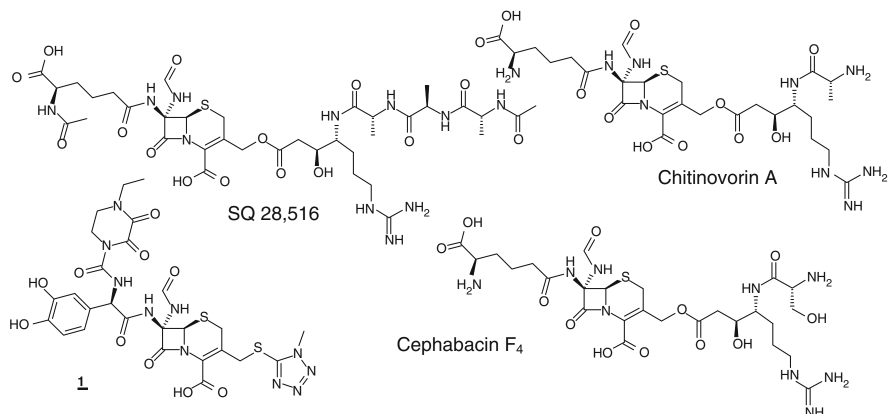

Fig. 3.16 Natural 7α-formamido cephems and a semi-synthetic analog

generation cephalosporins (e.g., cefotaxime and cefpirome) resulted in analogues that had markedly lower activity than the parent compound [85]. This result is reminiscent of the early attempts to make 6α-methoxy penicillins and suggests that, at least as far as antibacterial activity is concerned, the α-substituent is only compatible with certain acylamino side chains in the β-position. The rigid, bulky side chains of the third generation cephalosporins may well have steric conflicts with the α-substituent.

Cefoxitin has been associated with an elevated risk of beta-lactam associated coagulopathy [34] while ceftotetan, cefbuperazole and cefmetazole have the 1-methyltetrazol-5-yl thio substituent found in second-generation cephalosporins that is associated with alcohol incompatibility.

3.3.7 Chitinovorins and Cephabacins

Cephalosporins with a 7α-formamido substituent were discovered in the early 1980s in screening programs run at Shionogi, Squibb, and Takeda using tests designed to detect beta-lactam antibiotics stable towards beta-lactamase [93, 119, 120]. They were found in fermentation broths of several species of Gram-negative bacteria including *Flavobacterium* spp., *Lysobacter lactamgenus* [104] and *Xanthomonas lactamgena* [104].

The enhanced β-lactamase stability of the 7α-formamido cephems prompted several attempts to introduce this functional group into other cephems. but the results have been rather mixed. A series of semi-synthetic analogues of second generation cephalosporins were synthesized at Beecham during the 1980s and several (e.g., compound **1** in Fig. 3.16) showed potent broad-spectrum activity [19, 24]. In contrast, attempts at Hoechst-Roussell to produce analogues of the third-and fourth-generation cephalosporins cefotaxime and cefpirome resulted in compounds

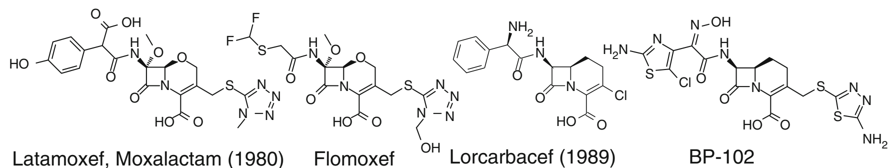

Fig. 3.17 Synthetic cephems

with disappointing lack of activity [85]. It seems that, as with the 7α-methoxy substituent, there can be steric constraints on the 7β-side chain for maintenance of biological activity.

3.3.8 Carbacephems and Oxacephems

The discovery at Merck that oxacephems exhibit stronger antibacterial activity than that of the corresponding cephalosporin [38] prompted many laboratories to attempt the total synthesis of such compounds, efforts which culminated in the introduction of latamoxef (Moxalactam) [98] and flomoxef [146] by Shionogi. Latamoxef had a broader spectrum of activity than the previously available cephalosporins including Gram-positive aerobic cocci, Enterobacteriaceae, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, and *Bacteroides fragilis*.

Latamoxef combines the higher reactivity of the oxacephem nucleus with the 7α-methoxy group of cephamycins and the α-carboxy substituent of carbenicillin in the 7β-acylamino side chain, which both confer beta-lactamase stability. Its activity profile was similar to that of the second-generation cephalosporins, and it is usually classified with these. Latamoxef has the 1-methyltetrazol-5-yl thio substituent found in many second-generation cephalosporins. Its use has been associated with prolonged bleeding time [34] and several fatal cases of coagulopathy were reported in the 1980s: it is no longer available in the USA.

Loracarbef (Lorabid®, Lilly) is the only carbacephem to have been marketed so far. The first attempts at total synthesis by the Merck group resulted in racemic carbacephems [57] that exhibited high chemical stability. Since this allowed structural manipulation in a manner that could not be done with the cephalosporin ring system, it encouraged efforts to develop efficient chiral syntheses. The chiral synthesis that led to loracarbef was developed at Kyowa Hakko Kogyo Co. [103]. The chemical stability also confers higher plasma stability and contributes significantly to the enhanced pharmacokinetic properties of carbacephems. Loracarbef has a broad-spectrum of action including Gram-negative and Gram-positive bacteria, including *E. coli*, *S. pyogenes*, *S. aureus*, *S. saprophyticus*, *S. pneumoniae*, *H. influenzae,* and *M. catarrhalis*. Adverse reactions are rare and consist primarily of hypersensitivity reactions with urticaria, nonspecific rash, and pruritus.

Blanca Pharmaceuticals has recently profiled BP-102 (Fig. 3.17) for use against beta-lactam resistant Gram-positive bacteria, including MRSA [59]. It is one of an

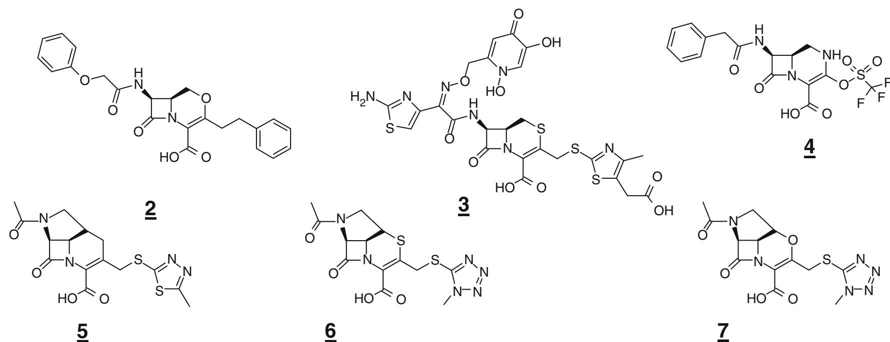

Fig. 3.18 Experimental cephems

experimental series of 3-heteroarythio-carbacephems that derive from compounds synthesized at Lilly Research Laboratories in the 1990s. It is interesting to note that the 3’ side chain is significantly smaller than that typical of the anti-MRSA cephalosporins, which suggests that there might be a different structure-activity relationship for the carbacephems [143].

3.3.9 Experimental Cephems

The chemistry team at Bristol Laboratories of Canada undertook the total synthesis of nuclear analogues of cephalosporins during the 1970s [40, 41], producing a series of isocephems, O-2-isocephems, N-2-isocephems and 2-substituted carbacephems (e.g., **2** in Fig. 3.18). The antibacterial activity of the O-2-isocephems appeared to be generally rather similar to the corresponding cephem and they have been investigated as antibiotics for oral administration [94]. Some of the series have been revisited more recently: for example, in the isocephem series, the third-generation cephalosporin analogue **3** shown in Fig. 3.18 had good activity against *P. aeruginosa* and *A. baumannii* [147], and compound **4** from the N-2-isocephem series had good activity against both Gram-positive and Gram-negative bacteria [74]. Tricyclic bridged carbacephems (Fig. 3.18, **5**) and isocephems (Fig. 3.18, **6**, **7**) with antibacterial activity were discovered at Hoffmann-La Roche during a chemical program developing broad-spectrum beta-lactamase inhibitors [11, 67]. Only those compounds with small 7-acylamino side chains had significant antibacterial activity, but all were inhibitors of serine beta-lactamases. The antimicrobial spectrum resembles that of third-generation cephalosporins, as they are more active against Gram-negative bacteria than Gram-positive organisms. They are, however, more active against strains with class A ESBLs or class C cephalosporinases than are the cephalosporins because of the beta-lactamase inhibitory properties. None of these more exotic variations of the cephem nucleus has yet been pursued in clinical trials.

3.4 Carbapenems

Beecham Laboratories developed a screen for beta-lactamase inhibitors towards the end of the 1960s that furnished a number of molecules that had a profound influence on antibiotic chemotherapy. Among these were the olivanic acids (e.g., MM 17880, Fig. 3.19), which were identified in fermentation broths of *Streptomyces olivaceus* Waksman, 1923 [33]. An extensive series of fermentation experiments resulted in the identification of several members of the olivanic acid family of carbapenems [32]. At about the same time, the Merck group was screening fermentation broths for inhibitors of cell-wall biosynthesis and discovered several representatives of the thienamycin family of carbapenems [8] in fermentation broths of *Streptomyces cattleya* Kahan et al. 1979. More than 50 carbapenems, belonging to at least five families (Fig. 3.19), have now been discovered in microbial fermentation broths using variations of the original screens. Most are very effective beta-lactamase inhibitors [107] but are too unstable for clinical use.

Chemists at Merck found that the solution instability of thienamycin could be overcome by derivatization to N-formimidoyl thienamycin (imipenem, Fig. 3.20) (Leanza et al. 1979). They were, however confronted with another problem, in that imipenem is a substrate for renal dehydropeptidase (DHP-I), as are all naturally derived carbapenems. The Merck team developed cilastatin, which is a competitive inhibitor of DHP-I that can protect imipenem against the enzyme. A fixed 1:1 combination of the two compounds was launched in 1985 under the name of Primaxin® (Zienam® in Europe).

The potent antimicrobial activity and beta-lactamase stability of the carbapenems attracted a lot of attention, firstly to try to improve fermentation yield, for the streptomycetes do not produce large amounts of any single compound, and then to attempt total synthesis of the system. Panipenem from Daiichi Sankyo Co., followed some years after Primaxin, in 1993: it, too, is marketed as a fixed combination, in this instance with the renal transport inhibitor betamipron, because of its instability towards the DHP-I enzyme. A solution to the DHP-I problem came with the discovery by the Merck team that synthetic analogues with a 1β-methyl substituent were

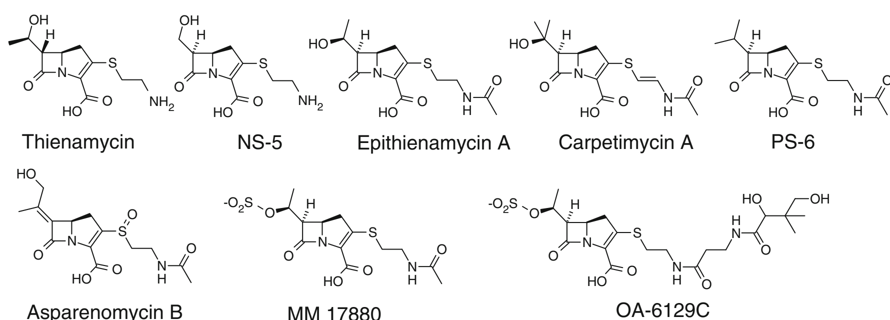

Fig. 3.19 Representative natural carbapenems

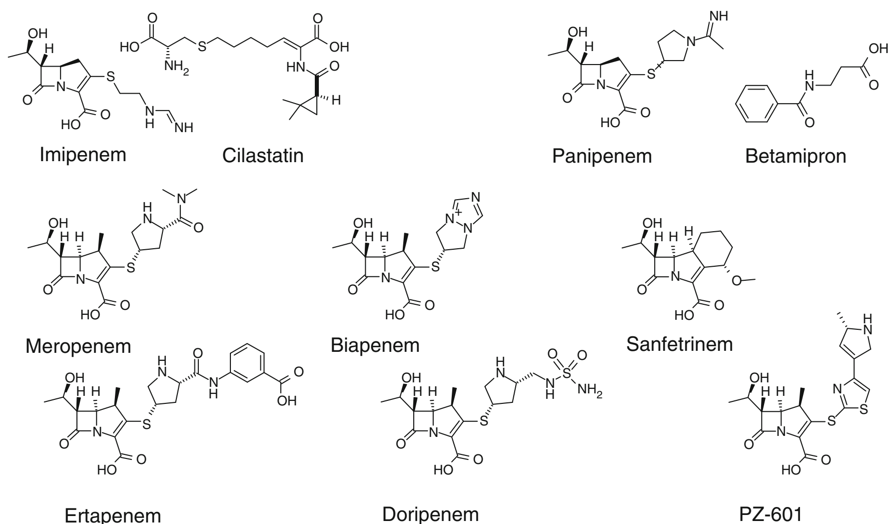

Fig. 3.20 Synthetic carbapenems and combination partners

stable towards DHP-I but still possessed potent antibacterial activity [10]. This observation paved the way for a second generation of carbapenems that could be administered as a single substances, the first of which was meropenem, to be followed by ertapenem and doripenem (Fig. 3.20). Sanfetrinem is an unusual carbapenem discovered at Glaxo Wellcome that has high stability towards many β-lactamases and to human renal DHP I. It has a broad spectrum of activity against Gram-positive and Gram-negative bacteria including penicillin-resistant *Streptococcus pneumoniae*.

PZ-601, formerly SM-216601 discovered at Sumitomo Pharmaceuticals [149], is an experimental compound in clinical development for treatment of infections caused by resistant bacteria, including MRSA and ESBL-producing Enterobacteriaceae. It has the same kind of long, relatively hydrophobic side chain, with a terminal basic group, as found in the anti-MRSA cephalosporins.

3.5 Penems and Oxapenems

Analogue synthesis was also extended to the preparation of penems [159] and oxapenems [45], but not many of these attempts have yielded useful antibiotics, largely because of stability and toxicity problems. Of the few penems that have been advanced through clinical studies, faropenem (Fig. 3.21) was marketed in Japan by Daiichi Asubio Pharma in 1997 as the sodium salt and ritipenem was introduced in Japan by Tanabe Seiyaku Co., as the sodium salt and as acetoxymethyl ester for oral administration. The orally administered ester faropenem medoxomil has been under investigation in the USA for use in community-acquired respiratory tract infections

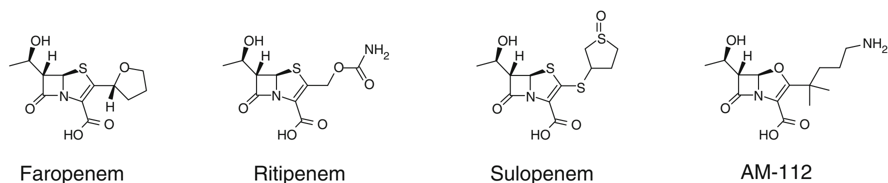

Fig. 3.21 Synthetic penems and an oxapenem

and skin infections. Sulopenem was discovered in the 1980s but not pursued until recently: Pfizer investigated sulopenem and a pro-drug (PF-03709270) for use in community-acquired pneumonias that require hospitalization. The penems have similar beta-lactamase stability to imipenem but are stable towards DHP-I. They have a broad spectrum of antibacterial activity but are not active against MRSA, enterococci, or *P. aeruginosa.*

The first oxapenems were very unstable but showed potential as beta-lactamase inhibitors. Hans Pfaendler and his team synthesized more stable analogs (including AM-112) that have some antibacterial activity against staphylococci and are potent inhibitors of serine beta-lactamases [78, 117]. AM-112 and its analogs are being profiled by Amura for use in combination with established beta-lactams.

3.6 Monocyclic Beta-Lactams

At about the same time that novel screens were being used to identify beta-lactamase inhibitors, workers at the Takeda company used a mutant strain of *E. coli* that was hyper-susceptible to beta-lactams to screen fermentation broths for inhibitors of cell wall biosynthesis [12, 14, 69]. They identified the first members of the nocardicin family of antibiotics from fermentation broths of an organism the referred to as *Nocardia uniformis tsuyamanensis* [75]. The antibiotic has moderate in vitro activity against Gram-negative bacteria including *Proteus* and *Pseudomonas*. Later, new monocyclic beta-lactam antibiotics with a formylamino substituent, named formadicins A, B, C and D. were found in fermentation broths of *Flexibacter alginoliquefaciens* YK-49. Formadicins have narrow antibacterial spectra including some species of *Pseudomonas*, *Proteus* and *Alcaligenes* but are stable to beta-lactamase because of the 3α-formamido substituent. No analogues have yet been brought to the clinic.

The Squibb Company used an assay based on the detection of beta-lactamase induction in a hypersensitive strain of *Bacillus licheniformis* to screen for novel beta-lactams [134]. Besides a number of new carbapenems, cephamycins and the 7-formamidocephems dealt with above, they found several new families of monobactams (e.g., SQ 26,180 [156] and SQ 26,970 from *Agrobacterium radiobacter* [160]). Screening at Beecham, using a differential assay for antibacterial activity against a

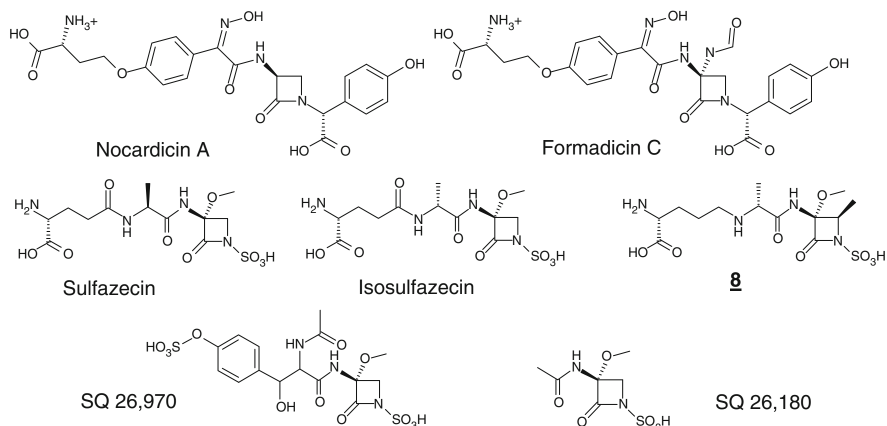

Fig. 3.22 Representative natural monocyclic beta-lactams

pair of *Acinetobacter* strains (one hypersensitive and the other resistant), yielded a number of new monobactams including compound **8** in Fig. 3.22 [32].

Squibb chemists found the azetidinone to be readily synthesized and followed up their screening discoveries with a series of totally synthetic molecules that resulted in aztreonam [135], which is marketed for use against infections caused by Gram-negative bacteria. Aztreonam (Fig. 3.23) has no useful activity against Gram-positive or anaerobic organisms but has potent activity against most species of aerobic Gram-negative bacteria. Carumonam, discovered at Takeda, was also investigated by Hoffmann-La Roche for use against infections caused by Gram-negative organisms: it has a very similar spectrum and beta-lactamase stability to aztreonam [76]. Aztreonam and carumonam are inhibitors of class C beta-lactamases, are hydrolyzed by the class A ESBLs but, remarkably, only very slowly hydrolyzed, if at all, by metallo-beta-lactamases. The latter is a unique property of the monocyclic beta-lactams. The inhibition of class C beta-lactamases is a common property of monobactams with a (S)-methyl group (as in aztreonam) or bulky (R)-substituent (as in carumonam) at the C4 position of the azetidinone ring [52, 65, 126] and is due to interference of the substituent with residues in the active site that prevents a rotation about the C3-C4 bond necessary for access of the water molecule that would hydrolyze the acyl-enzyme complex [69].

It was also found that the beta-lactam ring could be activated towards nucleophilic attack at the β-carbonyl by a variety different electronegative functions attached to the azetidinyl nitrogen. There followed the oxymazins (e.g., oximonam and its ester gloximonam) and monosulfactams (e.g., tigemonam), which have an O-atom attached to the azetidinone as well as compounds such as pirazmonam [17], which relate perhaps more to the nocardicin family in having a C-atom attached to the azetidinone. Like the monobactams, the oxymazins and monosulfactams have potent activity against aerobic Gram-negative bacteria, with good beta-lactamase

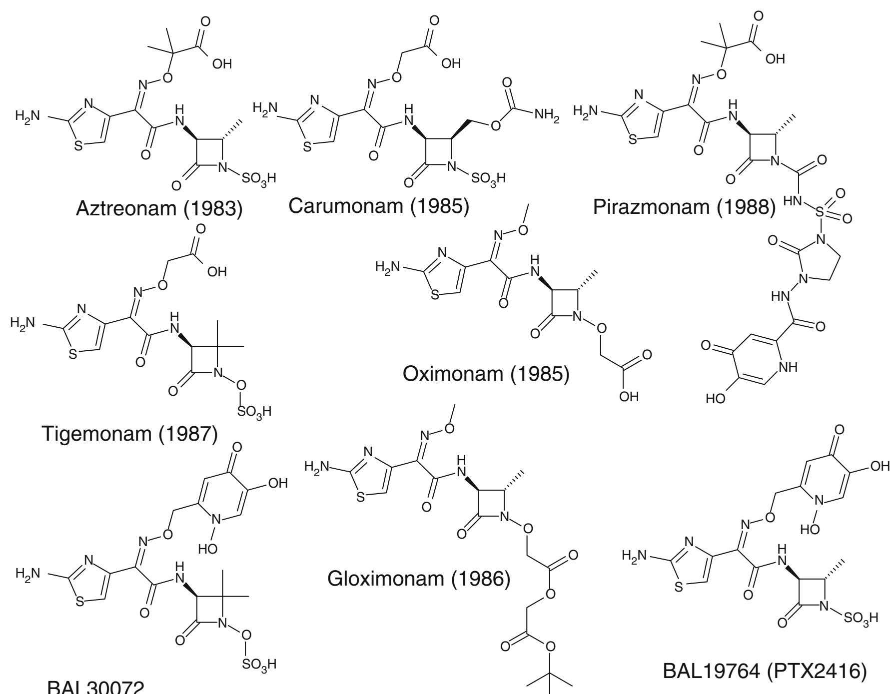

Fig. 3.23 Synthetic monocyclic beta-lactams

stability, but little activity against anaerobes and Gram-positive bacteria. Tigemonam has rather weak activity against *P. aeruginosa* compared to aztreonam or carumonam. Tigemonam, as its choline salt, and gloximonam were investigated for oral administration because of their greater bioavailability. Pirazmonam marks an attempt to improve activity against *P. aeruginosa* using an iron-chelating ("siderophore") group that might lead to its recognized and taken up by one of the essential iron-uptake systems. *P. aeruginosa* secretes a number of natural siderophores that bind and solubilize ferric or ferrous iron: the complexes are recognized by receptors in the outer membrane and then transported into the periplasm. Several attempts have been made to introduce natural siderophores or mimetic functional groups such as the hydroxypyridinone moiety used in pirazmonam, in order to exploit the endogenous transport systems [95]. BAL19764 (formerly Syn2416 or PTX2416) is an experimental monobactam (analogue of aztreonam) with such a siderophore moiety. It was shown to have enhanced activity against *P. aeruginosa* under conditions where the siderophore-uptake systems are expected to be induced. BAL30072 is an experimental monosulfactam (analogue of tigemonam) with the same siderophore. It has potent activity against *Acinetobacter* spp., which the monobactams do not, as well as *P. aeruginosa*, which tigemonam does not. It is under investigation for use against infections caused by multidrug-resistant Gram-negative bacteria.

3.7 Antibiotic Combinations with Beta-Lactamase Inhibitors

The screen for β-lactamase inhibitors devised at Beecham yielded a number of potent agents, especially carbapenems (see above). During the validation of their screening method, the Beecham group tested the cephamycin-producing *Streptomyces* cultures deposited by Merck and Lilly and discovered a potent beta-lactamase inhibitor in fermentation broths of *S. clavuligerus* [119]. This was isolated and characterized as clavulanic acid (Fig. 3.24), which has become the most widely used β-lactamase inhibitor in combinations with amoxicillin (Augmentin®) and ticarcillin (Timentin®). Clavulanic acid is a potent inhibitor of class A beta-lactamases and also inhibits some class D enzymes: it has little activity against class C, and no activity against class B beta-lactamases. The combination with amoxicillin therefore has improved activity against penicillinase-producing staphylococci and Gram-negative organisms, especially *E. coli* and *K. pneumoniae*, which have ESBLs. The combination with ticarcillin has improved activity against some strains of *Acinetobacter* spp., (against which clavulanic acid has modest antibacterial activity) as well as ESBL-producing Enterobacteriaceae.

The potent beta-lactamase inhibitory properties of clavulanic acid prompted chemical modification around the clavam nucleus as well as a search for synthetic molecules with similar activity. Sulbactam was prepared at Pfizer starting from 6-APA [52] and also showed interesting activity against class A beta-lactamases. Although not as potent as clavulanic acid, sulbactam is considerably more stable and is even available in Germany as a stand-alone product (Combactam®) for use in combination with beta-lactam antibiotics. Like clavulanic acid, it has little antibacterial activity itself, except against *Acinetobacter* spp., where it has quite respectable activity against strains that do not express beta-lactamases. Fixed combinations with ampicillin (Unacid®) and cefoperazone (Sulperazone®) are marketed in a number of countries and the mutual prodrug with ampicillin is also available (sultamicillin, Unasyn®). Tazobactam from Wyeth is a more recent extension of the penam sulfone class of inhibitors that has somewhat more potent activity than sulbactam and is active against some class C beta-lactamases [36]. It is available as a fixed combination with piperacillin (Zosyn®), which is widely used in hospitals for

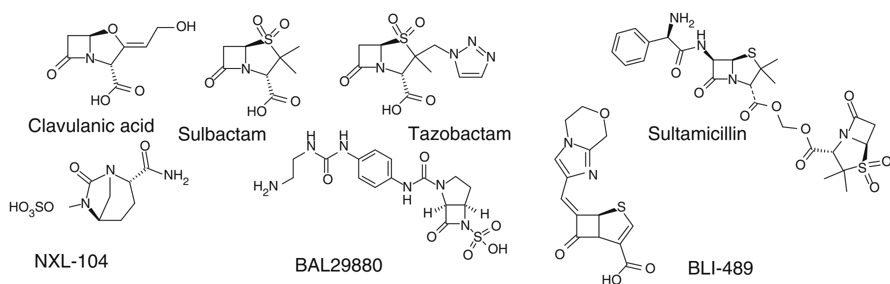

Fig. 3.24 Beta-lactamase inhibitors used in marketed combinations or recently under clinical investigation

treatment of infections caused by resistant Gram-negative pathogens, especially *P. aeruginosa*. Calexa have indicated that they would consider profiling a combination of their investigational cephalosporin CXA-101 with tazobactam.

There have been numerous attempts to discover broad-spectrum inhibitors that cover all of the serine beta-lactamases (classes A, C and D) but, as yet, none has come to the market. These experimental inhibitors are beyond the scope of this chapter and the reader should consult recent reviews for more details [33, 96, 109]. Three of experimental inhibitors have recently been profiled in combinations for treatment of resistant pathogens. NXL-104 (formerly AVE1330A) from Novexel is a unique representative of a novel class of diazabicyclo(3.2.1)octanones that are broad-spectrum inhibitors, active against class A, C and some D enzymes [23]. It is being investigated in combination with ceftazidime for use against Gram-negative pathogens [94] and as a combination with Forest's cephalosporin ceftaroline, which would make a broad-spectrum agent [42, 43]. BAL29880, a specific class C beta-lactamase inhibitor [65], was proposed as part of a unique triple combination, comprising also the siderophore monobactam BAL19764 (PTX2416) and clavulanic acid [112]. The combination exhibited activity against many beta-lactamase producer strains included those with class A ESBLs (protection afforded by clavulanic acid), class B (due to the intrinsic stability of the monobactam), class C (protection afforded by BAL29880) and class D enzymes (due to the intrinsic stability of the monobactam). BLI-489 from Wyeth [148] is a broad-spectrum inhibitor from the alkylidene penem family of inhibitors discovered at Beecham [105]. It is being profiled in combination with piperacillin as a successor to Wyeth's piperacillin-tazobactam combination [119].

3.8 Beta-Lactam Mimics

The search for easily synthesized surrogates of the β-lactam nucleus started almost as soon as the structure of penicillin was known but were not successful [49]; it was not until the 1980s that compounds with biological activity started to emerge. Lactivicin (Fig. 3.25) was discovered at Takeda Laboratories while screening fermentation broths of *Empedobacter lactamgenus* and *Lysobacter albus* for inhibitors of cell wall biosynthesis using a hypersensitive *E. coli* strain [59]. It was the first non-beta-lactam inhibitor of cell wall biosynthesis to be found: however, not only is it an inhibitor of the essential penicillin-binding proteins, but it is also a substrate for beta-lactamases and its activity is therefore limited. Nevertheless, its discovery prompted the investigation of synthetic analogues that might be more stable [92], including those such as **9** and **10** with side chains found in penicillins and cephalosporins [99; 135], which had some biological activity, and pyrrazolidinone derivatives such as **11** and **12**, which proved to be much less active [139].

In the mid-1980s, Jack Baldwin and his group were investigating the γ-lactam analogues of penems and carbapenems. The carbapenem analogue **13** proved not to be reactive enough to have biological activity but the analogues of the penems

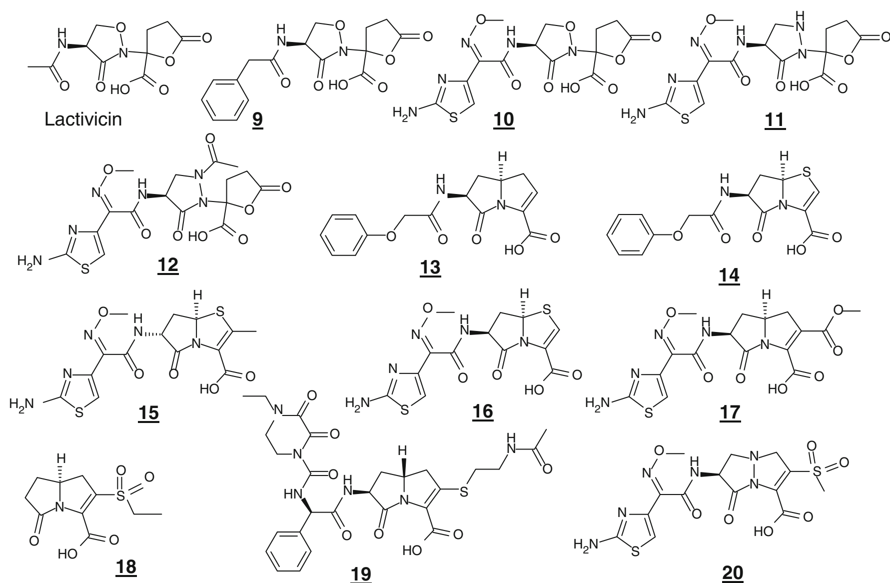

Fig. 3.25 γ-lactam analogues with antibacterial activity

synthesized by Woodward (see above) had better reactivity [15]. Eventually compound **14** was found with weak but broad-spectrum antibacterial activity, [16]. γ-Lactams were also pursued in the Lilly group, who also found that the analogues of penems (e.g., **15** and **16**) or carbapenem analogues electron-withdrawing substituents (e.g., **17** and **18**) had sufficient reactivity to confer weak biological activity [26, 27]. The Takeda group also investigated the γ-lactam analogues of carbapenems (e.g., **19**) with electron-withdrawing substituents at the 3-position [66], which are necessary to active the γ-lactam sufficiently for biological activity. The Lilly group also investigated (3.3.0) fused pyrazolidinones (e.g., **20**), which with appropriate electron-withdrawing groups at the 3-position, proved to have significant biological activity [142]. As yet, none of these surrogate molecules has been brought to the market.

3.9 Pharmacology

The beta-lactam antibiotics act on a family of enzymes, comprising transpeptidases and carboxpeptidases, that is involved in essential steps in the biosynthesis of the bacterial cell wall and its maturation [110]. The beta-lactam mimics part of the natural substrate of these enzymes and covalently modifies the target protein so that it cannot play its role in cell growth [155]. The target proteins (penicillin-binding

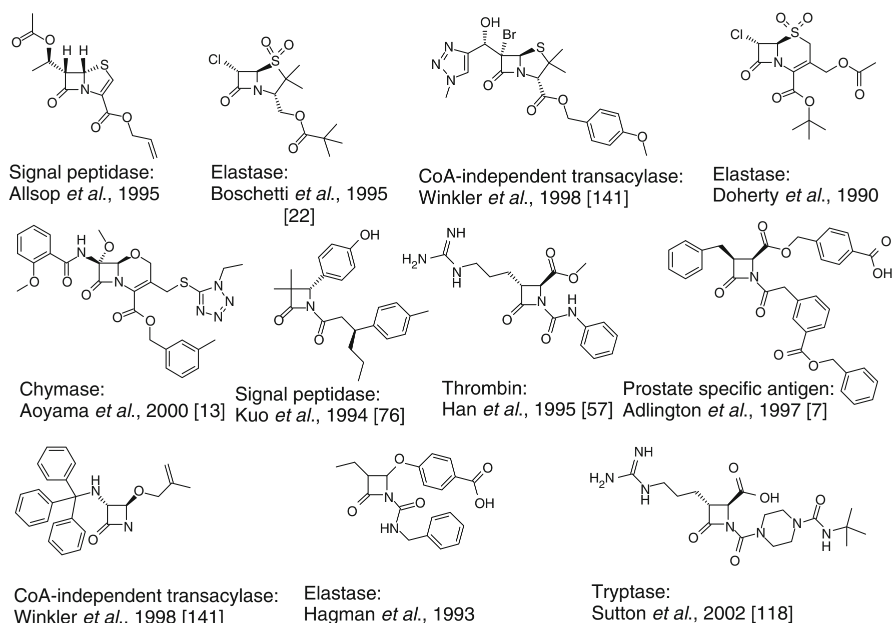

Fig. 3.26 Beta-lactams targeting proteins other than penicillin-binding proteins

proteins) are unique to bacteria, with a series of homologous sub-families being found in all bacteria with peptidoglycan cell walls. Thus, there is very little concern about specificity for bacteria versus reaction with a human equivalent of the target. In a broader sense, the penicillin-binding proteins, and the related serine beta-lactamases, belong to the serine protease family and some beta-lactams do inhibit other serine proteases. Indeed, following pioneering work by the Merck group, beta-lactams have been investigated as inhibitors of a number of other proteases including bacterial signal peptidase [9, 77], and other enzymes human cytomegalovirus protease [160] as well as human enzymes such as: γ-aminobutyric acid aminotransferase chymase [13], coenzyme A-independent transacylase [158], elastase [22, 49, 76], thrombin [58, 117], tryptase [130], fatty acid amide hydrolase [150], and phospholipase A2 [35]. The beta-lactams that have been found most versatile have been monocyclic azetidinones, but a number of bicyclic penem and cephem derivatives have also been described (see Fig. 3.26 for some examples). It has also been reported that many beta-lactam antibiotics are potent stimulators of GLT1 expression and thereby can exert a neuroprotective effect [118]. For example, ceftriaxone increased both brain expression of GLT1 and its biochemical and functional activity. Ceftriaxone was neuroprotective in vitro when used in models of ischaemic injury and motor neuron degeneration and when used in a mouse model of the fatal disease amyotrophic lateral sclerosis, the drug delayed loss of muscle strength and increased survival.

Generally, the constraints on functionalization of the monocyclic beta-lactams, and the stereochemistry of the bicyclic core as well as its functionalization, imposed by the requirements for biological activity are quite different between the beta-lactam antibiotics and the beta-lactams targeting human enzymes. Thus, there is little cross-reactivity between the different series and most of the adverse events linked to the use of the beta-lactam antibiotics are idiosyncratic reactions associated with individual molecules, as mentioned at the appropriate places in the preceding text.

The one common class effect associated with beta-lactams (≥1% of patients) is the induction of allergic responses including hypersensitivity, nausea, rash, and urticaria. Infrequent adverse effects (0.1–1% of patients) include fever, vomiting, erythema, dermatitis, and angioedema. Although penicillin is still the most commonly reported allergy, less than 20% of all patients that believe that they have a penicillin allergy are truly allergic to penicillin. Allergic reactions are primarily due to antibodies against the penicillin nucleus, but some patients have exhibited reactivity to the side chain, especially that of amoxicillin, in the absence of reaction towards penicillin G. Cephalosporin allergy occurs in about 10% of patients with penicillin allergy but in less than 2% of the general population. Anaphylactic reactions are extremely rare (<0.02%). The majority of allergic reactions appear to be due to antibodies to specific side chains rather than the cephalosporanic acid nucleus. The cephalosporins that employ the same side chains as the penicillins, tend to show the highest cross-reactivity with penicillins with 12–38% of cases showing clinical cross-reactivity. Carbapenems also show a high incidence of cross-reactivity with penicillins, whereas the carbacephem Lorcarbacef, and the monobactam aztreonam show much less. Aztreonam has the same side chain as the third-generation cephalosporin ceftazidime and cross-reactivity is more likely between these two compounds.

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Chapter 4
Review of the Quinolone Family

George A. Jacoby and David C. Hooper

4.1 Introduction

The quinolone family of antimicrobial agents was discovered inadvertently as a byproduct in the synthesis of chloroquine, an antimalarial. Nalidixic acid, the first member of the class to be used clinically, was approved in the United States in 1967 as an oral agent to treat urinary tract infections but had limited use because of only moderate activity, a strictly gram-negative spectrum of action, high protein binding limiting systemic use, and the ready appearance of bacterial resistance. Further manipulation of the molecule produced oxolinic acid, piromidic acid, and cinoxacin with increased gram-negative potency, and pipemidic acid with enhanced activity against *Pseudomonas aeruginosa*; but the most important modification was the addition of fluorine at the 6-position, which dramatically increased potency.

The 6-fluoroquinolones, especially those with a piperazinyl group or other amine-containing ring structure at position 7, combine this potency increase with enhanced gram-negative spectrum and include six agents currently available in the United States: norfloxacin (first approved in 1986), ciprofloxacin (1987), ofloxacin (1990), levofloxacin (1996), moxifloxacin (1999), and gemifloxacin (2003). Tens of thousands of other fluoroquinolones have been synthesized and evaluated. Some (temafloxacin, sparfloxacin, grepafloxacin, trovafloxacin, and gatifloxacin) have even been approved for use but withdrawn because of toxicity. Development continues especially for fluoroquinolones with enhanced activity against gram-positive organisms and anaerobes, fewer side effects, longer half-lives of elimination allowing once daily therapy, and reduced risk of resistance.

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4.2 Structure Activity Relationships

Figure 4.1 shows the general quinolone structure while Fig. 4.2 gives specific structures for selected quinolones. Nalidixic acid, trovofloxacin, and gemifloxacin have N rather than C at position R_8 and hence are properly 1,8-naphthyridones rather than pyridine-β-carboxylic acid derivatives, but all will be termed quinolones. The fluoroquinolones have a fluorine atom at R6. Garenoxacin and a few other investigational quinolones lack this substituent. The 3-carboxyl and 4-carbonyl groups and the double bond at 2–3 are required for antibacterial activity. The N at position 1 can be substituted with various groups including cyclopropyl (ciprofloxacin, grepafloxacin, moxifloxacin, gatifloxacin, and garenoxacin), fluorocyclopropyl, a benzoxazine ring also linked at position 8 (ofloxacin, levofloxacin), ethyl (norfloxacin, nalidixic acid), and difluorophenyl (trovafloxacin). An aromatic nucleus is found at position R7, either a piperazinyl derivative (norfloxacin, ciprofloxacin, levofloxacin, others), a pyrrolidyl derivative (gemifloxacin), or a bicyclic group (trovofloxacin, moxifloxacin). Various substituents are found at R8 including F (sparfloxacin), Cl (clinafloxacin), OCH_3 (gatifloxacin, moxifloxacin), and OCHF2 (garenoxacin). Some quinolones have a NH_2 (sparfloxacin), CH_3, or OCH_3 group at R5. Ofloxacin has a center of asymmetry and is racemic. Levofloxacin is the more active *l* isomer.

The 4-keto and 3-carbonyl groups are involved in binding the bacterial targets and are hence essential. Penetration into the bacterium and target binding are facilitated by the F at position 6 and the nature of the substituent at position 7. The steric hindrance of the substituents at positions 1 and 5 has a global effect on antibacterial activity. Bicyclic molecules with the core quinolone two-ring structure (nalidixic and

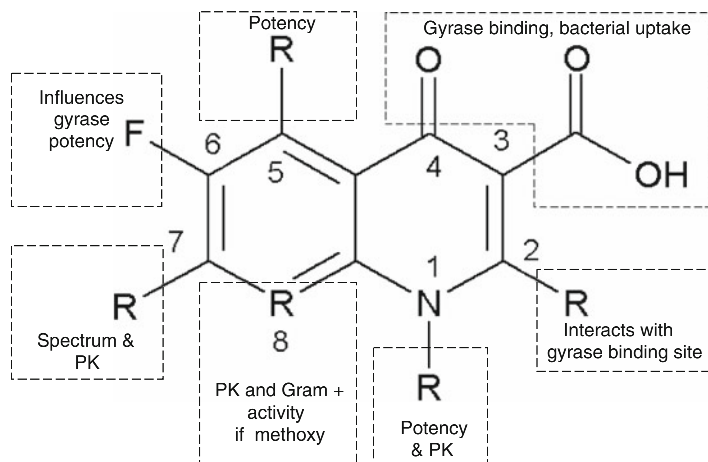

Fig. 4.1 Structure–activity relationships. If R8 is a carbon atom the molecule is a quinolone, and if R8 is a nitrogen atom the molecule is a naphthyridone. General influence of site modifications are listed on the figure

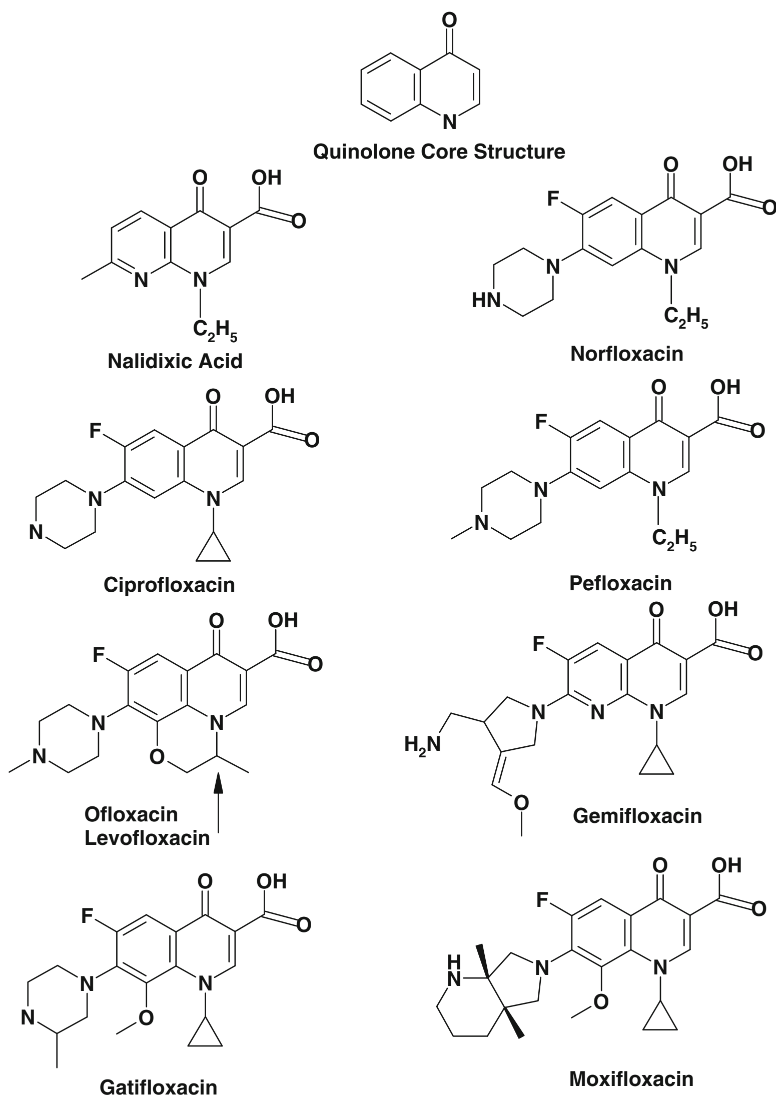

Fig. 4.2 Structure of representative quinolones. The asymmetric center in ofloxacin/levofloxacin is indicated by an arrow

oxolinic acids) are active only on *Enterobacteriaceae.* Molecules with an additional ring structure at position 7 (most of the rest in Fig. 4.2) have an increased gram-negative spectrum. Some with a non-piperzinyl ring at position 7 (moxifloxacin, gemifloxacin) or a tricyclic structure (ofloxacin and levofloxacin) have an even broader spectrum including streptococci, *Streptococcus pneumoniae*, and some strict anaerobes. Most fluoroquinolones have two ionizable groups: the 3-carboxyl and a

protonizable site on the heterocyclic side chain at position 7. At neutral pH nalidixic acid and many of its close relatives are acidic while newer quinolones are zwitterionic, which become important for membrane permeation. Permeation is also influenced by the lipophilicity of the molecule. Some quinolones are strongly hydrophilic (norfloxacin, ciprofloxacin) or strongly hydrophobic (nalidixic and oxolinic acids, clinafloxacin, trovafloxacin). Most in Fig. 4.2 are weakly hydrophilic.

4.3 Mechanism of Action

The quinolone targets are the essential bacterial enzymes DNA gyrase [66, 67] and DNA topoisomerase IV [100], which control the topology of the bacterial chromosome and thus facilitate DNA replication, recombination, and transcription [47]. Both enzymes are large, complex tetramers composed of two pairs of identical subunits. The subunits of DNA gyrase are 97-kDa GyrA and 90-kDa GyrB, encoded by the *gyrA* and *gyrB* genes. The corresponding subunits of topoisomerase IV are 75-kDa ParC and 70-kDa ParE. DNA gyrase introduces negative superhelical twists into DNA, can catenate and decatenate covalently closed circular DNA molecules, and unties knots in double-stranded DNA. DNA gyrase is also responsible for removing positive superhelical twists that accumulate ahead of the DNA replication fork. DNA topoisomerase IV mainly decatenates DNA and can also remove positive and negative supercoils.

Both enzymes make a pair of staggered, single-strand breaks or nicks in DNA and bind covalently via a pair of active-site tyrosine residues to the 5'-ends of the cleaved DNA [50, 133]. In an ATP-dependent reaction a second double-stranded DNA strand is passed through the break, which is then resealed [98, 200]. Quinolones act by binding to the DNA/gyrase or DNA/topoisomerase complex, preventing religation and thus forming a quinolone-gyrase-DNA complex that blocks DNA replication and cell growth.

This bacteriostatic effect is followed by a bactericidal one. With some quinolones, inhibition of protein synthesis by chloramphenicol blocks the lethal effect, suggesting that synthesis of a "suicide factor" is necessary for lethality. With other quinolones protein synthesis is not required, as though DNA released from quinolone-gyrase-DNA complexes with double-strand breaks was directly bactericidal [50, 114]. Structures associated with chloramphenicol-insensitive killing and a rapid bactericidal effect include the N-1 cyclopropyl and C-8 methoxy groups, and the nature of substituent at C-7 [50]. A role for peroxide in the bactericidal action of quinolones has recently been proposed since deficiency in bacterial peroxidase enhances quinolone lethality [202]. In *S. pneumoniae* there is evidence that autolytic amidases are involved in the bactericidal activity of quinolones [144].

A few pathogens (e.g., *Mycobacterium tuberculosis* and *Treponema pallidum*) are able to function with only DNA gyrase, but most bacteria have both enzymes. In gram-negative bacteria gyrase is more susceptible to inhibition by quinolones than is topoisomerase IV, whereas, in gram-positive bacteria topoisomerase IV is usually

the prime target, and gyrase is intrinsically less susceptible. This preference can be influenced by fluoroquinolone structure. For example, for *Streptococcus pneumoniae* addition of a bulky benzenesulfonylamido group at C-7 shifts preference from topoisomerase IV to gyrase [4]. Presence of a C-8 halogen or methoxy group has a similar effect [49].

Eukaryotic cells also contain topoisomerases with similar domain structure and limited amino acid sequence homology to the bacterial quinolone targets; however, those antibacterial quinolones in current clinical use have only minimal activity against mammalian topoisomerase II [89].

4.4 Mechanisms of Resistance

Rates of quinolone resistance have risen steadily over the past two decades paralleling increasing use of fluoroquinolones [137] and also the emergence of plasmid-mediated quinolone resistance [118]. For example, while the frequency of fluoroquinolones resistance in *Escherichia coli* is <10% in many parts of the world, in China more than 50% of *E. coli* clinical isolates are now ciprofloxacin-resistant [104] as are 48% of *Klebsiella pneumoniae* isolates [65]. Resistance is rising as well for the pneumococcus and may appear within days of starting therapy [112].

Bacteria acquire resistance by spontaneous mutations in chromosomal genes that alter the target enzymes, DNA gyrase, and topoisomerase IV, or affect drug accumulation in the cell [88]. Recently, several mechanisms for plasmid-mediated quinolone resistance have been recognized in gram-negative bacilli. These horizontally acquired genes do not themselves confer clinical resistance but enable survival under drug exposure and facilitate the selection of additive chromosomal mutations [92]. Lateral DNA transfer may be partly responsible for quinolone resistance in the pneumococcus and *Streptococcus pyogenes* as well [51, 186].

4.4.1 Chromosomal Mechanisms

Since quinolones have two target topoisomerases in most bacteria that vary in susceptibility, resistance mutations will be selected first in the more sensitive target. Furthermore, the level of resistance produced by mutation in the primary target will depend on the susceptibility of the second target. The less intrinsically susceptible the second topoisomerase, the higher the resistance level that can occur with mutation in the primary target, and the more balanced is the quinolone attack on both targets, the more difficult for the cell to mutate in one step to high-level resistance [85, 147, 188].

The lowest concentration of an antibacterial agent that prevents the appearance of mutants in a sizeable population of bacteria (10^{10} cells) has been termed the mutant prevention concentration (MPC) [48, 214]. At concentrations lower than

the MPC down to the lowest preventing growth (the MIC) mutant selection can occur (the mutant selection window). If the serum or tissue concentration of a quinolone can be maintained at a level greater than the MPC, no selection of resistance should occur. In addition, the best quinolone will be one with the narrowest mutant selection window, and the best schedule for drug administration should be one that maintains the quinolone concentration above the MPC for as much of the dosage interval as possible, thus minimizing the opportunity for mutant selection.

In gram-negative bacteria resistance mutations in GyrA are seen more often than mutations in the GyrB, ParC, or ParE subunits, reflecting the higher MICs produced by mutations in GyrA as compared to alterations in the other target enzyme subunits. In *E. coli* resistance mutations cluster at the amino terminus of GyrA in the quinolone-resistance-determining-region (QRDR) between amino acid positions 67 and 106 and are thus located near to the Tyr122 residue, which is covalently bound to DNA [211]. In the crystal structure of a 59-kDa N-terminal fragment of GyrA, the amino acids of the QRDR formed a positively charged surface along which DNA has been shown to bind [45, 131]. Resistance mutations are believed to alter the structure of the site for quinolone binding at this interface, and have indeed been shown to cause reduced quinolone binding to gyrase-DNA complexes [14, 204]. Two amino acids, Ser83 and Asp87, are most commonly mutated in resistant isolates. A Ser83Leu alteration produces a 128-fold increase in resistance to nalidixic acid but only a 16- to 32-fold increase in resistance to many newer quinolones, paralleling the greater propensity for resistance selection with nalidixic acid. Recently the crystal structure of a complex of ParC and ParE fragments of topoisomerase IV, DNA, and moxifloxacin has been solved and demonstrates the proximity of the equivalent Ser and Asp residues to the moxifloxacin binding site, thus defining directly the quinolone binding site on the enzyme-DNA complex and the molecular mechanism by which changes in these amino acids can reduce quinolone binding [107].

In other gram-negative bacteria, mycobacteria, and *Chlamydia trachomatis* substitutions at amino acid positions equivalent to Ser83 and Asp87 are also associated with resistance. For many gram-positive bacteria DNA gyrase is a secondary target and mutations in the ParC (Ser80Phe or Ser80Tyr) and ParE subunits of topoisomerase IV occur first. The changes in GyrA, however, that cause incremental resistance when present together with ParC or ParE mutations in S*taphylococcus aureus, Streptococcus pneumoniae, Enterococcus faecalis*, or *Mycoplasma. hominis* are analogous to those seen in gram-negatives.

Increased expression of efflux pumps and decreased expression of proteins forming porin channels in the outer membrane of gram-negative bacteria promote resistance by limiting quinolone access to their cytoplasmic targets. Often the two mechanisms are regulated together. The efflux pumps can be grouped into superfamilies. Those affecting quinolone access in gram-negative bacteria include members of the resistance-nodulation-division (RND), major facilitator (MFS), and multidrug and toxic compound extrusion (MATE) superfamilies with only the MFS and MATE transporters having been found associated with resistance to quinolones

Table 4.1 Efflux pumps mediating decreased quinolone susceptibility[a]

Organism	Efflux component MFP[b]	RND[c]	OEP[d]	MFS[e]	MATE[f]	Regulatory gene(s)	References
E. coli	AcrA	AcrB	TolC			*acrR, marA, robA, soxS*	[96,145, 199, 210]
	AcrE	AcrF	?			*acrS*	[96],
					YdhE	?	[142, 210]
				MdfA		?	[56, 142, 210]
K. pneumoniae	AcrA	AcrB	TolC			*acrR, marA, ramA, soxS*	[22, 123, 181]
P. aeruginosa	MexA	MexB	OprM			*mexR*	[121, 158]
	MexC	MexD	OprJ			*nfxB*	[121, 159]
	MexE	MexF	OprN			*mexT*	[105]
	MexX	MexY	OprM			*mexZ*	[121, 128]
S. enterica serovar Typhimurium	AcrA	AcrB	TolC			*ramA, rma*	[30, 54, 61, 216]
S. maltophilia	SmeA	SmeB	SmeC			*smeRS*	[212, 213]
	SmeD	SmeE	SmeF			*smeT*	[3, 179]
S. aureus				NorA		*arlS*	[64, 139
				NorB		*mgrA*	[194]
				NorC		*mgrA*	[195]
S. pneumoniae				PmrA		?	[71]

[a] Adapted and updated from Poole [160, 161]
[b] Membrane fusion protein
[c] Resistance-nodulation-division superfamily
[d] Outer membrane efflux protein
[e] Major facilitator superfamily
[f] Multidrug and toxic compound extrusion superfamily

in gram-positive organisms [97, 160, 161]. Selected examples are shown in Table 4.1. Depending on the efflux system, other antimicrobial agents, dyes, detergents, disinfectants, and solvents may be actively exported as well, so that the broad substrate spectrum of these pumps provides cross-resistance to a number of structurally unrelated agents.

Some quinolone resistance mutations reduce the fitness of *E. coli* [116] or *Salmonella enterica* [73] slowing growth and reducing virulence. Mutations at loci regulating efflux are particularly likely to exert a fitness cost, but additional mutations can improve fitness and even boost resistance further.

Resistant clinical isolates usually have more than one mechanism for quinolone resistance with multiple mechanisms present in more resistant isolates and evidence that such strains have an increased mutation rate [106]. *E. coli* isolates with a ciprofloxacin MIC of 500 μg/ml or more can be found in clinical specimens and typically have mutations in both *gyrA* and *parC* targets as well as in the increased expression of the AcrA efflux pump [31, 132]. In fact, known mechanisms account for fluoroquinolones resistance in only 50–70% of such isolates implying that additional mechanisms remain to be discovered [132].

4.4.2 Plasmid-Mediated Mechanisms

Plasmid-mediated resistance to quinolones, long thought unlikely to exist [36], was discovered in a clinical strain of *Klebsiella pneumoniae* isolated in 1994 in Alabama that could transfer low-level quinolone resistance along with a plasmid carrying resistance to other antibiotics to *E. coli* and other gram-negative bacteria [118]. In *E. coli* the plasmid caused an 8- to 32-fold decrease in susceptibility to nalidixic acid and fluoroquinolones. Although the level of resistance did not reach the CLSI defined breakpoint for loss of susceptibility, the plasmid raised the mutant protective concentration and facilitated the selection of fully quinolone-resistant mutants [92, 172]. It could also further augment resistance in *E. coli* strains with *gyrA*, *gyrB*, *parC*, *ompF*, *ompC*, or *marR* mutations [119].

The plasmid-encoded gene was named *qnr* and was found to encode a 219 amino acid protein belonging to the pentapeptide repeat family that could bind to and protect both DNA gyrase and topoisomerase IV from inhibition by ciprofloxacin [191–193]. Structural study of a pentapeptide repeat protein from mycobacteria (MfpA) that contributes to quinolone resistance revealed that it formed a rod-like dimer with surface charge and dimensions similar to double-stranded DNA [86] and could thus act as a DNA mimic. Qnr may have a similar structure but the two proteins differ in their action on DNA gyrase in vitro: MfpA only inhibits the enzyme [86, 124] while Qnr protects from quinolones at low concentrations and only inhibits at very high concentration [93, 124, 191]. Qnr also differs from MfpA in having a glycine residue that divides the protein into two domains.

Many Qnr proteins have subsequently been discovered. There are presently five Qnr families differing from each other by 40% or more in sequence (QnrA, QnrB, QnrC, QnrD, and QnrS) with minor sequence variation (<10%) defining alleles within each *qnr* family [91] *qnrB* with more than 20 alleles is the most varied. Strains containing plasmid-mediated *qnr* genes have been reported from around the world [26, 117, 170, 189] with the earliest currently known strains isolated in 1988, not long after the introduction of fluoroquinolones into clinical use [94].

qnr-like genes have also been found on the chromosome of both gram-positive [7, 173] and gram-negative bacteria [178]. In particular, many aquatic organisms (*Aeromonas* spp., *Photobacterium profundum*, *Shewanella* spp., and *Vibrio* spp.) [153, 155, 176] have *qnr*-like genes with *Shewanella algae* the leading candidate for the origin of *qnrA* [34, 156], and *Vibrio splendidus* a possible source of *qnrS* [25]. The native function of these *qnr*-like genes is not known.

The clinical importance of *qnr* is emphasized by a study in mice in which *qnrA* or *qnrS* was just as effective as a *gyrA* mutation in blocking ciprofloxacin treatment of *E. coli* urinary tract infections [2].

Most *qnr* genes are found on multiresistance plasmids linked to genes for extended-spectrum β-lactamase (ESBL), AmpC β-lactamase, and resistance to aminoglycosides, sulfonamides, trimethoprim, and other antimicrobial agents. Such linkage and the facilitation of higher level quinolone resistance mutations make therapy of *qnr*-containing organisms difficult.

Table 4.2 Susceptibility of *E. coli* J53 derivatives to quinolones

	MIC (μg/ml)		
	Ciprofloxacin	Levofloxacin	Nalidixic acid
CLSI susceptibility breakpoint	≤1	≤2	≤16
Resistance mechanism			
None	0.008	0.015	4
gyrA (S83L)	0.25	0.38	≥256
qnrA	0.25	0.5	16
aac(6')-Ib-cr	0.047	0.015	4
qepA	0.064	0.032	4

[a]Determined by etest on Mueller-Hinton agar

A second type of plasmid-mediated quinolone resistance was discovered while analyzing a Qnr-encoding plasmid that conferred unusually high ciprofloxacin resistance [171] The extra resistance was due to a second gene coding for a variant of a common aminoglycoside acetyltransferase that with two amino acid substitutions had acquired the ability to acetylate fluoroquinolones with an amino nitrogen on the piperazinyl ring, such as ciprofloxacin and norfloxacin. The responsible gene, *aac(6')-Ib-cr*, is part of a cassette found in an integron on plasmids worldwide [189].

A third variety of plasmid-mediated quinolone resistance (the newest to be recognized and currently the least common) is produced by a plasmid-encoded multidrug efflux pump. Two pumps that reduce fluoroquinolone susceptibility are presently known: QepA belonging to the major facilitator superfamily [149, 209] and OqxAB of the RND family [83, 102].

Table 4.2 shows the effect of these resistance mechanisms on the quinolone susceptibility of an *E. coli* laboratory strain. Plasmid-mediated *qnr* decreases susceptibility as much as a single mutation in *gyrA*, while *aac(6')-Ib-cr* and *qepA* provide less loss of susceptibility.

4.5 Antimicrobial Activity

Currently available quinolones are most active against aerobic gram-negative bacilli, especially members of the *Enterobacteriaceae* family and *Haemophilus* spp., and against gram-negative cocci such as *Neisseria* spp. and *Moraxella catarrhalis*. Ciprofloxacin is the most potent marketed fluoroquinolone against gram-negative bacteria. It and levofloxacin can be used against susceptible strains of *P. aeruginosa* or *Acinetobacter baumannii*, although resistance can emerge quickly when these quinolones are used alone in treating serious infections. With the exception of norfloxacin, fluoroquinolones are active against *S. aureus* and coagulase-negative staphylococci, but methicillin-resistant strains are very likely to be resistant to quinolones as well. Ciprofloxacin, norfloxacin, and ofloxacin have limited activity against streptococci and anaerobes, but levofloxacin, moxifloxacin, and especially gemifloxacin have greater gram-positive potency. *Bacillus anthracis* is fluoroquinolone susceptible, but enterococci are likely to be resistant. Fluoroquinolones are

also active against some mycobacteria, including *M. tuberculosis*, *Mycobacterium kansasii*, and *Mycobacterium fortuitum*, but not *Mycobacterium marinum*, *Mycobacterium chelonae*, *Mycobacterium abscessus*, or the *Mycobacterium avium-intracellulare* complex [90]. Other bacteria inhibited by fluoroquinolones include agents of atypical pneumonia such as *Legionella pneumophila*, *Mycoplasma pneumoniae*, and *Chlamydophila pneumoniae* and such genital pathogens as *Chlamydia trachomatis*, *Ureaplasma urealyticum*, and *M. hominis. T. pallidum*, however, is not susceptible.

4.6 Pharmacokinetics

Quinolones are well absorbed after oral administration, and for some can be administered parenterally. They are eliminated in the urine or in the bile. Some are metabolized in the liver. Peak serum concentrations usually occur within 1–3 h of an oral dose and reach 2.9 µg/ml for 500 mg ciprofloxacin bid, 5.2 µg/ml for 500 mg levofloxacin qd, and 3.5 µg/ml for 400 mg moxifloxacin qd [41]. Binding to serum proteins is generally low (26–37%) except for gemifloxacin (60%) [41]. Neither food nor achlorhydria have much effect on quinolone absorption, but enteral feedings given orally may reduce absorption [84]. Oral bioavailability is markedly reduced by coadministration of aluminum, magnesium, or calcium-containing antacids [164]. Sucralfate also reduces quinolone absorption, as does concurrent administration of $FeSO_4$, multivitamin preparations containing zinc [157], and the buffered formation of dideoxyinosine.

The volumes of distribution for quinolones are high and often exceed the volume of total body water, indicating accumulation in some tissues. Concentrations in prostatic tissue, feces, bile, lung, macrophages, and neutrophils usually exceed serum concentrations. Concentrations in urine and kidney tissue are especially high for quinolones with a major renal route of elimination (levofloxacin but not moxifloxacin). Concentrations in saliva, prostatic fluid, bone, and cerebrospinal fluid are generally lower than drug concentrations in serum. The distribution in the brain of levofloxacin and other quinolones is restricted by the action of multiple efflux transporters [190]. Ciprofloxacin, ofloxacin, pefloxacin, and probably other quinolones are concentrated in the breast milk of lactating women [70].

The half-lives of elimination from serum range from 3 h for norfloxacin and ciprofloxacin to 11 h for pefloxacin and 13 h for moxifloxacin, thus allowing once- or twice-daily dosing. Levofloxacin is eliminated predominantly by the kidneys while nalidixic acid, pefloxacin, and moxifloxacin are eliminated by nonrenal pathways. Other quinolones have mixed excretion by both renal and nonrenal routes. Renal clearance of norfloxacin, ciprofloxacin, and levofloxacin exceeds the glomerular filtration rate (GFR), indicating net tubular excretion. Renal clearance of pefloxacin is below or equal to GFR, indicating net tubular reabsorption [185].

Nalidixic acid and pefloxacin are metabolized in the liver with their active metabolites contributing to their antibacterial effects. Conversion of norfloxacin and

ciprofloxacin to less active metabolites accounts for 10–20% of elimination. There is little (<10%) biotransformation of levofloxacin. Hepatic metabolism and biliary excretion are the main routes of elimination for moxifloxacin [185].

Because of the pharmacokinetic differences dosage adjustment is necessary in the face of renal insufficiency for levofloxacin and, to a lesser extent, for ciprofloxacin but not for moxifloxacin. With mild to moderate hepatic disease no dose adjustment is thought to be necessary, even for moxifloxacin.

4.7 Pharmacodynamics

Quinolones exhibit concentration-dependent killing over a wide concentration range so that increases in the dose and drug concentration result in a faster and more extensive lethal effect. They have a post-antibiotic effect of 1–3 h in vitro that is enhanced in vivo, especially with adequate numbers of neutrophils. The PK/PD (pharmacokinetic/pharmacodynamic) parameter best predicting efficacy is the ratio of the 24-h area under the concentration-time curve (AUC_{24}) to minimum inhibitory concentration (MIC), although the peak concentration/MIC ratio also correlates well, since high-peak concentrations are important in preventing the emergence of resistant bacterial subpopulations [38].

Study of seriously ill patients (mainly with pneumonia) treated with intravenous ciprofloxacin indicated a significant improvement in outcome for AUC_{24}/MIC of 125 or greater and also a progressively more rapid bacterial eradication as the AUC_{24}/MIC ratio increased to ≥ 125 [62]. In a study of mixed infection types treated with levofloxacin, 100% pathogen eradication occurred with peak/MIC ratios of 12.2 or higher compared to 80.8% when the peak/MIC ratio was < 12.2, a breakpoint also corresponding to an AUC_{24}/MIC value of 100 [163]. In treating pneumococcal pneumonia a lower AUC_{24}/MIC ratio of 35 seems sufficient [5]. In terms of the mutant prevention concentration, the emergence of quinolone resistance should be prevented by an appropriate AUC_{24}/MPC or time > MPC [215]. The bacterial inoculum also plays a role with more resistance emerging with a high than a low *E. coli* inoculum despite comparable time within the mutant selection window in a mouse thigh infection model [60].

4.8 Toxicity

Adverse drug reactions to currently available quinolones are generally in the range of 2–10% with GI disorders (nausea, diarrhea, abdominal pain, vomiting, dyspepsia), CNS effects (dizziness, headache), and skin disturbances (mainly rash) predominating [10]. In addition there are reactions common to the class but more frequent and more severe with particular fluoroquinolones.

For example, grepafloxacin was withdrawn because an association with sudden cardiac death and cases of torsade de pointes related to QT interval prolongation. Similar cardiac effects have been reported with other fluoroquinolones but are rare [9]. Phototoxicity with most quinolones is uncommon, but potential serious phototoxicity has been seen with multiply-fluorinated and 8-halogenated fluoroquinolones and contributed to the abandonment of clinafloxacin and sparfloxacin. On the other hand, 8-methoxy derivatives are unlikely to cause phototoxicity [120]. Gatifloxacin was withdrawn because of an unacceptable incidence of hypo- and hyperglycemia [148]. Dysglycemia also occurs with levofloxacin but not with ciprofloxacin [8]. A few quinolones have had increased CNS side effects such as confusion, insomnia, and other sleep disturbances associated with ofloxacin or dizziness with trovafloxacin. Tendinitis, usually affecting the Achilles tendon, is also a rare class effect. It is more common in the elderly, in those receiving corticosteroid treatment, in patients with renal failure, and in recipients of organ transplants. Tendinitis may be bilateral and can lead to rupture [154]. Juvenile animals given quinolones develop arthropathy with cartilage erosion and defects in epiphyseal growth plates. Such changes have not been seen in human adults given quinolones, but the concern has restricted pediatric use of these agents. In limited experience in children with cystic fibrosis given quinolones joint symptoms have been uncommon and reversible [180].

Severe immunologically mediated adverse reactions have occurred specifically with the 1-(2,4)-difluorophenyl quinolones temafloxacin and trovafloxacin. The temafloxacin syndrome involved fever, chills, and jaundice a week into therapy with associated hemolysis and in about half the cases renal failure and hepatitis [20]. Trovafloxacin was associated with eosinophilic hepatitis with liver necrosis and, in a few cases, the need for transplantation [29]. Both side effects were rare but led to the abandonment of these agents.

4.9 Clinical Uses

4.9.1 Urinary Tract Infections

For uncomplicated urinary tract infections, usually in symptomatic young women with cystitis caused by susceptible organisms such as *E. coli*, most fluoroquinolones are likely to be effective. Three-day regimens of norfloxacin, ciprofloxacin, ofloxacin, and other fluoroquinolones have produced cure rates of 81–96% [82]. Single-dose therapy with ciprofloxacin, ofloxacin, or norfloxacin resulted in cure in 75–96% of patients [63, 152, 165]. For *Staphylococcus saprophyticus*, however, a 7-day course is recommended because of failure with shorter courses [166, 203]. Cystitis in elderly women is more likely to be caused by organisms less sensitive to antimicrobial agents. Longer courses of therapy have been recommended [140] but have no clear advantages [113].

Uncomplicated acute pyelonephritis also responds well to ciprofloxacin (either orally or parenterally), norfloxacin, levofloxacin, or ofloxacin [130, 150, 169]. Guidelines of the Infectious Disease Society of America recommend a fluoroquinolone as first-line treatment because of the increasing resistance of uropathogenic *E. coli* to trimethoprim-sulfamethoxazole [203]. Unfortunately, quinolone susceptibility cannot always be assumed. Ciprofloxacin-resistant *E. coli* causing community-acquired urinary tract infections have emerged in Europe [23] and are even more common in parts of China [201].

For complicated urinary tract infections occurring in men and in patients with catheters or structural or functional abnormalities of the urinary tract, more resistant pathogens are expected along with a higher incidence of relapse and reinfection. Ciprofloxacin has given better short-term bacteriological eradication rates than aminoglycoside therapy in such patients, but long-term efficacy has been equivalent [59]. For patients with spinal cord injuries ciprofloxacin for either 3 or 14 days produced similar short-term cure rates but long-term microbiological cure was better with the longer course of treatment [46]. *P. aeruginosa* infections have responded to ciprofloxacin or norfloxacin therapy, but failure can occur with the selection of resistant organisms [136].

Quinolones have also been used prophylactically in patients with bladder dysfunction due to spinal cord injury who use intermittent suprapubic taps or self-catheterization for bladder emptying. Relative to placebo 100 mg ciprofloxacin qhs reduced episodes of infection tenfold [18]. In addition quinolones, as well as other agents, have been used successfully for prophylaxis after transurethral prostate resection or transrectal prostate biopsy [17, 206].

Fluoroquinolones have proven valuable in treating prostatitis as well, since they are concentrated in prostatic tissue [40]. In patients with chronic prostatitis at least 14-day and often longer courses of norfloxacin, ciprofloxacin, or ofloxacin have produced bacteriologic cure in more than 60% of cases [135].

4.9.2 Sexually Transmitted Diseases

Neisseria gonorrhoeae is a major pathogen in urogenital, anogenital, and pelvic inflammatory disease (PID) and until recently could be reliably treated with fluoroquinolones. Because of rising resistance in some parts of the world and in certain patient groups, such as men having sex with men, quinolones have been removed from the recommended treatment guidelines, although they remain effective against susceptible isolates [138]. Quinolones are active against the sexually transmitted pathogens *C. trachomatis* and *Haemophilus ducreyi* but lack activity against *T. pallidum*. For chlamydial infections a 7-day course of ofloxacin or levofloxacin is as effective as a treatment with doxycycline [103, 127].

In the treatment of PID a 14-day course of IV and oral ciprofloxacin produced clinical resolution in 94% of patients, a response similar to that seen with clindamycin/gentamicin [39]. In another study 14 days of oral ciprofloxacin plus clindamycin

gave cure rates similar to doxycycline plus im ceftriaxone [6]. Fourteen days of po moxifloxacin has also been shown equivalent to ofloxacin plus metronidazole in patients with uncomplicated PID and produced fewer side effects [175]. In treating chancroid *H. ducreyi* was eliminated from genital ulcers in 100% of patients by 3 days of ciprofloxacin, a response equivalent to that produced by trimethoprim-sulfamethoxazole [134]. Ciprofloxacin 500 mg po bid for 3 days is one of the CDC-recommended treatment options for chancroid [207]. For bacterial vaginosis ofloxacin is less effective than metronidazole [37].

4.9.3 Gastrointestinal and Abdominal Infections

When they were first introduced, fluoroquinolones were highly active against all bacterial enteric pathogens and consequently quickly became mainstays for the treatment of bacterial gastroenteritis and enteric fever [16] Fluoroquinolones have been routinely used to treat infections with *Shigella*, *S. enterica* serovar Typhi, severe infections with nontyphoidal *Salmonella*, and for travelers' diarrhea. Use for *Campylobacter* infections has been abandoned because of resistance, and resistance is emerging with the other GI pathogens as well.

For travelers' diarrhea 3–5 day courses of norfloxacin or ciprofloxacin, begun soon after the onset of symptoms, have shortened the duration of loose stools by 1–3 days relative to placebo [57, 205]. Single-dose therapy with ciprofloxacin [151, 177] or ofloxacine with or without loperamide has also been effective [58]. In patients with shigellosis fluoroquinolones have been notably effective, and in comparative studies with other agents they have usually proved at least as effective and often more so both bacteriologically and clinically [16]. For enteric fever due to *S. enterica* serovar Typhi or *S. enterica* serovar Paratyphi ciprofloxacin [187] or ofloxacin [198] was highly effective. Typhoid strains with reduced quinolone susceptibility have, however, been reported from a number of Asian countries [35] due mainly to mutations in *gyrA* [28]. In patients with nontyphoidal *Salmonella* gastroenteritis, treatment with norfloxacin or ciprofloxacin has shortened symptoms in some, but not all, studies [16]. Few investigators have found reduced duration of fecal *Salmonella* excretion. Treatment of *Salmonella* gastroenteritis is generally not indicated, except for elderly and immunocompromised patients with enhanced risk of invasive disease. Limited data suggests that prolonged treatment with fluoroquinolones can eliminate chronic *Salmonella* carriage [16].

In treating cholera fluid replacement is the cornerstone of therapy, but antimicrobial agents, by reducing the duration and volume of diarrhea, are important adjuncts. Both norfloxacin [52] and ciprofloxacin [77, 197] are effective in shortening diarrhea and eradicating *Vibrio cholerae* from the stool. In patients with diarrhea caused by *Yersinia enterocolitica*, *Plesiomonas shigelloides*, or *Aeromonas* spp. quinolones have eliminated the organisms from the stool but have not yet been shown to shorten clinical illness [16]. Quinolones are active against *Helicobacter pylori* in vitro. Levofloxacin- or moxifloxacin-based combination regimens given for 7–10 days

have been successfully used for first-line or second-line rescue therapy [74, 75, 126]. Moxifloxacin penetrates well into the gallbladder tissue [143]. Limited data suggest that quinolones are effective in treating biliary sepsis [33]. Complex intra-abdominal infections have also been successfully treated with quinolones, usually combined with metronidazole for improved coverage of bowel anaerobes [184].

4.9.4 Respiratory Tract Infections

Many respiratory tract pathogens are susceptible to the fluoroquinolones including *H. influenzae*, *M. catarrhalis*, *M. pneumoniae*, *C. pneumoniae*, and *L. pneumophila.* Least susceptible among common respiratory pathogens is *S. pneumoniae* [109], but levofloxacin, gemifloxacin, and moxifloxacin have improved antipneumococcal activity and consequently have been termed respiratory fluoroquinolones.

Treatment of patients with a respiratory fluoroquinolone either alone (outpatient), combined with aztreonam (inpatient), or combined with an antipneumococcal, antipseudomonal β-lactam (in a patient in whom *P. aeruginosa* is suspect) is one of the options in the current IDSA/ATS guidelines for community-acquired pneumonia [115]. A number of studies have documented clinical success in treating community-acquired pneumonia with quinolones via either the oral or parenteral route with results comparable to treatment with amoxicillin or clarithromycin (oral therapy) or with a cephalosporin or amoxicillin-clavulanate (intravenous therapy) [11]. Levofloxacin, gemifloxacin, and moxifloxacin have also been shown to eradicate pneumococci from the lung, although a few patients with pneumococcal pneumonia have failed levofloxacin therapy because of preexisting or acquired resistance [42].

Quinolones also have a role in the treatment of health care or hospital-acquired and ventilator-associated pneumonia both for their activity against typical respiratory tract pathogens and also against *Enterobacteriaceae* and *P. aeruginosa*. In patients with late onset disease or risk factors for multidrug-resistant pathogens, an antipseudomonal fluoroquinolone (ciprofloxacin or levofloxacin) is one component recommended for empiric combination antimicrobial therapy [141].

Respiratory quinolones are at least as good, if not superior, to macrolides for treatment of *Legionella* pneumonia [19, 55]. Pneumonia due to *M. pneumoniae* and *C. pneumoniae* has also responded well [111]. Patients with acute bacterial exacerbations of chronic bronchitis have responded to treatment with quinolones, even with non-respiratory quinolones, thanks to their activity against *H. influenzae* [11]. For aspiration pneumonia and lung abscess moxifloxacin was as effective as ampicillin/sulbactam and more convenient to administer [146].

In the treatment of drug-sensitive pulmonary tuberculosis substitution of a first-line agent with ciprofloxacin or ofloxacin offers no therapeutic advantage and has been associated with increased treatment failures and slower sputum conversion rates. Some case series support the use of fluoroquinolones for multidrug-resistant tuberculosis, but controlled trials are lacking. Since they are well tolerated, quinolones do have a role, however, as substitutes for a first-line agent in patients intolerant

of standard regimens for tuberculosis [129] For nontuberculous mycobacteria a 4-drug regimen including ciprofloxacin did less well than a 3-drug regiment with clarithromycin in patients with AIDS and *M. avium-intracellulare* bacteremia [182].

Ciprofloxacin, levofloxacin, and moxifloxacin have been found equivalent to other agents for the treatment of acute bacterial sinusitis [162, 183].

4.9.5 Bone and Joint Infections

Quinolones have been useful for the necessarily prolonged treatment of chronic osteomyelitis due to susceptible gram-negative bacilli and methicillin-susceptible *S. aureus*. Failures have been associated with incomplete debridement, the presence of foreign bodies, and the development of resistance, especially with infections due to *P. aeruginosa* and *S. aureus* [68, 79]. Oral quinolones also have a role in the treatment of infections associated with prosthetic joints [44]. For staphylococcal joint infections regimens have often included rifampin as well as a quinolone [12, 13].

4.9.6 Skin and Soft-Tissue Infections

Comparative trials indicate that fluoroquinolones are as effective as other oral agents (usually cephalexin) in the treatment of uncomplicated skin infections with β-hemolytic streptococci and methicillin-sensitive *S. aureus* as the dominant pathogens [99] They offer no special advantages for uncomplicated infections but are particularly useful in treating complex, complicated infections where their broad spectrum can cover the polymicrobial etiology of surgical or traumatic wound infections, bites, or infected decubitus or diabetic foot ulcers [69, 72, 78] The emergence of community-associated methicillin-resistant *S. aureus* as a common pathogen in skin and soft-tissue infections has altered recommendations, however, since many of these isolates are quinolone-resistant [1] so that other agents are preferred for initial therapy until quinolone susceptibility is proven by culture.

4.9.7 Other Infections

Ciprofloxacin is indicated for the treatment of cutaneous or inhalation anthrax and for anthrax prophylaxis [15]. Pefloxacin and ofloxacin have been used in combination regimens to treat lepromatous leprosy [80], and moxifloxacin has antileprosy activity in a mouse model [81]. Patients with tularemia have been cured with levofloxacin [110] or ciprofloxacin [32]. The first-line treatment of *Coxiella burnetii* infection is a tetracycline, but ofloxacin for 14–21 days is an alternative for acute Q fever [122]. Mediterranean spotted fever due to *Rickettsia conorii* has also been successfully treated with pefloxacin, ofloxacin, or ciprofloxacin although doxycycline is the treatment of choice [174].

Quinolones have been used for prophylaxis in neutropenic patients with reduction in the occurrence of gram-negative bacteremia, but breakthrough bacteremia by streptococci has been a problem [95, 167] as has the emergence of quinolone-resistant *E. coli* [24]. Quinolones have also been used in oral therapy of low-risk febrile, neutropenic patients with results comparable to intravenous alternatives [101].

Fluoroquinolones penetrate fairly well into cerebrospinal fluid and have good in vitro activity against meningitis pathogens but have been infrequently used to treat central nervous system infections. Limited data suggest that they are useful for meningitis due to susceptible gram-negative organisms that are resistant (*P. aeruginosa*) or slow to respond (*Salmonella*) to other drugs. They are also useful for prophylaxis of individuals exposed to patients with *H. influenzae* or *N. meningitidis* meningitis [196], although a few ciprofloxacin-resistant *N. meningitidis* have been found recently in the United States [208].

Ciprofloxacin, levofloxacin, and ofloxacin have been clinically efficacious in treating bacteremia due to *Enterobacteriaceae*, but less so when bacteremia was caused by *Acinetobacter* spp. or *P. aeruginosa* [21, 43, 168]. With levofloxacin, the lower the MIC of the organism causing the bloodstream infection, the better the outcome, even if the organism tested susceptible [43]. In neutropenic patients with fever, ciprofloxacin combined with an aminoglycoside produced comparable response rates to piperacillin plus an aminoglycoside [27], but in another study ciprofloxacin monotherapy was inferior to piperacillin combined with amikacin [125].

Experience with quinolones in bacterial endocarditis is quite limited [108]. Selected patients with right-sided endocarditis due to methicillin-susceptible *S. aureus* have been cured with oral ciprofloxacin combined with rifampin [53, 87], but ciprofloxacin alone has failed with persistent bacteremia and the development of increased ciprofloxacin resistance [76].

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Chapter 5
Tetracyclines

Patricia A. Bradford and C. Hal Jones

5.1 Introduction

For more than half a century, tetracycline antibiotics have been used to treat infectious diseases. As one of the earliest antibiotics to be marketed following penicillin and streptomycin, and because of their convenient oral dosing, tetracyclines quickly achieved wide clinical usage. Unfortunately, this extensive use in clinical practice and agriculture has resulted in widespread resistance that ultimately has limited the clinical utility of the entire family of tetracycline antibiotics [114]. Tetracyclines inhibit bacterial growth by inhibiting protein synthesis. In general, they bind to the bacterial 30S ribosomal subunit and prevent aminoacyl-t-RNA binding to the ribosomal A site, thus preventing addition of amino acids to the growing polypeptide chain [18]. In the past decade, there has been renewed interest in this antibiotic class, with attempts being made to modify existing compounds so that they are not affected by common bacterial tetracycline resistance mechanisms. This chapter will review the history of tetracycline discovery from the early natural products through the newest tetracycline-like derivatives, the glycylcyclines and aminomethylcycline.

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Fig. 5.1 Dr. Benjamin Duggar

5.2 History of Tetracycline

5.2.1 The Natural Products Approach to Antibiotic Discovery

The discovery and clinical use of the tetracycline family of antibiotics emerged from efforts that began in the 1930s, using what were regarded then as cutting edge technologies. In 1935, the chemical giant American Cyanamid acquired Lederle Antitoxin Laboratories, a pharmaceutical company founded in 1906 to manufacture antisera in horses for the treatment of infectious diseases. American Cyanamid already had made substantial profits from the manufacture of calcium cyanamide, a synthetic fertilizer; now the company was interested in diversifying its markets by entering the pharmaceutical field. Following that decision, American Cyanamid increased their research efforts at Lederle's production site in Pearl River, New York, initiating a systematic search for life-saving drugs, particularly antibiotics.

A worldwide search for antibiotic-producing microorganisms began in earnest following Alexander Fleming's announcement of his discovery of penicillin [38]. Given Lederle's extensive participation in the U. S. government-sponsored penicillin research and development program during World War II, antibiotic discovery seemed a natural direction for Lederle to pursue. In 1944, the general manager of Lederle initiated a research effort to search for antibiotics that would be superior to streptomycin. Streptomycin, a natural product aminogylcoside antibiotic which was discovered by Selman Waksman and Albert Schatz at Rutgers University, was marketed by Merck in 1946 [110]. Outside of Waksman and his group, there were very few experts in antibiotic screening during the early 1940s. As a part of the effort at Lederle, 71 year-old Benjamin Minge Duggar (Fig. 5.1) was hired in 1943 to head the soil-screening department in an effort to identify therapeutic substances produced by soil microorganisms. Duggar, a retired professor of plant physiology and economic botany at the University of Wisconsin, was an energetic and scholarly scientist world-renown for his studies on fungal taxonomy and the invention of

Fig. 5.2 *Streptomyces* spp. in tube cultures. Different species of *Streptomyces* can produce a wide variety of colors and morphologies when grown on solid medium

novel methods for mass production of edible mushrooms [29]. Antibiotic discovery research was not within Duggar's previous expertise; however, he vigorously threw himself into the new challenge and his perseverance and scientific insight ultimately bore fruit for Lederle [46].

Duggar implemented his soil-screening program by consulting with an extensive network of colleagues who resided in diverse locations from whom he requested soil samples from sites that had not recently been disturbed. Upon receipt, soil suspensions were prepared and inoculated onto agar media to permit growth of the indigenous microflora. In that era, potentially new organisms were selected almost exclusively on a basis of their morphological properties (Fig. 5.2). Today, culture and microscopic morphologies, while still extremely important for recognizing unique isolates, have been supplemented with newer tools, including analysis of 16S rRNA sequences for bacteria and internal transcribed spacer (ITS) sequences for fungi, thereby enabling more precise discrimination of unique taxonomic groups. In addition, selection of cultures using DNA probes for polyketide synthetase (PKS) and nonribosomal peptide synthetase (NRPS) may increase the likelihood of finding novel antibiotics. [99]. However, armed only with the tools of his time, Duggar proceeded to screen 3,500 organisms comprised of eubacteria (B cultures) and actinomycetes (A cultures).

To test fermentation samples for antimicrobial activity, Duggar applied aliquots of spent medium into wells cut into agar plates that were inoculated for confluent growth with indicator organisms. Following incubation, the growth on the plates was scored for the appearance of zones of inhibition around the wells (Fig. 5.3). Duggar used both Gram-positive and Gram-negative indicator organisms in his screens, and he prioritized hits that were active against both bacterial types (i.e., antibiotics with a broad spectrum of activity). Many antibiotic-producing organisms and their active substances were discovered through Duggar's efforts at Lederle, but most were thought to be too toxic for medical use and were quickly discarded. For example, Duggar originally selected strain B-71 as producing a lead of interest that showed potent antibacterial activity; however, there was limited interest in pursuing this compound because of its nephrotoxicity. This strain was identified later as

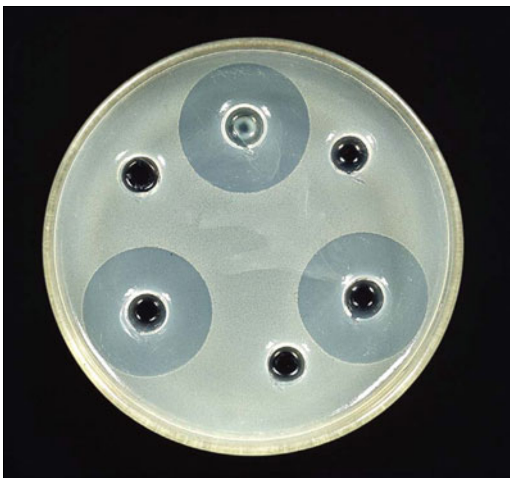

Fig. 5.3 Natural products assay plate. Wells were dug into an agar plate and extract from shake flask cultures was placed into the well. A zone of inhibition around the well indicated that the extract contained a compound that had antibacterial activity against the indicator organism

Bacillus polymyxa, which produced polymyxin. In recent years, the challenges posed by multi-resistant Gram-negative pathogens have caused a resurgence of interest in polymyxins [34, 35, 54].

5.2.2 The Discovery of the First Tetracycline

In August 1945, Duggar tested an unusual bronzed-colored actinomycete culture designated A-377 that was isolated from a soil sample collected by William Albrecht, a soil microbiologist and friend of Duggar, in a dormant timothy hay field in Sanborn Fields outside Columbia, Missouri. Duggar named this organism *Streptomyces aureofaciens* to reflect the golden (*aureus*) color of both the culture and its antibiotic product. The compound resulting from this culture had very different properties compared to the toxins usually recovered from Duggar's soil screens in that it demonstrated a broad-spectrum of antibacterial activity by inhibiting both Gram-positive and Gram-negative bacteria. This included strains resistant to sulfa drugs and other antibiotics known at that time. The antibacterial activity of this new substance was confirmed in rodent models of infection, and the compound appeared to be relatively nontoxic [86].

With the excitement of this initial discovery came the realization that fermentation scale-up of strain A-377 and chemical isolation of the compound needed to occur quickly. In 1946, Lederle chemist Joseph Niedercorn was assigned the task of producing the compound of interest (the structure was uncharacterized) in 5-gal stirred bottles. This assignment turned out to be quite challenging, because strain A-377 did not exhibit the same fermentation characteristics that were common to other organisms that he had experienced. One of the issues was that producing the golden compound in any appreciable quantities resulted in considerable autotoxicity for the producing organism. In early 1947, Niedercorn discovered that increased yields of the compound could be obtained when the fermentation medium was buffered with calcium carbonate, which caused the antibiotic compound to precipitate

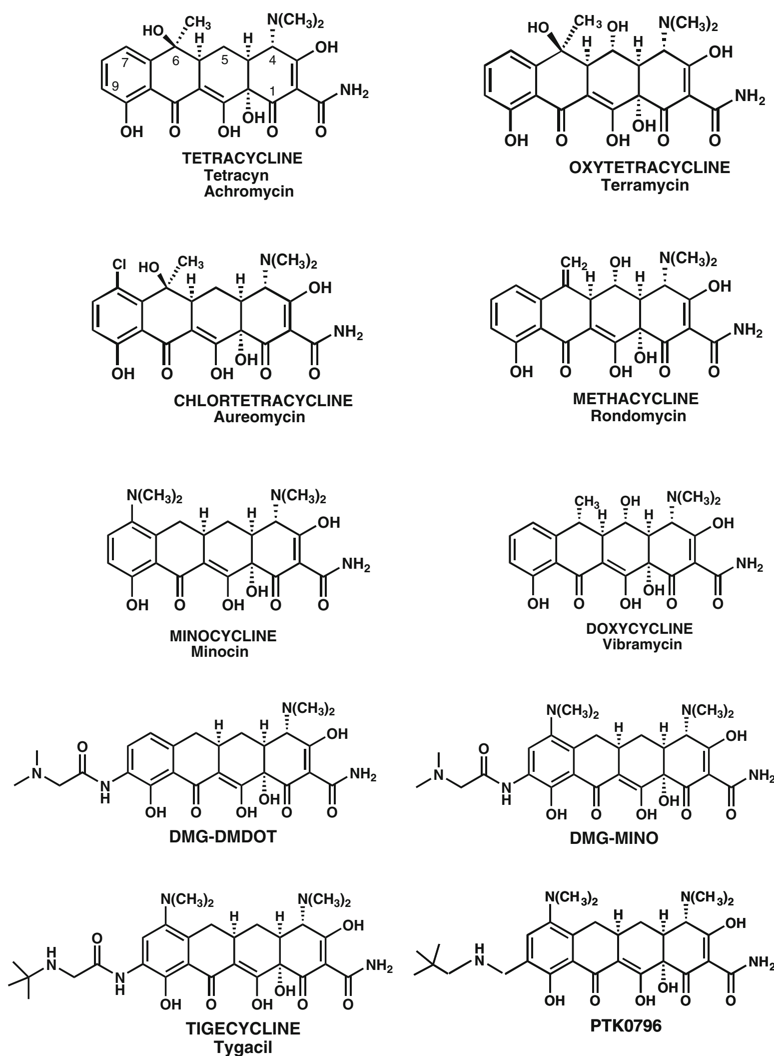

Fig. 5.4 Chemical structure of tetracycline derivatives, selected glycylcyclines and an aminoamethylcycline. The numbering convention for ring carbons is indicated for tetracycline

as an insoluble calcium salt, leaving little of it in solution to inhibit growth of the producing organism [84]. In addition, by employing previous experiences with penicillin fermentations, microbiologists at Lederle isolated mutants of strain A-377 that had improved yields of the novel compound. In April 1947, a pilot plant was established at Lederle that was devoted to development of the new antibiotic. It took nearly a year to solve problems associated with downstream processing, but finally the compound was recovered in crystalline form and was subsequently identified as chlortetracycline (7-chlorotetracycline, Fig. 5.4). Strain and fermentation improvements continued over many years to produce dramatically higher yields commensurate with commercial demands for the drug.

After mass production of chlortetracycline started in 1947, chlortetracycline became available in sufficient quantities to allow further animal testing. Later that year, the orally active drug was used for the first time as active therapy on a compassionate basis in patients with Rocky Mountain Spotted Fever, a rickettsial disease with a high mortality rate for which there was no treatment at that time. Following successful outcomes in these patients, it was also used in patients at Boston City Hospital suffering from a variety of bacterial infections [86]. Chlortetracycline met with immediate success for treatment of numerous previously intractable diseases such as typhoid fever (*Salmonella typhi*), typhus (*Rickettsia prowazekii*), and infections caused by invasive *Streptococcus pneumoniae* and β-hemolytic streptococci, which earned it the status of a "wonder drug." Following its approval in late 1948 by the United States Food and Drug Administration, Lederle launched chlortetracycline under the trade name Aureomycin® (Fig. 5.4), in deference to the golden color of the compound and to the bronze-colored soil bacterium that produced it. Duggar reported the process for fermentation and isolation of chlortetracycline, as well as some preliminary structural data, in 1948 [30]. The first tetracycline patent, "Aureomycin and preparation of the same" was issued in September 1949 [31]. As part of the patenting process and at the request of the United States Patent Office, a culture of *S. aureofaciens* A-377 (now the type strain *S. aureofaciens* subsp. *aureofaciens*) was deposited in the Agricultural Research Service Culture Collection at the Northern Regional Research Laboratory in Peoria, Illinois (accession number NRRL-2209).

During this same period, other pharmaceutical companies were diligently bioprospecting for their own novel antibiotics. During the mid-1940s, Chas. Pfizer & Sons, Inc., a Brooklyn, New York-based chemical company, also initiated a discovery campaign for new antibiotics. At that time, Pfizer manufactured citric acid for the food and beverage industry by deep-tank fermentation of molasses with *Aspergillus niger,* and was also involved in the wartime penicillin production program. After extensive screening of soil samples obtained from all over the world, Pfizer identified a strain of *Streptomyces rimosus* from a sample collected in Terre Haute, Indiana. This culture produced an antibiotic compound structurally related to chlortetracycline, which was later identified as oxytetracycline (5-hydroxytetracycline, Fig. 5.4). Following FDA approval, Pfizer launched oxytetracycline in 1950 under the trade name Terramycin®, from *terra*, Latin for earth, and perhaps in acknowledgement of its producer strain's source, Terre Haute, Indiana. Like chlortetracycline, oxytetracycline was orally bioavailable, and proved to be slightly more active than the Lederle drug [37]. Moreover, oxytetracycline possessed fewer side effects. Within months of its introduction, oxytetracycline became a serious competitor to chlortetracycline, assuming a large proportion of the American antibiotic market.

Even though both chlortetracycline and oxytetracycline were on the market by 1950, their chemical structures were still not completely known, and a competition to solve the structure of these molecules arose between the leading organic chemists of the time; the Lederle team led by James H. Boothe, and the Pfizer team led by Lloyd H. Conover. By the end of 1953, the Pfizer team proposed chemical structures were

for both oxytetracycline and chlortetracycline [50, 51, 117, 118]. These structures contained a naphthacene core, and the term "tetracycline" was coined to describe this family of antibiotics [50]. It was noted that oxytetracycline possessed a hydroxyl group at C-5 lacking in chlortetracycline whereas chlortetracycline possessed a chlorine atom at C-7 absent from oxytetracycline. In 1954, the complete structures of chlortetracycline and oxytetracycline were solved and revealed in a landmark paper, "The Structure of Aureomycin" (Fig. 5.4)[119]. As part of their structural proof the Pfizer chemists chemically modified chlortetracycline to generate an even more active antibiotic, tetracycline (later trade named Tetracyn®). Tetracycline was the structurally simplest member of this antibiotic class, and became the generic structure for this antibiotic class (Fig. 5.4). Subsequently, tetracycline was isolated in small quantities from spent broth of both *S. aureufaciens* [5] and *S. rimosus* [88].

Dehalogenation of chlortetracycline to produce tetracycline, which represented an important breakthrough in synthetic organic chemistry, became the subject of a lengthy patent dispute between Lederle and Pfizer. This conflict evolved, in part, because Lederle chemists were also working on semi-synthetic analogs of chlortetracycline and in the course of their investigations independently and concurrently discovered tetracycline. The Lederle team named their version of tetracycline Achromycin,® because of its pale yellow color compared to the deep gold of chlortetracycline. Furthermore, the Pfizer chemists had used Lederle's patented compound chlortetracycline as their starting material for production of tetracycline. This dispute was finally resolved through an amicable agreement between the two companies. By the mid 1950s, chlortetracycline, oxytetracycline, and tetracycline had become widely prescribed antibiotics.

5.2.3 Biosynthesis of the Tetracyclines

While structural studies were in progress, work began at both Lederle and Pfizer to characterize the biosynthetic routes for the tetracyclines. At Lederle, work was principally conducted with *S. aureofaciens*, optimizing fermentation conditions for and yields of chlortetracycline whereas Pfizer scientists devoted their attention to production of oxytetracycline by *S. rimosus* [74, 80]. A pioneer in the field of elucidating secondary metabolite biosynthetic pathways, Lederle scientist J. R. D. McCormick isolated numerous mutants blocked in the production of chlortetracycline. Using an innovative agar plate technique still employed today, McCormick was able to determine the precise sequence of each intermediate in the chlortetracycline pathway by determining which blocked mutants (secretors) produced diffusible compounds that enabled other blocked mutants (converters) to produce the final product. In the course of such "cross-feeding" experiments, the pathway intermediates elaborated by secretors were isolated and identified [75]. In this manner, the Lederle team discovered that tetracycline was a precursor of chlortetracycline. Today, McCormick's cross-feeding strategy has been augmented by the capacity to isolate and sequence the genetic pathway for biosynthesis of an antibiotic.

Subsequently, incorporation experiments with radiolabeled malonate and acetate showed that the naphthacene moiety is formed through successive addition of one malonyl ~ SCoA unit and 8 acetyl ~ SCoA units, followed by cyclization through a concerted series of enzyme-mediated foldings, ring closures, and stereospecific transformations. The total synthesis of tetracycline was finally achieved in 1959 [11]; however, as a result of extensive strain selections, improved fermentation conditions, and optimized downstream processing, tetracyclines are now produced solely by fermentation. The complete tetracycline biosynthetic pathway was later cloned and characterized at Wyeth (formerly Lederle Laboratories), and the functions of the genes comprising this pathway were deduced through bioinformatics and selective disruption [106].

5.2.4 Expanding the Utility Through Semi-Synthesis

Soon after the discovery of tetracyclines, chemists at both Lederle and Pfizer began tinkering with their structures in an effort to increase potency, decrease toxicity, and improve pharmacokinetic properties. Towards this end, each company chose different starting materials and used different semi-synthetic pathways. Naturally occurring tetracyclines can be altered either by modifying existing functional groups or by introducing novel functionalities, which could then be further modified. Both methods were used in the search for new tetracycline derivatives.

Pfizer chemists used oxytetracycline as the starting material for further development of this compound class. Oxytetracycline was converted to 11a-chlorotetracycline-6,12-hemiketal, oxidized and dehalogenated to form methacycline (Fig. 5.4), a broad-spectrum antibiotic that had improved stability and pharmacokinetic properties [10]. Subsequently, many different companies worldwide produced methacycline, which was sold under various trade names such as Rondomycin. Methacycline was effective against a variety of pathogens impacting human and animal health as well as pathogens of interest in the agricultural sector [23, 141]; however, Pfizer voluntarily withdrew it from the market in 2001 (http://www.fda.gov/OHRMS/DOCKETS/98fr/081601a.pdf).

Pfizer used methacycline as the precursor for another valuable compound. Catalytic reduction of methacycline produced a mixture of α-6-deoxyoxytetracycline and β-6-deoxyoxytetracycline, from which the 6 α-epimer (variant lacking they hydroxyl at the 6β position) was found to be a highly bioactive. The name "β-6-deoxyoxytetracycline" was shortened to doxycycline and given the trade name Vibramycin®. Today, the one-step stereospecific reduction of methacycline to doxycycline is used for the industrial manufacturing of doxycycline [36, 91]. Doxycycline is used widely for treatment of a variety of community-acquired bacterial infections, including Lyme disease; an increasingly common tick-borne bacterial infection caused by the spirochete *Borrelia burgdorferi* [53]. A single dose of doxycycline following a tick bite often can preclude emergence of borreliosis [82]. Recently

doxycycline has achieved a high profile as a treatment for anthrax, one of only two drugs (the other being ciprofloxacin) approved by the FDA for treatment of *Bacillus anthracis* infections [21]. Additionally, doxycycline, under the trade name Periostat® (Collagenex Pharmaceuticals) is approved for treatment of periodontitis, by virtue not only of its anti-anaerobe activity but also due to its inhibition of gingival matrix metalloproteases [44, 66, 128].

In 1956, demeclocycline (6-demethyl-7-chlorotetracycline) was discovered at Lederle during a screening program with blocked mutant strains of *S. aureofaciens* [73]. Initially marketed in 1959 as Declomycin®, the antibacterial properties of this substance were only marginally better than those of tetracycline, and its increased phototoxicity led to restricted use. However, demeclocycline proved quite important as a starting material for semi-synthetic manipulations of tetracycline derivatives. Moreover, it was at the center of an incident of corporate espionage, in which the producing strain was sold to European industrialists by a disgruntled Lederle chemist [86]. Sidney Fox and John Cancelarich stole a number of cultures that produced high yields of chlortetracycline, tetracycline and demeclocycline as well as trade secrets for production processes. The cultures were sold to several companies with production plants in Italy, as well as Miles Laboratories. Miles obeyed a court order to return the stolen cultures and the Italian companies were prosecuted after scientists detected effluent containing the Lederle cultures in the Bay of Naples. American Cyanamid pursued the case in criminal court resulting in Fox's conviction and 2-year prison sentence following Cancelarich turning state's evidence to assist in the conviction of several others for conspiracy (United States v. Bottone, 365F.2d 389, 1966).

Despite all of this, the main commercial importance of demeclocycline was that it could be reduced to 6 α-deoxy-6-demethyltetracycline (sancycline), the tetracycline structure with the minimal chemical features necessary for antibacterial activity [75]. Lederle chemists then converted sancycline to 7-aminosancycline, called minocycline (trade name Minocin®), which turned out to be the most potent antibacterial tetracycline described at that time (Table 5.1, Fig. 5.4) [70]. Church et al., then described a cost-effective, scalable process for the manufacturing of minocycline [25]. Minocycline had enhanced lipophilic properties compared to other tetracyclines available at that time [129], which increased its bioavailability and tissue penetrability. In addition, the drug proved effective against some strains of tetracycline-resistant bacteria [1, 78]. Minocycline is also used as an oral treatment for moderate acne, and the anti-inflammatory properties of the molecule are believed to contribute to its overall efficacy [137].

Extensive use of tetracyclines over the years led to widespread dissemination of genes encoding tetracycline resistance; as a result, the utility of this family of antibiotics became limited for many clinical indications [24]. A comprehensive review of tetracycline resistance genes and encoded mechanisms will be discussed in a subsequent chapter. However, the increase in tetracycline resistance drove the need to discover even more potent tetracycline antibiotics that could overcome the diverse mechanisms responsible for resistance.

Table 5.1 Comparative activity of selected tetracycline, glycylcycline and aminomethylcycline antibiotics (Data from [12, 15, 22, 68, 89, 90, 96, 130])

Organism	Antibiotic	MIC (µg/mL)		
		Range	MIC_{50}	MIC_{90}
S. aureus	Chlortetracycline	0.8–50	0.8	50
	Oxytetracycline	0.4–200	0.8	200
	Methacycline	0.2–>6.25	0.4	>6.25
	Tetracycline	0.25–>64	0.5	4
	Minocycline	≤0.06–8	0.12	0.12
	Doxycycline	0.25–8	0.25	0.5
	DMG-MINO	0.25–0.5	0.5	0.5
	DMG-DMDOT	0.5	0.5	0.5
	Tigecycline	0.06–1	0.12	0.25
	PTK 0796	0.06–0.25	0.12	0.12
S. aureus **(MRSA)**	Chlortetracycline	ND	ND	ND
	Oxytetracycline	ND	ND	ND
	Methacycline	ND	ND	ND
	Tetracycline	0.25–>64	0.5	32
	Minocycline	≤0.06–32	0.12	4
	Doxycycline	0.12–16	0.25	2
	DMG-MINO	0.12–2	0.25	2
	DMG-DMDOT	0.25–2	0.5	2
	Tigecycline	0.06–1	0.12	0.25
	PTK 0796	0.12–1	0.25	0.5
E. faecalis	Chlortetracycline	0.8–50	3.1	12.5
	Oxytetracycline	0.8–100	1.6	12.5
	Methacycline	1.6–>12.5	>12.5	>12.5
	Tetracycline	0.06–>64	64	>64
	Minocycline	≤0.06–16	4	16
	Doxycycline	0.12–32	8	16
	DMG-MINO	0.06–0.25	0.25	0.25
	DMG-DMDOT	0.12–0.5	0.25	0.25
	Tigecycline	0.03–0.12	0.06	0.12
	PTK 0796	0.06–0.5	0.25	0.5
E. faecium **(VRE)**	Chlortetracycline	ND	ND	ND
	Oxytetracycline	ND	ND	ND
	Methacycline	ND	ND	ND
	Tetracycline	0.25–>32	0.25	>32
	Minocycline	≤0.06–16	2	8
	Doxycycline	0.12–32	0.12	16
	DMG-MINO	0.06–0.25	0.12	0.25
	DMG-DMDOT	0.12–0.25	0.12	0.25
	Tigecycline	≤0.015–0.12	0.03	0.06
	PTK 0796	0.12–0.5	0.25	0.5

(continued)

Table 5.1 (continued)

Organism	Antibiotic	MIC (μg/mL)		
		Range	MIC_{50}	MIC_{90}
S. pneumoniae	Chlortetracycline	ND	ND	ND
	Oxytetracycline	ND	ND	ND
	Methacycline	0.2–0.8	0.4	0.8[c]
	Tetracycline	0.5–32	32	32
	Minocycline	≤0.06–16	0.06	8
	Doxycycline	0.12–16	0.25	16
	DMG-MINO	0.03–0.12	0.06	0.06
	DMG-DMDOT	0.06–0.12	0.12	0.12
	Tigecycline	≤0.004–0.06	0.015	0.03
	PTK 0796	≤0.06–0.25	≤0.06	0.12
E. coli	Chlortetracycline	6.25–25	12.5	25
	Oxytetracycline	0.6–12.5	3.1	12.5
	Methacycline	0.8–3.1	1.6	1.6
	Tetracycline	1–>64	2	>64
	Minocycline	0.12–32	0.5	8
	Doxycycline	0.5–64	1	64
	DMG-MINO	0.25–4	0.5	4
	DMG-DMDOT	0.25–4	1	4
	Tigecycline	0.06–0.5	0.12	0.25
	PTK 0796	0.5–2	1	2
K. pneumoniae	Chlortetracycline	25–50	NA[a]	NA
	Oxytetracycline	6.25–12.5	NA	NA
	Methacycline	ND	ND	ND
	Tetracycline	1–>64	2	16
	Minocycline	1–64	2	32
	Doxycycline	1–16	2	16
	DMG-MINO	1–16	4	8
	DMG-DMDOT	0.12–2	1	1
	Tigecycline	0.25–4	0.5	0.5
	PTK 0796	1–8	2	4
A. baumannii	Chlortetracycline	ND[b]	ND	ND
	Oxytetracycline	ND	ND	ND
	Methacycline	ND	ND	ND
	Tetracycline	0.12–>64	1	>64
	Minocycline	≤0.06–16	0.12	8
	Doxycycline	0.25–>32	1	32
	DMG-MINO	ND	ND	ND
	DMG-DMDOT	ND	ND	ND
	Tigecycline	0.03–4	0.25	2
	PTK 0796	0.12–16	2	8

(continued)

Table 5.1 (continued)

Organism	Antibiotic	MIC (μg/mL)		
		Range	MIC_{50}	MIC_{90}
P. aeruginosa	Chlortetracycline	3.1–>100	50	>100
	Oxytetracycline	3.1–50	12.5	25
	Methacycline	6.2–25	25	25
	Tetracycline	8–>64	32	64
	Minocycline	0.25–64	8	32
	Doxycycline	8–>32	16	16
	DMG-MINO	4–8	8	8
	DMG-DMDOT	4–16	8	8
	Tigecycline	0.25–32	8	16
	PTK 0796	16–64	32	32

[a]NA- not applicable, too few strains reported to calculate MIC_{50} and MIC_{90} values
[b]ND – no data
[c]Data on older tetracyclines generated before the emergence of resistance for some bacterial pathogens

5.2.5 A New Generation of Tetracyclines: The Glycylcyclines and Aminomethylcyclines

In 1988, Lederle Laboratories renewed its tetracycline research program, the principal aim of which was to obtain novel broad-spectrum tetracyclines active against clinically important pathogens harboring tetracycline resistance elements thereby restoring the therapeutic utility of this class of antibiotics. The research program was a multidisciplinary one involving the chemistry, molecular biology, biochemistry, and microbiology departments [125].

Initial stages of the program focused on extensive historical reviews of antibacterial data of older tetracyclines and involved some researchers who were part of Lederle's early tetracycline research programs. A series of new tetracyclines as well as "archived tetracyclines" prepared during earlier research programs, were reexamined in terms of binding to bacterial ribosomes, inhibition of protein synthesis, and transit across the outer membrane of Gram-negative bacteria. Results from these assays were coupled with determinations of in vitro activity using a set of isogenic strains of *Escherichia coli, Staphylococcus aureus* and *Enterococcus faecalis* that expressed well-characterized efflux or ribosomal protection resistance determinants [121]. Early structure-activity studies as well as those derived from new testing paradigms confirmed observations that changes in the hydrophobic domain of the tetracycline molecule led to compounds with enhanced antibacterial activity, whereas changes in the hydrophilic domain of the molecule resulted in loss of activity [125]. Efforts were thus focused on modifying the C-7 and C-9 positions of the D ring of the 6-demethyl-6-deoxytetracycline nucleus.

The first breakthrough came with the synthesis of the 9-amino series of tetracyclines, which had activity against *S. aureus* strains carrying the *tet*(M) determinant,

encoding ribosomal protection. These compounds were not stable under the assay conditions, however, and further structure-activity studies led to the preparation of *N*,*N*-dimethylglycylamido(DMG)derivatives *N*,*N*-dimethylglycylamidominocycline (DMG-MINO) and *N*,*N*-dimethylglycylamido-6-demethyl-6-deoxytetracycline (DMG-DMDOT, Fig. 5.4) [122]. Due to its increased antimicrobial activity towards some strains of tetracycline-resistance bacteria, minocycline was chosen as a starting point for chemical modifications. Lederle chemists made many modifications, finally resulting in a series of 9-aminotetracyclines bearing a glycyl moiety[122]. These compounds were referred to as "glycylcyclines" and this name has been accepted to describe this new class of tetracyclines (Table 5.1, Fig. 5.2).

Studies conducted with the early glycylcycline derivatives DMG-MINO and DMG-DMDOT demonstrated that the glycylcyclines efficiently (<30 μg/mL) blocked translation in extracts containing 30S ribosomes [97]. Moreover, the glycylcyclines were as effective in blocking translation of TetM- protected ribosomes and fourfold to eightfold better inhibitors than tetracycline or minocycline. Similarly, when tested by monitoring macromolecular synthesis in vivo, the glycylcyclines showed a 90% inhibition of translation on both tetracycline sensitive and TetM-protected ribosomes [97]. A later study, using filter binding to capture 70S ribosomes and monitor [^{3}H]-tetracycline binding to the ribosome, demonstrated that DMG-DMDOT bound to the same high-affinity binding site as tetracycline and bound approximately fivefold more tightly [9]. Using cell free translation, this study went on to show that the IC_{50} for translation inhibition was approximately tenfold higher for the glycylcyclines and further that inhibition was unaffected by the addition of semi-purified TetM or TetO to the in vitro translation system.

In the course of defining structure-activity relationships for glycylcyclines, it was observed that relatively small substituents at the C-9 position led to increased potency towards bacterial strains harboring tetracycline efflux proteins [121, 126]. This key observation directed the chemistry so that hundreds of derivatives were synthesized and tested against a panel of strains carrying every major tetracycline resistance gene. In this manner, the ideal chain length and substituent at position C-9 were established. A glycyl derivative of minocycline with an attached *t*-butyl amine group was identified as one of the most potent antibacterial compounds synthesized, and this compound 9-*t*-butyl glycylamido minocycline, named tigecycline, was chosen as a clinical candidate (Fig. 5.4) [90]. Tigecycline (trade name Tygacil®) was the first "glycylcycline" to be developed by Wyeth, who purchased Lederle in 1995. The attributes of tigecycline are discussed in detail below.

Paratek Pharmaceuticals, Inc., a biotechnology company in Boston, Massachusetts led by Stuart Levy, has developed another tetracycline antibiotic substituted at the C-9 position with a structural resemblance to tigecycline [7]. This compound, PTK-0796, lacks a glycylamido moiety, and Paratek refers to this class of tetracycline as an "aminomethylcycline" (Fig. 5.4). In preclinical studies PTK-0796 had a similar in vitro potency and antibacterial spectrum to tigecycline, though perhaps with somewhat less potent towards Gram-negative pathogens (Table 5.1) [68, 130], and proved efficacious in a number of animal models of infection [76, 77]. Recently, the safety and efficacy of PTK-0796 was demonstrated in a phase 2 clinical trial for complicated skin and skin structure infections [3].

5.3 Tigecycline

5.3.1 Mechanism of Action

The inhibitory activity of tigecycline was tested in a coupled in vitro transcription/translation system [85]. These studies revealed that the IC_{50} for tigecycline (0.75 μM) was approximately 20-fold and 2-fold lower than that of tetracycline and minocycline, respectively. In addition, these investigators monitored direct binding of tigecycline, minocycline, and tetracycline to 30S and 70S ribosomes by taking advantage of the intrinsic fluorescence of the tetracycline molecule. Based on the binding studies, tigecycline bound to the 30S and 70S ribosome with 5-fold and >100-fold greater affinity than minocycline and tetracycline, respectively. Using [^{14}C] tigecycline, competition studies demonstrated that the three tetracycline derivatives bound to the same or overlapping sites on the ribosome [85].

Using the 3.4-angstrom X-ray diffraction structure of the 30S ribosome from *Thermus thermophilus*, computational models of tetracycline, minocycline, and tigecycline bound to the ribosome were constructed [85]. In support of biophysical analyses described above, the interaction of tigecycline with the ribosome intersects with that seen for tetracycline; however, additional interactions were also mapped that are unlike any seen with other A-site binding molecules. Specifically, substantial hydrogen binding interactions were noted between the aminoglycyl tail of tigecycline and base C1054 in the 16S rRNA. In addition, the t-butyl group made additional van der Waals interactions with helices H34 and H18 in the 16S ribosomal RNA.

In order to map the interaction of tigecycline with 16S rRNA, Bauer et al., [8] utilized dimethylsulphate (DMS) and Fe^{2+} mediated probing of close contact sites comparing tetracycline and tigecycline interaction with the 70S ribosome. Using Fenton Chemistry, the investigators substituted the resident divalent cation, Mg^{2+}, chelated by tigecycline and tetracycline, with Fe^{2+}, to enable probing interaction sites between the antibiotics and the 70S ribosomes via H_2O_2 mediated RNA cleavage. These data showed, from a qualitative standpoint, that tetracycline and tigecycline interact with 16S rRNA with overlapping contact points. However, when tetracycline and tigecycline were titrated in the assay, difference were noted in the interactions with the target, suggesting that tigecycline binds with a higher affinity and with a different orientation of the molecule in relation to the binding site on the 16S rRNA. Compensation for the large bulky substituent at the nine position of tigecycline may necessitate approaching the 16S rRNA binding sites with a different orientation of the molecule. Further analysis, DMS probing and site directed mutation, supported the findings of the Fe^{2+} mediated cleavage studies. Mutations in helix 34 and helix 31, both of which showed identical patterns of Fe^{2+} mediated cleavage with tetracycline and tigecycline, resulted in fourfold to eightfold increases in MIC_{90} for both antibiotics [8].

5.3.2 Spectrum of Activity

5.3.2.1 Reference Susceptibility Test Method

During the establishment of the quality control (QC) ranges for tigecycline, inconsistencies were noted in the MIC limits obtained from different testing laboratories as well as over time from the same laboratory. It was determined that the relative age of the Mueller-Hinton broth (MHB) used in the MIC experiments and the effect media age had on the amount of dissolved oxygen in the media could account for the testing discrepancies [14]. HPLC analysis of tigecycline exposed to fresh (<12 h old) and aged media (>24 h old) revealed a novel species, believed to be a tigecycline degradant, that was present at a significantly higher concentration in aged media. Previous studies had shown that the addition of a biocatalytic oxygen-reducing reagent, such as Oxyrase®, could be employed to control dissolved oxygen concentration in broth media [98, 116]. It was demonstrated that addition of Oxyrase® to media prevented the accumulation of the tigecycline degradant and permitted the establishment of reproducible QC ranges for tigecycline [14, 89]. Moreover, controlling oxygen content in MHB was determined to be essential for maintaining QC, and recommendations from CLSI (Clinical Laboratory Standards Institute) state that MHB used for susceptibility testing must be less than 12 h old at the time of use when testing is performed by reference broth microdilution [26]. However, this requirement for fresh media does not affect the use of agar based tests such as Kirby-Bauer disk diffusion or Etest. In addition, there is no special requirement for tests performed with automated susceptibility test systems.

5.3.2.2 Susceptibility Test Data

Susceptibility data for bacterial isolates was obtained during the four pivotal clinical trials (two of complicated skin and skin structure infections, two of complicated intra-abdominal infections) and two recent clinical trials designed to determine the efficacy of tigecycline in community acquired pneumonia [15, 16]. Tigecycline is active against strains of *Staphylococcus* spp., regardless of the susceptibility to methicillin. For *S. aureus*, the tigecycline MIC_{90} for both MSSA (459 strains) and MRSA (143 strains) was 0.25 μg/mL. Similarly, for methicillin-susceptible (76 strains) and methicillin-resistant (74 strains) strains of *S. epidermidis* the MIC_{90} values of tigecycline were 0.25 μg/mL and 0.5 μg/mL, respectively. Of the 44 isolates of *S. haemolyticus* tested, all of the isolates were inhibited by 2 μg/mL or less of tigecycline. Tigecycline MICs for clinical isolates of *S. aureus* were unaffected by the presence of the *tet*(K) efflux determinant (48 isolates, MIC_{90} 0.25 μg/mL), the *tet*(M) (12 isolates, MIC_{90} 0.25 μg/mL) ribosomal protection determinant or both genes (5 isolates, MIC range 0.25 μg/mL) [57], as has been described in earlier studies [39, 90]. In a large collection of bloodstream infection isolates representing 29 countries from six continents for which the tigecycline susceptibilities for 5718

MSSA and 3047 MRSA isolates were virtually identical (MIC_{90} values were 0.25 μg/mL and MIC_{90} 0.5 μg/mL for MSSA and MRSA)[108]. Of particular interest, was a recent study looking at a global collection of nosocomial pneumonia isolates that included 1543 *S. aureus* of which 49.4% were oxacillin resistant [40]. Tigecycline susceptibility for this collection of pathogens was consistent with isolates from other infection types (MIC_{90} 0.5 μg/mL).

Importantly, tigecycline has shown potent activity against the recent threat of CA-MRSA that has arisen in the hospital and community setting [120]. A recent analysis of MRSA isolates from North American medical centers reported the tigecycline susceptibility of a large collection (1989) of epidemiologically defined CA-MRSA isolates [79]. Tigecycline susceptibility of the CA-MRSA subset of strains was no different from that of the concurrent collection (1907) of HA-MRSA isolates (MIC_{90} 0.5 μg/mL). Of the CA-MRSA strains, 71% were SCC*mec* type IV and of these 88% belonged to the USA300 clone and nearly 95% were PVL positive.

In another study, CA-MRSA were retrospectively genetically defined in isolates from phase 3 clinical studies for tigecycline [71]. All clinical isolates of MRSA and a set of clinically and genetically defined CA-MRSA control isolates (including USA-300 and USA-400) were ribotyped and the patterns compared using clustering software for genetic relatedness. The isolates were also evaluated based on the presence of the type IV SCC*mec* element and PVL using PCR. Of the 317 unique MRSA isolates collected during the phase 3 clinical trials, 81 isolates fulfilled all three of the criteria (clustering with known CA-MRSA PFGE types, SCC*mec* type IV element, PVL positive), suggesting that they were genetically similar to known, clinically defined CA-MRSA isolates. Tigecycline susceptibility of the genetically defined CA-MRSA subset (MIC_{90} 0.25 μg/mL) was identical to the entire MRSA collection as a whole, suggesting that MRSA isolates of community origin are as susceptible to tigecycline as MRSA isolates of nosocomial origin [71].

Tigecycline was demonstrated to be active against adherent bacteria growing in a biofilm model [65]. The growth of bacteria in a biofilm on the surface of a foreign body is often associated with infections of bloodstream catheters or prosthetic devices. The MIC_{90} values (determined by the non-reference method) of tigecycline were equal (MIC_{90}=0.5 μg/mL) for both planktonic and adherent cultures of *S. epidermidis*. Furthermore, the minimum bactericidal concentration (MBC) of tigecycline for adherent bacteria (8 μg/mL) was fourfold less than for growing bacteria and, interestingly, considerably lower than the MBC for vancomycin (32 μg/mL) [65]. In a more recent study, tigecycline was again shown to be more efficacious in clearing MRSA embedded in a biofilm. In a comparative study, tigecycline, minocycline and daptomycin performed better than linezolid and vancomycin in eradication of the biofilm [95].

The in vitro activity of tigecycline was determined against 301 recent clinical isolates of *Enterococcus* spp [15]. The MIC_{90} was 0.25 and 0.12 μg/mL for *E. faecalis* and *E. faecium,* respectively, and all of the isolates of enterococci were inhibited by ≤0.5 μg/mL of tigecycline. Tigecycline activity was also determined against defined VRE isolates was reported in a study in which the genetic determinant (*van*A, *van*B) encoding vancomycin resistance was defined for 202 *Enterococcus* spp. isolates [107].

For the 179 *van*A encoding strains, the tigecycline MIC range, MIC_{50} and MIC_{90} were ≤0.12–0.5, ≤0.12 and 0.25 μg/mL, respectively. Tigecycline MIC_{50} and MIC_{90} values for the 23 *van*B encoding *Enterococcus spp.* were identical to the *van*A encoding strains.

In another study, the activity of tigecycline was measured against a collection of 1832 *Enterococcus faecium* isolates from North America and Europe over 50% of which were vancomycin resistant [55]. These findings also demonstrated that tigecycline susceptibility was unaffected by the presence of vancomycin resistance mechanisms in enterococci (MIC_{90} 0.25 μg/mL). The collection of isolates included 678 that were phenotypically characterized as being of the CC-17 lineage: vancomycin resistance due to VanA and resistance to both ampicillin and ciprofloxacin. PFGE and PCR were used to further characterize a subset of the strains to identify CC-17 isolates based on genotype and encoding a specific pathogenicity island (PAI). Twenty-three of the isolates encoded the specific *esp* (enterococcal surface protein) variant gene that is a component of the PAI correlated with the CC-17 lineage. The phenotypically defined isolates (MIC_{90} 0.25 μg/mL) and genotypically defined isolates (MIC_{90} 0.12 μg/mL) were equivalently susceptible to tigecycline.

Tigecycline demonstrated good activity against the streptococci [15]. The 37 strains of *S. agalactiae* and the 86 strains of *S. pyogenes* and the 246 isolates of the *S. anginosus* group (*S. anginosus, S. constellatus*) collected were inhibited by 0.25 μg/mL of tigecycline. Similarly, *S. oralis* isolates from both the skin and intra-abdominal protocols were fully susceptible with a MIC_{90} value of 0.12 μg/mL.

Tigecycline has also shown good activity against gram-negative and gram-positive pathogens associated with respiratory infections as was demonstrated in the recent clinical trial for community acquired pneumonia (CAP) [16]. The 184 *S. pneumoniae* isolates collected during the CAP trial were 100% susceptible to tigecycline at 0.12 μg/mL (MIC_{90} 0.06 μg/mL) and susceptibility was unchanged when comparing penicillin–intermediate and penicillin-resistant *S. pneumoniae*. A recent analysis confirms that tigecycline has potent activity against *S. pneumoniae* that is not impacted by the penicillin susceptibility status of the organism [41]. The MIC_{90} was ≤0.03 μg/mL for 662 PSSP, 203 PISP and 169 PRSP. Early studies also demonstrated that tigecycline activity was unaffected by carriage of tetracycline resistance determinants in *S. pneumoniae* [40, 107]. In a large survey of 6,991 *S. pneumoniae* clinical isolates collected throughout Canada, the MIC_{50}, MIC_{90} and MIC range for tigecycline were reported to be 0.03, 0.06 and ≤0.015–0.25 μg/mL, respectively [140].

The expanded broad-spectrum activity of tigecycline includes gram-negative pathogens of the Enterobacteriaceae family and has the potential for use in infections where an ESBL or AmpC producing isolate might be suspected. The activity of tigecycline was determined for 1,062 isolates of *E. coli* from the skin and intra-abdominal clinical trails [15, 16]. All *E. coli* isolates were inhibited by 2 μg/mL of tigecycline and the MIC_{90} was 0.5 μg/mL. The clinical isolates collected from the cIAI trial included nine ESBL producers for which the MIC range (0.25–1 μg/mL) was the same as for the ESBL non-producers [4]. A recent survey of ESBL and AmpC producing clinical *E. coli* isolates support the finding that the expression of

these β-lactamases does not impact tigecycline susceptibility for *E. coli* [58, 132]. A genetic analysis of 98 phenotypically defined ESBL encoding *E. coli* isolates showed that 83% encoded a bla_{CTX} β-lactamase (CTX-M-1 or CTX-M-2 family) and 41% encoded a bla_{SHV} (SHV-2, SHV-5, SHV-12) β-lactamase. All 98 isolates were susceptible to 2 μg/mL tigecycline with an MIC_{90} 0.5 μg/mL. Likewise, the presence of bla_{AmpC} (ACT-1, MIR-1, CMY-2), alone or in combination with an ESBL, did not impact tigecycline susceptibility (MIC_{90} 0.5 μg/mL).

Tigecycline MICs for clinical isolates of *E. coli* were also unaffected by the presence of efflux determinants (*tet*(A)–*tet*(E)) or the *tet*(M) ribosomal protection determinant [133]as has been described in earlier studies [39, 48, 90]. In a recent study, 452 tetracycline resistant isolates were positive by PCR for one of six tetracycline resistance determinants (*tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(M)) with 33% of the isolates shown to encode more than one resistance gene. The tigecycline MIC_{90} for this collection of strains was 0.5 μg/mL with all of the isolates susceptible to 2 μg/mL [133].

Tigecycline activity also extends to *K. pneumoniae*: when tested against 199 clinical isolates of *K. pneumoniae*, the MIC_{90} for the clinical isolates was 1 μg/mL and 99% of strains were found to be inhbited by 2 μg/mL of tigecycline. However, 4 μg/mL was required to inhibit all isolates, reflecting the tendency of some strains of *K. pneumoniae* to show reduced susceptibility to tigecycline [103]. Nevertheless, the clinical isolates collected from the cIAI trial included 6 *K. pneumoniae* isolates that were ESBL producers for which the MIC range was the same as for the ESBL non-producers [4]. A recent survey of ESBL and AmpC producer clinical strains support the finding that the expression of ESBLs or AmpC β-lactamases does not impact the susceptibility of *K. pneumoniae* to tigecycline [58, 132]. A genetic analysis of 153 phenotypically defined ESBL encoding *K. pneumoniae* isolates showed that 71% encoded a bla_{CTX} β-lactamase (CTX-M-1 or CTX-M-2 family) and 28% encoded a bla_{SHV} (SHV-2, SHV-5, SHV-12) β-lactamase. Likewise, the presence of bla_{AmpC} (ACT-1, DHA-1), alone or in combination with an ESBL, did not impact tigecycline susceptibility (MIC_{90} 2 μg/mL). Although the MIC range for the 153 isolates was 0.25–8 μg/mL, the MIC_{90} was 2 μg/mL and the majority of isolates were fully susceptible to tigecycline (MIC ≤2 μ/mL) [58]. The reduced susceptibility to tigecycline in the eight isolates with an elevated tigecycline MIC (8 μg/mL) was not correlated with expression of an ESBL or AmpC and is proposed to be through up regulation of a multidrug efflux system.

The Tigecycline Evaluation and Surveillance Trial (T.E.S.T) collected tigecycline susceptibility data for ESBL-producing *K. pneumoniae* and demonstrated that the 126 ESBL positive organisms had equivalent susceptibility as the 1,334 non-ESBL producing strains (MIC_{90} for both subsets of strains was 2 μg/mL) [135]. A recent study by Morosini et al., investigated the activity of tigecycline against a collection of ESBL producing *Enterobacteriaceae* [81]. Tigecycline showed potent activity against this collection with 97.5% of isolates fully susceptible and the MIC_{90} was 1 μg/mL (MIC range 0.12–4 μg/mL). Additional recent large surveys of tigecycline activity have included ESBL producing *E. coli* and *K. pneumoniae* found that the MIC range, MIC_{50} and MIC_{90} were unaffected by the presence of an ESBL

determinant [13, 42, 49, 115]. Tigecycline has also shown potent activity against carbapenem resistant isolates as recently demonstrated during a carbapenem-resistant *K. pneumoniae* producing the KPC-2 β-lactamase outbreak in Brooklyn, NY hospitals [17]. Ninety-six isolates were obtained from ten hospitals and analyzed for susceptibility against a large panel of antimicrobials of which only tigecycline provided 100% coverage (MIC_{90} 1 μg/mL). The only other agent that provided better than 90% coverage against the outbreak strains was polymyxin B (MIC_{90} 2 μg/mL). Another large-scale study from the SENTRY antimicrobial surveillance program reported on the tigecycline susceptibilities of 104 carbapenemase-producing Enterobacteriaceae [20]. Tigecycline was the most active agent against this collection of pathogens, including *E. coli, K. pneumoniae,* and *Enterobacter* spp., with all isolates susceptible to 2 μg/mL and the MIC_{90} 1 μg/mL. The encoded carbapenemases in the strain collection included, KPC-2, KPC-3, IMP-1, VIM-1, NMC-A, and SME-1. Likewise, a study of 109 *Enterobacteriaceae* isolates expressing the VIM-1 metallo-β-lactamase, showed that 99% of isolates were susceptible to tigecycline at 2 μg/mL [92]. The tigecycline MIC_{90} was 1 μg/mL for the 31 *C. freundii* isolates analyzed; 2 μg/mL was required to inhibit all of the isolates from the intra-abdominal trial. Similarly, the 82 *E. cloacae* isolates tested were fully inhibited by exposure to 2 μg/mL tigecycline (MIC_{90} 1 μg/mL).

Tigecycline susceptibility testing of the *Proteeae* has consistently resulted in higher MICs than for other members of the *Enterobacteriaceae*. For 85 strains of *Proteus mirabilis* and 12 strains of *Proteus vulgaris* collected during the pivotal clinical trials, the tigecycline MIC_{90} was 4 and 2 μg/mL, respectively, with 8 and 4 μg/mL required, respectively, to inhibit all isolates [15]. Based on the FDA approved susceptibility breakpoint of ≤2 μg/mL, 47% of *P. mirabilis* and 8.0% *P. vulgaris* isolates would be classified as being non-susceptible to tigecycline [139]. Similarly, when tested against 22 strains of *Morganella morganii* the tigecycline MIC_{90} was 2 and 4 μg/mL was required to inhibit all strains; therefore, 9.0% of isolates would fall into the intermediate category. The activity of tigecycline has also been determined for a number of non-Enterobacteriaceae gram-negative pathogens [15]. As reflected in the MIC_{50} and MIC_{90} values of 16 and 32 μg/mL, respectively, tigecycline is less active against *Pseudomonas aeruginosa* than against some of the other non-Enterobacteriaceae [28]. Recently published tigecycline susceptibility studies on *A. calcoaceticus-baumannii* complex, including MDR strains, have shown variable results [19, 27, 47, 52, 56, 67, 83, 113, 127]; and as such may reflect differences in methodology as well as differences in region, site and associated epidemic clone. A recent study from the T.E.S.T. program evaluated 851 isolates from the US and reported an MIC_{90} of 2 μg/mL [47]. Nearly 30% of the isolates in the study were classified as multi-drug resistant. In a similar fashion, data on the utility of tigecycline for therapeutic intervention in infections caused by *A. baumannii-calcoaceticus* complex show mixed results [2, 87, 109, 123, 124].

Tigecycline was uniformly active against isolates of *H. influenzae* and isolates of *H. parainfluenzae* collected during the CAP clinical trials, with MIC_{90}s for both pathogens of 0.5 μg/mL [16]. These results were identical to preclinical data for that showed *H. influenzae* to be fully susceptible to tigecycline with an MIC_{90} 0.5 and

0.06–1 μg/mL [89]. A recent survey from Canada of 429 *H. influenzae* isolates using the reference test method showed similar results (MIC_{90} 0.25 μg/mL) [49]. In addition, against a small collection of *M. catarrhalis* isolates collected during the CAP trial, tigecycline inhibited all of the isolates at 0.12 μg/mL [16]. Similarly, for *M. catarrhalis* susceptibility testing, using the reference method resulted in values of 0.06 and 0.03–0.06 μg/mL, respectively, for the MIC_{90} and MIC range [89].

Tigecycline has shown good activity against anaerobic pathogens in preclinical studies and this was borne out in the recent clinical trials[15]. All of the *C. perfringens* and *P. micros* isolates collected were susceptible to tigecycline with 2 and 0.12 μg/mL of drug required to inhibit all isolates, respectively. Against *Bacteroides* spp., a wide range of MICs was seen, especially against isolates from the intra-abdominal trial. In contrast, the 33 strains of *B. vulgatus* in the collection, all from the cIAI protocol, were 100% susceptible to drug with an MIC_{90} of 2 μg/mL. A recent survey of anaerobes from Belgium supports the clinical data, as susceptibility among 443 anaerobes was 84% (MIC_{50} 0.5 μg/mL, MIC_{90} 8 μg/mL) [138]. The MIC_{90} for 238 *B. fragilis* group strains was 8 μg/mL; whereas, for 50 isolates of *Prevotella* spp., and 57 isolates of *Clostridium* spp., the MIC_{90}s were 0.5 and 4 μg/mL, respectively.

Furthermore, tigecycline has been shown to be active against several "atypical" bacterial species involved in respiratory infections such as *Mycoplasma* spp., and *Chlamydia* spp., [62, 100]. Showing comparable activity to comparator agents doxycycline, clarithromycin, and ofloxacin, tigecycline MIC ranges against 10 isolates of *C. pneumoniae* and five isolates of *C. trachomatis* were 0.125–0.25 and 0.03–0.125 μg/mL, respectively [100]. Tigecycline showed potent activity when tested against isolates of *M. hominis* and *M. pneumoniae* with all isolates inhibited at the MIC_{90} for each organism: 0.5 and 0.25 μg/mL, respectively [62]. Rapidly growing *Mycobacteria* spp., were also susceptible to tigecycline with all isolates of *M. abscessus* susceptible to 1 μg/mL and all isolates of *M. chelonae* and *M. fortuitum* susceptible to 0.25 μg/mL [136]. Importantly, an analysis of tetracycline resistant isolates of each species tested in this study resulted in identical tigecycline susceptibility data when compared to the tetracycline susceptible strains.

5.3.3 Mechanisms of Resistance to Tigecycline

Tigecycline is active against bacterial strains harboring all of the known tetracycline-resistance genes encoding efflux or ribosomal protection mechanisms tested to date; however, the possibility that these genes could readily mutate to a form capable of rendering cells resistant or, alternatively, that novel mechanisms could arise during exposure to tigecycline was explored in depth and remains the subject of ongoing research.

In preclinical studies, it was previously reported that certain efflux-encoding, tetracycline-resistance genes, *tet*(A) and *tet*(B), could mutate under selection to yield bacteria with reduced susceptibility to the earlier generation of glycylcyclines: DMG-MINO, MICs 8–16 μg/mL, DMG-DMDOT, MICs 4 μg/mL [45, 131].

However, these mutations resulted in strains with only a fourfold to eightfold increase in the tigecycline MIC (0.25–2 μg/mL) with the elevated MICs still falling within the susceptible category for tigecycline [139]. The clinical experience with tigecycline has yet to yield an isolate of *E. coli* with a tigecycline MIC that crosses the resistance breakpoint (≥4 μg/mL).

5.3.3.1 *S. aureus*

S. aureus mutants with reduced susceptibility have been raised in several laboratories following multiple serial passages on tigecycline [64, 72]. Investigators at Wyeth identified point mutations and deletions in the *mep*R gene that resulted in elevated tigecycline MICs. MepA, a novel efflux pump in the MATE family of multi-drug efflux pumps is regulated, at the transcriptional level, by the MepR repressor [72]. Presumably, the absence of an active repressor protein would have the consequence of over-expression of the cognate efflux pump, MepA, resulting in the increased MICs of tigecycline. Efflux may only be part of the story; however, this data suggests that the emergence of resistance in gram-positive organisms may involve multiple step mutations and will not arise rapidly.

5.3.3.2 The Proteeae and *P. aeruginosa*

Isolates of the family Proteeae appear to be intrinsically less susceptible to tigecycline. The MIC_{90}s for *P. mirabilis* and *P vulgaris* were 8 and 2 μg/mL, respectively, with the MIC range 0.5–8 μg/mL for both organisms [89, 134]. Likewise, *Providencia* spp., and *M. morganii* have broad ranges of susceptibility to tigecycline with MIC_{90}s of 8 and 2 μg/mL, respectively, and an MIC range of 0.12–8 μg/mL for both organisms [89, 102]. Using transposon inactivation, a tigecycline susceptible mutant was derived from a clinical isolate of *P. mirabilis* with reduced susceptibility (Table 5.2) [134]. It was determined that the transposon had inserted into the *acr*B gene, which is a component of the AcrAB efflux system. This efflux system, which pumps out a broad range of antibiotics, detergents, and dyes, is a member of the RND (resistance, nodulation, cell division) pump family [63, 94] and has been well characterized in other genera of Enterobacteriaceae, but had not previously been identified in *P. mirabilis*. RND pumps have broad specificity and expression of these efflux systems usually results in a multi-drug resistance (MDR) phenotype. Although close homologues of the AcrAB efflux system are found in *E. coli*, *K. pneumoniae* and *E. cloacae*, wild-type strains of these organisms do not show decreased susceptibility.

In a similar fashion, transposon mutagenesis of a *M. morganii* isolate with reduced susceptibility to tigecycline (MIC 4 μg/mL), resulted in the selection of two tigecycline-susceptible mutants (MIC 0.03 μg/mL) [102]. In both cases, the transposon insertion was mapped to the *acr*A gene. Northern blot and RT-PCR analysis of *acr*A expression showed that *acr*AB is expressed in higher amounts in the isolate with reduced tigecycline susceptibility compared to the tigecycline-susceptible isolate.

Therefore, increased expression of the AcrAB pump is the likely cause of reduced susceptibility of *M. morganii* to tigecycline (Table 5.2).

The decreased susceptibility to tigecycline in *P. aeruginosa* (MIC range 0.25–32 μg/mL) is also related to an RND efflux system [28]. Using a panel of well-characterized mutants, it was determined that the MexXY-OprM pump mediates intrinsic resistance to tigecycline, as the *mex*XY mutant strain resulted in an MIC of 0.5 μg/mL for tigecycline compared to 8 μg/mL for the wild-type parent strain. The MexXY-OprM pump is one of four pump systems in *P. aeruginosa* that belongs to the RND pump family [93]. Spontaneous resistant mutants of the *mex*XY deletion strain could be selected after a single passage on tigecycline, indicating that when challenged *P. aeruginosa* could easily compensate with another efflux system [28]. It was determined that when sufficiently over-expressed, both the MexAB-OprM and MexCD-OprJ pump systems could efflux tigecycline sufficiently to boost the tigecycline MIC over the breakpoint (8 μg/mL).

5.3.3.3 Other Enterobacteriaceae

In spite of the inability to easily select tigecycline-resistant mutants in the laboratory, several isolates of *Enterobacteriaceae* with reduced susceptibility to tigecycline were obtained from patients enrolled in clinical trials [60, 61, 101, 103, 105]. Acquired decreased susceptibility to tigecycline has been noted in *K. pneumoniae, E. aerogenes, E. cloacae*, and *E. coli* (Table 5.2). Although the majority of *Klebsiella pneumoniae* isolates are susceptible to tigecycline (MIC_{90} 2 μg/mL), a few clinical strains with reduced susceptibility to tigecycline have been isolated; such as strain G340, which has a tigecycline MIC of 4 μg/mL [103]. Indicative of a multidrug resistance (MDR) mechanism, MICs of tetracycline, minocycline, chloramphenicol, nalidixic acid, and trimethoprim were also elevated in isolate G340. Transposon mutagenesis of strain G340 resulted in tigecycline-susceptible mutants with MICs of 0.25 μg/mL. Mapping of the transposon insertion identified *ramA,* a gene previously identified in both *K. pneumoniae* and *E. cloacae* as an MDR determinant [111]. RamA is a transcriptional activator and member of a protein family that includes MarA, SoxS and Rob, which have been shown to promote antibiotic resistance due to up-regulation of the AcrAB-TolC pump [43]. As discussed above, the AcrAB efflux system is associated with reduced susceptibility to tigecycline in *P. mirabilis*. Constitutive over-expression of *ramA* in *K. pneumoniae* isolate G340 resulted in increased tigecycline MICs, suggestive of a role for RamA as an activator of AcrAB expression. In another study, elevated levels of *ramA* transcription coincided with increased expression of AcrAB in *K. pneumoniae* that were resistant to fluoroquinolones [111]. In addition, clinical isolates of *Klebsiella pneumoniae* were tested for a correlation between tigecycline MIC and expression of *ramA* by using real-time PCR. At MICs of 4 and 8 μg/mL, the expression of *ramA* was statistically significantly different from MICs of 2 μg/mL or less, supporting the tigecycline susceptibility breakpoint of <2_μg/mL for *K. pneumoniae* [105]. To determine the frequency of spontaneous mutations with reduced susceptibility to

Table 5.2 Organisms with decreased susceptibility to tigecycline

Organism	Intrinsic/ Acquired	Resistance determinant[a]	Efflux pump component(s)	Efflux pump expression[b]	Tigecycline MIC (μg/mL)
M. morganii	Intrinsic	*acr*A	AcrAB	+	8
				–	0.5
P. aeruginosa	Intrinsic	*mex*XY	MexXY	+[c]	8
				–	0.5
P. mirabilis	Intrinsic	*acr*B	AcrAB	+	4
				++	16
				–	0.25
E. coli	Acquired	*acr*A	AcrAB	+	1–2
		*acr*B		+	
		*mar*A		+++	
		*mar*B		++	
E. aerognes	Acquired	*acr*A	AcrAB	++	4
E. cloacae	Acquired	*acr*A	AcrAB	+	8
		*acr*B		–	0.5
				+	8
				–	0.5
K. pneumoniae	Acquired	*ram*A	AcrAB	+	4
				–	0.25
Acinetobacter calcoaceticus/ baumannii complex	Acquired	*ade*B	AdeABC	+	4
				–	0.5

[a]The gene that was identified by transposon mutagenesis as critical for reduced tigecycline susceptibility. In the case of *P. aeruginosa* the *mexXY* locus was deleted

[b]Level of expression of genes encoding efflux pump components was monitored by transcriptional profiling, northern blotting and/or RT-PCR

[c]Expression levels were not measured. The strains tested either contained a wild-type copy of *mexXY* or the locus was deleted

tigecycline, two susceptible *K. pneumoniae* clinical isolates were plated at 10^9CFU on tigecycline-containing medium at 16 times the MIC of the strain. Tigecycline-resistant colonies arose at a frequency of 3.5×10^{-8} and 4.4×10^{-8}, respectively [103]. While this result suggests that less susceptible strains can arise as a result of single-step mutations, the majority of clinical isolates tested have MICs values in the 0.25–1 μg/mL range.

Isolates of *E. cloacae* from four patients have been identified as having decreased susceptibility to tigecycline [60]. Comparison of the amounts of the *acr*AB transcript by northern blot analysis showed an increase in the amount of transcript in the clinical isolate with reduced tigecycline-susceptibility compared to a tigecycline susceptible isolate. Transposon mutagenesis of the isolate with reduced susceptibility mapped to the *acr* locus, insertions mapped to *acr*A and *acr*B. Similar to other species, inactivation of *acr* genes in *E. cloacae* resulted in significantly lower MICs

of tigecycline and other classes of antibacterial agents, such as Ethidium bromide and SDS.

A strain of *Enterobacter aerogenes* with reduced susceptibility to tigecycline was isolated from a single patient in a phase 2 clinical trial [60]. Two isolates, G587 (tigecycline MIC 0.25 μg/mL) and G666 (tigecycline MIC 4 μg/mL), from this patient were determined to be identical by ribotyping; therefore, it appeared that decreased susceptibility to tigecycline developed on therapy. Using RT-PCR and northern blotting, the level of *acr*A expression was significantly higher in G666 (tigecycline MIC 4 μg/mL) than in G587 (tigecycline MIC = 0.25 μg/mL). Therefore, the decreased susceptibility in *E. aerogenes* G666 was associated with the overexpression of the AcrA efflux pump.

Six *E. coli* isolates from an individual participant in a phase 3 clinical trial were examined for their differing susceptibility patterns for tigecycline [59]. Two isolates, G5048 and G5049, resulted in MICs of tigecycline of 1 and 2 μg/mL, respectively, whereas tigecycline MICs were 0.5 μg/mL for the remaining four isolates. All of the isolates from this patient were determined to be identical by ribotyping. In the two strains with elevated MICs, northern blot analysis demonstrated increased expression levels of the AcrAB efflux pump and MarA, a global response regulator that activates AcrAB [6]. Results were confirmed by transcriptional profiling that showed members of both the *mar* regulon and *acrAB* locus to be up regulated. Collectively, this data confirms the involvement of AcrAB and transcriptional activators, such as MarA, in decreased susceptibility to tigecycline that was established in earlier studies and extends these findings to clinical *E. coli* strains.

5.3.3.4 *Acinetobacter baumannii*

Two isolates of *A. baumannii* with reduced susceptibility to tigecycline (MIC 4 μg/mL) were identified in isolates from the phase 3 clinical trials for tigecycline [104]. As multidrug efflux pumps have been previously identified as contributing to tigecycline resistance in other organisms, the AdeABC pump, which is homologous to the AcrABC and MexAB-OprM pumps was targeted in *A. baumannii.* Using RT-PCR, it was shown that the expression of *ade*A was increased 27- and 37-fold in the two reduced susceptibility isolates relative to susceptible isolates. Therefore, the AdeABC pump was targeted for insertional inactivation and resulted in isolates fully susceptible to tigecycline. The regulatory region, *Ade*RS, that controls AdeABC expression encodes a two-component regulatory system [69]. Upon sequencing this region, it was discovered that the IS_{ABA-1} insertion element had inserted into the regulatory region [112]. The precise mechanism accounting for the increased expression of AdeABC in the resistant isolates is not fully elucidated at this time.

In summary, it is interesting to note that reduced susceptibility to tigecycline among clinical isolates is associated with up regulation of multidrug efflux pumps rather than by mutation of known, dedicated tetracycline resistance genes. This serves to underscore the distinct and improved properties of tigecycline as compared to the tetracycline antibiotics.

5.3.4 Clinical Indications

5.3.4.1 Complicated Skin and Skin Structure Infections

Two phase 3, randomized, double-blind trials were conducted to investigate the efficacy of tigecycline versus the standard combination of vancomycin/aztreonam in hospitalized patients with complicated skin and skin-structure infections [33]. Both protocols were international, muticenter studies enrolling patients from a total 118 centers representing 29 countries. Patient enrollment was initiated in August 2001 and the study ended in February 2004. Of the patients enrolled in the trials, 540 met the criteria for both clinical and microbiological evaluability: 279 treated with tigecycline and 261 treated with vancomycin/aztreonam. For both the clinically evaluable and microbiologically evaluable study populations, tigecycline met the statistical criteria for efficacy and non-inferiority when compared to vancomycin plus aztreonam. Cure rates for the clinically evaluable population were 86.5% and 88.6%, respectively, for tigecycline and comparator. Similarly, for the microbiological evaluable population, cure rates were 79.7% and 81.9% for tigecycline and comparator, respectively. With respect to microbiologic eradication as a measure of efficacy, tigecycline monotherapy was shown to be non-inferior to the standard therapeutic combination of vancomycin plus aztreonam. Specifically, for all MRSA, tigecycline, and comparator had eradication rates of 78.1% and 75.8%, respectively. In addition, tigecycline (77.8% cure) was as effective as comparator (75% cure) for treatment of patients with community acquired MRSA strains.

5.3.4.2 Complicated Intra-Abdominal Infections

Two phase 3, randomized, double blind trials were conducted to investigate the efficacy of tigecycline versus imipenem plus cilastatin in hospitalized patients with complicated intra-abdominal infections [4]. Enrollment in the study initiated in November 2002 and continued until May 2004. Both protocols were muticenter and international, enrolling patients from 190 centers located in 34 countries. A total of 512 tigecycline treated patients and 513 imipenem plus cilastatin treated patients met the criteria for both clinical and microbiological evaluability. For both the microbiologically evaluable (86.1% vs. 86.2%) and the modified microbiologic intent to treat (80.2% vs. 81.5%) (m-mITT) populations, the clinical cure rates were virtually identical for tigecycline and imipenem plus cilastatin. Tigecycline met the criteria for efficacy and non-inferiority when compared to imipenem plus cilastatin for both clinical populations studied. Additionally, for those patients with a positive pre-therapy blood culture (40 in tigecycline group, 50 in imipenem plus cilastatin group), clinical cure rates were 82.5% and 80.0% for tigecycline and imipenem, respectively. Similarly, tigecycline was efficacious and statistically non-inferior to imipenem/cilastatin when looking at microbiological eradication as an endpoint. In the case of *E. coli*, the most commonly isolated aerobe, eradication rates were

86.2% for tigecycline and 87.1% for comparator. Likewise, for the most commonly isolated anaerobe, *B. fragilis*, eradication rates were 78.2% and 80.8% for tigecycline and imipenem plus cilastatin, respectively. The in vitro spectrum of activity for tigecycline includes gram-negative pathogens expressing extended-spectrum β-lactamases (ESBLs). In the case of ESBL producing *E. coli,* tigecycline treatment resulted in clinical cure or eradication in 78% of patients; likewise, 83% of patients with ESBL producing *K. pneumoniae* resulted in a clinical cure or eradication following tigecycline therapy.

5.3.4.3 Community Acquired Pneumonia

Two phase 3, randomized, double blind trials were conducted to investigate the efficacy of tigecycline versus levofloxacin in hospitalized patients with community acquired pneumonia [32]. At the test of cure visit for the clinical evaluable population, tigecycline cured 89.7% of patients compared to levofloxacin, which cured 86.3% of patients. Similarly, at the test of cure visit for the clinical modified intent to treat population, tigecycline cured 81% of patients and levofloxacin cured 79.7% of patients. In both cases, tigecycline met the both the co-primary endpoints for efficacy and was non-inferior to the comparator.

5.4 Concluding Remarks

The tetracyclines were one of the first antibiotic classes discovered, and they were one of the first antibiotic classes to be mass-marketed. The history of tetracyclines represents a chronological progression, leading from the discovery of natural products with antibacterial activities to the development of these natural products (most notably chlortetracycline and oxytetracycline) as drugs, and thence to the improvement of the antibacterial, pharmacokinetic, and toxicological properties *via* semisynthesis. Lederle Laboratories led the discovery effort, while Pfizer and more recently Paratek Pharmaceuticals, have added value to the tetracycline family of antibiotics. The diligence and tenacity of scientists at both Lederle/Wyeth and Pfizer were responsible for bringing newer, more efficacious tetracyclines to the clinic. Through competition, Lederle and Pfizer challenged each other to further their research efforts in the antibacterial arena, to the benefit of patients for whom their drugs proved lifesaving. Wyeth recently developed and marketed a new generation of tetracycline antibiotics, the glycylcyclines, exemplified by tigecycline. Sadly, during the past decade there has been a sharp decline in efforts to discover and develop new antibacterial agents. Nonetheless, as the scourge of infectious bacterial diseases continues into the twenty-first century, the need for concerted efforts in antibacterial research remains unabated.

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Chapter 6
Macrolides and Ketolides

Ze-Qi Xu, Michael T. Flavin, and David A. Eiznhamer

6.1 Introduction

Macrolide antibiotics are a well-known class of antimicrobial agents and have long been considered drugs for the treatment of upper- and lower-respiratory tract infections caused by typical (such as *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Moraxella catarrhalis*, and *Haemophilus influenzae*) and atypical pathogens (such as *Mycoplasma pneumoniae*, *Legionella peumophila*, and *Chlamydophila pneumoniae*). They have been used as an alternative for patients allergic to β-lactams. However, worldwide resistance to these antibiotics is increasing at alarming pace. By 2005, 29.5% of *S. pneumoniae* strains isolated in the USA were found nonsusceptible to macrolides [1]. Many macrolide-resistant organisms are also resistant to β-lactams and some other antimicrobial classes, thereby limiting treatment alternatives for orally administered agents, particularly in children in whom fluoroquinolones are not approved for use [2]. In order to meet the challenges brought on by macrolide and other multidrug resistance, ketolides have been designed and developed. Ketolides are not only generally more potent than macrolides against susceptible microorganisms but are also active against species that are resistant to macrolides. Ketolides have a low propensity for selection of resistant mutants and have shown less frequent development of resistance in vitro. Therefore, ketolides may represent the future of macrolides and be an ideal replacement for the currently used macrolides.

The intent of this review is to focus on the current state of three macrolides erythromycin, clarithromycin, and azithromycin that have been used extensively in North America, the only approved ketolide telithromycin and the late stage development ketolide cethromycin. Future directions and challenges for the discovery and development of new macrolides and ketolides are also discussed.

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6.2 Development History and Chemistry

The first macrolide antibiotic, erythromycin (**1**), was discovered over 60 years ago in 1949 at Eli Lilly by McGuire, who isolated erythromycin from the metabolic products of a strain of the actinomycete *Saccharopolyspora erythraea*, formerly known as *Streptomyces erythreus*, found in soil samples collected from the Philippines by the Filipino scientist Abelardo Aguilar. It was first marketed by Eli Lilly in 1952 under the brand name Ilosone to overcome penicillin-resistant *S. aureus* [3]. Erythromycin is unstable in acidic environments and is poorly absorbed by the oral route, resulting in erratic pharmacokinetic behavior and gastrointestinal intolerance due to the acid degradation products. In acidic media, the 3-L-cladinose can be hydrolyzed and cyclization between the 6-OH and 9-keto groups can take place to form a hemiketal [4]. In addition, cyclization can occur between the 12-OH and 9-keto groups [4].

In the late 1970s, the outbreak of Legionnaire's disease, the discovery of *L. pneumophila* and the implication of *Chlamydia trachomatis* in sexually transmitted diseases led to renewed interest in the macrolide class of antibiotics. Clarithromycin (**2**) was then invented by scientists at Taisho Pharmaceutical, a Japanese drug company, in the 1980s [5]. In 1991, it was introduced to the Japanese market under the brand name of Clarith and also gained FDA approval as Biaxin through Abbott Laboratories [6].

Azithromycin (**3**) was discovered by a team of Croatian scientists at Pliva in the 1980s and marketed 8 years later in Central and Eastern Europe under the brand name of Sumamed [7]. In 1991, azithromycin gained FDA approval and was brought to the US market by Pfizer under the brand name of Zithromax [8].

The prevalence of *S. pneumoniae* resistant to multiple antibacterial agents, including macrolides, has spurred the search for new antibiotics to combat multidrug-resistant organisms and has led to the development of ketolides. Telithromycin (**4**) was synthesized by the French pharmaceutical company Hoechst Marion Roussel (later Sanofi-Aventis) in the 1990s [9]. Telithromycin was first approved by the European Commission in 2001 and subsequently by the FDA in 2004 under the brand name of Ketek [10].

Cethromycin (**5**) was created at Abbott Laboratories in the late 1990s [11, 12] and then licensed to Advanced Life Sciences in 2004 [13]. An NDA of cethromycin for the treatment of mild to moderate community-acquired pneumonia was submitted to the FDA in September of 2008.

As can be seen from the molecular structures (Fig. 6.1), the second generation of macrolides and ketolides are all semisynthetic derivatives of erythromycin. Clarithromycin differs only in the 6-*O*-methyl group while azithromycin has a 15-membered lactone, with a methylamine inserted between the C-9 and C-10 positions and the ketone group at the C-9 position reduced. The substitution at 6-O in clarithromycin or removal of the 9-keto group in azithromycin either decreases or prevents the acid-catalyzed hemiketal cyclization seen in erythromycin, resulting in increased acid stability in the digestive tract, improved oral bioavailability,

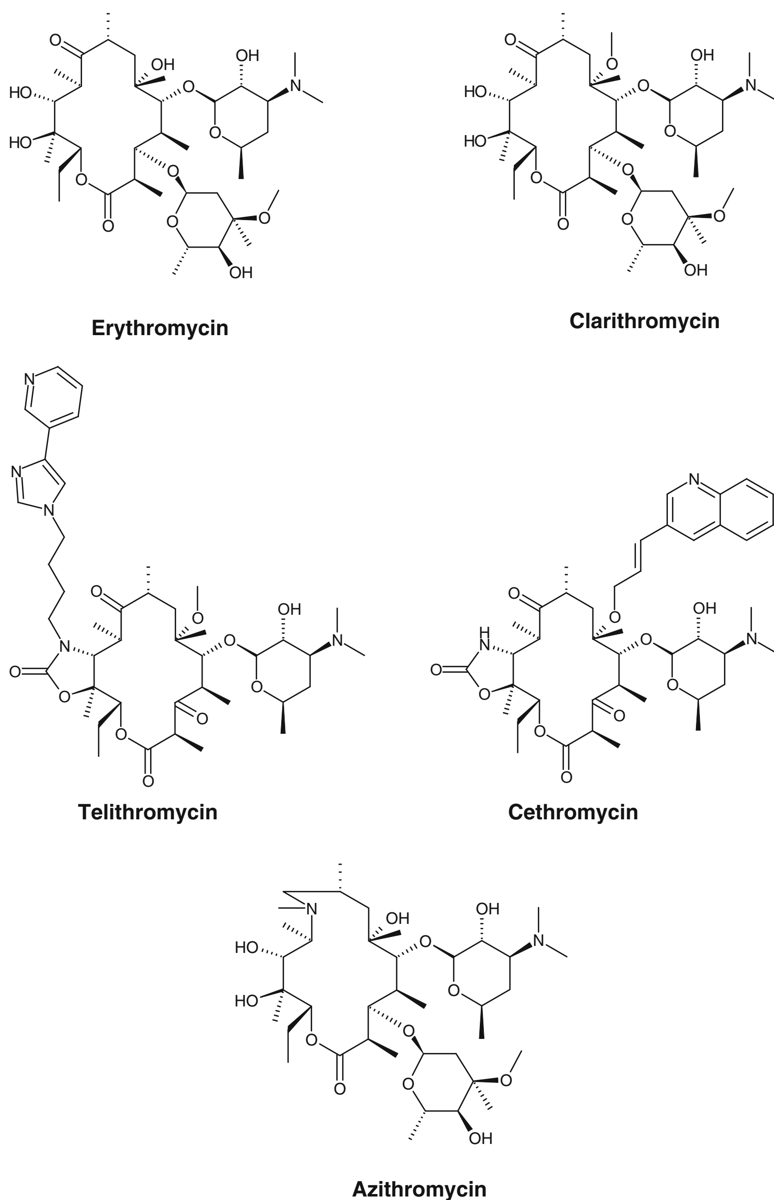

Fig. 6.1 misses the chemical structures of telithromycin and cethromycin

prolonged elimination half-life, and diminished gastrointestinal-related side effects such as nausea and stomach ache. However, the 3-L-cladinose present in both clarithromycin and azithromycin is still prone to hydrolysis in acidic media.

The ketolide antibiotics telithromycin and cethromycin contain the core 14-membered lactone ring. The chief structural characteristic of a ketolide is the

replacement of L-cladinose at the C-3 position by a ketone group, hence giving rise to the name ketolide, the incorporation of a carbamate side ring at C-11 and C-12, and the substitution at the 6-O position. These changes have not only eliminated acid-labile functionalities, thus further improving the chemical stability, but, more importantly, they have rendered significant increases in the antibacterial activity. For instance, the presence of a 3-keto group is responsible for the potent activity against inducible *erm*-containing *S. pneumoniae* and *S. aureus* and enhanced activity against strains of *S. pneumoniae* and *S. pyogenes* with *mef*-mediated macrolide efflux. The cyclic 11,12-carbamate moiety and the aryl side chain stabilize the conformation of the ketolide skeleton and provide additional interactions with the bacterial ribosome, thus improving the antibacterial activity [14].

It is also worth noting structurally significant differences between telithromycin and cethromycin. Telithromycin has a pyridinyl-imidazolyl-butyl group attached at N-11 of the carbamate ring, whereas cethromycin possesses a quinolinyl-allyl side chain substituted at the C-6 position, similar to clarithromycin. These structural differences may account for different side-effect profiles observed in the clinical use of telithromycin and cethromycin.

6.3 In Vitro Antibacterial Activity

The in vitro activities of macrolides and ketolides are shown in Tables 6.1–6.4, as represented by the range of MIC values and the minimum concentration of antibacterials required to inhibit growth in 90% of the tested isolates (MIC_{90}) for each organism.

The ketolides display potent activities against most of the Gram-positive aerobic bacteria (Table 6.1) [15]. Even though macrolide MICs generally rise with those of penicillin resistance, ketolides are consistently active against all pneumococci, irrespective of their penicillin-susceptibility. All of the penicillin-nonsusceptible isolates of *S. pneumoniae* were inhibited by the two ketolides cethromycin and telithromycin (MICs of ≤2.0 μg/mL), 97% of which were inhibited by cethromycin and 87% by telithromycin at a concentration of 0.125 μg/mL. In comparison, 71% of penicillin-nonsusceptible pneumococci were nonsusceptible to erythromycin and azithromycin [16].

The two ketolides, cethromycin and telithromycin, are very potent against *S. pneumoniae*, regardless of macrolide susceptibility. Both agents inhibited 100% of erythromycin-susceptible isolates of *S. pneumoniae* at a concentration of 0.125 μg/mL. Against erythromycin-nonsusceptible isolates, cethromycin inhibited 97% of the isolates at an MIC of ≤0.125 μg/mL and telithromycin inhibited 83% of the isolates at this concentration [16]. The superiority of cethromycin against *mef*(A,E) strains suggests that cethromycin is a poor substrate for this family of efflux pumps [17].

The activity of ketolides against *S. pneumoniae* isolates carrying both *erm*B and *mef*A is very significant, as this genotype exhibited multidrug resistance and its prevalence increased from 9.7% to 18.4% from 2000 to 2004 in the USA [18].

Table 6.1 In vitro activity of macrolides and ketolides against aerobic Gram-positive bacteria

Organism	Phenotype	MIC_{90} (μg/mL)					Reference
		Erythromycin	Clarithromycin	Azithromycin	Telithromycin	Cethromycin	
S. pneumoniae	PSSP	0.06	≤0.03	0.12	0.008	≤0.008	[17, 33]
	PISP	128	>64	128	0.125	0.125	[16, 34]
	PRSP	128	>64	128	0.125	0.125	[16, 34]
	Ery-S	0.03	0.03	0.12	0.015	0.004	[16, 31]
	Ery-*mef*A	8	4	8	1	0.06	[17, 31]
	Ery-*mef*E	16	32	16	0.5	0.125	[35]
	Ery-*erm*B	>128	>128	>128	0.5	0.25	[19]
	Ery-*erm*B/*mef*A	>128	–	–	0.25	0.25	[17]
	Ery-*erm*B/*mef*E	>128	>128	>128	1	0.5	[35]
	Ribosomal mutation	>128	32	>64	0.12	0.12	[17, 36]
	Tet-R	0.5	0.5	1	0.12	0.06	[19]
	TMP/SMZ-R	0.5	0.5	0.5	0.12	0.06	[19]
	Cipro-R	0.5	0.5	1	0.12	0.06	[19]
S. aureus	MSSA	64	16	32	0.06	0.06	[22, 23]
	MRSA/Ery-S	0.125	0.063	–	------	≤0.031	[24]
	MRSA/MLS_B inducible	>128	>128	–	------	≤0.031	[24]
	MRSA/MLS_B constitutive	>128	>128	–	>128	>128	[24, 37]

PSSP; penicillin-susceptible *S. pneumoniae*
PISP; penicillin-intermediate *S. pneumoniae*
PRSP; penicillin-resistant *S. pneumoniae*
Ery-S; erythromycin-susceptible
Ery-I; erythromycin-intermediate
Ery-R; erythromycin-resistant
*mef*A or *mef*E; macrolide efflux
*erm*B; macrolide methylase
Tet-R, tetracycline-resistant
TMP/SMZ-R, trimethroprim/sulfamethoxazole-resistant
Cipro-R, ciprofloxacin-resistant
AMP-R = ampicillin-resistant
Vanc-S, vancomycin-susceptible
BLNAR = β-lactamase-negative ampicillin resistant

Table 6.2 In vitro activity of macrolides and ketolides against aerobic Gram-negative bacteria

		MIC$_{90}$ (μg/mL)					
Organism	Phenotype	Erythromycin	Clarithromycin	Azithromycin	Telithromycin	Cethromycin	Reference
H. influenzae	β-lactamase negative	16	16	2	2	4	[33, 49]
	BLNAR	16	16	4	4	4	[27, 50]
	β-lactamase positive	16	16	2	2	4	[33, 49]
M. catarrhalis	β-lactamase negative	0.25	0.5	0.06	0.12	0.12	[19]
	β-lactamase positive	0.25	0.25	0.06	0.12	0.12	[19]
N. gonorrhoeae	–	0.5	0.25	0.12	0.06	0.03	[24, 31]
N. meningitidis	–	0.25	0.06	0.12	0.03	0.008	[24, 53]
B. pertussis	–	0.06	0.06	0.06	0.03	-----	[52]
H. pylori	Ery-S	0.25	0.015	0.5	0.125	0.06	[31, 51]
	Ery-R	>128	128	>128	32	64	[31, 51]

BLNAR; β-lactamase-negative ampicillin resistant
Ery-S; erythromycin-susceptible
Ery-R; erythromycin-resistant

Table 6.3 In vitro activity of macrolides and ketolides against anaerobic bacteria

		MIC_{90} (μg/mL)					
Organism	Phenotype	Erythromycin	Clarithromycin	Azithromycin	Telithromycin	Cethromycin	Reference
B. fragilis	–	>32	8	>32	32	8	[54]
C. difficile	–	>32	>32	>32	>32	>32	[54]
C. perfringens	–	2	0.125	4	0.25	≤0.06	[54]
Fusobacterium spp.	–	8	4	2	1	0.5	[54]
Peptostreptococcus spp.	–	2	0.5	4	≤0.06	≤0.06	[54]

Table 6.4 In vitro activity of macrolides and ketolides against other clinically important bacteria

Organism	Phenotype	MIC_{90} (μg/mL)					Reference
		Erythromycin	Clarithromycin	Azithromycin	Telithromycin	Cethromycin	
C. pneumoniae	TWAR	0.06	0.06	0.125	0.06	0.015	[57]
M. pneumoniae	–	≤0.004	≤0.001	≤0.001	≤0.015	≤0.001	[58, 60]
Ureaplasma spp.	–	1	0.063	1	0.03[a]	0.016	[58, 60]
L. pneumophila	Serogroup 1	1	0.06	2	0.25	0.06	[59]
	Serogroups 2-7, and 15	0.5	0.03	0.25	0.125	0.03	[59]

[a] *Ureaplasma urealyticum*

Ribosomal mutations have been described as a rare cause of clinical macrolide resistance in *S. pneumoniae*. The data suggest that mutations in streptococcal rRNA or ribosomal proteins, most likely selected by macrolide exposure, do not typically result in resistance to ketolides [17].

Macrolides and ketolides are generally susceptible in *S. pneumoniae* isolates resistant to other classes of antibiotics including fluoroquinolones, tetracyclines, trimethoprim/sulfamethoxazole, and second- or third-generation cephalosporins [19–21].

Against methicillin-susceptible *S. aureus* (MSSA) strains, ketolides exhibited excellent in vitro activities, which are at least 256-fold more active than the macrolides and are comparable to the fluoroquinolones [22]. Different MIC_{90} values of cethromycin against methicillin-resistant *S. aureus* (MRSA) strains were reported, with one being 0.06 μg/mL [22] and the other >128 μg/mL [23], reflecting different endemic MRSA in the two testing centers. However, when the *S. aureus* isolates were classified regarding their MLS_B phenotype, it is evident that cethromycin was very potent, with the MIC_{90} being ≤0.031 μg/mL, against methicillin-resistant but macrolide-susceptible or -inducible MLS_B resistant *S. aureus* [24]; it was the MRSA isolates with constitutive MLS_B phenotype, due to constitutive production of *erm*A or *erm*C methylase, that were very resistant to both ketolides (MIC_{90} of >128 μg/mL) [20, 24, 25].

Erythromycin resistance is invariably present in MRSA. It was reported that there was 8.2% resistance to erythromycin among MSSA and 87.9% erythromycin resistance among MRSA [26]. Due to the apparent inability of ketolides to act as inducers of methylase production, they maintain good in vitro activity against *S. aureus* strains with inducible methylase but lose activity against strains constitutively producing *erm*A or *erm*C methylase [17].

In addition, cethromycin was effective (MIC_{90} 0.06 μg/mL) against staphylococcal strains that were macrolide-resistant but clindamycin-susceptible, although much less effective (MIC_{90} >16 μg/mL) against staphylococcal strains that were resistant to macrolides and clindamycin [27].

Cethromycin was also effective against the USA300 clone of community-associated MRSA (CA-MRSA) [28], which has been implicated in recent outbreaks within the USA and worldwide and is resistant to many currently marketed antimicrobial agents. Ketolides are active against macrolide-susceptible and -inducible MLS_B resistant coagulase-negative *Staphylococcus* spp. (CoNS) but again lack activity against constitutive MLS_B resistant CoNS [24, 29]. The methicillin susceptibility alone had no effect on ketolide MIC values for CoNS.

Cethromycin is the most active agent against *S. pyogenes* among the macrolides and ketolides; it is also extremely active against macrolide-resistant *S. pyogenes* due to the *mef*A efflux, the inducible *erm*A, or the constitutive *erm*B methylase [17, 38]. In comparison, telithromycin MIC values increased to resistant levels against *S. pyogenes* isolates harboring the *erm*B determinant, either alone or in combination with the *mef*A [30, 39]. There is no cross-resistance between macrolide/ketolide and the fluoroquinolone classes of antibiotics [19].

Against other *Streptococcus* spp. such as *S. agalactiae* and Viridans group streptococci, ketolides are generally active regardless of macrolide susceptibility [27, 40–42, 44]. The macrolides had poor activity against *Enterococcus* spp. and ketolides were in general more active. However, none of the agents possessed activity against erythromycin- or vancomycin-resistant *E. faecalis* and *E. faecium* [25, 27, 43, 45, 46]. Both *Listeria monocytogenes* and *Corynebacterium* spp. showed susceptibility to all agents, with ketolides being more potent than macrolides [27, 31, 32].

Even though the majority of Gram-negative aerobes including *Pseudomonas aeruginosa*, *Enterobacteriaciae*, and *Acinetobacter* are intrinsically nonsusceptible to macrolides and ketolides, both classes of agents were active against a number of clinically important Gram-negative aerobic bacteria (Table 6.2). Ketolides and azithromycin had similar activity against *H. influenzae*, which were fourfold more active than erythromycin and clarithromycin [33, 49]. Their in vitro activities were not affected by the presence of β-lactamase production or by ampicillin susceptibility [27, 50]. This is significant as the prevalence of ampicillin-resistant *H. influenzae* due to β-lactamase production among the nontypable strains responsible for community-acquired respiratory infections is very high and resistance to β-lactams due to modification of penicillin-binding proteins (β-lactamase negative, ampicillin resistance – BLNAR) has also been reported.

Over 90% of all *M. catarrhalis* isolates, a common respiratory tract pathogen, produce β-lactamases that render them resistant to antibacterial agents such as amoxicillin. Ketolides had activity against *M. catarrhalis*, identical to the activity of azithromycin but twofold more active than erythromycin and clarithromycin. No difference in susceptibility was seen between β-lactamase-positive and β-lactamase-negative strains [19].

Clarithromycin is the most active macrolide against *Helicobacter pylori* and limited data indicated that ketolides have activity against clarithromycin-susceptible strains but are nonsusceptible to clarithromycin-resistant isolates [31, 51]. Both macrolides and ketolides displayed good in vitro activity against *Neisseria* spp. and *Bordetella pertussis* [24, 31, 52, 53]. MIC_{90} values of ketolides are generally one to two dilutions lower than macrolides. There was no data published for cethromycin against *B. pertussis*.

MIC data demonstrate the excellent activity of ketolides against a variety of clinically important anaerobic pathogens (Table 6.3), including Gram-positive anaerobes such as *Peptostreptococcus* spp. and *Clostridium* spp., as well as Gram-negative anaerobes such as *Fusobacterium* spp. and *Prevotella/Porphyromonas* spp. Macrolides are generally less active than ketolides and cethromycin is the most active [54]. Against 110 unique toxigenic *C. difficile* isolates of differing REA (restriction endonuclease analysis) type known to have caused *C. difficile*-associated disease, cethromycin showed a bimodal distribution with MIC values of 0.03–4 μg/mL against 80% of the isolates but an $MIC_{90} = 128$ μg/mL [55]. Both macrolides and ketolides have a poor activity against *Bacteroides* spp., a Gram-negative anaerobic pathogen [54, 56].

As shown Table 6.4, both macrolides and ketolides are very effective against the clinically important intracellular and atypical pathogens *C. pneumoni*ae, *L. pneumo-*

Table 6.5 Comparison of macrolide and ketolide IC_{50} (μg/mL) values for inhibition of cell growth and function in *S. aureus* cells [61] and apparent dissociation constants K_d (nM) in other microorganisms

Antimicrobial	Growth Rate	Protein Synthesis	50S Formation	K_d [a]
Erythromycin	0.25	0.1	0.2	41[b]
Clarithromycin	0.1	0.075	0.15	9.5[c]
Telithromycin	0.1	0.04	0.08	1.3[c]
Cethromycin	0.035	0.02	0.035	0.61[b]

[a]K_d: apparent dissociation constant = reverse rate constant (K_{-1})/ forward rate constant (K_1)
[b]Organism: *S. pneumoniae* [68]
[c]Organism: *E. coli* [69]

phila, *M. pneumoniae*, and *Ureaplasma urealyticum* [57–60]. Against *L. pneumophila* and other *Legionella* species [59], cethromycin (MIC_{90} = 0.06 μg/mL) and clarithromycin (MIC_{90} = 0.06 μg/mL) were the most active agents. These antimicrobial agents were more active than telithromycin (MIC_{90} = 0.25 μg/mL), and at least eightfold more active than the other macrolides tested (azithromycin MIC_{90} = 2.0 μg/mL and erythromycin MIC_{90} = 1.0 μg/mL). Most of the *L. pneumophila* strains tested belonged to Serogroup 1, but similar MIC results were observed for all agents, except for azithromycin (MIC_{90} = 0.25 μg/mL), for Serogroups 2–7, and 15. Cethromycin also maintained good activity against *Legionella* species other than *L. pneumophila*.

6.4 Mechanism of Action

Macrolides and ketolides exert their antimicrobial effects by preventing the bacterial ribosome from translating its messenger RNAs into new proteins. The bacterial ribosome is composed of two subunits, the small 30 S subunit made of 16 S ribosomal RNA and 21 ribosomal proteins, and the large 50 S subunit consisting of 5 S and 23 S rRNA and more than 30 ribosomal proteins. Macrolides and ketolides bind to the 23 S rRNA of the 50 S ribosomal subunit, which blocks the peptide exit channel and stimulates the dissociation of peptidyl-tRNA from the ribosome during the translocation process [61].

A second and potentially equally important mode of action of macrolides and ketolides is their ability to interact with partially assembled 50 S subunit precursors and to inhibit the complete formation of bacterial ribosomes with the unassembled precursor particles undergoing nucleolytic degradation. By contrast, 30 S particle formation was generally unaffected. As shown in Table 6.5, the ketolides were better inhibitors of cell growth and function than the macrolides, with cethromycin showing the best inhibition of protein synthesis and 50 S particle formation in *S. aureus* cells [61, 62]. Cethromycin was threefold more effective than telithromycin in *S. pneumoniae* cells [63, 64]. However, both ketolides appeared to be ineffective

in inhibition of 50 S particle formation in *H. influenzae* cells. Erythromycin was also ineffective in this regard, whereas azithromycin was a good inhibitor, explaining the superiority of this drug against *H. influenzae* [65, 66].

Cethromycin demonstrated a twofold higher binding affinity with *S. pneumoniae* ribosomes compared to that of telithromycin and a tenfold improvement in binding affinity over erythromycin [17]. The binding affinity of cethromycin with *H. influenzae* ribosomes was more than 20-fold tighter than that of erythromycin [67]. Cethromycin had a rapid association with ribosomes and a very slow dissociation compared to those of erythromycin in both *S. pneumoniae* and *H. influenzae*, resulting in smaller K_d (Table 6.5) [67, 68]. Telithromycin and cethromycin demonstrated a similar level of inhibitory effect on the *H. influenzae* protein synthesis with an IC_{50} value of 1.25 μg/mL [65].

Footprinting (chemical protection) experiments have shown that both macrolides and ketolides interact with domains II and V of the 23 S rRNA. These compounds protected residues A2058, A2059, and G2505 in the central loop of domain V from chemical modification, indicating direct contact between drug and rRNA [70]. Even though both macrolides and ketolides influenced residue A752 in hairpin 35 of domain II in the 23 S rRNA, their footprints were distinctively different [71]. Erythromycin led to enhanced modification at position A752 by dimethylsulfide (DMS) and removal of cladinose caused the loss of this footprint. The enhanced accessibility of A752 appeared not to be due to the direct contact of the cladinose, but via allosteric interaction elsewhere. In contrast, telithromycin and cethromycin protected A752 through the 11,12-carbamate side ring, suggesting direct interaction [69, 71]. This additional interaction with domain II may account for the tighter binding to ribosomes observed for ketolides [72].

Furthermore, ribosomal crystallography studies of bacterial ribosomes complexed with macrolides or ketolides have confirmed the binding sites and detailed the interactions between the antibiotics and the ribosome. The crystallographic structures of complexes of the 50 S ribosomal subunit from *Deinococcus radiodurans* with erythromycin, clarithromycin, azithromycin, telithromycin, and cethromycin reveal that all these drugs bind in the upper chamber of the exit tunnel adjacent to the peptidyl-transferase center near the constriction formed by the extended loops of proteins L4 and L22 [73–75]. The majority of the binding to the ribosome occurs via the macrocyclic lactone ring and the desosamine sugar moiety, with the former associating with the tunnel wall formed by positions 2057–2059 and the latter orienting toward the peptidyl-transferase center. There are no major interactions between the cladinose sugar in macrolides and the 23 S rRNA, implying that cladinose can be dispensable. As shown above, ketolides, which have a ketone group at the C-3 position instead of the cladinose sugar, bind tighter to ribosomes than macrolides. The nucleotides implicated in macrolide and ketolide binding (A2058, A2059, and G2505) are predominantly located in domain V of the 23 S rRNA [76] and there are no interactions with ribosomal proteins [73–75]. Surprisingly, no direct contact with A752 was observed with ketolides. However, the studied ketolides contact domain II via their side chains with the nucleotide residue U790, which is located at a distance of 11 Å from A752.

Even though it is fairly certain that the general location of the macrolide/ketolide-binding site is the same in ribosomes, the orientation of an individual drug inside the binding pocket and its interactions with the target may vary and may not be preserved among all ribosomes from different species. Compared with macrolides and other ketolides, cethromycin is shifted 3 Å deeper into domain V, creating stronger interactions via additional hydrophobic contacts with the carbamate group and desosamine. The quinolylallyl side chain of cethromycin forms an additional hydrogen bond with O2' of U790 in domain II of the *E. coli* 23 S rRNA through the quinolyl N3. In addition, the quinolylallyl group provides an unexpected interaction with domain IV through hydrophobic contacts with U1782, which had not previously been reported to participate in the binding of macrolides and other ketolides [74].

Azithromycin contains an additional nitrogen atom that increases the size of its macrocyclic lactone ring; however, this nitrogen does not directly contribute to the binding of the drug. Rather, it appears to alter the conformation of the macrocyclic lactone ring enough to induce novel contacts with a putative magnesium ion that serves as a bridge between the azithromycin molecule and the ribosome. The orientation of the cladinose sugar in azithromycin appears also to be different from that in erythromycin, enabling an additional hydrogen bond with U2586. The second binding site seen may be specific to *D. radiodurans*, as no other bacterial organism has a Gly60 residue, and this second binding was not seen in the *Haloarcula marismortui* complex [77].

In the telithromycin-bound ribosome from *H. marismortui*, the alkylaryl side chain of telithromycin was folded over the plane of the macrocyclic lactone ring oriented in the direction opposite to that observed in a complex with the *D. radiodurans* ribosome [77].

6.5 Mechanism of Resistance

There are three major mechanisms by which bacteria become resistant to macrolides:

- rRNA methylation
- Macrolide efflux
- Ribosomal mutation

The rRNA methylation process is accomplished by an adenosine-N^6 methyl transferase, coded for by the *erm* (*e*rythromycin *r*esistance *m*ethylase) family of genes, which either mono- or dimethylate the adenine residue A2058 in the peptidyltransferase center of the 23 S rRNA. Methylation of A2058 disrupts hydrogen bonding between the 2'-OH group of the desosamine sugar and A2058, producing a posttranslational conformational change in the 50 S ribosomal subunit. The added one or two methyl groups at N^6 of A2058 sterically hinders the access of macrolides to the ribosomal binding site. Monomethylation confers a low level of resistance to macrolides, whereas dimethylation confers high levels of resistance to all the

macrolides, lincosamides (clindamycin), and streptogramin B antibiotics (MLS_B phenotype) [78].

There are 21 different classes of *erm* genes which are carried on either plasmids or transposons, with the resultant MLS_B resistance either constitutively or inducibly expressed [79, 80]. Constitutive *erm* expression is often associated with structural changes to the *erm* mRNA attenuator and with the production of an active mRNA, resulting in high-level cross-resistance to MLS_B drugs. Inducible expression is associated with the production of an inactive mRNA encoded for methylase production. Upon ribosome stalling in the presence of drug, a translation attenuation mechanism promotes a conformational change in the mRNA that allows a formerly sequestered Shine–Dalgarno (SD) sequence to become accessible. The structure of the attenuator differs for the various classes of *erm* genes, resulting in a few major phenotypes of inducible MLS_B resistance. The *erm*B gene, carried on the constitutive transposons Tn*1545* and Tn*3872*, is the most clinically significant gene occurring typically in streptococci. The *erm*A and *erm*C genes occur in staphylococci, and the *erm*TR gene in *S. pyogenes* [81]. The *erm*B gene can be transmitted between organisms by transposons in addition to clonal spread.

Although ribosome-based mutations conferring macrolide resistance are common for microbes with one or two rRNA alleles, these mutations are found in less than 5% of the macrolide-resistant pathogens with four or more alleles. Ribosomal mutations at nucleotides directly involved in drug binding in domain V of 23 S rRNA by base substitution A2058G confer MLS_B resistance in *S. pneumoniae*. The exocyclic amino group at the 2-position of guanine disrupts the hydrogen bonding between the 2’-OH group of desosamine and the N^1 position observed for adenine. This 2-amine group would also alter the nature of the hydrophobic tunnel wall, making it less suitable for interaction with the hydrophobic face of the macrocyclic lactone ring. In the same way, point mutations at the adjacent nucleotide A2059G present a ML or MLS_B resistance phenotype. The change of C→T at 2611 results in the disruption of a Watson-Crick base pairing between 2057 and 2611 at the end of helix 73 before it opens into the central loop of domain V. It is conceivable that this base pair is essential for maintaining the conformation of A2058 and A2059 within the ribosomal binding site [82].

A single base deletion (A752) in a stretch of highly conserved adenines in hairpin loop 35 of domain II of 23 S rRNA was selected in vitro by clarithromycin and was resistant to all macrolides [83]. The deletion may have resulted in structural changes to the domain II ribosomal binding site by disruption of helix 35 [82]. Mutations in ribosomal proteins L4 and L22 have also been implicated in macrolide resistance. Common mutations in L4 occur in a highly conserved stretch of amino acids ($_{63}$KPWRQKGTGRAR$_{74}$), including single amino acid changes (G69C, G71R), substitutions, and insertions, while mutations in L22 all appear to be single amino acid substitutions and can occur in combination with L4 mutations [83]. As there are no direct contacts between ribosomal proteins and macrolides, the mechanism of resistance must be due to conformational changes to the residues of the binding site. The tip portion of L4 forms a portion of the polypeptide exit tunnel just below where the macrocyclic lactone binds. The large β-sheet of L22 interacts with the rRNA hairpin 35 in domain II and forms part of the hydrophobic tunnel wall.

The conformational changes induced by L22 mutations may disrupt the interaction of the hairpin tip with nucleotides C748, A750, and A751 of domain II which is, in turn, in strong contact with U2609 in domain V. The conformational change of the tip of the hairpin may also result in a large opening of the tunnel, rendering the macrolide unable to block the peptide exit tunnel [82].

The macrolide-specific efflux pump encoded by *mef* (*m*acrolide *ef*flux) genes binds macrolides and pumps them out of the cell. A *mef* gene is homologous to genes encoding membrane-associated transport proteins of the major facilitator superfamily [84]. The *mef*A gene was first identified in 1996 in *S. pyogenes* and was also subsequently found to be common in *S. pneumoniae*. The *mef*E gene was detected a year later in *S. pneumoniae* and found in a variety of other *Streptococcus* species. The *mef*A gene is carried on the transposon Tn*1207.1* in *S. pneumoniae* and Tn*1207.3* in *S. pyogenes*, while the *mef*E gene is carried by a different chromosomal element called "mega." Organisms expressing *mef* gene display a low level of resistance to only macrolides (M-phenotype). A related ATP-binding cassette gene, *msr*A, is found in *S. aureus* and is also associated with macrolide efflux. An *msr*D gene with homology to *msr*A was identified in *S. pneumoniae*, which is located immediately downstream of the *mef* gene and can be cotranscribed with *mef* [81].

Ketolides retain activity against strains of *S. pneumoniae* that have become resistant to macrolides via *mef*-encoded efflux or by possessing an inducible or constitutive *erm* gene that confers resistant to MSL_B drugs (Table 6.1). The potent activity of ketolides against constitutive *erm*B streptococci may be due to their higher affinities for methylated ribosomes than macrolides. Even though it is less effective in binding to methylated ribosomes, cethromycin binds to methylated ribosomes as effectively as erythromycin binds to unmethylated ribosomes [68]. Mutations in streptococcal rRNA or ribosomal proteins, most likely selected by macrolide exposure, do not typically result in resistance to ketolides [17]. In studies of *S. pyogenes*, ketolides are effective against almost all erythromycin-resistant clinical isolates. Cethromycin maintains activity against MRSA isolates that are macrolide-susceptible or -inducible MLS_B resistant [24] but loses activity against MRSA isolates with the constitutive MLS_B phenotype, due to constitutive production of *erm*A or *erm*C methylase (Table 6.1). The efflux pump encoded by the *msrA* gene in the USA300 clone of CA-MRSA can pump out azithromycin, clarithromycin, and erythromycin, but cannot eliminate cethromycin [28].

In contrast to macrolides, ketolides do not induce higher levels of 23 S rRNA methylation, and thus MLS_B resistance, in strains of *S. pneumoniae*, *S. aureus*, and *S. pyogenes* with an inducible *erm* gene [31, 68, 86]. This unique property is due to the lack of the 3-cladinose sugar in the ketolides [87]. A more recent report showed that ketolides may induce a very low level of *ermC* expression in *E. coli* [88].

Although reported ketolide resistance is rare [18], a few laboratory-derived and clinical isolates of *S. pneumoniae* have been documented [89, 90]. A deletion of the upstream region of the *erm*B attenuator resulted in a highly telithromycin-resistant strain. This deletion has been associated with the change from inducible to constitutive resistance in streptococci. Clinical isolates with mutations in the *erm*B leader sequences, a mutation (AT → AG) in the second Shine–Dalgarno (SD2) site of *erm*B and a 136 bp deletion in the *erm*B promoter region have displayed either decreased

susceptibility or resistance to telithromycin. A deletion of A752 showed a 500-fold increase in telithromycin MIC [83]. An *E. coli* mutation in domain V (U2609C) resulted in resistance solely to ketolides and not macrolides [71]. This base is located behind A752 of domain II and mutation of this base may change the conformation around A752 such that the ketolide can no longer bind to domain II. Clinical isolates with mutations in the ribosomal proteins have also been reported. Ribosomal insertion ($_{71}$REKGTG$_{72}$) and mutations (S20N or $_{69}$GTG$_{71}$ to $_{69}$TPS$_{71}$) in L4, insertions ($_{92}$VRPR$_{93}$ or $_{108}$RTAHIT$_{109}$) and a mutation (K68Q) in L22 have shown reduced susceptibility to telithromycin [89, 90]. Combinations of alterations in the *erm*B gene, the *erm*B control region, and the ribosomal proteins L4 or L22 have been observed in the pneumococcal isolates which exhibited the highest telithromycin resistance [91–93].

Other less common mechanisms of macrolide resistance include esterases that hydrolyze the macrocyclic lactone ring, phosphotransferases which phosphorylate the 2'-OH functional group, and the production of short peptides that are capable of expelling macrolides from their drug-binding site [94]. Gram-negative bacilli, particularly *Enterobacteriaceae*, *Pseudomonas* spp., and *Actinetobacter* spp., are intrinsically resistant to macrolides and ketolides. These hydrophobic agents are unable to pass through the outer membrane of the bacterial cell.

6.6 Pharmacokinetics and Pharmacodynamics

Pharmacokinetics measures the physiologic distribution of a drug over time. Generally, these measurements are taken from serum concentrations versus sampling time. The pharmacokinetic parameters for macrolides and ketolides following administration of multiple doses are summarized in Table 6.6.

Erythromycin base, the only active form in vivo, has low bioavailability because it is inactivated by gastric acid. It has a short half-life of 1.5–2 h that requires dosing four times a day. The absorption of erythromycin base is erratic and produces peak concentrations approximately 4–5 h after administration [95]. To improve the bioavailability and pharmacokinetics of erythromycin, enteric-coated dosage forms, acid-stable salts (stearate), esters (ethylsuccinate), and salts of an ester (estolate) were developed. Food may hinder the absorption of erythromycin base and stearate, whereas the ethylsuccinate ester is best taken just after a meal [96]. Erythromycin binds strongly to and markedly inhibits CYP3A4 and, therefore, it is expected that drug–drug interactions may occur when coadministered with a drug primarily metabolized by CYP3A [97]. Erythromycin is principally excreted in the bile. After oral administration, less than 5% of the administered dose was recovered in the active form in urine.

Clarithromycin appeared to be rapidly absorbed with a 55% bioavailability. Food delays the absorption of clarithromycin and may slightly increase its bioavailability, although not to a clinically significant extent. On average, 25% of a systemically available dose is metabolized by CYP3A4 to the active 14-hydroxy clarithromycin, the only substantial metabolite found in plasma. The formation of the microbiologically

Table 6.6 Pharmacokinetic parameters of macrolides and ketolides in plasma after multiple oral doses

Drug	Dosage (mg)	T_{max} (hr)	C_{max} (µg/mL)	AUC_{0-24} (µg•h/mL)	$t_{1/2}$ (hr)	CL[a] (L/h)	V_d[a] (L)	Protein binding (%)	F[b] (%)	Reference
Erythromycin	500[c]	4 – 5	0.3 – 3.5	1.04 – 8.48[d]	1.5 – 2	–	–	65 – 90	Varies[c]	[95]
Clarithromycin	250 QD	2.0	1.14	6.86	3.7	20.41	107.42	42 – 70	55	[99, 100]
	250 BID	2.6	2.85	20.84	4.8	13.22	91.79			
	500 BID	2.1	3.51	46.1	–	–	–			
	1000 QD[e]	7.8	2.59	42	–	–	–			
Azithromycin	250 QD[f]	----	0.23	15.9[g]	66.1	11.4	1114.46	7 – 50	37	[102, 103]
	500 QD	12	0.26	20.48[g]	65.6	8.84	840.91			
Telithromycin	800 QD	1.0	2.27	12.5	9.81	28.69	512.36	60 – 70	57	[105]
Cethromycin	300 QD	3.1	0.69	5.24	6.12	71.9	652	87 – 95	----	[107]

[a]Calculated [111]
[b]Bioavailability
[c]Varies based on dosage form
[d] AUC_{0-12}
[e]Extended-release formulation
[f]500 mg QD on first day
[g]$AUC_{0-\square}$

active metabolite is reduced when clarithromycin is administered with food [98]. The rise in clarithromycin peak plasma (C_{max}) concentration and area under the plasma concentration versus time curve (AUC) were disproportionate to increases in dose, suggesting nonlinearity in pharmacokinetics, a phenomenon not seen with other macrolides. The nonlinearity allowed accumulation to higher plasma concentrations than predicted, but steady state was readily achieved by the fifth dose. Clarithromycin terminal disposition half-life ($t_{1/2}$) also exhibited dose dependency, ranging from harmonic means of 2.7–4.8 h. Generation of the metabolite was reasonably rapid with T_{max} of 1.8–2.9 h. In contrast, nonlinearity in the 14-hydroxy metabolite pharmacokinetics was not observed. Plasma accumulation of this metabolite occurred to a lesser degree than that of the parent compound, despite a substantially longer $t_{1/2}$ for the metabolite, suggesting that formation of this metabolite is capacity-limited and that this may in part account for the nonlinearity observed in clarithromycin pharmacokinetics. Clarithromycin and its metabolite both undergo significant renal and nonrenal elimination. Fecal excretion of both is approximately 40%, while urinary excretion is 46–53%. Clearance by the renal route appeared to be linear, with no significant overall trend to dose dependency [99]. Since clarithromycin undergoes significant renal elimination, dosage adjustments must be made for renal insufficiency. No dosage adjustment is necessary in the elderly or for those with liver dysfunction. However, liver dysfunction does decrease formation the of metabolites, thereby potentially decreasing its activity against *H. influenzae* [100].

An extended-release (ER) formulation of clarithromycin resulted in prolonged absorption from the gastrointestinal tract after oral administration and produced significantly lower peak plasma concentrations (C_{max}) and a later onset (T_{max}) of the maximum concentration for both parent and the metabolite than does an equipotent dose of immediate release (IR) form; however, the ER clarithromycin exhibited $AUC_{0\text{-}24}$ equivalent to that of IR form. Food appeared to reduce the AUC of the ER formulation by approximately 30% and ER clarithromycin is recommended to be administered with food [100, 101].

Azithromycin has a bioavailability of approximately 37%. Food was shown to increase C_{max} by 23% but had no effect on AUC. The reported time to C_{max} varies from 2 to 12 h [102–104]. Azithromycin is eliminated from the serum in a polyphasic manner. The initial rapid decline in drug plasma levels is indicative of a rapid distribution phase, followed by a second component of distribution and elimination. The terminal half-life of azithromycin in serum is substantially longer than other macrolides, which is characterized by the extraordinary distribution from the tissue compartments into vascular compartments. The protein-bound azithromycin at clinically achievable serum concentrations is about 50%, which significantly diminishes as the concentrations increase. Azithromycin interacts poorly with the cytochrome P450 system in vitro [97] and the majority of absorbed azithromycin is found in the bile unchanged; ten inactive metabolites have been found in feces. Urinary excretion accounts for only a minimal route of elimination for azithromycin. The mean C_{max} and AUC values of azithromycin increased 61% and 35%, respectively, in subjects with severe renal impairment compared to subjects with normal renal function, while

mild to moderate renal impairment had an insignificant impact on the exposure. No dosage adjustment is recommended for subjects with renal insufficiency.

Following oral administration, telithromycin was rapidly absorbed, reaching C_{max} within 1 h of dosing [105]. Steady-state plasma concentrations of both telithromycin and its main circulating metabolite, formed by hydrolysis of the aryl rings of the carbamate side chain, were reached within 2–3 days of multiple dosing, regardless of the dose. Due to a slight decrease in nonrenal clearance with multiple dosing, there was moderate accumulation of both telithromycin and its metabolite, with AUC values approximately 1.5-fold higher than those attained after a single dose. The AUC of telithromycin deviated moderately from dose proportionality; a doubling of dose resulted in approximately a threefold increase in AUC. This may reflect a decrease in the metabolic clearance of the drug and a slight increase in the bioavailability of telithromycin with an increasing dose. The C_{max} values deviated only slightly from dose proportionality. Telithromycin exhibited biphasic elimination, with a short but predominant $t_{1/2}\lambda_1$ followed by a longer $t_{1/2}\lambda_2$. The systemically available telithromycin is eliminated by multiple pathways: 7% of the dose is excreted unchanged in feces by biliary and/or intestinal secretion, 13% in urine by renal excretion, and 37% of the dose is metabolized by the liver. Approximately 50% of metabolism is mediated by CYP3A4 and the remaining 50% is cytochrome P450-independent. Hepatic insufficiency, regardless of severity, had no impact on the C_{max}, AUC and $t_{1/2}$ of telithromycin. An increase in renal elimination was observed in hepatically impaired patients, suggesting that this pathway may compensate for some of the decreased metabolic clearance. However, a 1.4-fold increase in C_{max} and 1.9-fold increase in $AUC_{0\text{-}24}$ were observed in patients with severe renal impairment. It is recommended that the dosage of telithromycin be reduced in half in severely renally impaired patients.

The pharmacokinetics of cethromycin appeared to deviate from dose proportionality and time-linearity [106]. Cethromycin systemic exposure (AUC) increased more than proportionally with increasing dose, and was greater following repeated dosing than following a single dose. The nonlinearity may be a result of increasing oral bioavailability with an increasing dose rather than the saturation of drug elimination from the systemic circulation. Steady-state for cethromycin was reached by Day 4 and the mean cethromycin half-life at steady-state ranged between 6.0 and 8.8 h [107]. Food appeared to have little or no effect on the pharmacokinetics of cethromycin [108]. Cethromycin was principally metabolized by CYP3A, with the primary N-desmethyl metabolite present in plasma and feces and the primary route of elimination being presumably biliary excretion (87% of the dose was recovered in feces). Cethromycin was about 87–95% protein bound at clinically relevant concentrations, with binding to α_1-acid glycoprotein being of higher affinity than to albumin and accounting for most of the binding. While the steady-state exposure to cethromycin appeared to be greater for subjects with moderate hepatic impairment than for subjects with mild hepatic impairment or subjects with normal hepatic function, the data suggests that no dose adjustment in cethromycin is required for subjects with either mild or moderate hepatic impairment [109]. When cethromycin was administered to subjects with severe renal impairment, the exposure to

cethromycin was two- to fourfold greater than exposure in subjects with normal renal function [110].

One characteristic common to both macrolides and ketolides is that they are lipophilic in nature and have a low degree of ionization, which allows extensive penetration into tissues and fluids to occur, as indicated by their large volumes of distribution (Table 6.6). This is significant, as the majority of infections and the antimicrobial actions take place in tissues, mucosa, or interstitial space fluid. For example, concentrations of antibacterial agents in epithelial lining fluid (ELF) for extracellular pathogens and in alveolar macrophage cells for intracellular pathogens are corroborated with the efficacy for the treatment of pulmonary infections such as community-acquired pneumonia [112]. All other four macrolides/ketolides, except for erythromycin, notably accumulate in epithelial lining fluid and alveolar macrophages (Table 6.7).

Macrolides and ketolides also demonstrated higher concentrations in tissues of the upper respiratory tract such as bronchial mucosa, bronchial secretions, tonsils, and sinus fluid [96, 111]. Concentrations of clarithromycin and telithromycin were measured in peripheral soft tissues such as muscle and the subcutis. Data indicated that telithromycin concentration in interstitial space fluid was twice the free drug concentration in plasma, while clarithromycin achieved only approximately 50% of the free drug concentration in plasma [111].

The ability to enter and survive within host cells offers a pathogenic advantage to some microorganisms and, therefore, the intracellular concentrations of antibacterial agents are important in the defense against intracellular pathogens such as *L. pneumophila*, *C. pneumoniae*, *M. pneumoniae*, *U. urealyticum*, *Rickettsia*, and *Brucella*. In addition, extracellular pathogens such as *S. aureus* frequently invade the phagolysosomal compartment of phagocytes, resulting in recurrence of the infection or treatment failure. Macrolides and ketolides, unlike β-lactams and aminoglycosides, penetrate host defense cells, particularly neutrophilic granulocytes, macrophages, and polymorphonuclear leucocytes (PMNs) [96, 111, 118–120]. High concentrations of macrolides and ketolides have also been detected within lysosymes due to a lower pH (4 to 5) in lysosymes compared with the cytoplasm (pH 7). The effects of macrolides and ketolides as lysosome fusion with the phagosomes are an essential event in the phagocytic killing process [96, 111].

Pharmacodynamics correlates the antibacterial activity of a compound with its concentration profile at the site of action. Use of pharmacokinetic/pharmacodynamic parameters could help to optimize dosing, maximize efficacy, and prevent the emergence of resistance. The extensive distribution of macrolides and ketolides into tissues, fluids, and cells as discussed above, however, makes predictions of pharmacodynamic activity difficult, as plasma pharmacokinetic parameters frequently used as predictors do not necessarily represent the concentrations of these agents at the site of action [121]. Nevertheless, various models have demonstrated the pharmacodynamics of erythromycin to be time-dependent. The survival rate of the treated animals was associated with the time during which erythromycin concentrations remained above the MIC and, hence, more frequent administration of the same total

Table 6.7 Concentrations (μg/mL) in plasma, epithelial lining fluid (ELF) and alveolar macrophages (AM) after multiple oral doses

Time[a] (h)	Erythromycin[b] Plasma	ELF	AM	Clarithromycin[c] Plasma	ELF	AM	Azithromycin[d] Plasma	ELF	AM	Telithromycin[e] Plasma	ELF	AM	Cethromycin[f] Plasma	ELF	AM
2	ND	ND	ND	ND	ND	ND	ND	ND	ND	1.86	14.89 (8)	69.32 (37)	0.25	2.5 (10)	22.6 (90)
4	0.7	0.8 (1)	0 (0)	2.2	29.3 (13)	505.8 (230)	0.08	1.01 (13)	42.7 (534)	ND	ND	ND	0.38	2.7 (7)	48.5 (128)
8	0.1	0 (0)	0.1 (1)	2.6	72.1 (28)	256.7 (99)	0.09	2.18 (24)	57.2 (636)	0.63	3.7 (6)	81 (129)	0.09	0.9 (10)	22.6 (251)
12	0.04	0 (0)	0.8 (2)	0.8	48.6 (61)	236.5 (296)	0.04	0.95 (24)	40.4 (1010)	0.23	3.27 (14)	318.1 (1383)	0.1	0.8 (8)	33.6 (336)
24	ND	ND	ND	0.5	11.9 (24)	360.7 (721)	0.05	1.22 (24)	41.7 (834)	0.08	0.97 (12)	161.57 (2020)	0.01	0.1 (10)	6.7 (670)
48	ND	ND	ND	0.01	23.4 (2340)	17.0 (1700)	ND	ND	ND	<0.03	0.17 (>6)	2.15 (>72)	0	0	3.7 %)

ND – not done; the numbers in parentheses are fold increases over plasma levels

[a]Time from drug administration to time broncoalveolar lavage (BAL) fluid sample was taken

[b]Erythromycin was administered orally at a dose of 250 mg every 6 h for a total of nine doses [113]

[c]Clarithromycin was given orally at a dose of 500 mg every 12 h for a total of five doses [113, 114]

[d]Azithromycin was given orally with a dose of 500 mg on the first study day followed by a dose of 250 mg once daily for the next 4 day for a total of five doses [114]

[e]Telithromycin was given orally at a dose of 800 mg once daily for five days [115, 116]

[f]Cethromycin was given orally at a dose of 300 mg once daily for 5 days [117]

Table 6.8 $AUC_{0\text{-}24\ free\ drug}$/MIC for susceptible and resistant *S. pneumoniae*

Drug	Susceptible	Resistant (*erm*)	Resistant (*mef*)
Clarithromycin	24	NM	27
Azithromycin	24	NM	20
Telithromycin	121	132	86
Cethromycin	34	50	56

daily dose was significantly more effective than larger doses given less frequently [9, 122, 123].

Azithromycin pharmacodynamics appeared to be concentration-dependent, with efficacy being best correlated with the ratio of C_{max}/MIC [104] or AUC/MIC [122, 124]. The concentration-dependent behavior was greatest during the initial exposure to azithromycin which may be caused by an initial concentration-dependent uptake into cells. In contrast, for clarithromycin, interdependence among the pharmacodynamic parameters seems to exist, with time above MIC, C_{max}/MIC, and AUC/MIC all being reported to closely correlate with the reduction in bacterial density and survival [125].

Ketolides have consistently exhibited a concentration-dependent pharmacodynamics. Both telithromycin and cethromycin achieved bactericidal activity against macrolide-susceptible and -resistant *S. pneumoniae* in animal models with increased survival rates and lower ED_{50} than macrolides. In a neutropenic mouse thigh model, telithromycin demonstrated that both AUC/MIC and C_{max}/MIC ratios correlated well with antibacterial activity against *S. pneumoniae*, regardless of the phenotypic-resistant profile [126]. A bacteriostatic effect appeared to occur with a free drug AUC/MIC ratio of approximately 200 or a free drug C_{max}/MIC of 11, while bactericidal effects were observed when a free drug AUC/MIC ratio was >1,000 or a free drug C_{max}/MIC ratio was 90. Similarly, an approximate cethromycin free drug AUC/MIC ratio of 50 or C_{max}/MIC ratio of 1 resulted in bacteriostatic effects, as shown in a murine pneumococcal pneumonia model, where the maximal survival was achieved with a free drug AUC/MIC ratio of 100 or C_{max}/MIC ratio of 2 [127]. In an immunocompetent murine pneumococcal pneumonia model, the cethromycin free drug AUC/MIC ratio was upheld as the most predictive pharmacodynamic parameter; however, in the presence of neutrophils, lower drug exposure was required to produce the same degree of bacterial killing. Compared with a neutropenic host, the $AUC_{free\ drug}$/MIC ratio required to achieve a bacteriostatic effect was 2.5 times lower and the ratio needed to achieve a bactericidal effect was 4 times lower in the immunocompetent host [128].

Table 6.8 summarizes the targeted ratios of free drug $AUC_{0\text{-}24}$/MIC required to achieve a bacteriostatic effect for susceptible and resistant *S. pneumoniae* based on the neutropenic mouse model [129, 130]. It should be noted that, based on the pharmacodynamics of free drug concentrations, none of the macrolides and ketolides should be adequate for treatment of *H. influenzae*, as the drug concentrations do not reach the MIC_{90} for this pathogen. However, they are all clinically effective in treating and eradicating this pathogen.

Another feature that contributes to the antibacterial activities of macrolides and ketolides is their prolonged post-antibiotic effect (PAE). Generally speaking, the persistent suppression of bacterial growth requires the organism to be exposed to supra-inhibitory concentrations of the antibacterial agent. However, the suppression of bacterial growth can persist after the short exposure of organisms to antimicrobial agents. This is known as the PAE. The PAE is particularly important when the concentration of a drug declines below the MIC and may allow intermittent administration. Ketolides generally have PAEs equal to or greater than macrolides. Maximum PAEs induced by 1 h of exposure to telithromycin at concentrations ranging from 0.5× MIC to 32× MIC were 3.7 h for *S. aureus*, 8.9 h for *S. pyogenes*, and 9.7 h for *S. pneumoniae* [131]. At an exposure to 10× MIC of telithromycin, the reported PAE against *H. influenzae* was ≥6.7 h and ≥1.3 h against *M. catarrhalis* at 4× MIC [132]. At 10× MIC, cethromycin displayed a PAE of ≥1.7 h against macrolide-susceptible and -resistant *S. pneumoniae*, ≥3.4 h against *S. aureus*, ≥3.8 h against *M. catarrhalis*, and ≥4.9 h against *H. influenzae* [34, 67, 133, 134]. Cethromycin also demonstrated a PAE of ≥2 h against *L. pneumophila*, 1.5–2.7 h against anaerobic *P. anaerobius*, and 2.1–3.3 h against anaerobic *B. fragilis* [135].

6.7 Clinical Use

Due to their range of antibacterial activity, the macrolide/ketolide agents (erythromycin, clarithromycin, azithromycin, and telithromycin) have been utilized clinically in a variety of indications. In addition, this class of antibiotic has demonstrated immunomodulatory properties independent of their antibacterial effects. This provides additional opportunities for utility in a variety of both infectious and noninfectious disease states. Considerable literature exists detailing the clinical effects of the macrolides and a brief summary of their use in the treatment of a variety of relevant medical conditions follows.

6.7.1 Upper Respiratory Tract Infections

Macrolides, due to their causative pathogen coverage, are efficacious in treating common bacterial pharyngitis, sinusitis, and otitis media.

6.7.1.1 Bacterial Pharyngitis

While penicillin (intramuscular or oral) is generally considered to be the drug of choice for the treatment of streptococcal infections and the prophylaxis of rheumatic fever, the macrolides azithromycin and clarithromycin are indicated in patients who cannot use first-line penicillins [136, 137]. Both drugs have been demonstrated

to be effective in the eradication of *S. pyogenes* from the nasopharynx; however, their action on the subsequent prophylaxis of rheumatic fever has not been critically established. Erythromycin is indicated for all mild to moderate respiratory tract infections caused by *S. pyogenes*, but is additionally indicated for the prophylactic prevention of both initial and recurrent attacks of rheumatic fever in penicillin-allergic patients [138, 139].

6.7.1.2 Acute Maxillary Sinusitis

All of the macrolides are currently approved for the treatment of acute maxillary sinusitis due to infection by *M. catarrhalis*, *S. pneumoniae*, or *H. influenzae*. When using erythromycin in the treatment of a suspected *H. influenzae* infection, an adequate dosage of a sulfonamide antibiotic, administered concomitantly, is indicated [138, 139]. Both the immediate release and the extended-release formulations of azithromycin and clarithromycin are indicated for the treatment of acute sinusitis [136, 140, 147].

6.7.1.3 Acute Otitis Media

Both azithromycin and clarithromycin are available in a pediatric formulation and are indicated for the treatment of acute otitis media due to infection by *M. catarrhalis*, *S. pneumoniae*, or *H. influenzae* in children [136, 137]. No macrolide products are currently labeled for the treatment of otitis media in adults.

6.7.2 Lower-Respiratory Tract Infections

Macrolide/ketolide agents are efficacious in treating mild to moderate lower-respiratory tract infections due to their causative pathogen coverage. While azithromycin, clarithromycin, and telithromycin are labeled for their activity in specific clinical conditions, erythromycin is instead suggested for use in all lower-respiratory tract infections of mild to moderate severity caused by *S. pyogenes* or *S. pneumoniae* [138, 139]. The use of erythromycin is also indicated in the treatment of respiratory tract infections due to *M. pneumoniae* and listeriosis caused by *L. monocytogenes* [138, 139].

6.7.2.1 Acute Bacterial Exacerbations of Chronic Obstructive Pulmonary Disease

Azithromycin is indicated for adults with acute bacterial exacerbations of chronic obstructive pulmonary disease due to *H. influenzae*, *M. catarrhalis*, or *S. pneumoniae* infection [136].

6.7.2.2 Acute Bacterial Exacerbation of Chronic Bronchitis (ABECB)

Clarithromycin immediate and extended-release formulations are effective in the treatment of acute bacterial exacerbations of chronic bronchitis caused by *H. influenzae*, *H. parainfluenzae*, *M. catarrhalis*, or *S. pneumoniae* infection in adult patients [137].

6.7.2.3 Community-Acquired Bacterial Pneumonia (CABP)

The use of oral formulations of azithromycin (immediate release, extended release, and pediatric) is limited to adult and pediatric individuals with infection by *C. pneumoniae*, *H. influenzae*, *M. pneumoniae*, or *S. pneumoniae*. The use of the parenteral formulation of azithromycin in the treatment of CABP should be limited to those individuals requiring initial intravenous therapy for infection by *C. pneumoniae*, *H. influenzae*, *L. pneumophila*, *M. catarrhalis*, *M. pneumoniae*, *S. aureus*, or *S. pneumoniae* [141].

The immediate release oral formulations of clarithromycin (adult and pediatric) provide similar pathogen coverage to azithromycin as they are indicated for the treatment of individuals with CABP due to infection by *H. influenzae*, *M. pneumoniae*, *S. pneumoniae*, or *C. pneumoniae* (note: the pediatric formulation label omits infection by *H. influenzae*). The extended-release formulation of clarithromycin provides additional coverage for the treatment of patients with CABP caused by infection with *H. parainfluenzae* or *M. catarrhalis* [137].

Telithromycin represents the first agent in the ketolide class that has demonstrated efficacy in the treatment of CABP patients infected with multidrug-resistant *S. pneumoniae* (MDRSP) [142]. MDRSP is defined as isolates resistant to two or more of the following antibiotics: penicillin, second generation cephalosporins (such as cefuroxime), macrolides, tetracyclines, and trimethoprim/sulfamethoxazole. Telithromycin is indicated for the treatment of mild to moderate CABP due to infection with *S. pneumoniae* (both susceptible and MDRSP), *H. influenzae*, *M. catarrhalis*, *C. pneumoniae*, or *M. pneumoniae* in patients 18 years old or above.

Cethromycin is currently seeking FDA approval for the indication of CABP in adult patients. Cethromycin administered at 300 mg QD for 7 days was reported to have achieved noninferiority to clarithromycin in clinical cure rate. Similar results were also observed in bacteriological cure rate, pathogen eradication rate, and radiological success rate [143].

6.7.3 *Skin and Soft Tissue Infections*

Erythromycin, clarithromycin, and azithromycin are all indicated in the treatment of uncomplicated skin and skin structure infections caused by *S. aureus* or *S. pyogenes* (azithromycin provides additional coverage against infection by *S. agalactiae*)

[136–139]. Clarithromycin is the only macrolide indicated for infections of this type in the pediatric population. In cases of abscess, surgical drainage is usually required prior to the initiation of treatment with a macrolide agent. A warning suggesting that resistant staphylococci may emerge during treatment is provided on the erythromycin label.

A topical erythromycin gel is indicated for the treatment of acne vulgaris [144].

6.7.4 Genital Infections

Both azithromycin and erythromycin have demonstrated considerable efficacy in the treatment of genital infections brought about by a number of pathogens.

The use of oral azithromycin in the treatment of urethritis and cervicitis due to *Chlamydia trachomatis* or *N. gonorrhoeae* is well documented [136]. Pelvic inflammatory disease caused by infection with these pathogens as well as *Mycoplasma hominis* can be treated with intravenous azithromycin; however, if an anaerobic microorganism is believed to be contributing to the infection, additional agents with anaerobic coverage should be administered simultaneously [141].

Alternatively, intravenously administered erythromycin followed by oral erythromycin can be used to treat acute pelvic inflammatory disease caused by *N. gonorrhoeae* in subjects who have previously demonstrated penicillin sensitivity [138, 139]. In cases of tetracycline sensitivity or other cases in which tetracyclines are contraindicated, erythromycin can be used to treat uncomplicated urethral, endocervical, or rectal infections due to infection with *C. trachomatis* in adults. Erythromycin can also be used to treat *C. trachomatis* urogenital infections during pregnancy [138]. These infections may result in both newborn conjunctivitis and/or pneumonia of infancy, both of which can be treated with erythromycin [139].

Azithromycin is not indicated for the treatment of syphilis [136]. Erythromycin, however, can be used orally in the treatment of primary syphilis caused by *Treponema pallidum* in patients allergic to penicillins [138, 139].

Additionally, erythromycin can be used in the treatment of nongonococcal urethritis caused by *U. urealyticum* in patients in which tetracyclines are contraindicated.

In male patients, genital ulcer disease (chancroid) due to infection by *Haemophilus ducreyi* can be successfully treated with azithromycin [136]. Insufficient data in female subjects prohibits the recommendation of this treatment in this population.

6.7.5 Mycobacterial Infections in AIDS Patients

Clarithromycin is indicated for the treatment of disseminated mycobacterial infections due to *Mycobacterium avium* or *Mycobacterium intracellulare* in both adult and pediatric patients [137]. In addition, treatment with clarithromycin is indicated for the prevention of disseminated *Mycobacterium avium* complex (MAC) in both adult and pediatric patients with advanced HIV infection [137].

6.7.6 Other Pathogens

Oral erythromycin is indicated for the treatment of intestinal amebiasis caused by infection with *Entamoeba histolytica* [138, 139]. Amebiasis occurring outside of the intestinal tract is not susceptible to treatment with erythromycin.

6.7.7 Other Proposed Antibacterial Uses of Macrolides

One proposed use for macrolide antibiotics involves the eradication of persistent pathogens believed to be responsible for a chronic inflammatory state. In these disease states, patients are chronically infected with bacterial pathogens whose presence results in the activation of pro-inflammatory pathways which contribute to the pathogenesis of the condition. Three chronic diseases – atherosclerosis, asthma, and Crohn's disease – will be discussed here as potential conditions which may be effectively treated with macrolide antibiotics. While no product has yet to receive regulatory approval for one of these indications, scientific evidence suggests that macrolides may at least play a role in the amelioration of disease symptoms in affected patients.

6.7.7.1 Atherosclerosis

The bacterium *C. pneumoniae* was originally associated with atherosclerosis and coronary artery disease in 1986 [145]. At that time, several lines of evidence pointed to a direct correlation between the presence of chronic *C. pneumoniae* infection and the incidence of atherosclerotic events [146]. Studies examining plaques taken from coronary artery walls demonstrated the presence of *C. pneumoniae* in the plaque [147, 148]. Animal models of atherosclerosis were employed to examine the effects of treatment with macrolide antibiotics and these results were positive [146]. As a result of these animal studies, several small-scale human clinical trials were initiated to examine the preventative benefit of treatment with macrolide antibiotics [149, 150]. Intriguing results in these studies resulted in the initiation of the WIZARD and the ACES trials, which were statistically powered to detect a clinically significant difference in the incidence of cardiac events in subjects receiving azithromycin versus subjects not receiving macrolide treatment [151, 152]. Unfortunately, these large-scale clinical trials did not demonstrate any significant effect of azithromycin treatment. Since these results were published, several reviews of all of the macrolide/atherosclerosis evidence have been prepared [146, 153, 154]. The current consensus among experts seems to be that while chronic infection with *C. pneumoniae* may play a role in the pro-inflammatory state seen in atherosclerosis, it is not sufficient to result in atherosclerosis or the clinical consequences of atherosclerosis in and of itself, and therapeutic eradication alone does not result in a clinically significant difference in outcome.

6.7.7.2 Asthma

Association of infection with the atypical bacteria *C. pneumoniae* or *M. pneumoniae* with asthma disease severity or exacerbation has been demonstrated [155, 156]. This would suggest a role for the macrolide agents with atypical pathogen coverage in the treatment of acute asthma attacks. In trials examining the effect of macrolide treatment in chronic asthma patients a generally positive correlation has been demonstrated [157, 158]. One recent study, using telithromycin, demonstrated statistically significant and clinically meaningful benefits in subjects receiving telithromycin treatment in addition to the standard of care therapy [159]. Challenges exist in teasing out the mechanism of effect of macrolide intervention in asthma patients. Many attribute the results demonstrated thus far to be simply the result of the general anti-inflammatory properties of the macrolide class and not directly related to an antibacterial mechanism of action.

6.7.7.3 Crohn's Disease

Several studies have demonstrated a larger number of *Mycobacterium avium paratuberculosis* bacteria in the intestines of patients with Crohn's disease when compared to ulcerative colitis patients or normal healthy control subjects [160–162]. Clinical trials examining the use of macrolide antibiotics combined with corticosteroids or sulfasalazine have yielded encouraging results [163, 164]. Several examples of long-term remission of symptoms have been demonstrated suggesting that further large-scale controlled trials are necessary to establish the role of antibacterial agents such as the macrolides which retain coverage against *Mycobacterium avium paratuberculosis* in the treatment of Crohn's disease.

6.7.8 Proposed Anti-inflammatory Uses of Macrolides

In addition to their direct antibacterial effects, macrolide antibiotics appear to function in a general anti-inflammatory role. Several targets in the inflammatory pathway have been identified as being affected by administration of macrolides. For example, macrolides have been demonstrated to reduce neutrophil chemotaxis, limiting the recruitment of neutrophils to the site of inflammation [165, 166]. Macrolides also affect neutrophil function via the reduction of the oxidative burst [167]. This results in a decreased production of several mediators including nitric oxide [168], neutrophil elastase [169], TNF [170], and prostaglandin E_2 [171]. Macrolides also suppress NF-κB activation in respiratory epithelial cells and monocytes [172].

6.7.8.1 Diffuse Panbronchiolitis/Cystic Fibrosis

Diffuse panbronchiolitis and cystic fibrosis are diseases characterized by persistent inflammation of the bronchioles accompanied by continuous recruitment of

inflammatory cells into the lungs [173]. Clinical trials of long-term and low-dose macrolide therapy (especially using azithromycin) have demonstrated clinical improvement in patients receiving therapy [174, 175]. As the doses of macrolides given in these trials were considerably lower than that required for antibacterial effects, the immunomodulatory activity of the macrolide is believed to be responsible for the therapeutic effect. Superinfection with *P. aeruginosa* is a common complication of both diffuse panbronchiolitis and cystic fibrosis, suggesting that the antibacterial effect of the macrolides may play a role in the therapeutic effect. In vitro activities of the macrolides against *P. aeruginosa*, however, are quite low, leading investigators to search for alternative mechanisms of action [176]. This interaction is generally inhibited by the biofilm preventing phagocytosis by host immune cells.

6.7.8.2 Other Inflammatory Conditions

Macrolide therapy has been examined, albeit in small numbers of patients, in several other conditions believed to have an inflammatory pathogenesis. Examples include middle-lobe syndrome [177], prurigo pigmentosa [178], and confluent and reticulated papillomatosis [179].

6.8 Safety Issues

The macrolides erythromycin, azithromycin, and clarithromycin are generally considered to be among the safest antibacterial agents available. The ketolide agent telithromycin also demonstrated the benign safety profile seen with the earlier generation macrolides, but due to isolated incidences of severe hepatotoxicity, it has been limited to the treatment of community-acquired pneumonia. Tolerability of all of these agents is good with few discontinuations seen due to adverse events. Less frequent adverse events such as cardiac toxicity (QT prolongation), hepatic effects, and visual disturbances have been reported with the use of macrolides and ketolides. Due to their interaction with the cytochrome P450 system, with the exception of azithromycin, the potential for drug–drug interactions must be monitored. The clinical safety profile of each agent is summarized below.

6.8.1 Erythromycin

Erythromycin has generally been shown to be a safe antibiotic with an adverse event profile limited to non-life-threatening events [180–182]. The most frequently seen adverse events are gastrointestinal in nature and include nausea, vomiting, abdominal pain, and diarrhea [138, 139]. These events, however, have been reported to occur in 20–50% of all patients receiving erythromycin [183]. Due to reports of hepatic dysfunction, including increased liver enzymes, and hepatocellular and/or

cholestatic hepatitis, with or without jaundice, occurring in patients receiving oral erythromycin, a warning is included in the erythromycin product monograph [138, 139]. Additionally, a warning is included pertaining to the possibility of patients developing pseudomembranous colitis [138, 139]. This warning is identical in all of the macrolides' product information.

6.8.2 Clarithromycin

The newer macrolide agents clarithromycin and azithromycin were developed in an attempt to minimize the incidence of gastrointestinal adverse events seen in patients taking erythromycin. Clarithromycin has demonstrated excellent tolerability in subjects receiving both the immediate and extended-release formulations of the drug. In comparative studies, patients receiving clarithromycin were shown to have fewer adverse experiences than patients receiving erythromycin [184]. The most common adverse events experienced were gastrointestinal in nature and included nausea (3.8%), diarrhea (3%), and abdominal pain (1.9%) [185]. Patients receiving the extended-release formulation of clarithromycin, however, did appear to experience less severe gastrointestinal side effects as well as experience fewer discontinuations of the drug due to intolerability [186]. Less than 3% of all subjects receiving clarithromycin in clinical trials withdrew participation due to an adverse event [187]. Most common among laboratory abnormalities were elevated liver function tests, decreased white blood cell counts, and elevated BUN [137]. Severe side effects such as pancreatitis [188], myasthenic syndrome [189], cholestatic hepatitis [190], and fulminant hepatic failure [191] have been seen in an isolated number of subjects receiving clarithromycin. Treatment of mycobacterial infections in elderly subjects with high-dose clarithromycin (1,000 mg BID) resulted in increased incidence and severity of adverse events [192].

Clarithromycin is warned to not be used in pregnant women unless there is no alternative therapy [137], due to adverse effects on the fetus seen in animal reproduction studies [193]. The pseudomembranous colitis warning is also present on this package insert [137].

6.8.3 Azithromycin

Azithromycin has perhaps the most benign safety profile of the currently marketed macrolide agents. Indeed, only 0.7% of subjects receiving azithromycin discontinued therapy due to adverse events, compared with 2.6% of subjects receiving comparator drugs [194]. A post hoc review of adverse events in over 6,600 patients reported that gastrointestinal side effects were the most frequently reported by approximately 12% of all patients examined, with diarrhea (3.6%), nausea (2.6%), and abdominal pain (2.5%) most frequently reported [195]. Over 60% of these reported adverse events

were considered mild. All other adverse events were reported in<2% of all patients [195]. Liver function test elevations were seen in 1.5% of patients [195]. Both pain at the injection site and local inflammation were reported for parenterally administered azithromycin in fewer than 10% of subjects receiving the drug [196]. Labeled warnings for azithromycin the pseudomembranous colitis warning found on all macrolide antibiotic product information monographs [136, 140, 141].

6.8.4 Telithromycin

The safety profile of the ketolide telithromycin is considerably more complex than that of the other previously discussed macrolides. Pre-approval clinical trial data suggested that telithromycin was as safe as comparator agents used in those trials and similar in adverse event severity and incidence to the marketed macrolide agent clarithromycin [197]. In controlled studies of over 2,700 patients receiving telithromycin, gastrointestinal side effects such as diarrhea (10.8%), nausea (7.9%), and vomiting (2.9%) were seen as were CNS side effects such as headache (5.5%) and dizziness (3.7%) [198]. These incidences were similar in both telithromycin and comparator-treated subjects as were the discontinuation rates due to adverse experiences (telithromycin: 4.4%, comparators: 4.3%) [198]. Other less commonly seen but notable adverse events in the controlled trials included visual disturbance, which was experienced in 1.1% of telithromycin subjects compared to 0.28% of patients receiving other antibiotics [198], a small increase in QT interval (~1.5 ms) in telithromycin-treated subjects [198] and increased liver enzymes and hepatocellular and/or cholestatic hepatitis (with or without jaundice) [142]. The incidence of ALT levels above three times the upper limit of normal was 1.6% in telithromycin-treated subjects and 1.7% in comparator-treated subjects [198]. Reversible hepatitis was experienced by 0.07% of all subjects treated with telithromycin in the controlled Phase 3 program [142]. The risk benefit profile of telithromycin was determined to be favorable and in April of 2004 telithromycin was approved by the FDA for the treatment of community-acquired pneumonia, acute exacerbation of chronic bronchitis, and acute sinusitis.

Post-marketing surveillance studies, however, have resulted in the discovery of several safety issues prompting worldwide regulatory agencies to issue serious warnings concerning the use of telithromycin, as well as limit the approved indications for the drug. In 2006, Clay et al. reported on three cases of severe hepatotoxicity in subjects who were taking or who had taken telithromycin [199]. One of these subjects fully recovered, one required liver transplantation, and one died. In 2007, the regulatory agencies, e.g., EMEA and FDA, and the sponsor agreed to limit the indicated use of telithromycin to the treatment of mild to moderate community-acquired pneumonia, considerably narrowing its use [200, 201]. Since that time, post-marketing surveillance has resulted in an additional warning box added to the telithromycin labeling. This warning concerns patients with myasthenia gravis and states in part that telithromycin is “contraindicated in patients with myasthenia gravis. There have been reports of fatal and life-threatening respiratory failure in

patients with myasthenia gravis associated with the use of Ketek" [142]. Also included are warnings related to hepatotoxicity, QTc prolongation, visual disturbances, loss of consciousness, and pseudomembranous colitis [142, 202].

6.8.5 Drug–Drug Interactions

Erythromycin, clarithromycin, and telithromycin are all primarily metabolized by the CYP3A4 subclass of the cytochrome P450 hepatic enzyme system [203, 204]. Azithromycin is primarily eliminated unchanged, and does not interact with the cytochrome P450 system [205]. All three drugs metabolized via CYP3A4 also act as inhibitors of the enzyme adding to their drug–drug interaction potential [203, 204].

Administration of erythromycin has been reported to increase the plasma levels of a variety of drugs including theophylline [206], warfarin [207], triazolam [208], alfentanil [209], bromocriptine [210], carbamazepine [211], and cyclosporine [212]. Clarithromycin use may also increase the plasma levels of multiple medications [203, 213–224]. Once again, caution must be taken when coadministering clarithromycin with any other agent that interacts with the CYP3A4 enzyme [137].

Azithromycin does not interact with the cytochrome P450 system and thus its potential for drug–drug interactions is greatly reduced [205]. Interestingly, there still are anecdotal reports of toxicity seen when azithromycin is coadministered with lovastatin [225], warfarin [226], cyclosporine [227], disopyramide [228], and theophylline [229]. Telithromycin interacts with CYP3A4 as both a substrate and an inhibitor of the enzyme [204]. Additionally, telithromycin also competitively inhibits the CYP2D6 enzyme system [204]. These interactions result in the contraindication of administration of telithromycin with a number of other agents [142, 197, 198, 204, 230, 231]. Due to the interaction of telithromycin with two separate cytochrome P450 hepatic enzymes, additional opportunities for drug–drug interactions exist with this drug. Caution is warranted when administering telithromycin to patients taking concomitant medications [142]. Similarly, cethromycin has also been shown to be a substrate and an inhibitor of CYP3A [232–235].

In summary, due to their interactions with the cytochrome P450 hepatic enzyme system, macrolide/ketolide agents including erythromycin, clarithromycin, telithromycin, and cethromycin may present challenges in patients taking medications which also interact with this system. The lack of interaction with P450 enzymes renders azithromycin less potential for drug–drug interaction although care is still warranted with certain concomitantly administered medications.

6.9 Future Directions

The understanding of structure–activity relationships and the advancement in synthetic methodology have led to the discovery of a variety of new macrolides and ketolides [236].

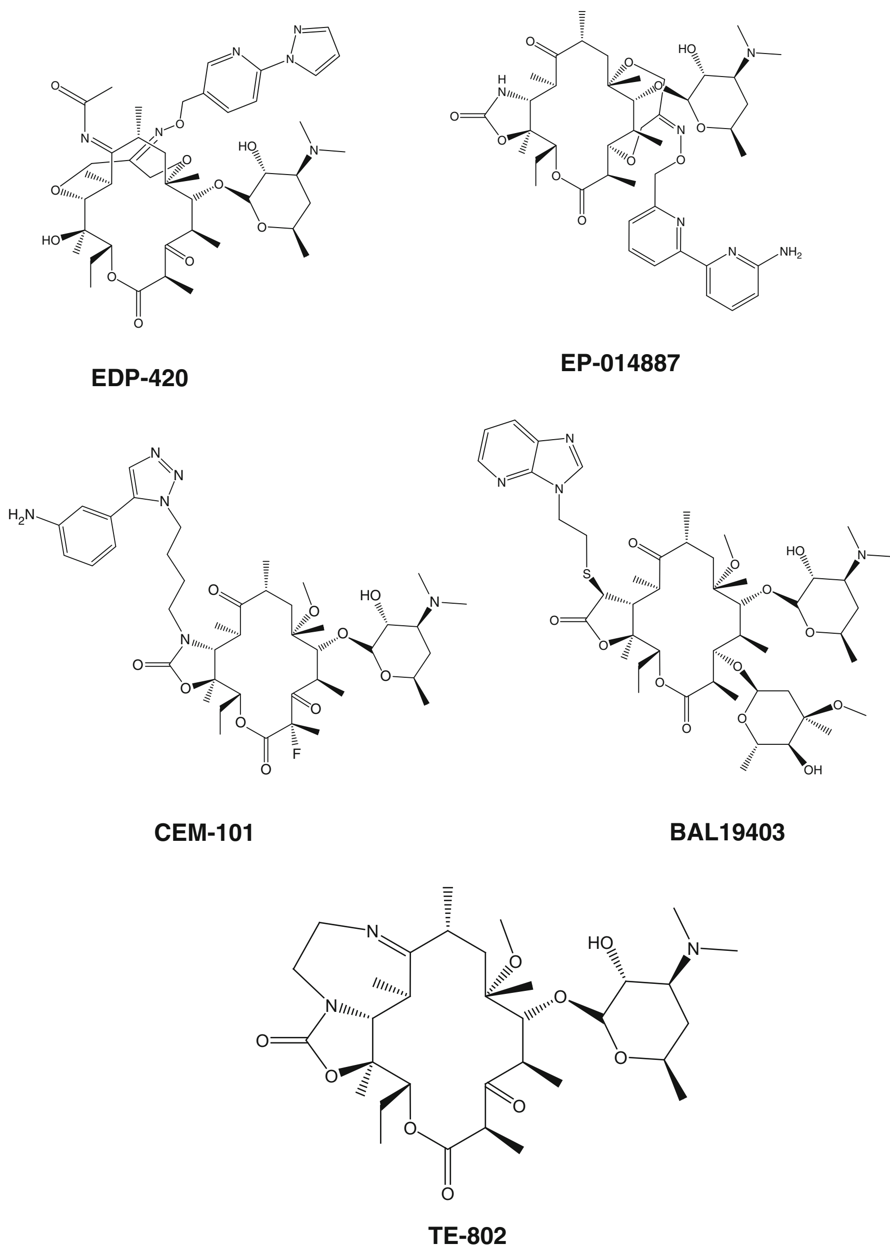

Fig. 6.2 misses the chemical structures of TE-802

EDP-420 (EDP-013420, S-013420) is a 6,11-bridged bicyclic ketolide (Fig. 6.2). The 6- and 11-hydroxy groups of the macrocyclic ring are linked by a three-carbon bridge which is attached by a heteroaryl oxime at the center carbon. In addition, the 9-keto group is replaced by an acetylamino functional group. These modifications

enhance the acid stability of the molecule. The in vitro spectrum of antibacterial activity of EDP-420 is very similar to that of telithromycin and cethromycin [202]. This molecule is in Phase II clinical development for community-acquired bacterial pneumonia (CABP). The reported clinical cure rates were 92.5% (37/40 cases) in CABP patients who were administered 300 mg on the first day followed by a 4-day 150 mg QD regimen and 85.0% (34/40 cases) in CABP patients who were given 400 mg on the first day followed by a 4-day 200 mg QD regimen. The bacteriological eradication rates were 81.8% and 88.2%, respectively, for the two dosing regimens [237].

EP-014887 is a 3,6-bridged tricyclic macrolide derived from an intensive medicinal chemistry investigation [238]. This compound showed activity against a broad spectrum of bacteria including macrolide-resistant *S. pneumoniae* and macrolide-resistant *S. pyogenes*. More interestingly, this agent exhibited activity against the MLS_B phenotype of *S. aureus* and MRSA at MIC values of <0.06 and 4 μg/mL, respectively. It is not known if the MLS_B phenotype is inducible or constitutive. The compound also displayed activity against vancomycin- and linezolid-resistant *E. faecalis* and *E. faecium* with MIC_{90} values of 1 and 2 μg/mL. In a mouse protection model against MRSA, EP-014887 demonstrated an ED_{50} value of 2.7 mg/kg after intravenous administration.

CEM-101 is a 2-fluoroketolide in early clinical development. Its MIC values against respiratory tract pathogens including macrolide-resistant *S. pneumoniae* and multidrug-resistant serotype 19A were two- to fourfold lower than telithromycin [239]. BAL19403 is a macrolide with an 11,12-lactone ring. Even though it has been optimized for antipropionibacterial and anti-inflammatory activities and is being developed as a topical treatment for mild to moderate inflammatory acne vulgaris, this macrolide demonstrated in vitro activity against *S. aureus* and *S. pyogenes* [240]. TE-802 is a tricyclic ketolide with an additional diazaheptene ring linked between the 9-position and the carbamate. Due to its poor activity against *H. influenzae*, it has not been further developed but has instead served as a lead molecule for further structural optimization [236].

The structure-based rational design of macrolides and ketolides by integrating the high-resolution structures of *H. marismortui* and *D. radiodurans* 50 S ribosomal subunits and computational modeling will undoubtedly produce novel molecules not only potent against wild-type bacteria but more specifically targeting drug-resistant mutant species. Molecules that irreversibly bind to bacterial ribosomes may offer additional advantages in overcoming resistance. In addition, the genes encoding the polyketide synthases (PKSs) responsible for the biosynthesis of erythromycin by the bacterium *Saccharopolyspora erythraea* have been elucidated, cloned, and sequenced; genetic manipulation of the PKS-encoding genes can result in novel macrocyclic core structures that are not easily achievable through conventional chemical synthesis [241, 242]. These core structures can then be further chemically modified and derivatized [243].

Macrolides and ketolides have shown the ability to inhibit the assembly of the 50 S ribosomal subunit in a number of organisms including *S. pneumoniae*, *S. aureus*, *H. influenzae*, and *E. coli*. However, preferential inhibition of ribosome assembly in growing cells by translational inhibitors has not been fully exploited.

This may represent a novel target for the development of important bactericidal antimicrobial agents [72].

The global increase in macrolide-resistant *S. pneumoniae* is associated with the wide clinical use of macrolides in the community, especially in the case of azithromycin [244–246]. The coverage of pathogens and the convenience of oral and once-daily administration render ketolides highly suitable for use in community-acquired respiratory tract infections where macrolide-resistant *S. pneumoniae* is prevalent. However, recent changes in the regulations have created a paradigm shift for the development of antimicrobials. It appears that active-controlled noninferiority study designs will no longer be adequate to support approval of drugs for the indications of acute bacterial sinusitis, acute bacterial exacerbation of chronic bronchitis, and acute bacterial otitis media; placebo-controlled or superiority designs are recommended [247–250]. For community-acquired bacterial pneumonia, even though placebo-controlled clinical trials are deemed to be inappropriate and active-controlled noninferiority study designs acceptable, as stated in the draft guidance document, it is recommended that at least 50% of the study population in pivotal trials should have pneumonia severity PORT scores of III or greater for an oral formulation and a minimum of 25% of the study population have PORT scores of IV or above for an intravenous formulation. The microbiological Intend-to-Treat (MITT) population will be the primary analysis and a 10% noninferiority margin should be met to demonstrate efficacy. Also, at least 30–40% of the enrolled patients should have confirmed bacteriological etiology, with atypical bacterial pathogens such as *L. pneumophila*, *M. pneumoniae*, and *C. pneumoniae* excluded from the trials [251]. These changes will inevitably pose challenges in developing antimicrobial drugs in general and macrolides/ketolides in particular for community use. These challenges include difficulties in patient enrollment and the requirement for larger trials – approximately 1,000 patients (500 patients per study arm) in the ITT population per trial may be needed, thus prolonging the development time and increasing the cost of antimicrobial drug development. The pharmaceutical industry will need to devise more innovative clinical approaches to meet these challenges.

On the other hand, "bad bugs" continue to evolve and confer resistance to currently available antibacterial treatments. Resistance to the so-called ESKAPE pathogens (*E. faecium*, *S. aureus*, *Klebsiella pneumoniae*, *Actinetobacter baumanii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) are considered by the Infectious Diseases Society of America (IDSA) to be clinical super-challenges in the twenty-first century [252, 253]. More people now die of MRSA infection in US hospitals than of HIV/AIDS and tuberculosis combined [252]. Inactivity against *S. aureus* with a constitutive MLS_B phenotype and intrinsic resistance by Gram-negative bacilli such as *Enterobacteriaceae*, *Pseudomonas* spp., and *Actinetobacter* spp. are among the drawbacks of the currently available macrolides and ketolides. Future generations of macrolides and ketolides able to fight the multidrug-resistant "ESKAPE" pathogens will certainly provide an enhanced armamentarium for twenty-first century physicians to defend public health.

Furthermore, the effect of macrolide/ketolide agents on long-term infections resulting in a chronic inflammatory state is promising, as is their low-dose effect as

immunomodulatory molecules. Continued investigation into these additional effects should prove fruitful in establishing this antibacterial class as a viable alternative in the treatment of a variety of troublesome medical conditions.

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Chapter 7
Aminoglycosides

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7.1 Introduction

Aminoglycosides are used by clinicians for many gram-negative infections in the hospital setting, particularly for complicated urinary tract infections (cUTI). For other serious infections such as hospital acquired pneumonia (HAP), complicated intra-abdominal infections (cIAI), and blood stream infections, they are frequently used in combination with other antibiotics to provide additional coverage of both gram-negative and selected gram-positive pathogens or for the promise of preventing the development of resistance. Guidelines from the American Thoracic Society and the Infectious Disease Society of America recommend inclusion of an aminoglycoside in combination treatment regimens for HAP and ventilator-acquired pneumonia (VAP), particularly in cases where the pathogen is presumed to be multidrug resistant [1]. Both intravenous (IV) and inhaled formulations of aminoglycosides, primarily tobramycin, are used in the management of cystic fibrosis patients who are colonized with *Pseudomonas aeruginosa*, and aminoglycosides remain an important second-line treatment for drug-resistant tuberculosis.

Aminoglycoside use has declined as older members of the class confront increasing levels of resistance. New aminoglycosides have not been developed recently, due to challenging synthetic chemistry, concerns about potential toxicity, and competition from other broad-spectrum antibiotics, including the later generation cephalosporins, carbapenems, and fluoroquinolones. As increasing resistance renders these agents ineffective, new aminoglycosides that overcome existing resistance mechanisms may be an attractive option for the treatment of serious infections due to gram-negative pathogens and/or methicillin-resistant *Staphylococcus aureus* (MRSA) in hospitalized patients.

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Our understanding of both the safety and efficacy of aminoglycosides has advanced considerably since they were first approved for human use over 50 years ago. Pharmacodynamic principles show that once-daily dosing reduces nephrotoxicity and ototoxicity, while maintaining and possibly improving efficacy [125]. Nonclinical and clinical studies have affirmed the hypothesis that less frequent aminoglycoside administration results in less aminoglycoside uptake in proximal renal tubule epithelial cells, and ultimately, a lower rate of nephrotoxicity during administration of reasonably short courses (<10 days) of therapy. Administering an aminoglycoside infrequently and briefly does not completely eliminate the risk of drug-induced nephrotoxicity but provides a window for safe treatment of patients administered these agents. Because aminoglycosides are concentration-dependent bactericidal drugs, relatively short courses (≤7 days) should provide near-maximal effect and near-minimal toxicity.

In this chapter, we describe the discovery and applications of the aminoglycoside antibiotics and also discuss a next-generation aminoglycoside, or neoglycoside, ACHN-490. ACHN-490 has potent activity against gram-negative and gram-positive bacterial species, including those that produce aminoglycoside-modifying enzymes, which inactivate earlier aminoglycosides. ACHN-490 is also active against Enterobacteriaceae resistant to other classes of antibiotics and against *S. aureus*, including MRSA. The first phase 1 clinical trial was successfully completed in June 2009 [2] and a phase 2 study in cUTI has been initiated as of this writing.

7.2 Aminoglycoside Mechanism of Action

Aminoglycosides kill bacteria by binding to the bacterial ribosome and inhibiting normal protein synthesis. Specifically, they bind tightly to the highly conserved A-site (the transfer RNA acceptor site) of bacterial 16 S ribosomal RNA of the 30 S ribosomal subunit [3]. Structural elucidation of multiple aminoglycosides bound to RNA oligomers mimicking this site [4] and to the 30 S subunit itself [5] has defined the molecular interactions and shown that aminoglycosides bind in a defined major groove pocket at this site (Fig. 7.1). The tight molecular interaction is driven primarily by multiple hydrogen bonds. In addition, electrostatic and hydrophobic interactions contribute to stability. In each structure known, the central 2-deoxystreptamine ring of the non-streptomycin aminoglycosides (see Fig. 7.4) adopts a highly conserved conformation and maintains the same interactions with the target RNA.

The molecular target of aminoglycosides is the 16 S ribosome, which is highly conserved across bacteria. As such, aminoglycoside antibiotics exhibit in vitro activity against a wide variety of clinically important gram-negative bacteria, including all Enterobacteriaceae (e.g., *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., *Yersinia* spp., *Salmonella* spp., *Shigella* spp.), *Acinetobacter* spp., *Pseudomonas* spp., *Franciscella* spp., and *Brucella* spp., as well as gram-positive *Bacillus* spp., *Staphylococcus* spp., and some streptococci. However, they lack predictable in vitro activity against *Bacteroides* spp. and other anaerobic microorganisms, *Streptococcus*

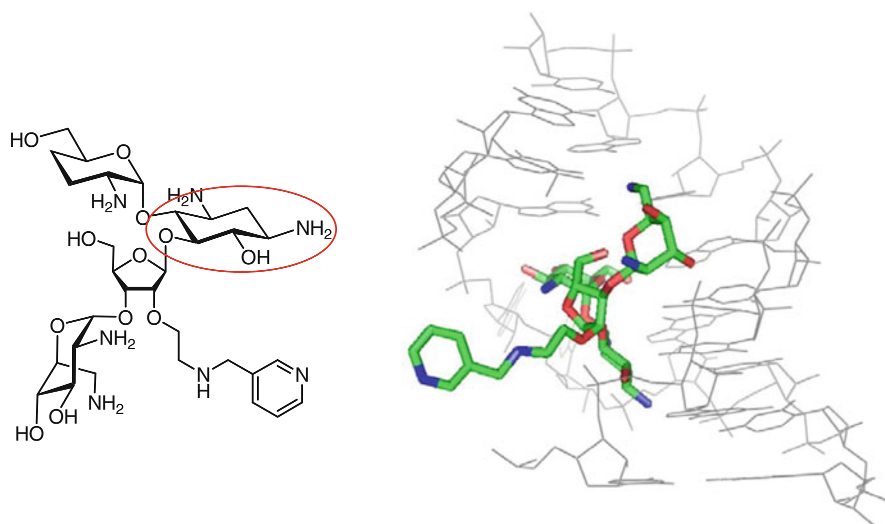


Fig. 7.1 Paromomycin analog with 2-deoxystreptamine circled and shown bound to its target A-site RNA

pneumoniae, *Burkholderia* spp., and *Stenotrophomonas maltophilia*. Their activity against enterococci is adequate only when they are used synergistically with a cell wall-active antibiotic, such as vancomycin or a penicillin. These holes in the otherwise broad spectrum of aminoglycosides occur not because of changes in the target but due to decreased uptake into and/or increased efflux out of the bacterial cell.

Aminoglycosides have been used to treat serious infections, both for empirical treatment and otherwise. The broad-spectrum activity and high level of bacterial cidality are important contributing factors to their clinical success. While the specific mechanism responsible for this bactericidal activity is not well defined, it has been suggested that the mode of aminoglycoside binding to the ribosome plays a key role by stabilizing both cognate and non-cognate transfer RNA, resulting in the insertion of random amino acids during protein synthesis [6]. It is believed that the bacterial lethality of aminoglycosides is correlated with the production of these mistranslated proteins. Similarly, the persistent activity of aminoglycosides after removal of the drug may be due to lingering effects of sublethal levels of these proteins, or the time required for the drug to dissociate from the ribosomal target [7]. This pronounced post-antibiotic effect permits once-daily dosing to minimize toxicity while not sacrificing efficacy [8]. Aminoglycosides also exhibit a post-adaptive response [9]. This effect is characterized by a period of time after a first exposure to an aminoglycoside in which a second exposure to an aminoglycoside has no effect on the organism. This usually lasts 4–6 h, after which the response to a second exposure is similar to the first exposure. In *Pseudomonas*, the effect has been shown to be due to a transient upregulation of the efflux pump, MexXY-OprM [10]. The post-adaptive response further supports once-daily dosing regimens for the aminoglycosides.

7.3 Mechanisms of Aminoglycoside Resistance

With over 50 years of widespread use, it may be expected that widespread resistance would have followed. However, although new aminoglycoside-modifying enzymes (AMEs) have emerged as each new compound has been introduced, resistance rates have not increased at the rapid pace observed for other classes such as the fluoroquinolones. It should be noted that despite continued worldwide aminoglycoside use, there have not been any newly described AMEs in over 20 years [11] and no descriptions of new mutations of known AMEs leading to new aminoglycoside substrate profiles. However, there has been a single mutation in a 6´ acetylating AME that now confers resistance to ciprofloxacin and other fluoroquinolones without altering the aminoglycoside substrate profile [12]. This has important implications for the development of new members of the aminoglycoside class. If chemical structures can be engineered to overcome all the current clinically important AMEs, then these compounds have the potential for a long life expectancy without cross-resistance from other classes.

AMEs are the most prevalent cause of aminoglycoside resistance in the clinic [13]. With the exception of *Pseudomonas*, *Acinetobacter*, and *Burkholderia pseudomallei*, the regulation of intracellular concentration by overexpression of efflux pumps plays a less important role as an aminoglycoside resistance mechanism (AGRM) [14]. Ribosomal target modifying enzymes have recently emerged, generating very high levels of resistance to all known 4,6-linked aminoglycosides, but they are currently of low prevalence [15]. AMEs are often plasmid mediated, frequently occurring on transposons in combination with resistance genes for other antibiotic classes, making them a considerable threat to the utility of aminoglycosides. AMEs inactivate aminoglycosides by *N*-acetylation (AAC), *O*-adenylation (ANT), or *O*-phosphorylation (APH). Figure 7.2 shows the AMEs and the affected functional groups on the kanamycin B scaffold. The aminoglycosides whose activity is altered are listed next to each enzyme. The position of the aminoglycoside that is modified is indicated by the number in parentheses. When an enzyme carries out the same transformation but on a different set of substrates, it is assigned a separate Roman numeral. AMEs are found in a broad range of both gram-positive and gram-negative organisms. Multiple different genes have been discovered encoding the enzymes that carry out the chemical transformation of the aminoglycoside. The different genes encoding the same enzyme type are indicated by the alphabetical designation in Fig. 7.2, e.g., the AAC(6')-Ia is encoded by a different gene than AAC(6')-Ib but both have the same spectrum of enzymatic activity.

Crystallographic structures have been determined for several AMEs. Interestingly, despite the lack of similarity at the amino acid sequence level, these structures show remarkable similarity to portions of various eukaryotic enzymes. Structural similarities exist between ANT(4',4")-Ia and cytochrome C [16]; APH(3')-IIIa and protein kinases [17]; AAC(3)-Ia [18] and AAC(6')-Ii; and the GCN5-related *N*-acetyltransferase superfamily. Detailed kinetic studies of the mechanisms of several AMEs have also been published, most suggest an ordered binding of cofactors

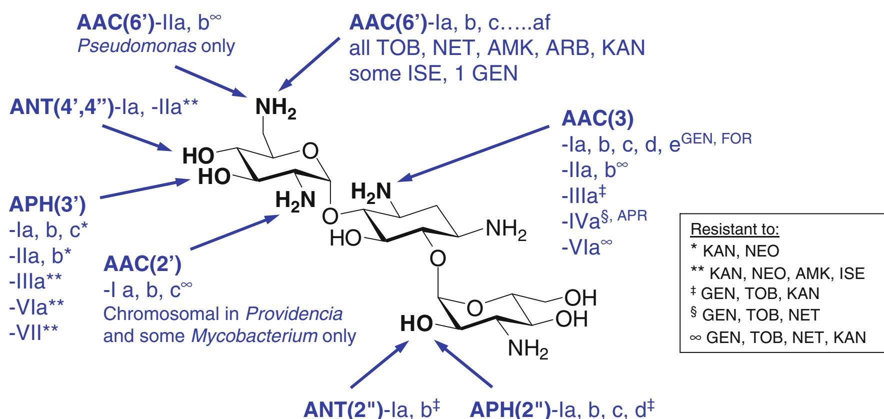


Fig. 7.2 AMEs shown with target functional groups of kanamycin B. Affected aminoglycosides are listed next to each enzyme. *AMK* amikacin, *ARB* arbekacin, *FOR* fortimicin, *GEN* gentamicin, *ISE* isepamicin, *KAN* kanamycin, *NET* netilmicin, *NEO* neomycin, *TOB* tobramycin. AMEs not shown in this figure, e.g., APH(3')-IV/-V are found in aminoglycoside-producing organisms but are not known in clinical isolates

and substrates indicative of Theorell–Chance kinetics. These include AAC(6')-Ib [19], ANT(2")-I [20], AAC(6')-Ii [21], APH(3')-IIIa [22], and APH(2")-Ia + AAC(6') Ie [23]. Thermodynamic studies of aminoglycoside binding interactions with APH(3')-IIIa [24], ANT(2")-Ia [25], and AAC(3)-IIIb [26] have also been published. These studies showed that there is often no difference between the binding affinities of 4,6-disubstituted 2-deoxystreptamine aminoglycosides and 4,5-disubstituted 2-deoxystreptamine aminoglycosides even if only the former are substrates for the enzyme (e.g., with ANT(2")-Ia). Similarly, kanamycin B and 3'deoxy-kanamycin B (tobramycin) have similar affinities for APH(3')-IIIa even though only kanamycin B is inactivated by the enzyme. Generally, amino groups at positions 2' and 6' result in higher affinity binding than seen with aminoglycosides having hydroxyls at these positions [27].

Other than the APH(3')-I/II enzymes, which were very common worldwide, aminoglycoside resistance in the USA in the mid-1970s was mainly caused by the gentamicin-modifying enzymes, AAC(3) and ANT(2"), with little or no AAC(6')-I. However, in Japan, kanamycin resistance in the form of ANT(2") and AAC(6')-I predominated [33]. In limited surveys, AMEs in Europe [28] and Chile [33] were found to be similar to those in the USA. In the late 1970s and early 1980s, tobramycin, netilmicin, and amikacin were introduced into clinical practice in the USA, Europe, and Latin America. All three of these aminoglycosides are substrates for AAC(6')-I and as a result, the distribution of AMEs began to change in these countries. Surveys of aminoglycoside resistance between 1984 and 1988 in the USA [29, 30], Europe [31], and Belgium [32] showed an increase in AAC(6")-I alone and frequently in combination with the previously prevalent gentamicin-modifying enzymes, AAC(3)-II and ANT(2")-I.

A large multinational study confirmed the multiple independent observations that enzymes that modify gentamicin are frequently found in combination with AAC(6')-I enzymes that inactivate the newer aminoglycoside amikacin [33]. An extensive collection of clinical isolates collected worldwide between 1988 and 1993 was examined for the presence of AMEs. Detection of an AME was conducted through susceptibility testing with aminoglycosides to generate an antibiogram that serves as a unique enzymatic "signature" [34]. When more than one enzyme was present, the interpretation of the antibiogram became complex, necessitating molecular techniques such as DNA hybridization to confirm the initial findings.

Recently a similar surveillance effort was conducted on a smaller scale to investigate whether the types of enzymes and the most frequent combinations had changed in the intervening 15 years. The results are summarized in Fig. 7.3 [35]. The survey demonstrated that the prevalence of the most common resistance mechanisms has remained constant. The enzymes ANT(2")-I, AAC(3)-II and AAC(6')-I (either alone or in combination) are the key resistance determinants among the Enterobacteriaceae. Ribosomal methyltransferases (RMT) were not detected in the earlier survey and the current incidence remains minimal worldwide [15]. Two enzymes dominate *P. aeruginosa* isolates, ANT(2")-I and AAC(6')-I. In *Pseudomonas*, one of the most common aminoglycoside resistance mechanisms is the upregulation of the MexXY efflux pump [36]. This is difficult to identify through susceptibility testing as it tends to alter the susceptibility to all aminoglycosides equally. Efflux also plays a leading role in *Acinetobacter*, in addition to the combination of multiple different AMEs [37].

7.4 Aminoglycoside Natural Products

The first successful use of natural product screening was the discovery of streptomycin by Schatz and Waksman [38]. As a consequence, there have been many natural product screening programs designed to find new aminoglycosides and these have been extensively reviewed [39, 40]. In his quest for antibiotics active against *Mycobacterium tuberculosis*, Waksman had previously identified compounds such as actinomycin in fermentation broths from Actinomycetes [40] but none had the desired *M. tuberculosis* activity. The discovery of streptomycin quickly led to the isolation, purification, and use of streptomycin for the treatment of tuberculosis in clinical trials in collaboration with Merck [41]. While streptomycin was remarkably successful in these initial trials, resistance developed quickly and vestibular and auditory toxicities were observed. Resistance in these trials was caused by target site mutations in a ribosomal protein closely associated with the binding site of streptomycin. While unfortunate, this rapid development of resistance led to the successful use of combination chemotherapy as other new antitubercular drugs were discovered [42]. Streptomycin has broad-spectrum activity against most gram-negative bacteria and several gram-positive bacteria but lacks clinically relevant activity against *Pseudomonas* [43].

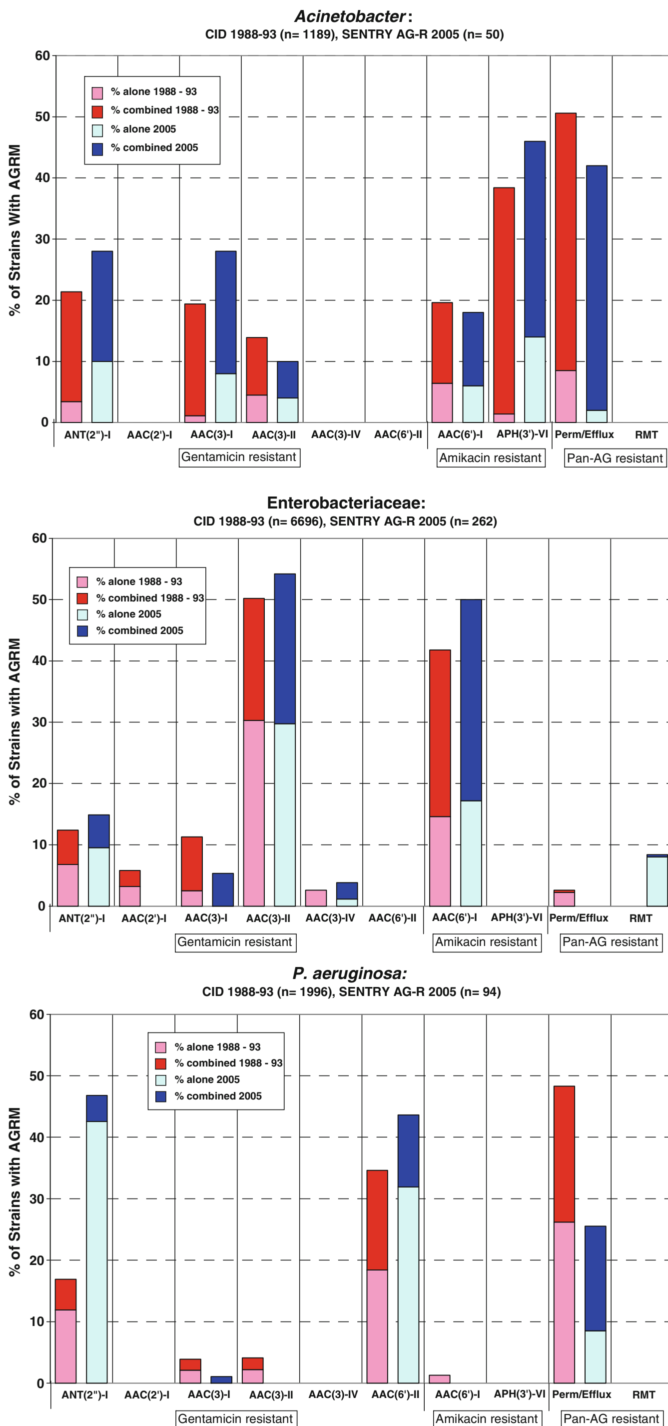


Fig. 7.3 Relative prevalence of AGRM alone or in combination with other AGRM in two time periods. (**a**) Enterobacteriaceae have three predominant AMEs and the percentage of isolates with each has remained largely unchanged. (**b**) *P. aeruginosa* resistance is commonly due to changes in permeability/efflux in addition to two predominant AMEs. (**c**) Resistance among *Acinetobacter* spp. is often caused by combinations of AMEs

Table 7.1 Aminoglycoside natural products

Aminoglycoside family	Date of discovery/ publication	Producing organism	Activity spectrum[a] (in the absence of AGRM)
Streptidine containing aminoglycosides			
Streptomycins	1943	*Streptomyces griseus*	*Mycobacterium tuberculosis*
2-Deoxystreptamine containing aminoglycosides			
Neomycins	1949	*Streptomyces fradiae*	–
Paromomycins	1956	*Streptomyces rimosus*	Protozoa
Kanamycins	1957	*Streptomyces kanamyceticus*	–
Gentamicins	1963	*Micromonospora purpurea*	*Pseudomonas*
Tobramycin	1968	*Streptomyces tenebrarius*	*Pseudomonas*
Sisomicin	1970	*Micromonospora inyoensis*	*Pseudomonas*
Ribostamycins	1970	*Streptomyces ribosidifcus*	–
Lividomycins	1971	*Streptomyces lividus*	–
Butirosins	1972	*Bacillus circulans*	*Pseudomonas*
Verdamicin	1975	*Micromonospora grisea*	*Pseudomonas*
Novel Aminoglycosides			
Hygromycins	1953	*Streptomyces hygroscopicus*	Protozoa
Spectinomycin	1961	*Streptomyces spectabilis*	–
Apramycin	1968	*Streptomyces tenebrarius*	–
Fortimicins	1977	*Micromonospora olivoasterospora*	–

[a]In addition to Enterobacteriaceae and staphylococci (see Sect. 7.2)

The discovery of streptomycin led to the widespread screening of natural products for antibiotic activity, and many new aminoglycoside families were discovered over the next 30 years. Several of these are listed in Table 7.1, with structures shown in Fig. 7.4. Most, if not all, of the aminoglycosides were found in mixtures of closely related secondary metabolites. The first of these, also from the Waksman laboratory, consisted of three principal components (neomycin A, B, and C), referred to here as the neomycin family [44]. Discovered in 1949, neomycin had a spectrum of activity against gram-positive and gram-negative bacteria similar to streptomycin but was approximately two-fold more potent [42]. Table 7.2 shows minimum inhibitory concentrations before (MICs) of aminoglycoside natural products in cation-adjusted Mueller-Hinton broth with susceptible recent clinical isolates. The MICs are very similar to the early MIC data [42] determined on agar. However, it should be noted that it was necessary to screen a large number of gram-negative bacteria in order to find recent isolates susceptible to both streptomycin and neomycin as resistance to these agents is now endemic. Clinical resistance to neomycin was first observed in 1959. In 1967, the molecular basis of this resistance was shown to be neomycin phosphotransferase (then called NPT, now APH(3')-I) [45]. Shortly thereafter, a second enzyme with a very similar resistance profile, APH(3')-II, was reported [46].

The discovery of the neomycin family was quickly followed by the paromomycin and kanamycin families [47, 48]. The early MIC data [42] showed that these

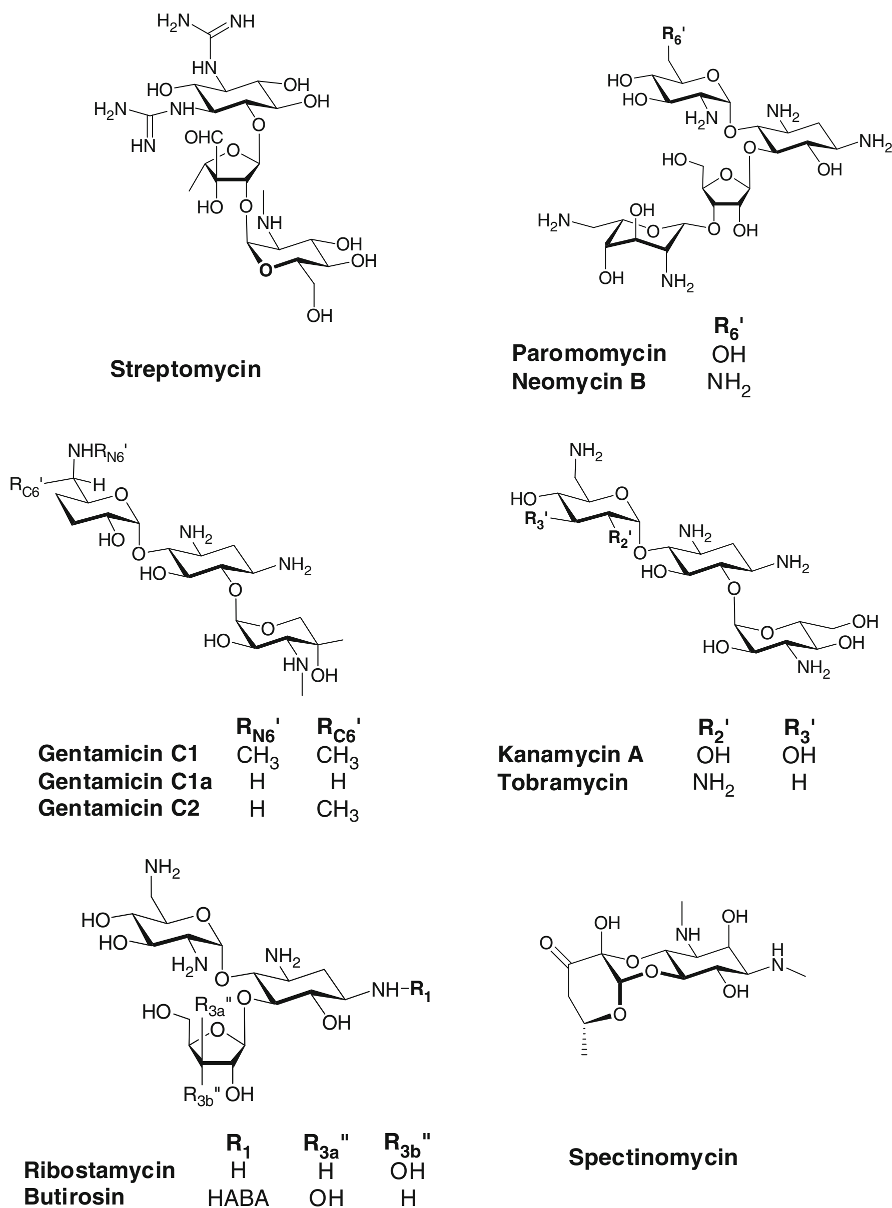


Fig. 7.4 Structures of naturally occurring aminoglycosides

compounds had similar activity to neomycin except they were less active against *Pseudomonas*. The 16-fold differential in antipseudomonal activity between neomycin and paromomycin highlighted the important role of the 6'-amino group present in neomycin over the 6'-hydroxyl group of paromomycin. Conversely, the greater

Table 7.2 Aminoglycoside natural product MICs against susceptible[a, b] gram-negative bacteria (Miller 2009, Achaogen, Inc.)

		Geometric mean MIC (μg/mL)								
Organism	n	STR	KAN	NEO	PAR	GEN	SIS	TOB	APR	FOR
E. coli[a]	16	5.9	5	1.8[c]	7.7	1	1.1	1.2	9.6	4.9
K. pneumoniae[a]	29	1.8	2.5	1.0[d]	2.9	0.3	0.4	0.6	3.8	3.4
Pseudomonas[b]	14	ND	ND	ND	ND	1.9	1.4	1	13.2	50

APR apramycin, *PAR* paromomycin, *SIS* sisomicin, *STR* streptomycin, *ND* no data

[a]Selected isolates with STR and KAN MICs <16 μg/mL

[b]Selected isolates with GEN and TOB MICs <8 μg/mL

[c]n = 6

[d]n = 16

activity of paromomycin against Protozoa suggested a different structure–activity relationship for these organisms. The discovery of the kanamycins in Japan in 1956 [48] was followed by the emergence of a kanamycin acetyltransferase (then called KAT, now known to be AAC(6')-I) that subsequently limited the clinical utility of kanamycin [49].

The 1963 discovery of the gentamicin [50–52] family ushered in another burst of aminoglycosides including tobramycin [53], sisomicin [51, 52, 54], and later verdamicin [55]. These aminoglycosides were more potent against Enterobacteriaceae and also had clinically useful activity (at the time) against *Pseudomonas*. The principal difference between these aminoglycosides (as well as the ribostamycins [56] and lividomycins [57] (which were discovered about 10 years later) and the older neomycins/kanamycins is that the newer compounds lacked a hydroxyl group at the 3'-position. The lack of the 3'-hydroxyl group was understood to correlate with antipseudomonal activity. Once again, enzymatic resistance emerged to these new agents; gentamicin adenylyltransferase, ANT(2") [58] was discovered in 1970, and gentamicin acetyltransferases, AAC(3)-I [59], AAC(3)-II, and AAC(2')-I [60] all emerged between 1972 and 1973.

The butirosins expanded on the idea of 3'-hydroxyl removal to enable *Pseudomonas* activity by incorporating "steric" protection of the 3'-hydroxyl (Table 7.1) [61, 62]. This family of aminoglycosides is closely related to the ribostamycins except for the acyl linkage of an (*2 S*)-2-hydroxy-4-aminobutyric acid substituent (HABA) on the C-1 amino group. The HABA group of the butirosins unexpectedly broadened activity against those Enterobacteriaceae able to phosphorylate the distal 3'-hydroxyl of ribostamycin (see APH(3'), Fig. 7.2) and also increased antipseudomonal activity dramatically. The molecular basis for this increased activity was explained in 1996 [63] when it was shown that the protein that phosphorylates the 3'-hydroxyl of the neomycin-kanamycin families of aminoglycosides (*APH(3')-IIb*) is chromosomally encoded in *P. aeruginosa*. Kawaguchi et al. [91] utilized this information to synthesize an analog of kanamycin A (amikacin) also with a HABA modification at the C-1 amino group. As with the butirosins, amikacin is active against *Pseudomonas* and Enterobacteriaceae with the APH(3') enzyme. Subsequently, derivatization of the 1-N-amino with acyl or alkyl groups was utilized by other investigators to make several clinically useful aminoglycosides (see Sect. 7.5).

Several families of aminoglycosides with novel structural features were discovered during the era of natural products screening, among which are the hygromycins [64], spectinomycins [65], apramycins [53], and fortimicins [66] (Table 7.1). Hygromycins like paromomycin and the subsequently discovered G-418 [67] have a 6'-hydroxyl group rather than a 6'-amino group. They are active against Protozoa and Saccharomyces but susceptible to phosphorylation by APH(3') and thus are now most commonly used as resistance markers in eukaryotes.

Spectinomycin, although active against a broad spectrum of susceptible gram-positive and gram-negative bacteria, has found its primary clinical use in the treatment of *Neisseria gonorrhoeae* infections. It shares a common binding site on the 30 S ribosomal subunit with streptomycin and is similarly affected by ribosomal protein mutations conferring streptomycin resistance. One of the AMEs that causes resistance to streptomycin, ANT(3")-I, also modifies spectinomycin. Interestingly, the phosphorylating enzyme, APH(3")-I, which modifies the same 3"-hydroxyl of streptomycin does not cause spectinomycin resistance. Conversely, the modifying enzyme ANT(9)-I modifies spectinomycin but not streptomycin. None of these resistance mechanisms confer resistance on the more broadly used neomycin, kanamycin, or gentamicin families of aminoglycosides and therefore are not discussed in Sect. 7.3.

The fermentation of *Streptomyces tenebrarius* to produce tobramycin also produces apramycin, which has been used in veterinary medicine. A unique modifying enzyme, AAC(3)-IV, which confers apramycin resistance was originally found only in veterinary isolates [68] but is now found in 2–5% of aminoglycoside-resistant Enterobacteriaceae from human clinical isolates [35]. The fortimicins are another structurally distinct group of pseudo-disaccharides that have broad-spectrum activity and are not modified by many of the AMEs found in gram-negative bacteria. However, their MICs against *Pseudomonas* are relatively high (Table 7.2).

7.5 History, the Semisynthetic Era

Natural sources provided a wealth of new aminoglycosides that have served both as clinical therapies and as tools to understand the mechanisms by which bacteria develop resistance. This knowledge was critical to the further expansion of the aminoglycoside class through chemical modification of natural products, termed semi-synthesis. The first semisynthetic compound, dihydrostreptomycin, was prepared in 1946 by a simple chemical reduction of the aldehyde group present in streptomycin. However, irreversible ototoxicity caused by this compound eventually led to a stop of its clinical use [69, 70].

The main semisynthetic era of aminoglycoside antibiotics was fueled by an increasing understanding of AMEs, still the predominant bacterial resistance mechanisms against aminoglycosides (see Sect. 7.3). Moreover, the observed nephro- and ototoxicity of existing aminoglycosides were of concern (see Sect. 7.9). The major challenge in developing semisynthetic aminoglycosides was to design a compound

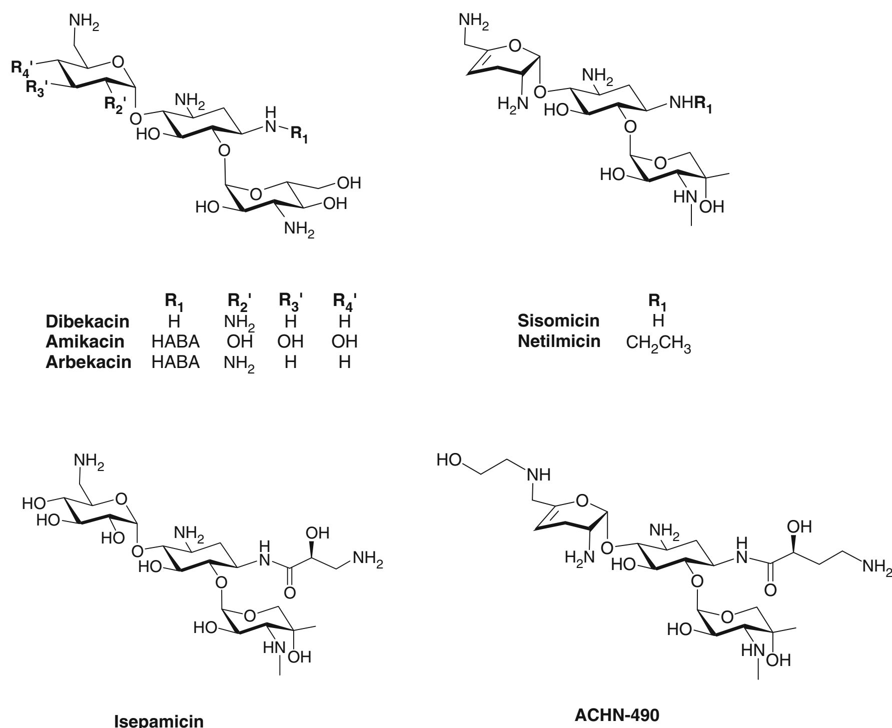
with improved activity against AMEs while retaining activity against susceptible organisms and providing a similar (or preferably improved) safety profile. The different resistance profiles of naturally occurring aminoglycosides such as tobramycin (3'-deoxy) and sisomicin (3'-4'-dideoxy) helped to define the precise targets of the APH(3') and ANT(4') enzymes. Butirosin incorporates a HABA, which highlighted the importance of N-1 acyl substituents in providing protection against ANT(2''), APH(2''), AAC(3), and APH(3') enzymes. Consequently, most of the synthetic efforts were focused on these critical functional groups.

Aminoglycosides are highly functionalized molecules that necessitate extensive protecting group strategies to isolate particular amino or hydroxyl groups for specific transformations. Some of these reactions are quite selective due to different levels of reactivity of functional groups driven by nucleophilicity and steric accessibility. The chemistry has been previously summarized in numerous reviews [71–80] and this chapter only summarizes key transformations that are considered cornerstones to access semisynthetic aminoglycosides of value to the antibacterial field.

- Ketal protection of vicinal hydroxyl groups.
- Oxazolidinone formation of 1,2-aminoalcohols by either basic ring closure of carbamoyl derivatives or reaction with carbonyl diimidazole.
- Selective protection of amino group(s) utilizing in situ protection by chelating metals such as zinc, copper, cobalt, and nickel.
- Deoxygenation or transformation of hydroxyl groups by activation, substitution, and – in case of deoxygenation – reduction.
- Acylation or alkylation of isolated functional groups.

Extensive modification of the pseudodisaccharide neamine, easily accessible by acidic hydrolysis of neomycin B, provided valuable insight into the importance of functional groups on antibacterial activity and resistance patterns. Simple methylation of either the 3'- or 4'-hydroxyl group either completely abolished or greatly reduced the antibacterial activity of the corresponding analogs [81]. A number of deoxygenated versions on both ring systems demonstrated that the removal of 3'- and 4'-hydroxyl groups led to compounds with comparable antibacterial activity and helped to subvert some of the resistance mechanisms [82]. Removal of the 5-hydroxyl group from neamine slightly enhanced the potency while the 5,6-dideoxy derivative had comparable activity to neamine [83]. A notable finding was the fact that 3'-epineamine was not a substrate for 3'-phosphotransferases and consequently retained activity against isolates with this resistance mechanism. However, the antibacterial activity against susceptible strains was reduced [84]. Replacement of any of the hydroxyl groups by amines reduced the intrinsic antibacterial activity of compounds [85, 86]. A marked enhancement of neamine's intrinsic activity was observed upon combination of 3'-, 4'-dideoxygenation, and N-1-acylation with HABA, the side chain identified in the natural product butirosin [87]. The combination of these derivatizations also added protection against most aminoglycoside-modifying enzymes with the exception of AAC(2')-I and AAC(6')-I.

Application of the 3'-, 4'-dideoxygenation to kanamycin B yielded dibekacin (Fig. 7.5), which has remarkable activity against resistant organisms, and against



	R_1	R_2'	R_3'	R_4'
Dibekacin	H	NH_2	H	H
Amikacin	HABA	OH	OH	OH
Arbekacin	HABA	NH_2	H	H

	R_1
Sisomicin	H
Netilmicin	CH_2CH_3

Fig. 7.5 Structures of semisynthetic aminoglycosides

P. aeruginosa [88]. Dibekacin was the first semisynthetic aminoglycoside approved for clinical use. It was introduced in the 1970s in Japan and Korea and limited European countries but eventually was replaced by arbekacin due to the latter's improved activity against isolates with AMEs.

The realization that butirosin (N-1-HABA ribostamycin) not only retained the intrinsic antibacterial activity of aminoglycoside derivatives but also was protected from different classes of aminoglycoside-modifying enzymes led to structural exploration at N-1 using ribostamycin as a scaffold [89, 90]. Overall, derivatives with 2-hydroxy-ω-aminoacyl moieties with three or four (as in HABA) carbon atoms displayed the best activity. The stereochemistry of the 2-position influenced activity, with the S-configuration preferred. Small alkyl groups were also tolerated at the N-1 position, not requiring an additional amino group for activity but providing less protection than HABA against the AAC(3) modifying enzymes.

N-Acylation with HABA was eventually applied to a large variety of aminoglycosides. The use of kanamycin A as a scaffold yielded amikacin (Fig. 7.5), which was demonstrated to have improved activity against kanamycin-resistant bacteria and had lower acute toxicity than its parent compound [91]. Amikacin was launched in 1976 by Bristol-Myers Squibb as an injection formulation for the treatment of serious infections due to amikacin-sensitive gram-negative organisms as well as

known or suspected staphylococcal infections. Modification of dibekacin with HABA yielded arbekacin (Fig. 7.5), which was launched in 1990 by Meiji-Seika in Japan for the treatment of staphylococcal infections. Unlike dibekacin, arbekacin is resistant to inactivation by the amikacin-modifying enzymes, APH(3')-III, -VI, and ANT(4',4")-I.

Another effort to find a safer semisynthetic aminoglycoside with improved activity against bacteria containing AMEs resulted in netilmicin (*N*-1-ethyl sisomicin, Fig. 7.5) [73]. The compound had activity against organisms containing 2"-*O*-modifying and many 3-*N*-acetylating enzymes, and was shown to be markedly less nephro- and ototoxic than gentamicin in all species of laboratory animals tested [92]. Netilmicin was launched by Menarini in Italy in 1982 and later in other countries; however evaluation in humans has not borne out the promise of a safer aminoglycoside that was suggested by the preclinical data [93]. More recently, isepamicin (Sch 21420, Fig. 7.5) was prepared from gentamicin B by installing the acyl substituent ((S)-3-amino-2-hydroxypropyl or HAPA, one carbon atom shorter than HABA) at the C-1 amino group. Isepamicin was launched by Schering in Japan in 1989, in Italy in 1996, and is still in clinical use in some countries today.

Substantial efforts also explored the 2-deoxy-5-epi-streptamines as the core scaffold [73]. This compound was made by activation and inversion [94]. It was originally produced using a mutated version of *Micromonaspora inyoensis*, the organism that produces sisomicin, with a blocked 2-deoxystreptamine biosynthesis. Five-epi-sisomicin was produced by feeding 2-deoxy-5-epi-streptamine to the fermentation process. The resulting compound displayed two- to four-fold elevated activity in vitro and in vivo compared to gentamicin with comparable nephro- and ototoxicity potential based on experiments in rats and cats. This was the first and possibly only time a modification was shown to substantially improve the intrinsic activity of an aminoglycoside against gram-negative organisms. The epimerization of the 5-hydroxyl group also provided protection from ANT(2"), AAC(2'), and some AAC(3) enzymes. A HABA analog was prepared to further enhance the resistance profile; it displayed excellent microbiological activity but also increased toxicity. A similar pattern was described for the N-1-HABA-5-epi-gentamicin B (5-epi-isepamicin), indicating tolerability issues with 5-epi derivatives. The golden area that produced all the semisynthetic compounds currently in clinical use came to an end. The activity of these compounds against susceptible strains is shown in Table 7.3, which demonstrates how the overall potency was generally maintained relative to the natural products while activity against AMEs was incorporated.

Subsequently, synthetic efforts focused on refinements of antibacterial activity and the design of molecules to disrupt biologically important protein–RNA interactions [77, 79, 80]. While this work expanded the knowledge of this compound class, it has not produced additional clinical candidates until very recently. The clinical need for novel antibiotics with potency against multidrug-resistant gram-negative bacteria led in 2006 to the initiation of a new project at Achaogen. Our goal was

Table 7.3 Geometric mean MICs (μg/mL) of semisynthetic aminoglycosides AMK, ARB, ISE, and NET compared to natural products (Miller 2009, Achaogen, Inc.)

			Geometric mean MIC (μg/mL)							
Organism	Sensitive to	n	GEN	TOB	NET	AMK	ISE	ARB	KAN	NEO
E. coli	GEN, TOB, NEO	14	0.9	1.3	1.3	4.3	1.0	1.5	5.9	1.9
Klebsiella spp.	GEN, TOB, NEO	15	0.4	0.5	0.4	1.7	0.8	0.6	1.9	0.9
Pseudomonas spp.	GEN, TOB, AMK	15	1.6	0.9	4.0	4.1	2.8[a]	1.9	>64	5.9
Staphylococcus spp.	GEN, NEO	16	0.5	0.25[b]	≤ 0.5[b]	1.5	1.7[b]	0.5	3.2[b]	0.4

[a]12 isolates
[b]4 isolates

to identify novel aminoglycoside analogs with improved safety compared to the legacy aminoglycosides, and with activity against multidrug-resistant gram-negative bacteria including those with aminoglycoside-inactivating enzymes. Structural information about aminoglycoside-ribosomal RNA interaction, novel synthetic transformations, and greatly enhanced analytical and purification technologies allowed us to evaluate this problem afresh.

Sisomicin was chosen as our starting point due to its intrinsically high potency against gram-negative bacteria and the lack of 3'- and 4'-hydroxyl groups and we focused on the discovery of additional modifications or alterations required to achieve our goal. After examination of a large number of derivatives at the 6' and/or 2' positions of sisomicin, we identified ACHN-490 as the compound with the optimal combination of broad antibacterial spectrum against aminoglycoside-resistant strains and safety profile [95].

7.6 The "Neoglycoside" ACHN-490 is Active Against Bacteria with AMEs

Although the means of generating resistance to aminoglycosides have remained largely unchanged for the past decades, the proportion of clinical isolates with those resistance mechanisms has increased. In the last 10 years, the percentage of isolates resistant to at least one aminoglycoside has surpassed 10% in North America and Europe, while in South America and Asia the numbers are even higher [96]. The extant need for new gram-negative agents has led scientists at several institutions to conduct a search for a new aminoglycoside that would retain the potency and spectrum of its predecessors but evade the growing threat of AMEs. ACHN-490 is the first of these next-generation aminoglycosides, or neoglycosides. ACHN-490 is now called Plazomicin. Multiple studies describing ACHN-490 were presented at the 49th ICAAC and are reviewed here.

ACHN-490 is a semisynthetic molecule derived from sisomicin. The modifications introduced are designed to provide protection from the plethora of AMEs described in Sect. 7.3. The structure of ACHN-490 is shown in Fig. 7.5. The elimination or shielding of key functional groups in ACHN-490 blocks all but one of the

AMEs shown in Fig. 7.2. The one known enzyme that does decrease the activity of ACHN-490 is AAC(2')-I. This enzyme is found chromosomally in *Providencia stuartii* and has not been transferred to other species [97]. As a result its current clinical significance is quite limited.

Sisomicin naturally lacks the 3'- and 4'-OH groups that protect it and ACHN-490 from the APH(3')-III, -VI, and -VII and ANT(4') enzymes that generate resistance to amikacin. The introduction of the HABA substituent at the C-1 amino group provides protection from the AAC(3), ANT(2"), and APH(2") AMEs. The hydroxyethyl substituent at the 6' position blocks the multitude of AAC(6') AMEs without the reduced potency that has been found with other efforts to shield this position [75].

The structural modifications introduced in ACHN-490 provide protection against the AMEs and thus its activity is maintained in isolates that are resistant to amikacin and/or gentamicin. Table 7.4 shows the activity of ACHN-490 compared to amikacin and gentamicin against multiple isolates with defined AMEs. In Enterobacteriaceae, the baseline potency may be defined by the geometric mean MIC against the *E. coli* strain ATCC 25922. In the absence of AMEs, this strain has equivalent susceptibility to gentamicin and ACHN-490 while amikacin appears to be three-fold less potent. The presence of an AME does not alter the ACHN-490 MIC but the three most common AMEs (Fig. 7.2) cause a 30-fold or greater decrease in the activity of the comparators.

The Proteae are intrinsically less susceptible to aminoglycosides, even in the absence of AMEs due to lower permeability compared to other Enterobacteriaceae [98]. The activity of ACHN-490 was comparable to amikacin against these organisms (Table 7.4). Target modification is the least common route to resistance toward the 2-deoxystreptamine aminoglycosides and has only recently surfaced in Enterobacteriaceae in the form of ribosomal methyltransferases [15]. These enzymes methylate the ribosomal binding site and uniformly generate high MICs to all 4,6-linked aminoglycosides including ACHN-490.

The staphylococci have a limited repertoire of AMEs but the three that are known are widespread. ACHN-490 activity is not altered by any of the AMEs found in staphylococci (Table 7.4). The Enterobacteriaceae possess a more diverse array of AMEs, none of which alter the activity of ACHN-490, while amikacin, gentamicin, and other aminoglycosides are modified such that their MICs are typically above established breakpoints. Similarly, AMEs in *P. aeruginosa* and *A. baumannii* do not change the activity of ACHN-490. The number of isolates available for testing with individual AGRM is lower within these two species as the majority of clinical isolates possess more than one AGRM (Fig. 7.2); however combinations of these enzymes leave the MIC of ACHN-490 similarly unaffected. Efflux of aminoglycosides is a common resistance mechanism in *P. aeruginosa* and *A. baumannii* and one to which all aminoglycosides are susceptible. Overall, ACHN-490 is broadly active against aminoglycoside-susceptible and -resistant Enterobacteriaceae and staphylococci. The activity is improved relative to other aminoglycosides against non-fermenters with AMEs alone and comparable to other aminoglycosides in the presence of upregulated efflux pumps.

Table 7.4 Analysis of ACHN-490 activity against clinical isolates with defined AMEs. Isolates with a single AGRM are included here, along with the two most common combinations in Enterobacteriaceae (AAC(6')-I with either AAC(3)-II or ANT(2")-I). APH(3')-I/II, which generates KAN/NEO resistance is excluded for clarity.

		Geometric mean MIC (µg/mL)				
Group	Phenotype	AMK	GEN	ACHN-490	n	Species
Enterobacteriaceae	Normal ATCC 25922	3	1	1	1	ECO
	AAC(3)-I	4	32	1	1	KPN
	AAC(3)-II	4	62	1	22	CFR, EAE, ECL, ECO, KOX, KPN, SLI
	AAC(3)-IV	2	16	1	2	ECO, KPN
	AAC(6')-I	30	1	1	26	CFR, EAE, ECL, ECO, KOX, KPN, SMA
	ANT(2")-I	2	35	1	7	EGE, KPN, ECL, ECO, SMA
	AAC(3)-II, AAC(6')-I	32	>64	1	18	CFR, ECL, ECO, KPN, SMA
	ANT(2")-I, AAC(6')-I	34	23	1	14	CFR, ECL, KPN
	Proteae	10	25	6	24	MMO, PMI, PRE
Staphylococci	Normal ATCC 29213	3	1	1	1	SAU
	ANT(4',4")-I	13	1	1	9	SAU, SEP
	APH(2")+AAC(6')-I	18	50	1	22	SAU, SEP
	APH(3')-III	4	1	1	14	SAU, SEP
P. aeruginosa	Normal ATCC 27853	2	1	3	1	PAE
	AAC(6')-II	14	64	6	5	PAE
	ANT(2")-I	8	56	3	5	PAE
A. baumannii	Normal	4	1	2	1	ACB
	AAC(3')-I	4	16	4	2	ACB
	ANT(2")-I	16	>64	2	1	ACB
	APH(3')-VI	>64	4	3	3	ACB

ACB A. baumannii, *CFR C. freundii*, *EAE E. aerogenes*, *ECL E. cloacae*, *ECO E. coli*, *KOX K. oxytoca*, KPN = *K. pneumoniae*, *MMO M. morganii*, *PAE P. aeruginosa*, *PMI P. mirabilis*, *PRE P. rettgeri*, *SAU S. aureus*, *SEP S. epidermidis*, *SLI S. liquifasciens*, *SMA S. marcescens*

7.6.1 ACHN-490 is Active Against Bacteria Resistant to Key Antibiotics

While the expansion of aminoglycoside resistance is cause for concern, many recent reports have sounded the alarm regarding the appearance and spread of extended-spectrum β-lactamases (ESBLs) and plasmidic AmpC enzymes [99, 100]. Many of these isolates are also resistant to the currently available aminoglycosides. The specter of widespread resistance to third-generation cephalosporins and fluoroquinolones has led to increased use of carbapenems, which in turn increases the selection pressure for metallo-β-lactamases and *Klebsiella pneumoniae* carbapenemases (KPCs) [101, 102]. The carbapenemase enzymes first reported in *K. pneumoniae* have rapidly spread to other Enterobacteriaceae [103], to *P. aeruginosa* [104], and to *Acinetobacter* [105]. The activity of ACHN-490 was examined against 235 organisms with ESBLs, chromosomal or plasmidic AmpC cephalosporinases, serine carbapenemases, and metallo-β-lactamases and none of these had an effect on observed MIC values [106]. The fact that ACHN-490 is not susceptible to these resistance mechanisms demonstrates its potential as a suitable alternative to the extensive use of carbapenems. In a separate study, the activity of ACHN-490 was determined against clinical isolates of *K. pneumoniae* with (n = 25) and without (n = 77) KPCs. The activity of ACHN-490 was the same against both groups of strains while the susceptibility to imipenem and other front-line agents was decreased in the KPC group [107].

Several areas of the world are known to have increased levels of antibiotic resistance, generally these are locations where large numbers of people and large numbers of resistant bacteria coincide. In North America, New York City is one such area [101]. Surveillance studies in these areas are valuable tools for the prediction of resistance trends and also to gather organisms for testing new agents against isolates that will likely represent the clinical flora in the future. ACHN-490 was tested against a subset of the isolates from the 2006 surveillance in Brooklyn, NY [108]. The isolates were selected in an effort to reflect the resistance patterns present in the broader 2006 collection. ACHN-490 was more active than aminoglycoside comparators against Enterobacteriaceae and *A. baumannii*. Only 55% of the *P. aeruginosa* group was susceptible to imipenem and 36% to ciprofloxacin while the activity of ACHN-490 was similar to the legacy aminoglycosides, which retained higher levels of susceptibility.

7.6.2 ACHN-490 is Rapidly Bactericidal

Bactericidality is an important feature of aminoglycosides, enhancing their utility in the treatment of serious infections. ACHN-490 was found to be bactericidal in a concentration-dependent manner against isolates with and without AMEs [109].

In time-kill assays, ACHN-490 demonstrated rapid bactericidal activity, achieving a 3-log decease in CFU/mL within the first 2 h of exposure against all eight of the organisms tested. At 24 h there was sporadic evidence of regrowth at concentrations two- and four-fold above the MIC with either ACHN-490 or the aminoglycoside comparator. Follow-up MIC determinations for representative ACHN-490 regrowth colonies from exposures of four-fold MIC or greater demonstrated a four- to eight-fold increase for all aminoglycosides tested, characteristic of changes in membrane permeability [9] rather than an ACHN-490-specific mechanism.

7.6.3 ACHN-490 is Synergistic with Other Agents Against MRSA

Aminoglycosides are often employed in combination with other agents to treat serious infections empirically and ensure broad-spectrum coverage, and also to exploit the potential for synergy with the second antibiotic. The activity of ACHN-490 alone and in combination against 47 MRSA isolates was confirmed by in vitro synergy time-kill experiments. The most striking synergy occurred in combination with daptomycin, where 91% of the isolates showed >2 $\log_{10}$ decrease in CFU/mL between the combination and its most active constituent after 24 h [110]. All of the clinical isolates tested had ACHN-490 MICs ≤4 μg/mL, including those with decreased susceptibility to vancomycin. The potent activity of ACHN-490 alone and in combination is currently being examined against other species.

7.7 Aminoglycoside Pharmacokinetics

This section describes the absorption, distribution, metabolism, excretion (ADME) [111, 112], and pharmacokinetics (PK) of aminoglycosides. All of the aminoglycosides achieve peak plasma concentrations at the completion of an IV infusion. Bioavailability (as measured by plasma concentration) after intramuscular (IM) injection is nearly 100%, and peak concentrations occur in plasma 30–90 min after administration. Aminoglycosides are highly polar cations and thus are very poorly absorbed from the gastrointestinal tract; less than 1% of a dose is absorbed following either oral or rectal administration. Systemic absorption of aminoglycosides has been reported after topical use on denuded skin and burns and following instillation into and irrigation of wounds, body cavities, and joints, but not the urinary bladder.

Except for streptomycin, there is negligible binding of aminoglycosides to plasma albumin. The apparent volume of distribution of these drugs is 25% of lean body weight, which approximates the volume of extracellular fluid. Aminoglycosides do not undergo metabolism and are excreted almost exclusively by glomerular filtration, achieving high urinary concentrations. Thus, the bulk of a parenterally

administered dose is excreted unchanged during the first 24 h, with most of this appearing in the urine in the first 12 h. The half-lives of the aminoglycosides in plasma are similar throughout the class, ranging between 2 and 3 h in patients with normal renal function. The rate of elimination of drug from tissue sites, primarily the kidney, is considerably longer than that of plasma; the half-life for tissue-bound aminoglycosides has been estimated to range from 30 to 700 h. A two-compartment model best describes the plasma PK of aminoglycosides [113].

Renal clearance of aminoglycosides is approximately two-thirds of the simultaneous creatinine clearance, suggesting some tubular reabsorption of these drugs. After a single dose of an aminoglycoside, disappearance from the plasma exceeds renal excretion by 10–20%; however, after 1–2 days of therapy, nearly 100% of subsequent doses are eventually recovered in the urine. Concentrations in the renal cortex approach 10% of the administered dose, but the uptake is saturable, and the renal accumulation depends on the rate of infusion, dosing frequency, and duration of therapy [114, 170, 172, 178].

High urinary concentrations of aminoglycosides contribute to the eradication of pathogens in the urine, just as high concentrations in the renal cortex are important in treating infections in renal tissue. Serum or plasma concentrations may be useful surrogate markers for renal tissue concentrations [115]. For example, renal concentrations of gentamicin were shown to be an important factor in treating *E. coli* in an experimental model of pyelonephritis and renal abscess [116]. A short (3-day) course of gentamicin, during which drug was shown to accumulate in the renal cortex, was used for the treatment of experimental pyelonephritis in rats. The short course of gentamicin was more effective than a 14-day course of cephalothin or trimethoprim and equivalent to ampicillin [116].

Small concentrations of aminoglycosides can be found in bile; however, the liver is a very minor excretory route for aminoglycosides. Inflammation increases the penetration of aminoglycosides into peritoneal and pericardial cavities. Because of their polar nature, aminoglycosides are excluded from the central nervous system and the eye. Concentrations of aminoglycosides in cerebrospinal fluid that are achievable with parenteral administration are usually subtherapeutic. Administration of aminoglycosides to women late in pregnancy may result in accumulation of drug in fetal plasma and amniotic fluid; only small amounts have been reported in breast milk. Diffusion into synovial and pleural fluid is relatively slow, but concentrations that approximate those in the plasma may be achieved after repeated administration. Aminoglycosides readily penetrate into pulmonary secretions at concentrations sufficient for treatment of HAP and VAP [117–119]. After a 3.5 mg/kg dose of gentamicin, the maximal alveolar lining fluid (ALF) concentration in patients with VAP was 4.24 ± 0.42 μg/mL [117]. There was a statistically positive correlation between serum and ALF concentrations in these patients, suggesting that higher doses could achieve higher ALF concentrations. In a dual pneumonia and thigh infection model in neutropenic mice, gentamicin was more rapidly bactericidal and had a significantly greater maximum efficacy ($p < 0.01$) in lung infections than in thigh infections. In addition, gentamicin has a greater maximum bactericidal effect in the lungs than that of imipenem and ceftazidime [120].

Table 7.5 PK parameter estimates for a single bolus IV injection of ACHN-490 administered at 10 mg/kg in mice, rats, and dogs

	No./Sex	C_0 (µg/mL)	$AUC_{0-\infty}$ (hr*µg/mL)	$t_{1/2}$ (h)[a]	CL (mL/h/kg)	V_{ss} (mL/kg)
Mouse [b]	15/M	88	19	1.4	541	340
Rat	3/M	38 ± 8	16 ± 3	0.9 ± 0	648 ± 96	437 ± 35
Dog	3/M	120 ± 14	83 ± 12	1.2 ± 0	123 ± 19	164 ± 16

$AUC_{0-\infty}$ area under the concentration-time curve from time 0 to infinity, C_0 initial drug concentration, *CL* clearance, t½ half-life, V_{ss} volume of distribution at steady state

[a]t½(beta) estimated using noncompartmental analysis

[b]Single values based on sparse sampling across multiple animals

7.7.1 Pharmacokinetics of ACHN-490

ACHN-490 maintains the favorable ADME properties and PK profile seen for the aminoglycosides. Noncompartmental analysis of the ACHN-490 concentration-time data in plasma allows for PK parameter comparisons across nonclinical animal species. As shown in Table 7.5, the PK profile of ACHN-490 after IV administration is similar in mice, rats, and dogs [121].

Initial plasma ACHN-490 concentrations and exposure (AUC) increased in proportion to dose after administration of single IV doses up to 75 mg/kg in rats. Elimination of ACHN-490 is fairly rapid in both rats and dogs (apparent half-life is approximately 1 h). The volume of distribution in mice, rats, and dogs is moderate and close to extracellular fluid volumes. Plasma protein binding of ACHN-490 is independent of total concentration and does not differ among species, with less than 20% of the drug bound in mouse, rat, rabbit, dog, cynomolgus monkey, and human plasma.

ACHN-490 does not show evidence of in vitro metabolism using mouse, rat, dog, cynomolgus monkey, and human liver microsomes and hepatocytes. ACHN-490 does not inhibit the human CYP isoforms 1A2, 2 C9, 2 C19, 2D6, or 3A4 in vitro. ACHN-490 is stable in plasma from mouse, rat, rabbit, dog, cynomolgus monkey, and human. Similarities between rats and dogs are observed in the urinary excretion of ACHN-490, with high excretion of ACHN-490 within the first 24 h after a single dose.

7.8 Aminoglycoside Pharmacodynamics

When aminoglycosides were first introduced into clinical practice several decades ago, detailed knowledge of their PK/PD did not exist. Consequently, the original strategy for dosing was to maintain the aminoglycoside serum concentration above the (MIC) of the infecting organism for as long as possible throughout the dosing interval. This approach resulted in dosing frequencies of every 8 h, long infusion

times, and occasional attempts at dosing patients by continuous infusion [122]. Unfortunately, this has resulted in suboptimal efficacy and increased toxicity. Despite nearly two decades of mounting scientific evidence in favor of once-daily dosing of aminoglycosides, in the year 2000 as many as 25% of 500 randomly selected acute-care hospitals in the USA had not adopted new dosing recommendations, and a large proportion had no written guidelines [123]. New knowledge and understanding of the PK/PD of the aminoglycoside class allows the design of optimal dosing regimens that notably improve the efficacy and safety of this class of drugs, and thus may optimize the clinical utility of neoglycosides such as ACHN-490.

Both peak serum concentration (C_{max}) and the area under the time-concentration curve (AUC) in relation to the MIC of the target pathogen are important indicators of the efficacy of aminoglycosides. In vitro and in vivo models have demonstrated that aminoglycoside bactericidal activity is concentration-dependent, i.e., higher concentrations of drug cause faster killing of exposed bacteria.

PK/PD investigations suggest that AUC is probably the best parameter in predicting efficacy for aminoglycosides in both animal experiments and clinical studies [124–127]. Dose fractionation studies in mice have been used to demonstrate that the AUC/MIC ratio predicts efficacy for aminoglycosides [124] and this ratio has also been shown to be a predictor of clinical efficacy [125, 133], but it should be noted that C_{max} and AUC are highly correlated in patients receiving once-daily regimens. In a study of patients with *P. aeruginosa* bacteremia secondary to pneumonia, infected vascular catheter, UTI, and other infected sites, a clinical cure was associated with an AUC/MIC ratio of 64 or greater, and clinical failure was associated with a ratio of 39 or less ($p = 0.002$) [133]. Optimal dosing has been described as producing at least a 90% chance of clinical cure [125]. For gentamicin, Monte Carlo simulations have shown that a dose of 5 mg/kg/day for 7 days should achieve an AUC of 60–75 and should be sufficient for the treatment of organisms with MICs of ≤1.0 μg/mL [125].

While AUC is an important determinant of efficacy, several investigators have also shown a relationship between C_{max} and clinical outcome, including mortality and clinical cure in patients with pneumonia and bacteremia [128–133]. A C_{max}/MIC ratio of at least 10 in the first 24 h of treatment is considered important [4]. A C_{max} for gentamicin or tobramycin of at least 7 μg/mL was also shown to be an independent predictor of clinical success ($p < 0.006$) in treating patients with pneumonia caused by gram-negative bacteria including *P. aeruginosa*, although the study did not examine C_{max}/MIC ratios [130]. In a subsequent study of over 200 patients with gram-negative infections including pneumonia, cIAI, and bacteremia, the same investigators showed that the C_{max}/MIC ratio of 8.5 ± 5.0 μg/mL had high statistical significance and was more predictive of clinical response ($p = 0.00001$) as compared to a C_{max} alone of 8.2 ± 2.8 μg/mL ($p = 0.013$) [129]. A statistically significant relationship between the C_{max}/MIC ratio and clinical response has also been demonstrated for patients with nosocomial pneumonia [131, 132]. In patients with pneumonia due to gram-negative pathogens, a 90% probability of temperature and leukocyte resolution by Day 7 of therapy was associated with a C_{max}/MIC ratio of ≥ 10 ($p < 0.00001$) [131, 132]. Similarly, clinical cure, defined as a complete

resolution of signs and symptoms of serious infections including *P. aeruginosa* bacteremia, was associated with a $C_{max/}$MIC ratio of 5.3 or greater, and a clinical failure was associated with a ratio of 3.2 or less (p = 0.001) [133].

A high C_{max} provides an independent and additive effect above the effect contributed by AUC alone [134]. It is possible to achieve a higher C_{max} for a given AUC by delivering the total daily dose in a single administration (i.e., once-daily dosing) and shortening the infusion time of the administration (i.e., 10-min infusion vs. 30-min infusion). For example, a dose of 80 mg of gentamicin delivered to human subjects as a 1-min or a 30-min infusion achieved a C_{max} of 15.6 or 6.2 μg/mL, respectively. When this rate of infusion and corresponding PK profile were generated in vitro with the AUC held constant, time-kill results demonstrated more rapid and deeper killing with the higher C_{max} delivered by the shorter infusion [134]. Additionally, a high C_{max} has been correlated with better tissue penetration [135]. Thus, it is likely that both AUC and C_{max} are important for efficacy.

7.9 Toxicity and Safety of Aminoglycosides

While aminoglycosides are excellent bactericidal agents against susceptible pathogens, a major challenge facing the older members of the class is diminishing efficacy resulting from the emergence of resistance. In addition, the use of aminoglycosides continues to raise concerns about safety, particularly when these compounds are dosed according to current labeling recommendations. The primary adverse reaction associated with the class is nephrotoxicity; however, ototoxicity and neuromuscular blockade have also been observed, albeit less frequently. Fifty years of clinical experience with aminoglycosides and increased knowledge of molecular and cellular mechanisms of toxicity have led to a better understanding of the class. This experience and knowledge supports the implementation of dosing strategies to minimize the incidence of these adverse reactions.

7.9.1 Aminoglycoside-Associated Neuromuscular Blockade

Neuromuscular blockade is a rare clinical event reported in conjunction with very high doses or overdoses of aminoglycoside therapy. This blockade at the neuromuscular junction is believed to be the result of disrupting acetylcholine neurotransmission. The decreased release and response of acetylcholine may result from a blockage of calcium channels by high serum concentrations of the drug [136]. Paralysis of the phrenic nerves of the diaphragm in rats and rabbits has been demonstrated [137, 138]. In animal models, the blockade leads to respiratory paralysis and death and is the dose-limiting readout in acute toxicity studies. It has been shown that blockade is correlated with peak serum concentration and is independent of either rate of clearance, route of administration, and duration of infusion [139]. In acute toxicity studies in mice, the serum concentration of dibekacin at the time of death exceeded 800 μg/mL [139]. This dose level is more than 20-fold higher than

the C_{max} (30–40 μg/mL) recorded for gentamicin at clinically relevant doses. In human cases, this transient and fully reversible condition is related to high serum levels of the drug, typically brought on by accidental overdose [136]. Because of the effect on neuromuscular junctions, patients receiving concomitant aminoglycoside therapy with general anesthesia may experience a minimal delay in recovery from general anesthesia.

7.9.2 Nephrotoxicity

Nephrotoxicity is the main use-limiting toxicity reported with aminoglycosides. Where it occurs, the resulting renal impairment typically appears several days (7–10 days) after the initiation of treatment [127]. Impairment is usually mild and reversible after drug withdrawal [140], although it may be more severe, especially in patients with underlying renal disease. It is non-oliguric in nature, and associated with a gradual rise in serum creatinine (Cr) and blood urea nitrogen (BUN) [141]. Several factors increase the risk of aminoglycoside-associated nephrotoxicity, including age of the patient, gender, aminoglycoside trough concentration, duration of therapy, concurrent nephrotoxic drugs (i.e., vancomycin, amphotericin B), liver disease, neutropenia, and peritonitis [142, 143]. Incidence rates for nephrotoxicity in patients have ranged from 3% to 28% [144–149, 160], but most studies appear to cluster around a rate of approximately 10% [145]. Unfortunately, most studies and meta-analyses do not clearly take into account other associated risk factors that may be contributing to or causing the nephrotoxicity.

Aminoglycosides vary in their intrinsic ability to cause nephrotoxicity in animals, and numerous models (e.g., mouse, rat, dog, and primate) have demonstrated the nephrotoxic potential of aminoglycosides. In rat models comparing azotemia and histopathology, there are measurable differences in nephrotoxicity among members of the class, with neomycin being the most nephrotoxic, gentamicin and tobramycin being intermediate, and amikacin the least nephrotoxic [150–152]. Other studies have shown that tobramycin is at least three-fold less nephrotoxic than gentamicin in rats [153, 154] as it does not increase serum creatinine even at three times the gentamicin dose. While tobramycin appears to be less nephrotoxic than gentamicin, the mechanisms are the same as confirmed by histology [154], and differences in toxicity may be due to differences in uptake and accumulation described in more detail below. Dibekacin showed similar animal nephrotoxicity [155] to gentamicin and tobramycin. Studies on sisomicin suggest that it is slightly more nephrotoxic than gentamicin in animals. While different aminoglycosides show different potencies of nephrotoxicity in animals, any comparison of safety profiles must also consider their relative antibacterial potencies. For example, amikacin appears the least nephrotoxic in animal models, but its lower antibacterial potency results in a similar therapeutic index to the other aminoglycosides.

It is important to note that relative nephrotoxicity of different aminoglycosides does not always correlate with relative renal tissue concentrations of the different

aminoglycosides [153, 156, 157], suggesting differences in cellular toxicity among aminoglycosides. Netilmicin was originally chosen for clinical development because of its lower toxicity in animals in comparison to other aminoglycosides [158]. However, in a well-designed study in rats, it was shown that netilmicin was more nephrotoxic than tobramycin and gentamicin and that the toxicity did not correlate with renal tissue levels [156]. Netilmicin concentrations were two- to threefold higher in rat kidney than those of tobramycin [156]. The authors hypothesized that these differences were based on net cationic charge, which led to differences in uptake into proximal tubule cells as well as differences in their ability to bind phospholipids [156].

Most animal models exploring nephrotoxicity have used evaluations of serum creatinine, BUN, and histology as measures of toxicity. Serum creatinine and BUN are not sensitive measures of renal damage, and additional metrics of renal toxicity, thought to be more sensitive, have been proposed, including the urinary enzymes β-glucuronidase, muramidase, and N-acetyl-β-glucosaminidase [158]. There is considerable variability with these markers, however, and their clinical utility has not been widely accepted. More recent genomics approaches have identified additional biomarkers of kidney damage such as Kim-1 that may warrant additional study [159].

In patients, all known aminoglycosides appear to have the potential to cause some degree of nephrotoxicity. However, as in animals, there are likely differences between members of the class in uptake, accumulation, and intrinsic cellular toxicity in humans. In humans, neomycin is reported as the most nephrotoxic aminoglycoside and streptomycin the least, with the others on a gradient between these two compounds.

Accurate estimates of rates of nephrotoxicity in humans are difficult to determine due to the lack of well-controlled trials, differences in patient populations being studied (i.e., intensive care unit [ICU] vs. non-ICU patients, comorbid conditions, and concomitant medication use), differences in the definition of nephrotoxicity, differences in length of treatment, and differences in dose regimens used in these trials. Most of the randomized, prospective studies date back to the 1970s and 1980s. Three such comparative trials reported on the rates of nephrotoxicity in these patients receiving various aminoglycosides (Table 7.6) [160–162]. Nephrotoxicity data from a meta-analysis of clinical studies published between 1975 and 1982 [163] are summarized in Table 7.7. Comparative rates of nephrotoxicity in a prospective trial of 87 patients suffering from mycobacterial infections (tuberculous and nontuberculous) treated with streptomycin, amikacin, or kanamycin for long periods of time (median duration, 15 weeks) have been compared to more typical indications for aminoglycosides [164]. In trials comparing netilmicin with tobramycin, gentamicin, and amikacin, a wide range of nephrotoxicity rates were observed (0–29% for netilmicin, 1–29% for gentamicin, 0–5% for tobramycin, and 0–11% for amikacin) [165].

To date there have been two prospective randomized trials specifically designed with a primary objective of assessing nephrotoxicity (and ototoxicity) in patients with serious infections treated with either once-daily or multiple-daily doses of

Table 7.6 Nephrotoxicity reported in comparative clinical trials

Study type	n	SIS (%)	TOB (%)	GEN (%)	AMK (%)
Retrospective, sepsis [a]	405	3.5	6	6.5	5.5
Prospective, randomized, serious infections [b]	107	17		25	
Prospective, double-blind, randomized, sepsis [c]	87	7	15.5		

AMK amikacin, *GEN* gentamicin, *SIS* sisomicin, *TOB* tobramycin
[a]Gram-negative sepsis; most patients were postsurgical [160]
[b]Bacteremia, pneumonia, intra-abdominal infections, "soft-tissue sepsis," and osteomyelitis [161]
[c]Intra-abdominal infections, soft-tissue infections, many with bacteremia [162]

Table 7.7 Nephrotoxicity from literature review of clinical trials 1975–1982 [163]

Drug	Dose (mg/kg/day)	Nephrotoxicity (%)
Gentamicin	3.9	14
Tobramycin	3.8	12.9
Netilmicin	5.2	8.7
Amikacin	15.4	9.4

gentamicin, tobramycin, or amikacin [127, 192]. Although not large, the first study showed a statistically significant difference in nephrotoxicity between the twice-daily group (15.4%) and the once-daily group (0%) ($p=0.026$). The mean onset of nephrotoxicity in the twice-daily group was 8.8 (± 3.4) days [127]. In the second and largest study investigating the issue, a single-center, open-label trial evaluated gentamicin administered once daily in over 2,100 adult medical-surgical patients. The patients dosed once daily demonstrated a 1.2% rate of nephrotoxicity compared with the study institution's historical rate of 3–5% [192].

7.9.3 Renal Uptake of Aminoglycosides

Aminoglycosides are filtered by the renal glomerulus into the proximal tubule of the kidney, where they are taken into proximal tubule cells. Uptake is thought to involve binding to the brush border and transport into the cell through an active process involving the megalin receptor. Megalin, a low-density lipoprotein receptor-related protein-2 (LRP-2), is expressed on the brush border of the proximal tubule epithelium of the kidney [166] and binds to aminoglycosides as they pass through the proximal tubule, facilitating transport by pinocytosis into endosomes [145, 167, 168]. Megalin-deficient knockout mice show a 20-fold lower uptake and accumulation than mice with normal megalin expression [169].

The rate of megalin-receptor-mediated uptake is relatively fast, but this uptake appears to be a saturable process [170, 171], in that the uptake of aminoglycosides by the proximal tubule epithelial cells plateaus even as the concentration in the proximal tubule continues to increase [170–172]. This saturable process can be modeled using Michaelis–Menten kinetics with a binding constant (K_m) associated with the saturation level. In the rat, the saturation K_m of gentamicin corresponds to

approximately 15 μg/mL in the serum [170]. For amikacin, tobramycin, and isepamicin, the binding to the brush border is also saturable at concentrations of approximately 40, 40, and 20 μM, respectively [173]. This variability in nonlinear saturation may partially explain the relative differences in renal accumulation and nephrotoxicity described for members of the class [170]. A saturation K_m for amikacin has been described for humans using mathematical modeling of nephrotoxicity [174]. At concentrations below the K_m, the uptake appears to be linear and more "efficient," with different rates for each member of the class [170, 174].

Because of the properties of the uptake process, dosing strategies should affect cortical accumulation, and hence nephrotoxicity, of aminoglycosides. Simulated models using saturable uptake demonstrate that a single-daily dose should lead to less kidney accumulation than multiple-daily doses of the same total amount of drug [172]. Less frequent dosing leads to higher peak proximal tubule lumen concentrations, but because these peak concentrations are above the saturation limit for uptake, this actually leads to a lower kidney cell accumulation of drug compared to an equal total dose given more frequently. Less frequent dosing may also allow more time below threshold trough level in the proximal tubule, again decreasing overall uptake and allowing for intracellular clearance between doses. Consistent with this model, animal studies have shown that there is no correlation between C_{max} and the development of nephrotoxicity [150]. In fact, once-daily dosing of gentamicin or tobramycin caused significantly less nephrotoxicity in rats and rabbits than twice-daily dosing, thrice-times daily dosing [150, 175], or continuous infusion [176]. This is consistent with the model of a saturable uptake with a prolonged clearance $T_{1/2}$ from proximal tubular epithelial cells [170].

Available data suggest this model also applies to humans. Continuous infusions of low doses of gentamicin and amikacin resulted in higher cortical levels [177, 178]. These principles may not hold for tobramycin within the recommended dosing range. Tobramycin cortical concentrations were similar regardless of the dosing strategy, which may reflect its higher K_m and the fact that it does not reach a saturation of uptake. While the human K_m for gentamicin has not been derived directly from primary data, based on the K_m of gentamicin in rats [170] and allometric scaling from the human accumulation data [178], the human K_m for gentamicin can be estimated at approximately 50-60 μg/mL. This may have important implications for dosing strategies. Specifically, our recent studies have demonstrated that the time above the uptake threshold should be minimized to minimize renal cell uptake and nephrotoxic potential. It follows that high-dose, once-daily therapy, in short infusions, is an optimal dosing profile for neoglycosides.

7.9.4 Mechanisms of Cellular Toxicity

In the kidneys, aminoglycosides preferentially accumulate in the proximal tubule, causing cellular damage and ultimately a reduction in the glomerular filtration rate (GFR) [179]. The molecular mechanism whereby aminoglycosides lead to damage of kidney tubule endothelium is a topic of ongoing study. Once inside the

proximal tubule cells, aminoglycosides are concentrated in intracellular lysosomes [180]. One proposed sequence of events leading to tubule endothelial cell damage after aminoglycoside exposure is lysosomal phospholipidosis followed by lysosomal rupture [177, 181, 182], leading to release of aminoglycoside into the cytoplasm of the cell and impairment of mitochondrial respiration [141, 183]. Other observed effects are alteration of normal protein tubular reabsorption, alteration of protein synthesis by endoplasmic reticulum, and depression of Na^+/K^+ pumps. Aminoglycoside-induced damage may be due to free-radical generation [184, 185]. Aminoglycosides have also been shown to bind to phospholipid membranes in vitro, and it has been postulated that they thereby interfere with phospholipid metabolism [141, 168, 183, 186, 187].

Whatever the specific cause, the consequent cellular damage appears to result in death of the kidney cells through apoptosis, and direct cellular injury seems to be the primary cause of acute renal toxicity associated with aminoglycosides. The presentation of non-oliguric kidney function impairment is postulated to be due to subsequent shutdown of individual nephrons in response to local damage, inflammation, and tubular-glomerular feedback.

7.9.5 Aminoglycoside Ototoxicity

While rare, aminoglycoside-induced ototoxicity is often irreversible, and this makes it a serious clinical concern. Aminoglycoside-induced ototoxicity occurs in a dose-cumulative and idiosyncratic fashion, the latter presumably due to genetic factors. While there are some similarities in the pathophysiology of aminoglycoside-induced nephrotoxicity and ototoxicity, they are not well correlated in clinical studies in humans [188]. High total cumulative dose [189] and long durations of therapy [127, 164] appear to increase the risk of ototoxicity. There have been reports of familial patterns of increased risk of aminoglycoside-induced ototoxicity, particularly in some Asian countries [190]. Some have proposed that the increased susceptibility to ototoxic effects of aminoglycosides and other ototoxic agents may be increased through a mutation on mitochondrial RNA [190].

Aminoglycoside-induced ototoxicity is due to destruction of hair cells in the cochlea and vestibula. Hair cell damage appears to be selective, beginning with the outer hair cells of the lower portion of the cochlea and working inward [191]. This process results in hearing loss primarily in the high-frequency range and subsequently affects lower-frequency ranges [191]. There is some evidence that ototoxicity, both cochlear and vestibular, is reversible in a proportion of affected patients [192, 193]. However, it tends to be irreversible once loss of the auditory and vestibular sensorineural cells in the cochlea and vestibule of the inner ear has occurred. In contrast to proximal tubule cells of the kidney, these cells have no potential for regeneration once lost.

While data in humans are sparse due to both the low occurrence and paucity of well-controlled trials (see Sect. 7.9.6), aminoglycosides seem to be differentiated in

their ability to cause either cochlear toxicity or vestibular toxicity. In a comparison of several different aminoglycosides, sisomicin caused the most cochlear and vestibular toxicity followed by gentamicin [194]. In animal studies, gentamicin and sisomicin were relatively more ototoxic than tobramycin and amikacin [195]. Studies in guinea pigs have shown that netilmicin is less ototoxic, including cochlear and vestibular toxicity, than gentamicin, tobramycin, and amikacin [196, 197]. Dibekacin is similar to amikacin, and half as ototoxic as gentamicin in animals. Sisomicin is reported to be slightly more ototoxic than gentamicin in animals. Arbekacin has been shown to be less ototoxic in rats than dibekacin and amikacin.

7.9.6 Ototoxicity in Humans

There is some discrepancy between rates of ototoxicity in animal studies and rates reported in human clinical trials [198, 199]. Factors complicating the assessment of aminoglycoside ototoxicity in the clinic include inconsistent patient reporting of high-frequency hearing loss (beyond the frequencies needed for daily functions like speech), unstandardized criteria for audiometric measurements, and the lack of patient baseline function data in many studies. The low incidence of ototoxicity results in few trials that are sufficiently powered to determine the rate of ototoxicity; many other trials do not have a suitable comparator arm to act as a control. Many of the reports of ototoxicity in the clinical literature use audiometric thresholds that are actually normal variations found in healthy individuals [198]. Background rates of cochlear and vestibular dysfunction are quite high even in healthy individuals, which complicates the measurement of drug-induced ototoxicity, particularly in the absence of appropriate baseline data and definition of thresholds for drug-induced ototoxicity. A recent National Health and Nutrition Examination Survey (NHANES) showed that 32% of healthy participants in the USA had 25-dB or greater hearing loss [200], and 35% of US adults aged 40 years and older showed evidence of vestibular abnormality [201]. Thus, many of the clinical studies may result in overestimates of drug-induced ototoxicity.

In general, ototoxicity has been reported to occur in between 2% and 5% of patients receiving an aminoglycoside, and is higher in some studies of patients who receive long durations of aminoglycoside therapy. Ototoxicity data from a meta-analysis of clinical studies published between 1975 and 1982 [163] are summarized in Table 7.8. In a more recent review of over 2,100 patients who received once-daily aminoglycoside therapy, only two patients were reported to have ototoxicity, of which only one patient, who received 5 weeks of therapy, had long-term deficits [192].

In another prospectively designed randomized trial specifically designed to assess the incidence of ototoxicity, patients with serious infections were treated with either once-daily or multiple-daily doses of gentamicin, tobramycin, or amikacin [127]. In patients who received two or more audiometric evaluations, one patient in the twice-daily group and none in the once-daily group developed ototoxicity. The mean duration of therapy in these groups was 10.6 ± 9.7 days and 8.2 ± 6.2 days,

Table 7.8 Reported toxicity from literature review of clinical trials from 1975 to 1982 [163]

Drug	Average dose (mg/kg/day)	Cochlear toxicity (%)	Vestibular toxicity (%)
Gentamicin	3.9	8.3	3.2
Tobramycin	3.8	6.1	3.5
Netilmicin	5.2	2.4	1.4
Amikacin	15.4	13.9	2.8

respectively. Although a small sample, it does suggest that once-daily dosing has an advantage in preventing ototoxicity.

A prospectively designed trial compared rates of ototoxicity in patients with mycobacterial disease (including tuberculosis) who received streptomycin, amikacin, or kanamycin for long durations (median 15 weeks) [164]. Results of this study, although in an atypical patient population treated with aminoglycosides, give a better understanding of the development, time course, and correlates of ototoxicity. This study defined ototoxicity as a hearing loss of at least 20 dB. Applying this widely used metric, ototoxicity occurred in 32 of 87 patients (37%) given this long-term aminoglycoside therapy.

7.9.7 Uptake into Inner Ear Cells

There are some similarities in the mechanisms of toxicity for aminoglycoside-induced nephrotoxicity and ototoxicity. As in renal proximal tubular epithelium, megalin is expressed in the labyrinth epithelium of the ear [166, 167, 190, 202] and thus megalin may also mediate uptake into the inner ear hair cells [167]. It appears that transmembrane transport into the cell is through endocytosis in the inner ear as it is in the kidney [170]. Alternative routes for aminoglycosides to enter inner ear (and renal) cells have been proposed, such as transmembrane cation channels like the Transient Receptor Potential Ca^{++} channel subtype V1 (TRP-V1) [203]. These channels may be able to mediate gentamicin uptake directly into the cytoplasm of the inner ear hair cells and proximal tubule epithelial cells. However, the bulk of intracellular aminoglycoside appears to be in the lysosomes in the inner ear cells [204–206]. While there are some similarities with the mechanism of nephrotoxicity, aminoglycoside-induced ototoxicity cannot be explained solely by high concentrations in the inner ear fluid or by high rates of uptake that lead to accumulation of drug within inner ear tissues [207]. Some have proposed that it is related to the slow clearance of aminoglycosides from hair cells (aminoglycosides have a clearance half-life of months in the inner ears of guinea pigs) [208, 209].

In animal models, frequency of administration appears to have less effect on ototoxicity than on nephrotoxicity, perhaps because of differences in tissue distribution kinetics involved in the inner ear as compared to uptake in the renal proximal tubule. High peak serum levels achieved after rapid IV injections did not result in either an increase in perilymph levels or inner ear damage in rabbits [210], when

compared to IM injections of the same dose. However, less drug accumulation has been observed in animal models with less frequent dosing [176, 211]. Some studies in guinea pigs have shown no difference in ototoxicity of aminoglycosides with different frequencies of dosing [212, 213] while others have shown less ototoxicity with once-daily dosing [214]. Ultimately, single high daily doses of aminoglycosides are not more ototoxic in animal models than smaller divided doses, supporting the concept of once-daily dosing.

7.10 Conclusion

Aminoglycosides continue to be critical therapeutics for the treatment of life-threatening infections, with many desirable characteristics including spectrum of activity, rapid bactericidal activity, predictable PK, excellent solubility and stability, and a lack of metabolism in humans and animals. The emergence and spread of plasmid-mediated enzymatic resistance mechanisms, however, has reduced the clinical utility of the legacy aminoglycosides. Fortunately, decades of experience with aminoglycosides have resulted in a wealth of knowledge surrounding the synthetic approaches to these scaffolds and a clear map of where resistance and other pitfalls lie. As we enter an era where multidrug-resistant pathogens are rapidly spreading around the globe, and where increased deployment of other drug classes (e.g., fluoroquinolones, cephalosporins and carbapenems) further elevate the likelihood of pan-antibiotic resistance, we must continue to capitalize on our understanding of the aminoglycosides to maximize options available to clinicians. The advent of the neoglycosides, of which ACHN-490 is the first, brings with it the prospect of broad-spectrum activity against pan-resistant organisms. Enhancing once-daily aminoglycoside therapy with a high dose, short course approach provides an additional opportunity to rejuvenate the promise of the aminoglycoside class.

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Chapter 8
Oxazolidinone Antibacterial Agents

Michael R. Barbachyn

8.1 Introduction

The "golden age" of antibacterial agent discovery commenced with the identification of the sulfa drugs in the mid 1930s and concluded with the emergence of the quinolones in the early 1960s (Fig. 8.1). It is interesting to note that the vast majority of these early antibiotics were derived from natural products. Somewhat surprisingly, at least through the beginning of the year 2000, all subsequent marketed antibiotics have been largely semi-synthetic or synthetic variations of pre-existing antibacterial scaffolds. While many of these next-generation agents have provided considerable clinical utility in treating infections caused by pathogenic bacteria, the fact remains that they do not represent truly novel mechanistic classes. Clearly, this "innovation time gap" suggests that the discovery and development of new antibacterial agent classes is a non-trivial endeavor and successes in the area have indeed been relatively rare.

The emergence of a plethora of clinically useful antibacterial agents in the twentieth century has sometimes been thought to mark an end to the need for additional new antibiotics, as the mortality burden associated with microbial disease has definitely been alleviated to a considerable extent [1]. However, the resiliency of bacteria to environmental pressures, including xenobiotics such as antibacterial agents, suggests that the situation is more dynamic than it appears on the surface. Indeed, the continued development of bacterial resistance to available antibacterial agents strongly suggests that there will be an ongoing need for the discovery and development of new antibiotics [2, 3]. For example, a recent retrospective study of the incidence, distribution and burden of invasive methicillin-resistant *Staphylococcus*

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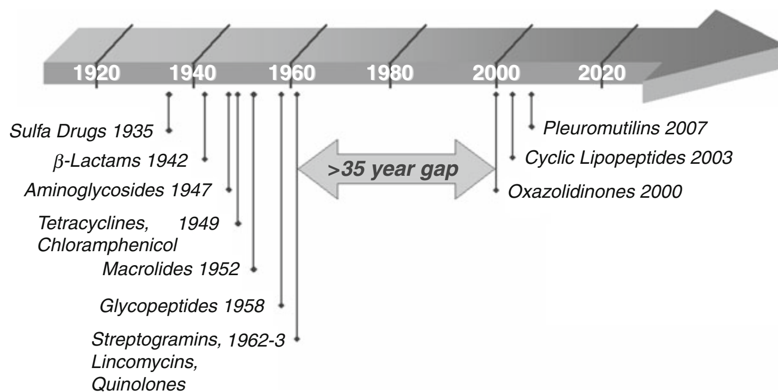

Fig. 8.1 Timeline depicting approximate dates for the first clinical use of agents from each respective antibacterial class and the >35 year innovation time gap that ended with the introduction of the oxazolidinones in 2000

aureus (MRSA) infections in the United States in 2005 revealed that approximately 1 out of every 5 such infections resulted in death, despite aggressive antibacterial therapy [4]. Overall, there were more deaths arising from invasive MRSA infections than from AIDS. Other multidrug-resistant (MDR) Gram-positive bacteria of particular concern include glycopeptide-intermediate *S. aureus* (GISA), vancomycin-resistant *S. aureus* (VRSA), vancomycin-resistant enterococci (VRE), and penicillin- and macrolide-resistant *Streptococcus pneumoniae* [5]. In addition, Gram-negative bacilli resistant to extended spectrum beta-lactam antibiotics, fluoroquinolones, aminoglycosides, and other antibacterial agents have become increasingly prevalent in the healthcare setting. Particularly problematic Gram-negative organisms include extended spectrum β-lactamase-expressing strains of *Klebsiella pneumoniae* as well as the non-fermenters *Pseudomonas aeruginosa*, *Acinetobacter baumannii,* and *Stenotrophomonas maltophilia* [6–8]. Management of nosocomial infections associated with all of the above organisms presents a difficult therapeutic challenge for critical care physicians. Indeed, infections caused by these bacteria are often associated with considerable morbidity and mortality. Because of this, there is an urgent need to identify new therapeutic agents with the ability to cover these MDR bacterial strains.

A number of potential solutions have been advanced for overcoming the bacterial resistance problem [9]. Certainly, the structural modification of existing antibacterial agents is a well proven approach but one that generally provides only transient relief. Combination drug therapy has a proven record of success in this area. A variation on this theme wherein a single agent employs two or more mechanisms of action has also been suggested as another potential solution [10]. Narrow spectrum agents with a companion diagnostic tool may have merit. Novel approaches involving immune system modulators and agents targeting bacterial virulence factors are also under active investigation. However, the best solution to the bacterial resistance dilemma remains the identification of novel antibacterial agents employing a unique mechanism of action.

This chapter will briefly chronicle the discovery of the oxazolidinone antibacterial agents, a relatively new class of totally synthetic compounds with potent activity primarily against clinically relevant Gram-positive pathogens. Considerable emphasis will be given to the prototypical oxazolidinone antibacterial agent linezolid, the first clinically useful member of this class, and the only marketed representative (Zyvox™). Linezolid serves as a useful vehicle to share the various properties and characteristics associated with the oxazolidinones class. An update and opinion on promising second-generation oxazolidinone prospects will also be provided. Finally, some thoughts will be shared on what the future may hold for this important class of antibacterial agents.

8.2 Discovery of the Oxazolidinones

8.2.1 Emergence of Antibacterial Oxazolidinones at DuPont

It is important to note that the first bioactive members of the oxazolidinone antibacterial agent class were identified at the E. I. du Pont de Nemours, Company (DuPont) and reported in 1978 [11]. The lead compounds emerged from a screening exercise aimed at identifying novel agents for treating selected plant diseases of fungal and bacterial origin. A representative compound from this early series, racemic 5-(chloromethyl)-3-(4-methylsulfonylphenyl)-2-oxazolidinone (**1**), is shown in Fig. 8.2. A subsequent patent reported additional 5-halomethyl and 5-hydroxymethyl variants (e.g., chiral, non-racemic **2**) that were claimed to have primarily Gram-positive antibacterial activity and utility in treating infections in mammals [12]. For example, orally administered oxazolidinone **2** was reported to be efficacious (ED_{50} = 9 mg/kg) against *S. aureus* in a murine lethal systemic infection model. It is important to note that **2** represents the first disclosure of the importance of the (5*R*)- absolute configuration for antibacterial activity. That is, the corresponding (5*S*)- enantiomer of **2** is totally inactive. Structure-activity relationships (SAR) within the 5-(hydroxymethyl) oxazolidinone series were further explored by Gregory [13]. This effort culminated in the identification of S-6123 (**3**). S-6123 exhibited modest in vitro activity (mean minimum inhibitory concentration or MIC = 22.3 and 7.7 µg/mL against *S. aureus* and *S. pneumoniae,* respectively).

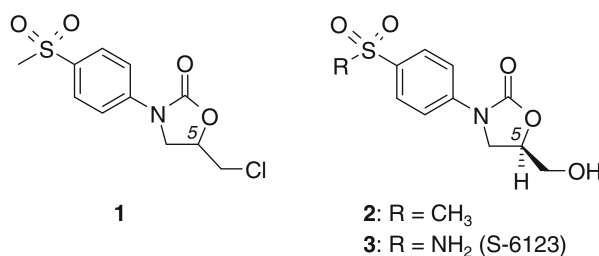

Fig. 8.2 Early DuPont oxazolidinones

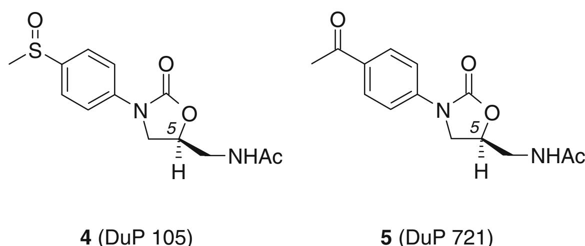

Fig. 8.3 Structures of DuPont lead oxazolidinones DuP 105 and DuP 721

Nevertheless, S-6123 demonstrated good oral efficacy (ED_{50} = 17.1 mg/kg) against *S. aureus* in a murine lethal infection model.

The first description of oxazolidinones bearing a C-5 acetamidomethyl side chain appeared in the patent literature in the mid-1980s [14]. Subsequently, at the 1987 Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) in New York City, workers from the E.I. du Pont de Nemours, Company (DuPont) formally reported the structure and antibacterial activity profiles of two new, apparently optimized oxazolidinones, Dup 105 (**4**) and DuP 721 (**5**), as shown in Fig. 8.3 [15]. Dup 721 and DuP 105 showed significantly improved characteristics relative to their progenitor compounds. Especially attractive characteristics of these novel, totally synthetic compounds included the following: (1) a unique mechanism of action involving inhibition of protein synthesis, (2) promising coverage of multi-drug-resistant Gram-positive pathogens, (3) an inability to generate resistant mutants in vitro, employing traditional, serial twofold dilution methods, (4) the availability of both oral (PO) and intravenous (IV) administration routes, and (5) favorable pharmacokinetic performance profiles that translated into PO/IV efficacy in relevant animal infection models.

Further oxazolidinone analog work was conducted at DuPont, which helped identify some of the fundamental structure-activity-relationships (SAR) in this area [16–18]. A comprehensive overview of the DuPont work is beyond the scope of this narrative, but the following generalizations can be gleaned: (1) the requirement for an N-aryl group, (2) the essentiality of the (5*S*)-absolute configuration for antibacterial activity, (3) the preference for a C-5 acetamidomethyl side chain, (4) the finding that unsaturated (e.g., an alkenyl group or a second aromatic or heteroaromatic ring) or electron-withdrawing groups (e.g., acetyl or methanesulfinyl) in the *para* position of the N-aryl ring generally potentiate activity, and (5) additional substitutions on the proximal aromatic ring or at C-4 or C-5 of the oxazolidinone ring usually have a detrimental, or at best, indifferent effect on the antibacterial activity. As subsequent events will illustrate, some of these dogmas are subject to revision (vide infra).

DuP 105 and DuP 721 were apparently briefly entered into human clinical trials but development of these agents was quickly terminated, as emerging safety data for the two compounds was deemed unsatisfactory [19]. A consequence of the Dup 105 and DuP 721 experience was the demise of the DuPont oxazolidinone research program.

8.3 The First Clinically Useful Oxazolidinone, Linezolid

8.3.1 Identification of Oxazolidinones with Improved Safety Profiles at the Upjohn Company

Immediately following the public disclosure of DuP 105 and DuP 721 by DuPont in 1987, Steven J. Brickner started a part-time, lead-finding oxazolidinone project in his chemistry laboratory at The Upjohn Company in Kalamazoo, Michigan. Taking advantage of Upjohn's rather remarkable policy of encouraging original exploratory research in an area outside of assigned project work, and engaging Upjohn colleagues in other relevant scientific disciplines, Brickner and co-workers were able to identify three distinct oxazolidinone scaffolds (**6–8**) within a period of just 18 months (see Fig. 8.4) [20, 21]. These early analogs were all prepared in racemic form, which represented an acceptable compromise at this early stage of the program, as the objective was to rapidly probe fundamental SAR for the oxazolidinone pharmacophore. It should be noted that all three compounds shown in Fig. 8.4 have antibacterial activity and oral efficacy comparable to or somewhat less than that of racemic DuP 721.

The indanone analog PNU-82965 (**6**) eventually assumed a pivotal role for the Kalamazoo oxazolidinone program. By 1989, fragmentary reports were obtained that DuPont had removed an oxazolidinone from clinical trials, due to observed toxicity in several animal models [19]. In order to probe this notion, a comparative in-house safety evaluation of racemic DuP 721 and PNU-82965 was conducted in the laboratory of Richard Piper [22]. In the study design, racemic DuP 721 and PNU-82965 were administered orally at a dose level of 100 mg/kg body weight twice a day for 30 days to three male and three female Sprague-Dawley rats. Rats treated with racemic DuP 721 fared poorly in the study. One death was noted and

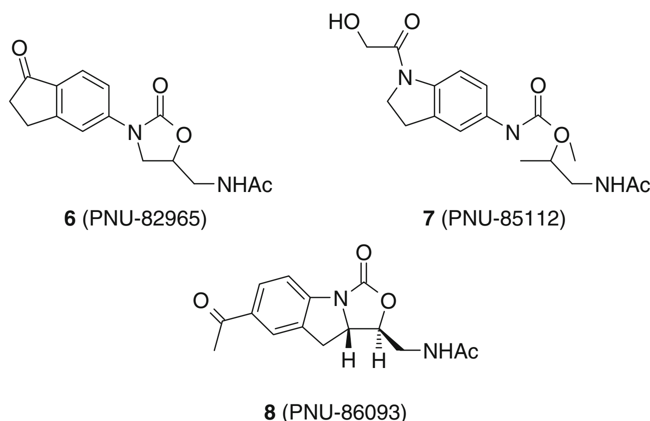

Fig. 8.4 Early Upjohn oxazolidinone lead compounds

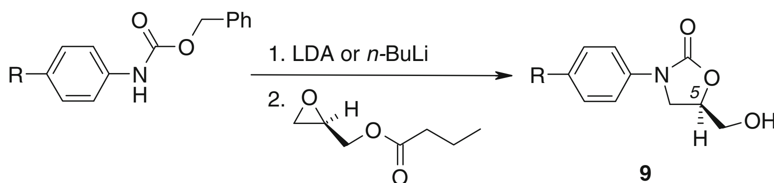

Fig. 8.5 The Manninen Reaction: a practical solution for the preparation of enantiomerically enriched 5-(hydroxymethyl)oxazolidinones

two rats were sacrificed in a moribund state. Additional findings for the racemic DuP 721 treated animals included severe progressive weight loss and evidence of bone marrow atrophy. In contrast, the PNU-82965 treated rats exhibited only a few adverse findings and they were judged to be very mild in nature. There were no clinical signs, serum, or urine chemistries or histopathological manifestations of drug-related toxicity. The major outcome of this comparative safety study was the finding that structure-toxicity relationships are operative within the oxazolidinone class.

Following up on the favorable safety findings with PNU-82965, and speculating that alternative fused bicyclic inserts might confer favorable properties to the oxazolidinone pharmacophore, the racemic indoline congener PNU-85112 (**7**) was also examined [23]. Like its indanone relative, PNU-82965, this PNU-85112 was found to exhibit an excellent safety profile when tested in an essentially identical chronic rat toxicity study.

8.3.2 Upjohn Studies Leading to the Identification of PNU-100480, Eperezolid and Linezolid

With the identification of structure-toxicity relationships putting the oxazolidinone class in a more favorable position, the research program at Upjohn underwent a significant expansion in late 1990/early 1991 [22].

One priority for the fledgling program was to develop a reliable, general method for the preparation of enantiomerically enriched oxazolidinone analogs. Previously described synthetic methods involving aryl isocyanates were effective, but not general [16, 24]. During extensive investigational studies at Upjohn, it was found that *N*-lithiated carbamate derivatives of anilines can be reacted with commercially available (*R*)-glycidyl butyrate under appropriate conditions to directly generate (*R*)-3-aryl-5-(hydroxymethyl)oxazolidinones (**9**), as shown in Fig. 8.5 [25, 26]. This step has become affectionately known as the Manninen Reaction, and it represents a general, scalable solution for the rapid construction of enantiomerically enriched oxazolidinone intermediates. These hydroxymethyl intermediates can be readily elaborated to the desired final oxazolidinone antibacterial agents.

By late 1992, the Upjohn discovery research effort encompassed primarily just three different subclasses of oxazolidinone analogs (see Fig. 8.6): (1) piperazinyl

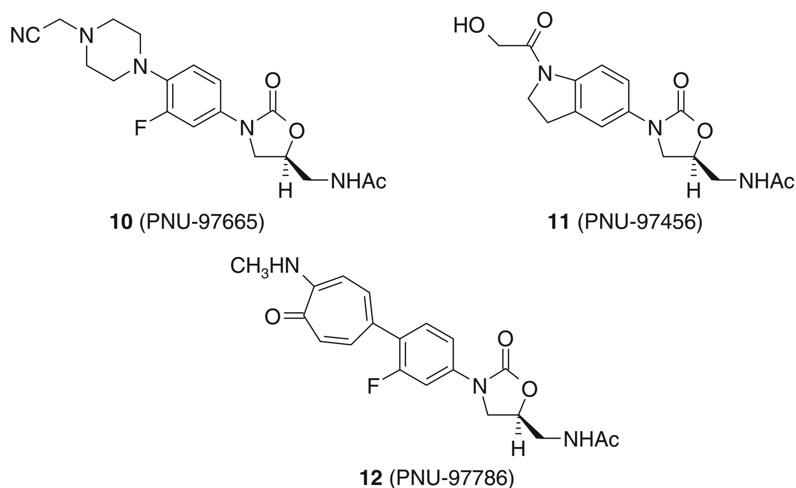

Fig. 8.6 Representatives of lead oxazolidinone series under consideration in 1992

phenyl oxazolidinones, represented by **10** (PNU-97665), (2) indolinyl oxazolidinones, exemplified by **11** (PNU-97456), and (3) the troponyl phenyl oxazolidinones, for example **12** (PNU-97786) [22, 23, 27, 28]. The indolines generally exhibited an excellent safety profile but demonstrated somewhat lower levels of antibacterial activity than desired. The tropone analogs were generally the most interesting compounds from an antibacterial activity standpoint but they displayed poor water solubility, they were somewhat challenging to synthesize, they sometimes had a negative safety signal, and they exhibited variable pharmacokinetic properties in the rat. In contrast, selected piperazine derivatives exhibited excellent in vitro and in vivo activity while also maintaining an acceptable safety profile, good water solubility, and excellent pharmacokinetic parameters. As a bonus, the piperazine analogs were also the easiest compounds to synthesize. Because of these and other characteristics, the piperazine series became the principal focus of the chemistry effort at Upjohn going forward.

An examination of the structural features of oxazolidinones **10–12** and the eventual clinical candidates (vide infra), suggests that the Upjohn group's principal contributions to advancing an understanding of SAR in the oxazolidinone area include the following: (1) the finding that one or two fluorine atoms flanking the substituent in the *para* position on the phenyl ring (e.g., **10** and **12**) usually exerts a significant potentiating effect on antibacterial activity and/or efficacy, [28] and (2) an electron-donating amino substituent in the *para* position on the phenyl ring (e.g., **10** and **11**) often confers a good safety profile while maintaining excellent antibacterial activity [29–32].

A complete accounting of the piperazinyl phenyl oxazolidinone SAR optimization story is beyond the scope of this narrative and, surprisingly, it has never been published in a comprehensive format. Nevertheless, in brief, it was found that a

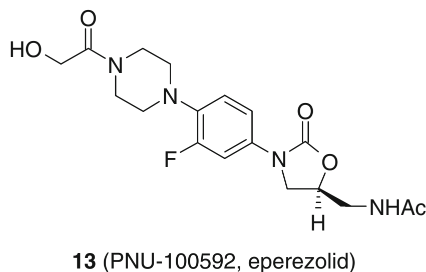

Fig. 8.7 Structure of eperezolid (PNU-100592)

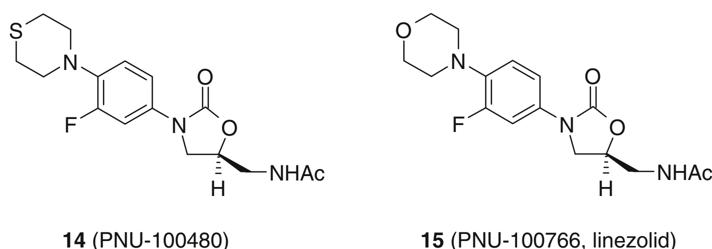

Fig. 8.8 Structures of PNU-100480 and linezolid (PNU-100766)

wide range of alkyl, carbonyl, and sulfonyl substituents were tolerated on the distal piperazine nitrogen atom [29–31]. Fluorinated derivatives again provided improved antibacterial activity, although the monofluoro congeners were generally found to have better water solubility profiles than their difluoro counterparts, an important consideration for the targeted intravenous route of administration. Many of the synthesized piperazine analogs exhibited interesting levels of in vitro activity and in vivo oral efficacy against problematic Gram-positive pathogens [29–31]. Following a number of iterative synthetic cycles, it was found that the hydroxyacetyl moiety was apparently the optimal nitrogen appendage. Ultimately, the monofluoro analog **13** (PNU-100592, formerly U-100592), which was subsequently named eperezolid, emerged as the analog with the best balance of antibacterial activity and efficacy, pharmacokinetics, water solubility, and other pertinent properties (see Fig. 8.7) [22, 32].

An awareness of available bioisosteric replacements for the piperazine ring system led to a concerted effort to prepare and examine some of these analogs [33, 34]. Again, the scope of the Upjohn analog effort was too broad to discuss in its entirety here. Focusing on key deliverables, this exercise led to the identification of the interesting antimycobacterial thiomorpholine derivative **14** (PNU-100480, formerly U-100480), [35] and also the morpholine analog **15** (PNU-100766, formerly U-100766), which subsequently became known as linezolid (see Fig. 8.8) [22, 32].

8.3.3 Linezolid Emerges as the Oxazolidinone with the Best Overall Profile

Eperezolid and linezolid were quite unusual in that they were almost identical in their overall preclinical profiles. Their equilibrium solubility in pH 7 phosphate buffer (4.2 vs. 3.7 mg/mL, respectively), their minimum inhibitory concentrations (MICs) against relevant Gram-positive organisms, their overall antibacterial spectrum, their efficacy in predictive murine infection models, their pharmacokinetic (PK) behavior in the dog, and their overall safety profile in 30-day rat and dog multiple dose toxicity studies were quite similar [22, 32]. Linezolid did exhibit a superior PK profile in the rat. When dosed orally in the rat at 25 mg/kg, linezolid exhibited improved clearance (10.5 vs. 24.9 mL/min/kg/) and oral bioavailability (109% vs. 56%) compared to eperezolid [36]. In contrast, differences between the two compounds were more equivocal in the corresponding dog studies, with clearance (1.99 vs. 6.3 mL/min/kg, respectively) and oral bioavailability (97% vs. 100%, respectively) in a favorable range for both compounds [36]. Since eperezolid and linezolid were members of a novel class, with no previous clinical experience, it was not known which preclinical species would be more predictive of the eventual human PK experience. Therefore, the Upjohn team undertook the unusual strategy of taking both eperezolid and linezolid through a Phase 1 clinical trial in order to determine if there might be a significant difference between the two compounds with respect to their PK behavior in humans. Of course, another important advantage of the envisioned parallel clinical development plan was that single ascending dose (SAD) and multiple ascending dose (MAD) safety profiles would be obtained for both drug candidates [37]. It should be mentioned that the thiomorpholine derivative PNU-100480, despite its comparable activity and good oral efficacy against Gram-positive organisms, was removed from further consideration as a drug candidate at the time because of its relatively poor water solubililty (0.2 mg/mL in pH 7 phosphate buffer), precluding it from IV administration, and a complex oxidative metabolism profile in rodents [35].

Eperezolid entered human clinical trials in October of 1994, with linezolid following just 6 months later in April of 1995. Ultimately, linezolid was selected over eperezolid for advancement into Phase 2 clinical trials in patients based on its superior pharmacokinetic profile (vide infra), which suggested twice daily dosing would be achievable [37]. Phase 3 trials began in January, 1998 and the NDA was filed in October, 1999. The FDA granted fast-track status to linezolid and the compound was approved on April 18, 2000 and is now marketed as Zyvox™. Interestingly, PNU-100480 (PF-02341272) was recently resurrected by Pfizer and entered into human clinical trials in 2009 as a novel agent for the treatment of infections caused by susceptible and multidrug-resistant strains of *Mycobacterium tuberculosis*.

8.3.4 Clinical Experience with Linezolid

Two randomized, double-blind, placebo-controlled, sequential dose-escalating Phase 1 trials in parallel groups of healthy human volunteers were conducted, one evaluating oral administration and the other intravenous administration of linezolid [38]. Linezolid was administered at oral doses of 375, 500 or 625 mg or intravenously at doses of 500 mg or 625 mg twice daily for up to 18 days. Linezolid was absorbed rapidly when administered orally, with complete bioavailability, suggesting that no dose adjustment would be needed when switching from IV to oral dosing. Drug concentrations remain above the MIC_{90} of targeted pathogens for most of the dosing interval, supporting a twice-daily dosing regimen [38]. In fact, the mean steady-state C_{max} achieved at the 625 mg BID dose level was 18.75 μg/mL [38, 39].

In a radiolabeled [^{14}C]linezolid study, involving a single 500 mg dose, linezolid was found to be very well distributed, [40] reminiscent of the prior experience in the rat [36]. Recovery of radioactivity totaled 94%, with a majority found in the urine. Linezolid was excreted largely intact, although two inactive morpholine ring oxidized metabolites were characterized. Interestingly, both of these metabolites arise from non-enzymatic oxidation processes involving a reactive oxygen species [36]. Linezolid was not found to be an inhibitor or inducer of CYP_{450} isozymes [41].

In a study probing the effect of food on the absolute oral bioavailability of linezolid, it was noted that there was no statistically significant difference in AUC, CL or half-life when administering linezolid to either fed (high fat meal) or fasted healthy volunteers [42]. The only statistically significant difference between the two arms was the effect on peak plasma concentration, which was 23% greater for fasted subjects. Overall, only the rate but not the extent of absorption was affected.

The steady-state intrapulmonary concentration of orally administered linezolid was examined in healthy volunteers [43]. Epithelial lining fluid (ELF) concentrations reached a lofty level of 64.3 ± 33.1 μg/mL, while plasma concentrations were 7.3 ± 4.9 μg/mL. Linezolid's long plasma and intrapulmonary half-lives as well as the percentage of time the drug's level remains above the MIC (100% of the dosing interval) provided a robust pharmacokinetic rationale for linezolid's likely effectiveness in treating lung infections.

Phase 3 clinical trials involved more than 2,000 patients in seven randomized, multicenter, comparative trials, along with an open-label non-comparative study [19]. Linezolid was administered for up to 28 days and at doses of 400 mg or 600 g twice a day. Linezolid performed well in these pivotal trials, with clinical outcomes generally comparable to that of the comparator agent regimens. The following indications were examined (clinical cure rates): outpatient community-acquired pneumonia (CAP) (90% vs. 91% for cefpodoxime), [44] hospitalized CAP (91% vs. 89% for ceftriaxone/cefpodoxime), hospital-acquired pneumonia (66% vs. 68% for vancomycin), [45] uncomplicated skin and soft tissue infections (SSTI) (91% vs. 87% for clarithromycin), complicated SSTI (89% vs. 86% for oxacillin/dicloxacillin), [46] MRSA infections (77% vs. 74% for vancomycin), and vancomycin-resistant enterococcal (VRE) infections (88% vs. 74% for linezolid 200 mg BID) [19, 47]. The VRE study is interesting because, in the absence of an approved

comparator agent, linezolid was compared to itself in what amounted to a dose-ranging study (600 mg BID vs. 200 mg BID).

Linezolid has been administered to several million patients and is generally considered to be well tolerated [48, 49]. The most common adverse events noted in the Zyvox™ package insert are (% incidence) the following: diarrhea (2.8–11%), nausea (3.4–9.6%), and headache (0.5–11.3%) [50]. Linezolid is a weak, reversible, non-selective monoamine oxidase (MAO) inhibitor (human MAO-A Ki = 55 μM) [51]. Therefore, linezolid has some potential for a reversible pressor response in the presence of adrenergic agents, including sympathomimetic amines such as tyramine. Linezolid also has some potential for interaction with serotonergic agents, such as selective serotonin reuptake inhibitors (SSRIs) and, in this context, there have been some reports of serotonin syndrome [52–54]. Linezolid use for greater than 2 weeks, or in patients with pre-existing myelosuppression, or in patients receiving concomitant administration of drugs that induce bone marrow suppression, has an association with reversible myelosuppression (including anemia, thrombocytopenia, leukopenia or pancytopenia). In these cases, complete blood counts should be monitored weekly. Lactic acidosis has been reported in some patients treated with linezolid [55, 56]. Patients receiving linezolid for longer than the maximum recommended duration of therapy, 28 days, are at increased risk for developing peripheral and optic neuropathy [57–61]. The causal relationship for the observed lactic acidosis and neuropathy safety signals has not been firmly established, although there are reports suggesting that inhibition of mitochondrial protein synthesis may play a role [56, 62, 63].

8.3.5 Linezolid Indications (Marketed as Zyvox™)

Zyvox™ has been approved in the U.S. for the treatment of the following indications: (1) vancomycin-resistant *E. faecium* infections, including cases with concurrent bacteremia, (2) nosocomial pneumonia caused by *S. aureus* (methicillin-susceptible and -resistant strains) or *S. pneumoniae* (including multi-drug resistant strains), (3) community-acquired pneumonia caused by *S. pneumoniae* (including multi-drug resistant strains), including cases with concurrent bacteremia, or *S. aureus* (methicillin-susceptible strains only), (4) complicated skin and skin structure infections, including diabetic foot infections, without concomitant osteomyelitis, caused by *S. aureus* (methicillin-susceptible and -resistant strains), *S. pyogenes,* or *S. agalactiae,* (5) uncomplicated skin and skin structure infections caused by *S. aureus* (methicillin-susceptible strains only) or *S. pyogenes*. Linezolid remains the only oral drug approved for treating infections caused by MRSA and VRE. Linezolid has also been approved for use in children and newborns, although the dosage may need to be adjusted upward due to the generally higher clearance observed in children. Linezolid is not approved and should not be used for the treatment of patients with catheter-related bloodstream or catheter-site infections. This is apparently because a Gram-negative organism is frequently associated with these infections and linezolid has no Gram-negative activity [50].

Zyvox™ is available for administration via a ready-to-use IV bag, a film-coated compressed tablet or via an oral suspension obtained by constitution of an orange-flavored granule/powder. The most common linezolid-dosing regimen is 600 mg administered twice daily.

8.4 Other Noteworthy Oxazolidinones

8.4.1 Additional Key Contributions to Oxazolidinone SAR

The oxazolidinone area is no longer the emerging antibacterial opportunity that it once was. In fact, at the time of this writing, there are now more than 30 companies that have established a visible track record of research in the area. Therefore, a complete accounting of all of the contributions in the oxazolidinone field is beyond the scope of this endeavor. What follows is a personal selection of what constitutes some of the more significant oxazolidinone SAR findings to date.

8.4.1.1 Oxazolidinone Ring Replacements

One area of research receiving considerable attention was aimed at identifying suitable bioisosteric replacements for the usual oxazolidinone ring. Early work in this area identified the apparent fundamental requirements for a successful ring replacement: (1) a 5-membered ring appears optimal, (2) a sp^2 center (or at least substantial sp^2 character through resonance delocalization or tautomerism) adjacent to the phenyl ring, (3) a strategically located ring oxygen and (4) a chiral center of appropriate absolute configuration bearing the essential acylaminomethyl side chain [64–66]. As subsequent events will illustrate, the latter requirement is subject to revision (vide infra). The most successful early surrogate for the oxazolidinone ring was found to be the butenolide system (e.g., **16** in Fig. 8.9). In vitro antibacterial activity for the butenolides was found to be directly comparable to that of the corresponding oxazolidinones [64–67].

Consideration of the above described principles led to conception of the isoxazoline ring system as an attractive oxazolidinone bioisostere [67]. Indeed, the isoxazoline moiety was subsequently found to be an acceptable ring replacement, affording compounds with activity closely approaching that of their oxazolidinone

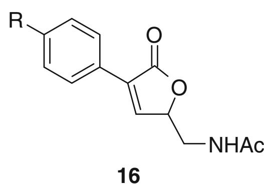

Fig. 8.9 Generic structure of butenolide **16**

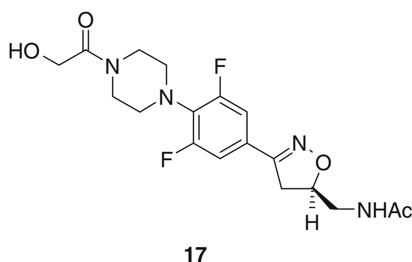

Fig. 8.10 Structure of piperazinyl difluorophenyl isoxazoline analog **17**

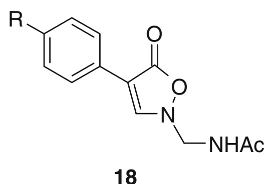

Fig. 8.11 Generic isoxazolinone structure **18**

congeners. A representative isoxazoline analog, **17** (see Fig. 8.10), exhibited MICs against *S. aureus* UC12673*, S. pneumoniae* UC9912 and *E. faecalis* UC9217 of 1, 0.5 and 2 μg/mL, respectively, while also demonstrating oral efficacy against *S. aureus* UC9213 in a murine lethal systemic infection model (ED_{50} = 5.0 mg/kg vs. 4.4 mg/kg for linezolid) [67].

Pioneering work by Snyder and co-workers led to the rational design and synthesis of novel analogs incorporating an isoxazolinone ring surrogate for the usual oxazolidinone moiety [68]. These isoxazolinone analogs retain all of the antibacterial activity of their oxazolidinone progenitors. Additional study led to an expanded series of isoxazolinones that confirmed this ring system as a successful replacement (see generic structure **18** in Fig. 8.11) [69]. Perhaps even more important was the finding that the C-5 chiral center of the oxazolidinone ring system could be replaced by a saturated, sp^3 hybridized nitrogen atom, rendering the resultant compounds achiral and, in principal, simplifying the overall synthetic process.

8.4.1.2 Evolution of the Oxazolidinone C-5 Side Chain

Workers at Zeneca and then AstraZeneca pioneered the identification of novel C-5 side chains wherein the usual acetamide moiety was replaced by either oxygen- or amino-linked heteroaromatic ring systems or a directly appended 1,2,3-triazole [70–72]. An early drug candidate in this series was AZD2563 (**19**), a promising isoxazolinyloxymethyl oxazolidinone with activity equivalent to or somewhat better than that of linezolid (see Fig. 8.12) [70, 73–77]. AZD2563 was briefly entered into human clinical trials in 2002 as an intravenously administered monophosphate prodrug (vide infra). The 1,2,3-triazole series (e.g., **20**) was found to provide good antibacterial

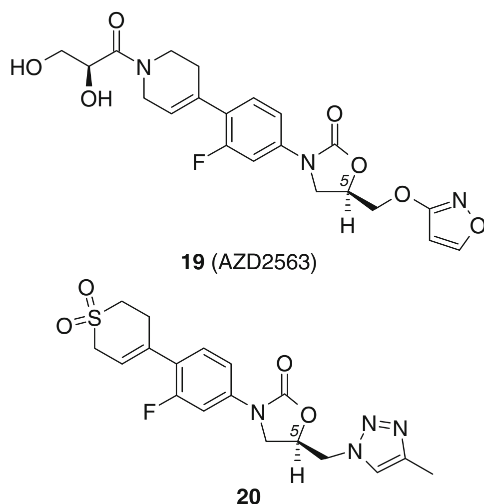

Fig. 8.12 Structures of AZD2563 and a 1,2,3-triazolyl oxazolidinone

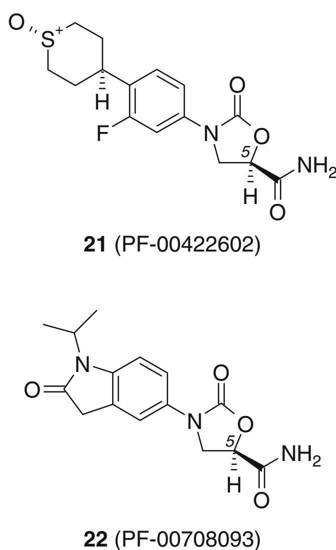

Fig. 8.13 Structures of reversed amide oxazolidinones PF-00422602 and PF-00708093

activity while exhibiting a greatly improved in vitro monoamine oxidase (MAO) inhibitory profile, at least with selected triazole substitution patterns [71, 72].

Perhaps an obvious evolution of the usual C-5 acetamidomethyl moiety would be to incorporate a reversed amide linkage. This notion was reduced to practice by workers at Pharmacia-Upjohn and the seminal characteristics of this series were subsequently described in a paper focused on the analog PF-00422602 (**21**, see Fig. 8.13) [78]. PF-00422602 exhibits in vitro Gram-positive activity roughly

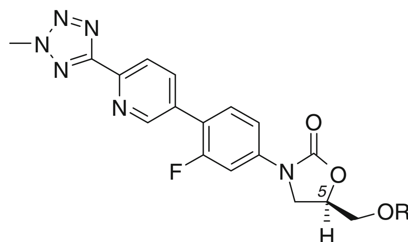

Fig. 8.14 Structures of torezolid (**23**) and its phosphate derivative TR-701 (**24**)

comparable to that of linezolid but has reduced activity against the fastidious Gram-negative organisms, *H. influenzae* and *M. catarrhalis*. Oral efficacy versus *S. aureus* in predictive animal infection models was found to be similar to that of linezolid. Pharmacokinetic performance in both rat and dog was found to be quite good. More importantly, PF-00422602 was reported to have greatly reduced inhibition of human MAO-A at the in vitro level ($K_i = 546$ vs. 53 μM) and significantly reduced myelotoxicity potential compared to linezolid in a rat 14-day safety study (NOAEL 200 mg/kg/day). An apparently optimized compound in this series, PF-00708093 (**22**), was eventually entered into human clinical trials (vide infra) [79].

In a rather remarkable return to early DuPont SAR, workers at Dong-A reported an interesting C-5 hydroxymethyl oxazolidinone, DA7157, with an extended tetrazolyl binding motif at the other end of the molecule (**23**, Fig. 8.14) [80]. Compound **23** was subsequently acquired by Trius and is now known as TR-700 or torezolid. A further enhancement of **23** led to the identification of the monophosphate prodrug TR-701 (**24**, DA7218), now known generically as torezolid phosphate. Torezolid is a promising second-generation oxazolidinone with a number of incremental improvements (vide infra) over linezolid that entered Phase 3 clinical trials in late 2010 and is on track to be the next marketed oxazolidinone.

8.4.1.3 Oxazolidinones with Extended Binding Motifs

The independent discovery of hybrid antibacterial agents comprised of a fluoroquinolone subunit and a partner oxazolidinone, has been reported [81–83]. These hybrid analogs incorporate chemically and metabolically stable linkages between the two partner antibiotics (see Fig. 8.15). One of these heterodimeric agents (MCB 3681, **25**) briefly progressed into human clinical trials as a phosphate ester prodrug (MCB 3837, **26**) [84, 85]. MCB 3681 exhibits broad-spectrum activity, including coverage of Gram-positive, most Gram-negative (but not *P. aeruginosa*), and anaerobic organisms. This reflects the hybrid compound's dual targeting of bacterial DNA gyrase and the ribosome (protein synthesis inhibition). MCB 3681 exhibits

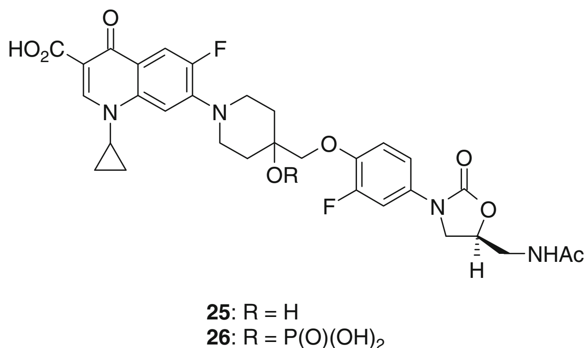

Fig. 8.15 Structures of oxazolidinone-quinolone hybrids MCB 3681 (**25**) and MCB 3837 (**26**)

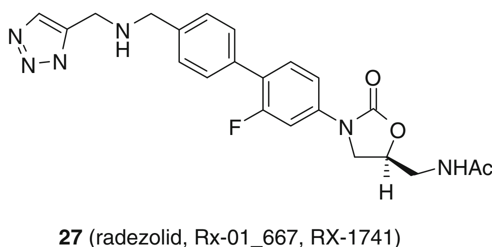

Fig. 8.16 The structure of radezolid (**27**, Rx-01_667 or RX-1741)

activity against both linezolid- and fluoroquinolone-resistant strains of pathogenic Gram-positive bacteria, including MRSA, GISA, VRE, and multidrug-resistant *S. pneumoniae* (MDRSP).

The most successful second generation oxazolidinone candidates to date have been biaryloxazolidinones with nitrogen-laden heterocycles appended to their distal aryl ring through either direct ring-to-ring connectivity (e.g., torezolid, see structure **23** in Fig. 8.14) or via an acyclic linking moiety (e.g., radezolid or Rx-01_667 or RX-1741, see compound **27** in Fig. 8.16) [86, 87]. The tetrazole and triazole of torezolid and radezolid, respectively, pick up additional binding site interactions with the ribosome and so confer increased potency, relative to that of linezolid. In addition, both torezolid and radezolid have enhanced activity against newly emergent linezolid-resistant strains of bacteria (vide infra).

8.4.2 Post-linezolid Clinical Oxazolidinones

When linezolid was approved in 2000, it became the reference oxazolidinone compound to beat going forward. Remarkably, despite being on the market some 11 years as of the writing of this chapter, linezolid remains the only commercial oxazolidinone. Despite this apparent void, a number of oxazolidinone agents have in

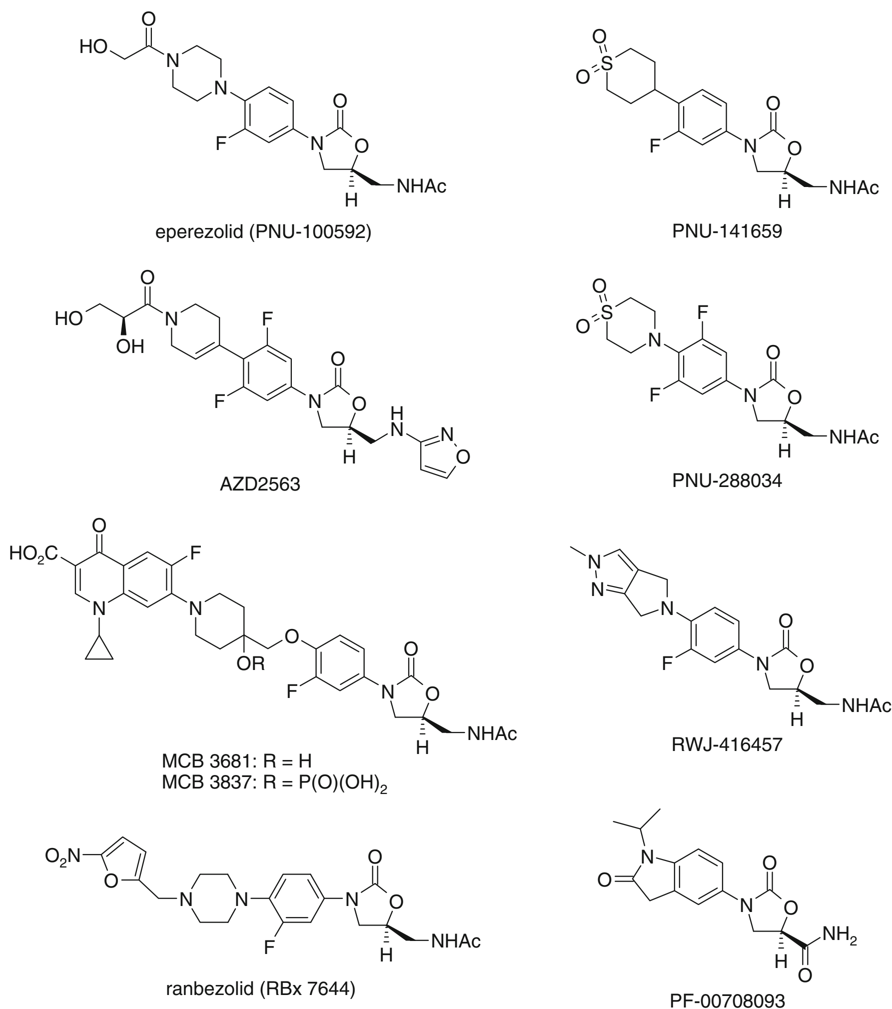

Fig. 8.17 Clinical oxazolidinones not progressing beyond phase 1

fact been advanced into human clinical trials. As shown in Fig. 8.17, eight oxazolidinones have been progressed into Phase 1 trials in healthy volunteers and then stopped. A detailed treatise on why these compounds were terminated is beyond the scope of this narrative. However, in general these compounds were not advanced into clinical trials involving patients because of the following reasons: (1) they possessed inadequate pharmacokinetic properties – duration of exposure was not in the desired therapeutic range or dosing frequency was unsatisfactory, and/or (2) they exhibited a poor safety profile, and/or (3) they just didn't adequately differentiate from linezolid. That is, they only offered incremental, marginal improvements over their progenitor. Overall, the greatest challenge in the oxazolidinone field lies in circumventing or at least minimizing the myelosuppression safety signal intrinsic to this class while

maintaining an acceptable antibacterial profile. Another significant hurdle is the achievement of adequate coverage against linezolid-resistant strains of bacteria.

8.4.2.1 Oxazolidinones Under Active Clinical Investigation

As mentioned previously (vide supra), the thiomorpholinyl oxazolidinone PNU-100480 (PF-2341272, see Fig. 8.8) was identified in the mid-1990s as a potent antimycobacterial agent with in vitro activity and in vivo efficacy superior to that of linezolid [35, 88]. Building on the foundational work of Cynamon and co-workers [88] Nuermberger and colleagues recently reported murine efficacy model results that indicated the addition of PNU-100480 (100 mg/kg of body weight per day) to the standard daily regimen of rifampin, isoniazid and pyrazinamide resulted in an additional 2 log reduction in lung CFU counts during the first two months of treatment [89, 90]. These results suggest that PNU-100480 may have the potential to significantly shorten the duration of therapy for drug-susceptible as well as multidrug-resistant tuberculosis. Pfizer initiated Phase 1 clinical trials with oral single ascending doses of PNU-100480 in healthy volunteers in April, 2009 [91]. In these studies, it was revealed that the principal circulating antibacterial agent was, in fact, the sulfoxide metabolite PNU-101603. Low levels of the corresponding sulfone metabolite PNU-101244 were also detected. Fortunately, all three sulfur oxidation states exhibit similar levels of antimycobacterial activity [35, 92]. In this trial, the bactericidal activity of linezolid, PNU-100480 and its metabolites was assessed via an ex vivo whole-blood culture test method. PNU-100480 exhibited time-dependent killing that was superior to that of linezolid. In the subsequent Phase 1 multiple ascending dose trials, PNU-100480 was very well tolerated and it was suggested that the compound provided an improved safety profile relative to that of linezolid [93]. This finding was rationalized by noting that the combined circulating steady-state levels of PNU-100480 and its two metabolites not only exceeded the MIC_{90} of *M. tuberculosis* (i.e., in the therapeutic range) but also remained below their respective mitochondrial protein synthesis (MPS) IC_{50} values [63, 94, 95]. In contrast, linezolid dosed at 600 mg twice daily provides steady-state concentrations that exceed its MPS IC_{50} throughout the entire dosing interval. Inhibition of mitochondrial protein synthesis is thought to be the proximal event leading to the various hallmark toxicities of the oxazolidinone class [63, 94, 95].

AstraZeneca has started clinical trials with an investigational anti-tubercular oxazolidinone of unknown structure, AZD5847, in December, 2009 [96, 97].

Radezolid (see Fig. 8.16) emerged from the Rx-01 discovery program at Rib-X [86, 87]. This “designer” oxazolidinone, so called because of its RNA modeling platform origins, is currently being studied in Phase 2 clinical trials, focusing on both CAP and uSSSI indications. Radezolid is generally twofold more active in vitro than linezolid against the staphylococci and 4–16-fold more potent against the streptococci and enterococci. Unlike linezolid and torezolid, radezolid does offer coverage of the fastidious gram-negative organisms *H. influenzae* and *M. catarrhalis* (MIC90s ≤1 μg/mL). Radezolid also exhibits improved intracellular killing relative to that of linezolid. Against the recently emergent staphylococci expressing the *cfr*

methyltransferase gene, [98, 99] radezolid is 2–8-fold more active than linezolid but 2–4-fold less active than torezolid [100]. Radezolid also offers the potential for once daily dosing with a relatively low dose (300 or 450 mg). It is unclear at this point whether radezolid has any appreciable safety advantages over linezolid. Overall, radezolid offers the potential for several incremental improvements but it is unclear how well it will compete against linezolid when it becomes a generic agent in 2015.

Torezolid (TR-700, see Fig. 8.14) originated at Dong-A and is undergoing further development by Trius. The orally bioavailable monophosphate prodrug TR-701 is rapidly converted to the bioactive parent TR-700 in vivo. In general, TR-700 is 4–8-fold more active in vitro than linezolid against the staphylococci, streptococci and enterococci [101, 102]. Importantly, focusing specifically on linezolid-resistant staphylococci, TR-700 is generally 8–16-fold more potent than linezolid. The phosphate prodrug moiety provides several fundamental advantages for torezolid. First, it effectively masks the potential liability of the parent'sC-5 hydroxymethyl side chain, which generally confers enhanced monoamine oxidase inhibitory activity to the oxazolidinones. Secondly, it also provides exquisite water solubility for parenteral administration of the agent. Thirdly, the sterically compact nature of the hydroxymethyl side chain is thought to be responsible for torezolid's greatly improved activity against staphylococcal strains possessing the *cfr* methyltransferase gene [100]. This recently emergent, plasmid-mediated resistance determinant effectively eliminates the therapeutic utility of all 50S protein synthesis inhibitors, including the lincosamides, pleuromutilins, streptogramins, phenicols, and linezolid [98, 99, 103]. Torezolid also maintains appreciable activity (MIC values ≤2 μg/mL) against *S. aureus* strains possessing both Cfr and L3 ribosomal protein mutations [104]. Torezolid is currently just starting Phase 3 clinical trials, with an initial focus on the cSSSI indication.

8.5 Oxazolidinone Mechanism of Action and Resistance Development

8.5.1 Mechanism of Action

For a comprehensive overview of the mechanism of action of the oxazolidinones the reader is referred to the summary of Leach and co-workers and references cited therein [105]. Seminal work in this area involved the use of a panel of bioactive and photoreactive oxazolidinone antibacterial agents of diverse structure which were irradiated in living bacterial cells to induce cross-linking [106]. The resultant cross-linking data, along with mapped point mutations in the 23S rRNA of resistant strains of bacteria, rapidly led to a model of oxazolidinone binding that implicated the peptidyl transferase center (PTC) as the site of action. More specifically, the model suggested that oxazolidinones occupy the A-site of the PTC, interfering with subsequent binding of aminoacyl-t-RNA and therefore inhibiting the overall protein synthesis process.

Importantly, when the same photoreactive oxazolidinones were reacted with human cells, it was found that cross-linking occurred to rRNA in the PTC of mitochondrial but not cytoplasmic ribosomes. Inhibition of mitochondrial protein synthesis has been linked to several of the side effects associated with linezolid therapy [63, 94, 95]. The accuracy of the predicted oxazolidinone binding motif was corroborated by subsequent linezolid single crystal X-ray co-crystal structures with the 50S ribosome subunits of either *Haloarcula marismortui* or *Deinococcus radiodurans* [107, 108].

8.5.2 Resistance Development

Linezolid resistance development was first reported in *E. faecium* in 1999 during the compound's compassionate use clinical trial [109–112]. The principal linezolid resistance mechanisms characterized thus far involve modification or mutations in and around the 23S rRNA peptidyl transferase center. As mentioned previously, methylation of adenosine A2503 by the Cfr methyltransferase confers pan-resistance to all of the marketed 50S protein synthesis with the exception of the macrolides and ketolides [98, 99]. Target mutations have been the principal source of linezolid resistance. The most prevalent target mutation observed in the clinical setting, in both the enterococci and staphylococci, has been a guanine to uracil nucleotide substitution at position 2576 (G2576U) in the gene region corresponding to domain V of the peptidyl transferase center of the 23S rRNA. It is interesting to note that G2576 is not in direct contact with linezolid but it apparently helps stabilize the positioning of G2505 and U2506, via either a stacking or hydrogen bond interaction, respectively, in the overall binding site ensemble [113]. Other 23S rRNA point mutations noted include: U2504G, G2447U, G2528U, G2505A, amongst others [108, 113]. Occasionally, point mutations in ribosomal proteins have been noted as a source of reduced linezolid susceptibility. For example, mutations in the ribosomal protein L4 have been shown to confer reduced susceptibility to linezolid in *S. pneumoniae* [114]. More recently, staphylococcal mutations in L3 have also been noted [104, 115]. Consistent risk factors for linezolid resistance development in the clinical setting include the following: (1) a long duration of therapy (>14 days), (2) immunocompromised patient status, (3) presence of a permanent in-dwelling device, and (4) sub-therapeutic levels of linezolid.

8.5.2.1 Staphylococci

Linezolid resistance development amongst the staphylococci remains at a low level, despite the agent being on the market and heavily used in the clinical setting for more than 10 years. For example, according to 2007 LEADER surveillance data (United States), >99.9% of *S. aureus* isolates (n = 3318, MRSA and MSSA) remain susceptible to linezolid [116]. There was a bit more erosion in the susceptibility of

coagulase-negative staphylococci to linezolid. Susceptibility of oxacillin-resistant strains (n=742) was 97.8% and oxacillin-susceptible strains (n=278) 99.3%. Similarly, 2007 ZAAPS surveillance data (non-US, 23 countries) revealed that >99.9% of *S. aureus* strains (n=3000) remain susceptible[117]. In this survey, 99.7% of coagulase-negative staphylococci (n=716) were susceptible. In a recent 2008 CANWARD surveillance report, 100% of *S. aureus* isolates (n=1007, 27% MRSA) remained susceptible to linezolid [118]. Another MRSA surveillance study involving 7,492 MRSA isolates collected in 48 Canadian hospitals from 1995–2008 indicated that 100% of strains were susceptible to linezolid [119]. A further study of a variety of marketed anti-staphylococcal agents against a worldwide contemporary panel of 10,000 isolates of *S. aureus* (5,000 MRSA and 5,000 MSSA) was also revealing [120]. Linezolid susceptibility against those MRSA and MSSA strains was >99.9% and 100%, respectively. Finally, a panel of methicillin-resistant (MRSA), vancomycin-intermediate (VISA), vancomycin-resistant (VRSA), heteroresistant VISA (hVISA) and daptomycin-nonsusceptible *Staphylococcus aureus* isolates was found to be 100% susceptible to linezolid [121]. A strong contributor to linezolid's slow development of resistance is no doubt due to the presence of multiple gene copies encoding for 23S rRNA [109, 111]. Multiple gene copies must suffer mutations before the MIC rises appreciably. The greatest threat to linezolid's coverage of the staphylococci appears to be the Cfr methyl transferase, which is transferrable by plasmid-mediated processes (see radezolid and torezolid discussion, vide supra) [98–100].

8.5.2.2 Enterococci

There has been some erosion in linezolid's effectiveness against the enterococci. LEADER data (United States) for 2007 indicated that 98.9% of enterococcal isolates (n=705) remain susceptible to linezolid [116]. This is consistent with 2007 surveillance data from 23 non-US countries (ZAAPS) where it was reported that 99.3% of the enterococci examined (n=906) remain susceptible[117]. Despite these encouraging numbers, the fact remains that linezolid is slowly losing its effectiveness against both *E. faecium* and *E. faecalis*. A few hospitals have reported occasional clonal outbreaks where higher incidences of enterococcal resistance, as much as 20%, are observed [122, 123].

8.5.2.3 Streptococci

Linezolid retains robust activity against the streptococci. Both LEADER and ZAAPS 2007 surveillance data revealed that *S. pneumoniae* (n=622 and 452, respectively), viridians group streptococci (n=249 and 155, respectively), and β-hemolytic streptococci (n=391 and 362, respectively) remain 100% susceptible to linezolid [116, 117]. In the CANWARD 2008 study (vide supra) 100% of the 544*S. pneumoniae* isolates were susceptible to linezolid [119]. Similarly, a study of 891 pneumococci

collected in 2008 from 22 centers in the United States were again uniformly susceptible to linezolid [124]. Two linezolid-resistant strains of *S. pneumoniae* (MICs of 4 μg/mL) have been reported in a study including 7,746 isolates [125].

8.6 Oxazolidinone Opportunities and Conclusions

By all accounts, linezolid has been a very successful antibacterial agent. Certainly, in a commercial sense it has been noteworthy, with worldwide sales exceeding $1 billion per year during the period 2007–2010. It has also been a timely and very effective addition to the physician's antibacterial armamentarium. Importantly, linezolid showed that a bacteriostatic agent could play an effective role in treating very serious infections caused by Gram-positive pathogens. While resistance development to linezolid has occurred, it remains remarkably modest in magnitude considering it has been on the market for more than 10 years. Two very distinct factors have contributed to this situation. First, there are multiple gene copies expressing the targeted 23S rRNA, rendering the need for multiple mutations before a rise in linezolid's MIC is realized. Secondly, there is definitely a significant economic factor involved in that the premium pricing of linezolid generally leads to its being used only when the medical need justifies it – potentially a new marketing paradigm for the entry of any novel antibacterial agent. The available intravenous to oral switch, without the need for a dose adjustment, has been a compelling cost-saving characteristic of linezolid, allowing for an early patient discharge from the hospital setting. At the time of this writing, linezolid remains the only oral agent approved by the FDA for treating nosocomial pneumonia and complicated skin and skin structure infections caused by MRSA, as well as bloodstream infections caused by vancomycin-resistant *Enterococcus faecium*. As linezolid enters the generic phase of its life cycle in the second quarter of 2015, it will likely remain a formidable factor in the overall Gram-positive antibacterial market. As a consequence, the requirements for a successful market entry by any nascent second-generation oxazolidinone are likely going to be rather stringent.

A second-generation oxazolidinone antibacterial agent will need to address one or more of the weaknesses of its progenitor, linezolid. Opportunities to improve on linezolid follow. Certainly, increased potency would be desirable in order to enable a lower dose. Enhanced intracellular killing would be advantageous in certain indications. More bactericidal character would be preferred, if possible for a 50S rRNA inhibitor. A once-daily dosing regimen would also be a step forward. Enhanced water solubility to reduce the IV administration volume from 300 mL to a more manageable amount would represent an advance. An expanded spectrum of activity, perhaps including better coverage of atypical and fastidious Gram-negative respiratory organisms would open the door to additional indications. Quite obviously, appreciable coverage of linezolid-resistant Gram-positive pathogens would also be a requisite. A reduction or elimination of linezolid's reversible myelosuppressive effects might enable extended use in the community setting for indications requiring

good safety (e.g., osteomyelitis, TB, etc.). The latter goal might require a better understanding of the structural differences between bacterial and mammalian mitochondrial ribosomes. Finally, an improved MAOI profile would also be ideal for broad community use.

Looking at oxazolidinones under active clinical investigation, torezolid appears to have the best chance of reaching the market. Whether or not it will be as commercially successful as its progenitor remains to be seen. While it offers a number of incremental advantages over its predecessor, they may not suffice in the face of readily available, cheap, generic linezolid. Intuitively, the development of higher levels of linezolid-resistance may be required in order to justify torezolid's inevitably higher price point.

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Chapter 9
Glycopeptides and Lipoglycopeptides

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9.1 Introduction

The scourge of resistance to antibacterial agents is a global problem of increasing severity. A recent review by Boucher and colleagues [33] summarized the alarming viewpoint of the Infectious Diseases Society of America (IDSA) that a stagnated development pipeline combined with continuing evolution of resistance, including emergence of pan-resistant strains, foretells of continued escalating healthcare costs and little reason for optimism in countering resistance to antibacterial agents.

Among the bacteria of heightened concern because of their propensity to cause nosocomial infections [200], methicillin-resistant *Staphylococcus aureus* (MRSA) continues to confound our efforts to limit its spread. In 2005, there were an estimated 368,600 hospital stays with MRSA infection in the U.S. alone, resulting in an estimated cost of hospital stays of over $5 billion [76]. Strikingly, this incidence tripled between 2000 and 2005. Another report confirmed the high burden of morbidity and mortality of invasive MRSA infection in the U.S., with 18,650 deaths attributed to an estimated 94,360 invasive infections in the U.S. in 2005 [126]. To further compound the problem, MRSA has emerged in the community over the past decade in individuals lacking established MRSA risk factors, including recent hospitalization, surgery, residence in a long-term care facility, dialysis, or the presence of an indwelling medical device. Such community-associated MRSA (CA-MRSA) infections initially had their onset in the community but CA-MRSA is now recognized to have established itself as a nosocomial pathogen [45, 62, 66].

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Of the few anti-MRSA agents that are currently in late-stage clinical development or awaiting regulatory approval, the lipoglycopeptides are of particular note, since they were either isolated or produced semi-synthetically in efforts to enhance the safety and efficacy of vancomycin, the prototypic glycopeptide and mainstay of anti-MRSA therapy since the 1960s. The Clinical and Laboratory Standards Institute (CLSI) recognizes dalbavancin, oritavancin, teicoplanin, and telavancin as members of the lipoglycopeptide subclass of glycopeptides [52]. This review aims to summarize the most salient features of dalbavancin, oritavancin, and telavancin, with reference to vancomycin and to teicoplanin. References are generally to the primary data.

9.2 Chemistry

The increased reliance on vancomycin for the treatment of serious Gram-positive infections and the gradual appearance of vancomycin resistance, in particular in enterococci, drove pharmaceutical discovery programs to look for improved glycopeptides. These efforts had emphasis on improved pharmacological properties and enhanced activity, particularly against antibiotic-resistant microorganisms.

Glycopeptide antibiotics form a large class of antibacterial agents loosely associated because they possess a peptide scaffold and are glycosylated. The term is generally applied to specifically designate the dalbaheptides [176]. These compounds have a central heptapeptidic core with at least five amino acid residues bearing aromatic side chains. These amino acids are crosslinked to form a unique trimacrocyclic or tetramacrocyclic structure. The structures may furthermore be glycosylated at selected hydroxyl groups on the scaffold, be they phenolic or aliphatic. A number of academic groups have investigated totally synthetic approaches to glycopeptide antibiotics [32, 176], in particular the very interesting work performed by Boger and colleagues on skeletal modifications to surmount resistance [57]. There are also attempts to reengineer the biosynthetic pathway of glycopeptides in an attempt to create novel derivatives [181, 256]. In practical terms however, investigators have mostly relied on semi-synthetic modifications to naturally occurring glycopeptides to investigate structure and function.

Despite their highly functionalized nature, a number of selective chemical modifications have been described (Fig. 9.1) [183]. In particular, the aglycon C-terminus can be esterified or converted to an amide [49, 150, 151], the N-terminus and the carbohydrate amino groups can be acylated or reductively alkylated [53, 54, 169, 170, 189, 240], the resorcinol can be aminomethylated [190] and, with proper protection procedures, one of the phenolic hydroxyls can be acylated [51]. The carbohydrate moiety can also be proteolytically removed, including with selective cleavage of a single glycosidic bond [50], and the resulting phenol can be modified to introduce novel glycosidic groups [101]. In addition, the terminal amino acid can be removed by Edman degradation [6, 241], and the resulting amino group can be alkylated [242] or reacylated [243]. Removal of amino acids 1 and 3 and the subsequent rebuilding of the dalbaheptide scaffold has been demonstrated [152]. The reductive alkylation reactions, of particular interest to the agents approved or

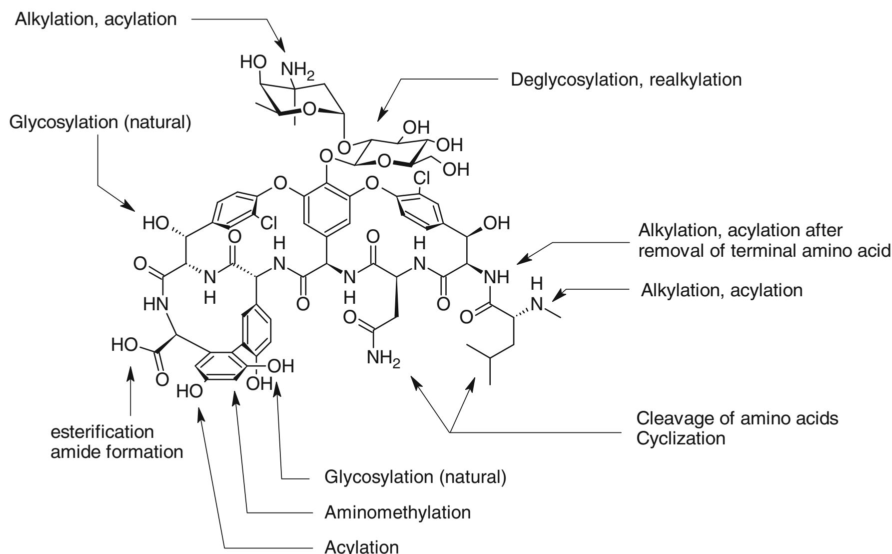

Fig. 9.1 Chemical and natural modifications to the glycopeptide scaffold

in development, have been optimized and are durable enough to be used at manufacture scale [26, 27, 143]. These selective chemical modifications have led to a better understanding of the impact of key structural elements in the pharmacology of glycopeptides [250] and have clearly established a role for hydrophobic groups in overcoming resistance mechanisms, leading to the discovery and development of dalbavancin, oritavancin, and telavancin.

Dalbavancin (Fig. 9.2) is derived from the naturally occurring teicoplanin-like glycopeptide parvocidin (A 40926) [149, 153]. Parvocidin is a lipoglycopeptide with a long half-life [94], three to four times longer than that of teicoplanin in mice, but with less activity against coagulase-negative staphylococci, which was the driving force for determination of structure-activity relationships. Dalbavancin is the dimethylaminopropylamide of parvocidin, and the conversion is afforded in three synthetic steps [149], largely relying on the ability to selectively esterify the carboxylate function of the N-acylglucuronic acid moiety without affecting the C-terminal carboxylate group.

Oritavancin (Fig. 9.2) is derived by the reductive alkylation of chloroeremomycin, a vancomycin-type glycopeptide, which lacks a hydrophobic tail [3]. Oritavancin was developed in a program aiming to maintain the activity of chloroeremomycin against *S. aureus* while simultaneously improving activity against vancomycin-resistant enterococci [2]. It differs from vancomycin in the presence of the (4-chlorophenyl) benzyl group, the different stereochemistry of the amino sugar, and the presence of a second amino sugar. As in other semi-synthetic lipoglycopeptides derived from the vancomycin scaffold, there is a clear correlation between the chain length of the hydrophobic tail and activity against antibiotic-resistant bacteria [53, 170, 183].

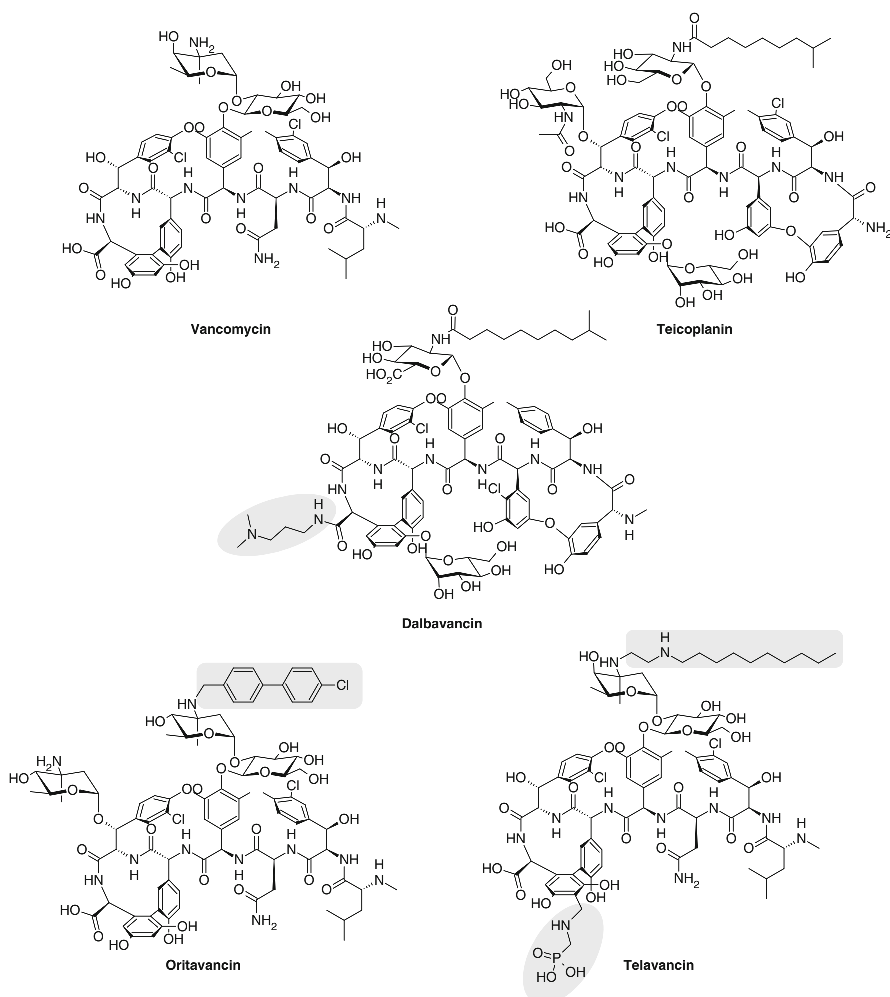

Fig. 9.2 Chemical structure of lipoglycopeptides in development

Telavancin (Fig. 9.2) is obtained in three synthetic steps from vancomycin itself [115, 133]. The reductive alkylation of vancomycin with N-protected N-decylaminoacetaldehyde furnishes, after deprotection, the telavancin precursor N-decylaminoethylvancomycin (THRX-689909). This latter compound is the result of a lead optimization program seeking to optimize activity against both MRSA and VanA type vancomycin-resistant enterococci (VRE). As in the case of oritavancin, there was a correlation between chain length and activity, with the N-decyl chain affording the best compromise. While the in vitro profile of this compound was very favorable, it tended to be poorly excreted and to accumulate in liver and kidneys. Addition of the phosphonomethylaminomethyl group on the resorcinol moiety of THRX-689909 led to the discovery of telavancin.

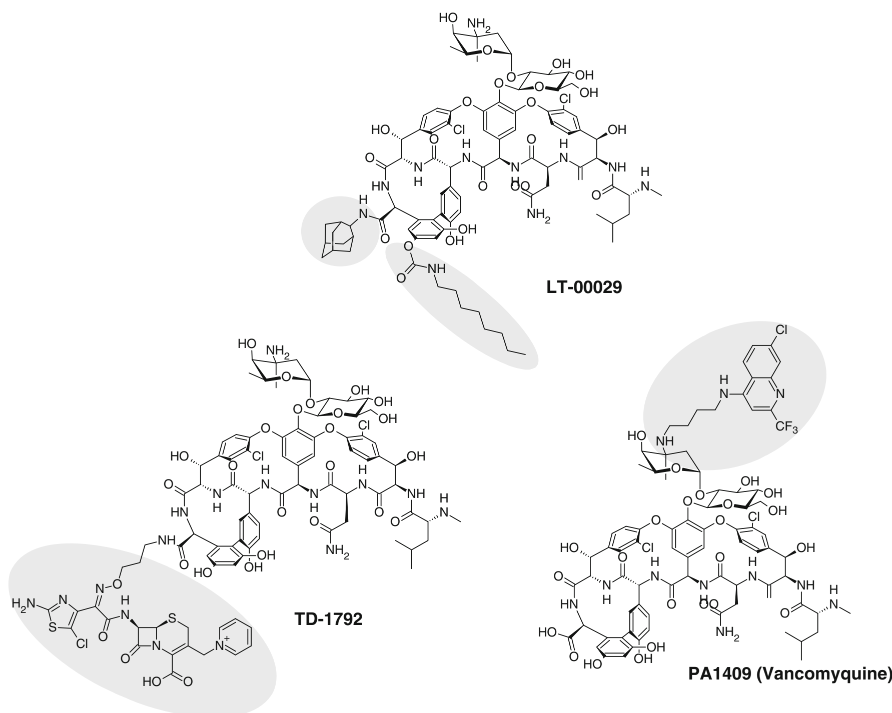

Fig. 9.3 Chemical structure of newer semi-synthetic glycopeptides in development

Beyond these three novel lipoglycopeptides there have been continued efforts in the discovery of novel semi-synthetic glycopeptides. Of particular interest, is the discovery of LT-00029 (Fig. 9.3) in which the lipophilic functional groups have been shifted away from the carbohydrate moiety, while maintaining antibacterial activity against *vanA* VRE (4 μg/ml for *vanA E. faecalis* and 0.25 μg/ml for *vanA E. faecium*) [51]. In addition to simple hydrophobic groups, the glycopeptide scaffold has also been modified in efforts targeting the formation of hybrid antibiotics. Thus structure-activity relationships have allowed the preparation of the glycopeptide-cephalosporin hybrid TD-1792 (Fig. 9.3) (Long et al. [145, 146]). This hybrid structure is extremely potent, with MIC_{90} values that are up to 64X lower than vancomycin in vancomycin-susceptible staphylococci and up to 500X lower in vancomycin-susceptible *E. faecalis*. On the other hand, it was not able to overcome high level vancomycin-resistance in enterococci. Similarly, vancomycin hybridization with 4-aminoquinolines has led to the discovery of PA1409 (Vancomyquine, Fig. 9.3) [47, 162, 163]. This compound displayed MIC values of 0.12–0.5 μg/ml against MRSA, 0.06–0.5 μg/ml against vancomycin-susceptible enterococci (VSE) and 4 μg/ml against VRE.

9.3 Mechanism of Action

9.3.1 *Cell Wall Synthesis Inhibition*

Glycopeptides are a class of natural products that impair cell wall synthesis in Gram-positive bacteria. The bacterial cell wall is a structural component external to the bacterial membrane that is vital to maintaining the structural integrity of the cell. The major component of the cell wall is peptidoglycan, a polymer composed of repeating disaccharide-pentapeptide units which are synthesized in the cytoplasm (Fig. 9.4). Transport of the disaccharide-pentapeptide units across the membrane occurs in form of a complex with a lipid carrier (undecaprenylpyrophosphate), collectively known as lipid II. Translocation of lipid II across the membrane provides the substrate for transglycosylase enzymes to incorporate the disaccharide-pentapeptide monomer into nascent peptidoglycan. The prototypic glycopeptide vancomycin is a substrate specific inhibitor of peptidoglycan synthesis and acts by specifically binding to the carboxyl-terminal *acyl*-D-alanyl-D-alanine (*acyl*-D-Ala-D-Ala) residues of the pentapeptide moiety of lipid II: bound vancomycin sterically hinders the transglycosylase enzyme from incorporating the disaccharide-pentapeptide monomer into nascent peptidoglycan [199]. Arrest of cell wall synthesis by vancomycin leads to relatively slow and concentration-independent bactericidal activity [209]. By comparison, the semi-synthetic lipoglycopeptides dalbavancin, oritavancin and telavancin (Fig. 9.2) typically exert more rapid killing that is concentration dependent [132, 160]. The focus of this section is to review the differences between the mechanisms of action

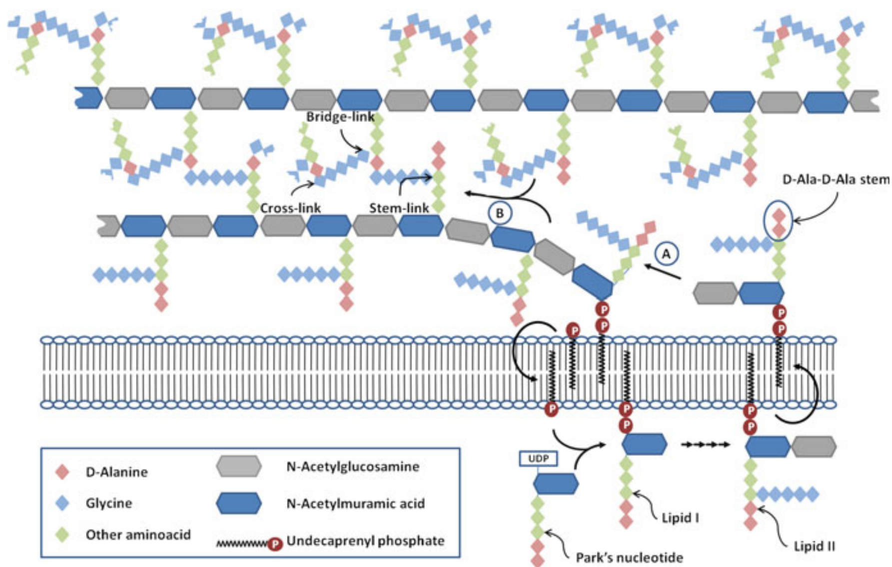

Fig. 9.4 Schematic representation of cell wall biosynthesis

of these antibacterial agents with special focus on mechanisms that contribute to the rapid bactericidal activity of lipoglycopeptides and their ability to overcome vancomycin-resistance mechanisms.

Modification of glycopeptides with additional hydrophobic side-chains has been shown to increase the capacity of the resultant semi-synthetic lipoglycopeptides to impair cell-wall synthesis likely as a function of increased binding affinity to the target molecule lipid II. The hydrophobic side-chains of lipoglycopeptides undergo membrane anchoring, a process in which lipophilic substituents embed in the bacterial membrane and is thought to stabilize the interaction with lipid II [22]. Lipoglycopeptides also self-associate into dimers, which likely promote cooperative interactions between dimers and adjacent pentapeptides of the peptidoglycan and accounts for increased binding avidity [261]. Concentration-dependent dimerization of dalbavancin in solution has been measured by mass spectrometry [40]. The 4'-chlorobiphenylmethyl hydrophobic side chain of oritavancin undergoes membrane anchoring and stimulates dimerization [4]. Consequently, oritavancin exhibited a greater capacity to impair cell wall synthesis than vancomycin in classical pulse-chase macromolecular synthesis assays [10]. Furthermore, addition of a 284,000-fold molar excess of exogenous peptide ligand *N,N*'-diacetyl-L-Lys-D-Ala-D-Ala was needed to block oritavancin antibacterial activity whereas only 27- and 302-fold molar excess of peptide ligand suppressed vancomycin and chloroeremomycin activity, respectively [4]. In what has been described as a multivalent interaction, the hydrophobic substituent of telavancin is also thought to interact with the bacterial membrane and enhance its interaction with lipid II [37]. The affinity constant of telavancin for lipid II in model membrane vesicles was fivefold higher than that of vancomycin, as measured by isothermal titration calorimetry [37]. Furthermore, telavancin binding affinity to a water-soluble variant of lipid II was 35-fold stronger than that of lipid II embedded in membrane vesicles: vancomycin binding affinity to the latter form of lipid II increased only threefold [37]. In radioactive pulse-chase experiments to monitor macromolecular synthesis, telavancin was shown to inhibit cell-wall synthesis in a concentration-dependent manner with a 50% inhibitory concentration (IC_{50}) of 0.14 μM compared to an IC_{50} of 2.0 μM for that of vancomycin, a difference that may be reflected in telavancin in vitro potency [109].

The pioneering work of Strominger and colleagues demonstrating that lipid II is the target for vancomycin [8, 157] stimulated investigation of glycopeptide inhibition of transglycosylation and transpeptidation (Fig. 9.4). Interestingly, telavancin and oritavancin have been described to inhibit both the transglycosylation step of cell wall synthesis and transpeptidation, the cross-linking of the stem pentapeptides with a pentaglycine bridge (in staphylococci) that yields fully mature peptidoglycan. The Schaefer group has established the use of solid-state NMR to describe the molecular interactions of lipoglycopeptides with the cell wall and changes in cell wall structure as a result of these interactions [88, 186, 187, 219, 222, 223, 244]. These studies rely on metabolic labeling of cell wall components with amino acids containing NMR-active nuclei, such as [^{13}C]-alanine, [^{13}C]-glycine or [^{15}N]-lysine. Advanced NMR experiments involving the rotational echo double resonance

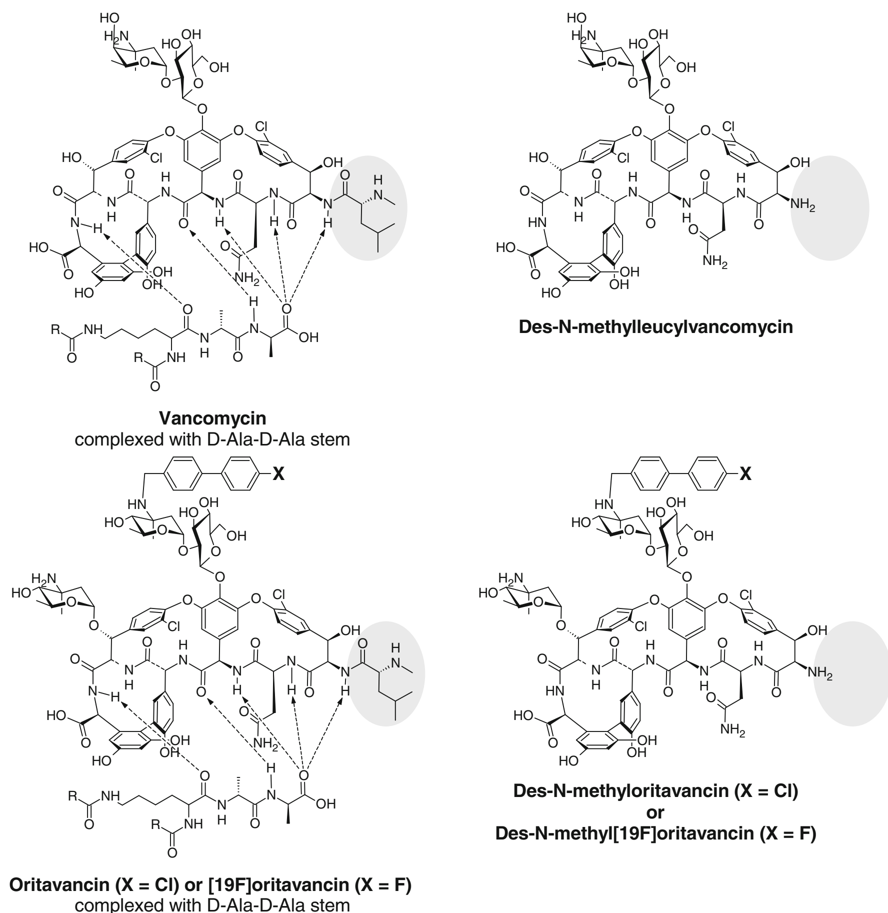

Fig. 9.5 Structures of vancomycin, oritavancin, [^{19}F]oritavancin and their des-*N*-methylleucyl derivatives

(REDOR) experiment were then used to determine the distance between these nuclei and lipoglycopeptides bearing an NMR-active fluorine atom. In particular, a number of the studies revolved around [^{19}F]oritavancin, an analog of oritavancin in which the chlorine on the hydrophobic moiety was replaced with a fluorine, as well as its des-N-methylleucyl parent, in which the D-alanyl-D-alanine binding pocket has been damaged (Fig. 9.5). These studies performed in either *S. aureus* or *E. faecium* demonstrated that lipoglycopeptides are able to interact with nascent cell-wall components in a more extensive manner than vancomycin (Fig. 9.6). In particular, lipoglycopeptides such as oritavancin were shown to bind the cross-linking portion of the peptidic component (the pentaglycine bridge) of the cell wall of *S. aureus* in addition to binding the D-alanyl-D-alanine termini [44, 120–122]. The fact that both [^{19}F]oritavancin and its des-N-methylleucyl parent are able to bind cross-links in the cell wall is intriguing, suggesting an alternate binding site in

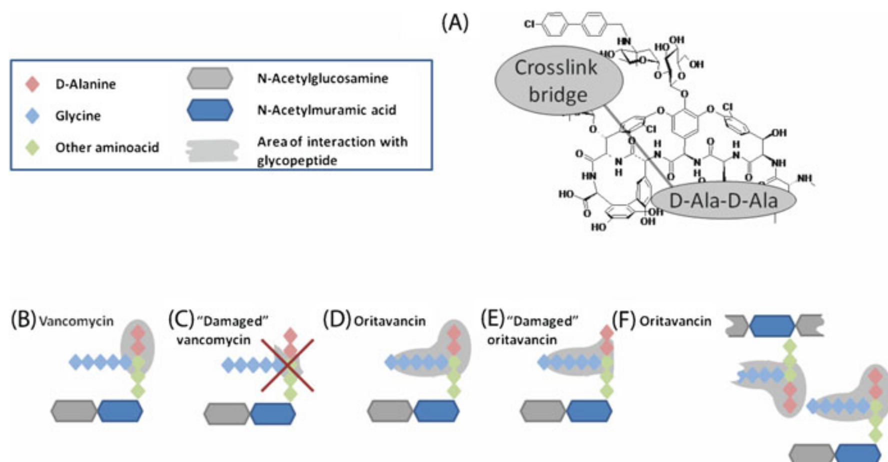

Fig. 9.6 Schematic representation of the interaction of glycopeptides with the cell wall

peptidoglycan and that interactions with the terminal peptides may not be required. Therefore, oritavancin may retain activity against vancomycin-resistant microorganisms in which the termini are modified to D-alanyl-D-lactate by instead binding the pentaglycine bridge in staphylococci. However, oritavancin also binds to models of cell-wall fragments involving the modified D-alanyl-D-lactate termini with near equal affinity [3, 173]. Thus, lipoglycopeptides are provided with additional opportunities to bind to the cell wall beyond lipid II, in particular the growing peptidoglycan chains. This was confirmed in pulse-chase cell wall labeling studies in *S.aureus* Mu50, a vancomycin intermediate *Staphylococcus aureus* (VISA) strain with 30% more uncross-linked stems than an methicillin-susceptible *Staphylococcus aureus* (MSSA) strain, which provides evidence that a significant proportion of the [^{19}F]oritavancin is bound to mature cell wall [124] and thus not confined to the nascent cell wall.

The effect of oritavancin on transpeptidation was further investigated by metabolic labeling of *S. aureus* simultaneously with amino acids containing a ^{13}C nucleus such as [^{13}C]alanine or [^{13}C]glycine, or a ^{15}N nucleus such as [^{15}N]lysine or [^{15}N] glycine. This labeling allowed pin-pointing of the NMR signals specifically associated with cross-links (the peptide bonds between a D-alanine carbonyl and the N-terminus of the pentaglycine bridge), bridge-links (the peptide bonds between the C-terminus of the pentaglycine bridge and the lysine side chain amino group), or stem-links (the peptide bonds between the carbonyl of the lysine and the amino group of the D-alanine in a D-alanyl-D-alanine stem) as described in Fig. 9.4. The impact of glycopeptides on the relative proportions of cell wall components and/or precursors was then studied [43, 188]. The ratio of D-alanyl-D-alanine stems to cross-links was determined to be 0.45 in *S. aureus* ATCC 6538P (MSSA). The level of cross-linking was slightly decreased in the presence of 20 μg/ml of vancomycin, giving a ratio of 1, which is consistent with the accumulation of D-alanyl-D-alanine

stems as a result of the sequestration of lipid II. This is an indication of inhibition of transglycosylation but not transpeptidation [43, 123]. In the presence of 0.15 μg/ml of penicillin, a known inhibitor of transpeptidation [123], the ratio obtained was also 1, consistent with the inhibition of transpeptidation that decreases in the extent of crosslinking in the cell wall. In contrast, oritavancin had an impact on cell wall cross-linking, which was intermediate between that of vancomycin and penicillin. At 20 μg/ml oritavancin, a ratio of 1.3 was obtained. This suggests that oritavancin has a more pronounced impact on transpeptidation and is consistent with the ability of oritavancin to bind both lipid II and nascent cell wall strands. Hence, the simultaneous accumulation of D-alanyl-D-alanine stems and decrease in the level of cross-linking is suggestive of an agent acting on both transglycosylation and transpeptidation.

Because the enterococcal cell wall undergoes a maturation step involving enzymatic removal of D-alanyl-D-alanine termini that are not consumed in the cross-linking processes, any measurable quantity of D-alanyl-D-alanine stems is attributable to the cytoplasmic pool of cell-wall precursors. Using parallel ^{13}C-^{15}N double-labeling experiments in *E. faecium* ATCC 49624 vancomycin susceptible enterococcus (VSE), the Schaefer group showed that exposure to 25 μg/ml of vancomycin resulted in a 50% increase in the level of D-alanyl-D-alanine stems with respect to cross-links, as measured by solid-state NMR [188]. This result is consistent with the accumulation of cytoplasmic cell-wall precursors associated with the sequestration of lipid II, and hence, inhibition of transglycosylation. Interestingly, exposure to 25 μg/ml of oritavancin did not significantly alter this ratio, suggestive that cytoplasmic cell-wall precursors do not accumulate in the presence of oritavancin and therefore binding to lipid II –and by extension, inhibition of transglycosylation – is not a significant contributor to its antibacterial activity. Vancomycin exposure gave rise to only a 25% decrease in the amount of lysine that was incorporated in the cell wall and an 18% decrease in the amount of lysine associated with D-alanyl-D-alanine. By comparison, the corresponding decreases were 62% and 81% with oritavancin. This marked decrease in the quantity of D-alanyl-D-alanine termini would suggest that a large number of these were not used in cross-linking the cell wall, but rather they were degraded as a result of cell-wall maturation. Oritavancin may therefore preferentially inhibit transpeptidation over transglycosylation in *E. faecium.*

A different approach was used to elucidate the effect of telavancin on tranglycosylation and transpeptidation of peptidoglycan in *S. aureus*. Using penicillin G to block the transpeptidation reaction, the effect of telavancin on transglycosylation of immature peptidoglycan was assayed by measuring the incorporation of [^{14}C]N-acetyl-glucosamine into 5% trichloroacetic acid-precipitable material. Conversely, synthesis (transpeptidation) of mature peptidoglycan was quantified by measuring the incorporation of the radiolabel into 4% hot sodium dodecylsulfate-insoluble material in the absence of penicillin G. From these studies, patterns of peptidoglycan inhibition by telavancin and selected agents were compared. Oxacillin selectively inhibited mature peptidoglycan synthesis with an IC_{50} of 2 μg/ml, an indication of its effects on transpeptidation. In contrast, telavancin had nearly an

equal effect on both immature (transglycosylation) and mature peptidoglycan synthesis (transpeptidation), inhibiting both reactions with IC_{50} values of 0.5 and 0.1 μg/ml, respectively. Vancomycin preferentially inhibited synthesis of mature peptidoglycan in these studies, with an IC_{50} value of 0.8 μg/ml compared to an IC_{50} value of 9.2 μg/ml for inhibition of immature transglycosylation reaction. This corroborates previous findings demonstrating inhibition of mature peptidoglycan synthesis by a similar method [89].

There is some evidence to suggest that lipoglycopeptides circumvent substrate binding and interact directly with transglycosylases to impair their activity. This may help explain, at least in part, why lipoglycopeptides retain activity against vancomycin-resistant strains with D-Ala-D-Lac as terminal peptides. Studies by the Kahne and Walker groups have shown that chlorobiphenyl-vancomycin and its modified counterpart chlorobiphenyl-des-methyl-vancomycin, that cannot bind to D-Ala-D-Ala, equally impair transglycosylation [89]. By inference, it is believed that lipoglycopeptides interact directly with transglycosylases to impair activity. This was followed-up by a study in a cell-free system showing that oritavancin and its des-methyl- counterpart also exhibit similar inhibitory activity [257].

9.3.2 Membrane Activities

Early studies on semi-synthetic derivatives of vancomycin and chloroeremomycin demonstrated membrane anchoring of hydrophobic side chains by these molecules [4, 5, 22]. These interactions are not only presumed to strengthen the intramolecular forces with peptidoglycan residues, but now have been shown to perturb bacterial membrane integrity. The bacterial membrane is a selective permeability barrier regulated in part by an electrochemical gradient, the membrane potential, which drives the uptake of ions and couples that to the production of the cellular energy currency, ATP. Although bacterial membrane potential is known to fluctuate during the growth cycle of bacteria and under different growth conditions [118], agents that cause depolarization of the membrane or increase membrane permeability can compromise the barrier function of the membrane and cause cell death.

The first study to demonstrate loss of membrane integrity upon exposure to lipoglycopeptides was with telavancin [109]. These studies used the fluorescent probe 3,3′-dipropylthiadicarbocyanine iodide ($DiSC_3$ [6]) as an indicator of the degree of depolarization of the plasma membrane following exposure to drug. Exposure of MRSA to telavancin at concentrations that resulted in cell killing also caused concentration-dependent membrane depolarization, as measured by an increase in fluorescence. Vancomycin did not cause fluorometric alterations; therefore, it did not depolarize the membrane under the conditions of the assay. Increased extracellular potassium and ATP were also measured fluorometrically, corroborating that the electrochemical gradient of the bacterial membrane was compromised following telavancin but not vancomycin challenge. In follow-on experiments, the permeability of the bacterial membrane was measured using the fluorescent dye pair SYTO 9 and

propidium iodide: bacterial membrane damage (increased permeability) allows the otherwise impermeant dye propidium iodide to enter the cell and displace the permeant dye SYTO-9 leading to a loss of fluorescence. Similar to its effects on membrane depolarization, telavancin caused a concentration-dependent decrease in SYTO 9 fluorescence, an indication that the bacterial membrane had become permeable to propidium iodide; vancomycin had no effect on membrane permeability. Increased membrane permeability preceded both membrane depolarization and cell death in these studies [109].

By flow cytometry, telavancin induced both time- and concentration-dependent depolarization of MSSA [147]. The IC_{50} was calculated to be 3.6 μg/ml for telavancin, comparable to an IC_{50} of 2.9 μg/ml for the positive control agent nisin. An IC_{50} could not be determined for vancomycin, suggesting that it had no effect on membrane potential under the conditions of the assay. Telavancin also depolarized membranes of vancomycin-intermediate *S. aureus* (VISA), heterogeneous VISA (hVISA) and a daptomycin non-susceptible isolate. Further exploration of the requirements to cause depolarization showed that binding to lipid II was necessary for telavancin effect since pretreatment of bacteria with agents (fosfomycin, D-cycloserine or bacitracin) that diminish cellular levels of lipid II decreased the effect of telavancin on membrane potential [147].

Oritavancin has also been shown to perturb membrane integrity in Gram-positive bacteria. In fluorometric assays with $DiSC_3$ [6] and SYTO 9/propidium iodide, oritavancin induced immediate, concentration-dependent effects on both membrane potential and permeability of *S. aureus* and enterococci of different resistance phenotypes, including MRSA and a VanA strain of VRE [24, 159]. Importantly, cell death was concurrent with perturbation of membrane integrity, independent of phenotype or genus. The 4'-chlorobiphenylmethyl group of oritavancin was critical for perturbation of bacterial membranes since exposure of cells to chloroeremomycin, which lacks the hydrophobic side chain, had no effect on membrane integrity nor cell viability within the time-frame of the assays [159]. Recent work has shown strong correlations between loss of membrane potential and cell death in hVISA, VISA, vancomycin resistant *Staphylococcus aureus* (VRSA) and VRE exposed to oritavancin [159]. The interaction of oritavancin with the bacterial membrane has been studied using liposomes and artificial membranes composed of the phospholipids cardiolipin, phosphatidylglycerol, and phosphatidylethanolamine [69]. The fluorescent probe calcein was immediately released from preloaded liposomes that were permeabilized upon exposure to oritavancin but not vancomycin. The rate of release of calcein was dictated by the phospholipid composition of the liposomes. Using atomic force microscopy, erosion and remodeling of supported lipid bilayers occurred in response to oritavancin but not vancomycin, again as a function of the phospholipid composition of the model bilayers. Interestingly, given the impact of lipoglycopeptides on membrane permeability, the NMR techniques described above did not detect interactions between fluorinated lipoglycopeptides and the membrane [125]. These interactions were not seen in whole cells or in *S. aureus* protoplasts but were only observed in vesicles obtained from protoplast membranes. Although interactions with the bacterial membrane may only occur above a threshold concentration of oritavancin, we believe its rapid bactericidal effect on Gram-positive pathogens

occurs by perturbing integrity of the membrane. This hypothesis is supported by studies demonstrating that oritavancin retained bactericidal activity against stationary-phase *S. aureus* inoculated into nutrient-depleted media [24, 155], conditions in which cell-wall synthesis is expected to be minimal and consequently killing by vancomycin and the β-lactam nafcillin was shown to be negligible [24, 155].

9.3.3 Ultrastructural Changes

Transmission electron microscopy (TEM) of thin sections of bacteria has offered insight into the mechanisms of antibiotic action by allowing visualization of the ultrastructural changes that occur following drug exposure. The pioneering work of Terry Beveridge and co-workers has significantly advanced our understanding of bacterial ultrastructure, including that of human pathogens at different stages of growth or exposed to antibiotics. In *S. aureus* and some other coccoid Gram-positive bacteria, cell-wall synthesis occurs only during the septation process of cell division [220]. Newly-synthesized cell wall originates from the outer wall bridge and extends inwards to form two nascent cross walls arranged in a parallel plane within the septum [156]. By TEM, the newly synthesized cross walls compose the midline, an electron-dense staining area within the septum [156, 245]. Both oritavancin and telavancin caused changes to the ultrastructure of the division septum of *S. aureus*. For example, MRSA exposed to the strain's MIC of telavancin for 15 min exhibited abnormal placement of initial septation sites [197]. Septa were abnormally thick and cells in advanced stages of septation often initiated asymmetrical daughter cell separation. In some instances, localized cell wall thinning resulted in buckling of the cell wall. Thickened and misshapen septa were also apparent in MRSA exposed to 1 μg/ml oritavancin for 10 min [23]; thickened septa were also apparent in a vancomycin-resistant *E. faecalis* strain exposed to 0.12 μg/ml oritavancin for 10 min. Interestingly, a cross-cut through the plane of septation in MRSA exposed to oritavancin revealed that only half the septum had formed, an indication that oritavancin may target the asymmetric initiation of septum formation that occurs in *S. aureus* [93]. Another striking feature of oritavancin exposure was a greatly decreased intensity of staining of the septal midline, which has also been described in cells exposed to penicillin [93]. Loss of midline staining could result from oritavancin inhibiting cell-wall synthesis [10, 257] or altering autolysin activity via its effects on membrane potential, believed to be important in regulating autolysis [119]. These ultrastructural changes were not observed in cells exposed to 16 μg/ml vancomycin for 3 h. Interestingly, oritavancin caused localized cell wall thickening in MRSA which contrasts with the thinning that was observed following telavancin exposure [197]. Such differences, as revealed by TEM, indicate subtleties in the mechanisms of action of these agents and their impact on the cell wall and membrane. Importantly, ultrastructural studies demonstrate that oritavancin and telavancin have pronounced effects on the division septum of *S. aureus* which are not seen with vancomycin. This is likely an indication that lipoglycopeptides target critical yet vulnerable sites to achieve their rapid bactericidal activity.

9.4 In Vitro Spectrum of Antibacterial Activity, Protein Binding, Time Kill Studies and Resistance Development

9.4.1 Spectrum of Activity and Potency

The relatively large size and chemical nature of the glycopeptides and lipoglycopeptides preclude their crossing of the Gram-negative outer membrane to reach their target. Thus, spectrum of activity of the glycopeptides and lipoglycopeptides is limited to Gram-positive organisms. However, activity encompasses a wide range of Gram-positive organisms, whether anaerobic or aerobic, including those that cause life-threatening infections such as staphylococci, enterococci and streptococci. For oritavancin and dalbavancin, guidelines for in vitro susceptibility testing require inclusion of 0.002% polysorbate-80 to prevent binding of drug to plastic [7, 11, 52]. This requirement has not been noted for vancomycin, teicoplanin, or telavancin [52]. Table 9.1 summarizes in vitro activity of glycopeptides and lipoglycopeptides against key disease-causing organisms as determined by minimum inhibitory concentrations (MIC). Due to the large number of studies in the literature, typically only the largest and/or most recent are summarized in the Table, for clarity.

By MIC_{90}, all the glycopeptides and lipoglycopeptides show equivalent activity (MIC_{90} identical or within one doubling dilution) against methicillin-susceptible *S. aureus* (MSSA) and MRSA, and against methicillin-susceptible coagulase-negative staphylococci (MS-CoNS) and methicillin-resistant coagulase-negative staphylococci (MR-CoNS) (Table 9.1). Dalbavancin, oritavancin, and telavancin are 2- to 16-fold, and 4- to 32-fold more potent than vancomycin and teicoplanin against MSSA/MRSA and MS-CoNS/MR-CoNS, respectively. For telavancin and oritavancin, robust activity has been demonstrated against both *pvl*$^{+}$ and *pvl* $^{-}$ community-associated MRSA isolates [12, 217]. Reduced activity is observed for the glycopeptides and lipoglycopeptides against VISA and hVISA strains when compared to MSSA/MRSA isolates. Telavancin MICs are generally one doubling dilution higher for hVISA and VISA isolates compared to non-hVISA, -VISA, and -VRSA strains. Vancomycin, teicoplanin, and oritavancin show MIC_{90} values that range from 2- to 16-fold higher against hVISA and VISA isolates compared to MSSA/MRSA (Table 9.1). There are no reports of dalbavancin activity against VISAs and hVISAs using the current approved methodology of susceptibility testing for dalbavancin. Vancomycin and teicoplanin show little or no activity against VRSA isolates (MIC_{90}s of >512 and >128, respectively). Oritavancin activity is reduced fourfold against the VRSA isolates (MIC_{90}=0.5) compared to MSSA/MRSA [13]. Telavancin MIC range against six VRSA isolates (2–4 μg/ml) is at least fourfold less potent than against MSSA/MRSA, VISA, and hVISA isolates [131]. There are no reports of dalbavancin activity against VRSA isolates using the current approved methodology of susceptibility testing for dalbavancin.

All the glycopeptides and lipoglycopeptides show activity against vancomycin-susceptible (VS) enterococci. Against VS *E. faecalis*, oritavancin, and dalbavancin are the most active (MIC_{90}=0.06 μg/ml) followed by teicoplanin (MIC_{90} =0.25 μg/ml),

Table 9.1 In vitro potency of glycopeptides and lipoglycopeptides

Organism	Antibiotic	n	MIC range (μg/ml)	MIC_{90} (μg/ml)	Reference
MSSA	Vancomycin	27,052	≤0.12–4	1	[30]
	Oritavancin	4,193	≤0.004–0.5	0.12	[16]
	Teicoplanin	4,193	≤0.06–4	1	[16]
	Dalbavancin	27,052	≤0.03–0.25	0.06	[30]
	Telavancin	1,217	≤0.03–1	0.5	[73]
MRSA	Vancomycin	19,721	0.25–4	1	[30]
	Oritavancin	4,882	≤0.004–4	0.12	[16]
	Teicoplanin	4,882	≤0.06–16	1	[16]
	Dalbavancin	19,721	≤0.03–0.5	0.06	[30]
	Telavancin	1,082	0.06–1	0.25	[73]
hVISA	Vancomycin	11	1–2	2	[13]
	Oritavancin	11	0.12–2	1	[13]
	Teicoplanin	11	1–8	8	[13]
	Dalbavancin	N/A	N/A	N/A	
	Telavancin	2	0.25–0.5	N/A	[131]
VISA	Vancomycin	14	4–8	8	[13]
	Oritavancin	14	0.5–4	2	[13]
	Teicoplanin	14	2–32	16	[13]
	Dalbavancin	N/A	N/A	N/A	
	Telavancin	26	0.25–1	1	[131]
VRSA	Vancomycin	10	16–>512	>512	[13]
	Oritavancin	10	0.12–1	0.5	[13]
	Teicoplanin	10	32–>128	>128	[13]
	Dalbavancin	N/A	N/A	N/A	
	Telavancin	6	2–4	NA	[131]
MS-CoNS	Vancomycin	2,836	≤0.12–4	2	[30]
	Oritavancin	213	0.008–1	0.25	[214]
	Teicoplanin	213	0.25–8	4	[214]
	Dalbavancin	2,836	≤0.03–1	0.06	[30]
	Telavancin	100	0.06–1	0.25	[73]
MR-CoNS	Vancomycin	9,472	≤0.12–8	2	[30]
	Oritavancin	649	≤0.004–1	0.25	[214]
	Teicoplanin	649	0.25–16	4	[214]
	Dalbavancin	9,472	≤0.03–0.12	0.12	[30]
	Telavancin	272	0.12–1	0.5	[73]
GAS	Vancomycin	959	≤0.06–1	0.25	[16]
	Oritavancin	959	≤0.0005–0.5	0.25	[16]
	Teicoplanin	29	0.001–0.25	0.06	[11]
	Dalbavancin	2,182	≤0.03–0.25	≤0.03	[30]
	Telavancin	68	0.015–0.12	0.06	[73]
GBS	Vancomycin	415	0.25–0.5	0.5	[16]
	Oritavancin	415	0.001–1	0.25	[16]
	Teicoplanin	29	0.03–0.12	0.06	[11]
	Dalbavancin	2,265	≤0.03–0.25	≤0.03	[30]
	Telavancin	45	0.03–12	0.06	[73]
VS – *E. faecalis*	Vancomycin	1,651	≤0.12–4	2	[16]
	Oritavancin	1,651	≤0.0005–1	0.06	[16]
	Teicoplanin	1,651	≤0.03–4	0.25	[16]
	Dalbavancin	10,025	≤0.03–0.5	0.06	[30]
	Telavancin	429	0.12–1	1	[73]

(continued)

Table 9.1 (continued)

Organism	Antibiotic	n	MIC range (μg/ml)	MIC_{90} (μg/ml)	Reference
VNS – *E. faecalis*	Vancomycin	87	8–>256	>256	[16]
	Oritavancin	87	0.015–1	1	[16]
	Teicoplanin	87	0.12–256	256	[16]
	Dalbavancin	349	≤0.03–>4	>4	[30]
	Telavancin	29	0.25–16	16	[72]
VanA *E. faecalis*	Vancomycin	65	>128–>256	>256	[16]
	Oritavancin	65	0.03–1	1	[16]
	Teicoplanin	65	32–256	256	[16]
	Dalbavancin	230	≤0.03–>4	>4	[30]
	Telavancin	22	4–16	16	[73]
VanB *E. faecalis*	Vancomycin	17	32–>256	>256	[16]
	Oritavancin	17	0.015–0.06	0.06	[16]
	Teicoplanin	17	0.12–8	8	[16]
	Dalbavancin	84	≤0.03–>4	4	[30]
	Telavancin	4	0.25–1	NA	[73]
VS – *E. faecium*	Vancomycin	350	0.03–4	1	[16]
	Oritavancin	350	≤0.0005–0.25	0.03	[16]
	Teicoplanin	350	≤0.015–8	1	[16]
	Dalbavancin	2,578	≤0.03–2	0.12	[30]
	Telavancin	92	≤0.015–0.5	0.25	[73]
VNS -*E. faecium*	Vancomycin	469	8–>256	>256	[16]
	Oritavancin	469	≤0.0005–1	0.25	[16]
	Teicoplanin	469	0.12–>256	128	[16]
	Dalbavancin	2,176	≤0.03–>4	>4	[30]
	Telavancin	245	<0.015–16	8	[72]
VanA *E. faecium*	Vancomycin	421	32–>256	>256	[16]
	Oritavancin	421	0.004–1	0.25	[16]
	Teicoplanin	421	32–>256	128	[16]
	Dalbavancin	1,744	≤0.03–>4	>4	[30]
	Telavancin	223	≤0.015–16	8	[73]
VanB *E. faecium*	Vancomycin	29	32–>256	256	[16]
	Oritavancin	29	0.004–0.06	0.06	[16]
	Teicoplanin	29	0.12–8	8	[16]
	Dalbavancin	134	≤0.03–>4	1	[30]
	Telavancin	17	0.12–4	2	[73]

MSSA methicillin-susceptible *S. aureus, MRSA* methicillin-resistant *S. aureus*, *hVISA* heterogeneous vancomycin-intermediate *S. aureus*, *VISA* vancomycin-intermediate *S. aureus*, *VRSA* vancomycin-resistant *S. aureus*, *MS CoNS* methicillin-susceptible coagulase-negative staphylococci, *MR CoNS* methicillin-resistant coagulase-negative staphylococci, *GAS* group A streptococcus, *GBS* group B streptococcus, *VS* vancomycin susceptible, *VNS* vancomycin non-susceptible, *N/A* not available, *NA* not applicable (MIC_{90}s are not reported when n ≤ 10 isolates)

telavancin (MIC_{90} =1 μg/ml) and vancomycin (MIC_{90} =2 μg/ml). Against VS *E. faecium*, oritavancin is the most active (MIC_{90} = 0.03 μg/ml), followed by dalbavancin (MIC_{90} = 0.12 μg/ml), telavancin (MIC_{90} = 0.25 μg/ml) and vancomycin and teicoplanin (MIC_{90} = 1 μg/ml). Vancomycin is inactive against both Van A- and VanB-expressing vancomycin-resistant enterococci (VRE) with MIC_{90} of >256 μg/ml.

Teicoplanin shows some activity against Van B-expressing VRE (MIC_{90}=8 μg/ml for both *E. faecalis* and *E. faecium*), consistent with the observation that it is not an effective inducer of this operon [79] but is inactive against Van A-expressing VRE (MIC_{90}s of 256 μg/ml and 128 μg/ml for *E. faecalis* and *E. faecium*, respectively). Only oritavancin retains strong activity against VanA *E. faecalis* and *E. faecium* strains with MIC_{90}s of 1 and 0.25 μg/ml, respectively. Oritavancin MIC_{90} values against Van B *E. faecalis* and *E. faecium* are within twofold of those of the respective VS counterparts. This is not found for the other glycopeptides and lipoglycopeptides where MICs against VS enterococci are lower than those against Van B isolates.

Glycopeptides and lipoglycopeptides show excellent activity against Group A streptococci, with MIC_{90}s ranging from ≤0.03 μg/ml for dalbavancin to 0.25 μg/ml for vancomycin and oritavancin (Table 9.1). Similar excellent activity is found against Group B streptococci with MIC_{90}s ranging from ≤0.03 μg/ml for dalbavancin to 0.5 μg/ml for vancomycin.

Glycopeptides and lipoglycopeptides demonstrate significant in vitro activity against *Clostridium difficile* [87, 95, 98, 179, 180]. Activity against *Bacillus anthracis* has been demonstrated for vancomycin, teicoplanin, oritavancin, telavancin, and dalbavancin in vitro [42, 106, 107, 117, 127, 248] and for dalbavancin and oritavancin in animal models [107, 108].

9.4.2 Protein Binding

Estimates of protein binding for the glycopeptides and lipoglycopeptides range from 46% to >95% [9, 17, 21, 246]. Because of the high percentage of protein binding, in vitro activity profiles of the glycopeptides and lipoglycopeptides are diminished up to eightfold in the presence of serum or purified albumin [14, 142, 161, 247]; however, recent findings of a lower-than-anticipated impact of serum binding on in vitro activity of vancomycin, teicoplanin, and telavancin support the concept of an "active fraction" [246] that may offer additional insight into the pharmacodynamic behavior of protein-bound drugs.

9.4.3 Time Kill Studies

In vitro time-kill assays elucidate the rate and extent of antibacterial activity and are recognized for their value in characterization of new agents. Such studies distinguish bacteriostatic (0–<99.9% kill relative to starting inoculum at 24 h) from bactericidal (>99.9% kill relative to starting inoculum at 24 h) antibiotics [172]. Time-kill studies with vancomycin and teicoplanin at concentrations that approximate their free maximal concentration (C_{max}) and free trough in plasma when administered at approved doses for complicated skin and skin structure infection have shown that both drugs are slowly bactericidal against vancomycin-susceptible *S. aureus* strains but had no effect on a vancomycin-resistant strain [160].

Against two VISA strains, teicoplanin was not bactericidal at both the free C_{max} and free trough concentrations while vancomycin was bactericidal only at free C_{max} against one of the strains [160]. Oritavancin demonstrated concentration-dependent and rapid bactericidal activity (within 15 min to 2 h) against vancomycin-susceptible strains and a strain of VRSA [160]. At its predicted free C_{max} from 200 mg once-daily dosing, oritavancin was bactericidal at 24 h against one of two VISA strains [160]. Telavancin demonstrated bactericidal activity in time-kill studies at or above 4X MIC against hVISA, VISA and VRSA strains [142]. Telavancin was also bactericidal at or above 2X MIC against MRSA, *Staphylococcus epidermidis*, *Streptococcus agalactiae*, *Streptococcus pneumoniae* and *Streptococcus pyogenes,* but showed bacteriostatic, concentration-independent activity against an *E. faecalis* strain [132]. Dalbavancin was bactericidal at ≥1 μg/ml in time-kill studies against *S. aureus* strains including a VISA strain [97].

Vancomycin and teicoplanin in time-kill studies at predicted free C_{max} and free trough concentrations were bacteriostatic or had no effect against enterococci, including VSE and VanA and VanB VREs [160] as well as against Group A streptococci [15]. Whereas oritavancin was bacteriostatic at both its free trough and free C_{max} levels predicted from 200 mg daily dosing against the enterococci, except for a VSE strain for which oritavancin was bactericidal at free C_{max} concentration, it achieved 99.9% killing of all enterococci tested, regardless of VanA or VanB phenotype, when tested at its free C_{max} from an 800 mg dose [160]. This bactericidal activity against enterococci is unique amongst the glycopeptides and lipoglycopeptides. Whether it is related to the interactions between oritavancin and branch peptides in enterococci as demonstrated by REDOR-NMR (as described above; Patti et al. [188]) or to more effective target binding relative to other glyco- and lipoglycopeptides remains to be demonstrated. Against Group A streptococci, oritavancin was bactericidal at both free C_{max} and free trough concentrations from 200 mg dosing [15].

9.5 Resistance Development

Compared to other classes of antibiotics such as the rifamycins and fluoroquinolones, resistance development to the glycopeptides and lipoglycopeptides has been slow to emerge. In vitro resistance development to dalbavancin in staphylococci by direct selection (single-step) or step-wise selection (serial passages) failed to yield stable mutants with elevated MICs relative to parental strains [97]. Stepwise selection for resistance to vancomycin and teicoplanin in *S. aureus* showed fourfold and an eightfold increases, respectively, in MIC relative to parental strains [131]. In another report, a derivative of a vancomycin-resistant strain that was serially selected on vancomycin was shown to have a 32- to 64-fold increase in vancomycin MIC [36]. This strain also showed a 16- to 128-fold increase in teicoplanin MIC, a 16- to 64-fold increase in dalbavancin MIC but only a 2- to 4-fold increase in oritavancin MIC [36]. For telavancin, an isolate with an eightfold increase in MIC was obtained in serial passages [131]. Stepwise selection for reduced susceptibility to oritavancin yielded

S. aureus isolates with MIC increases of maximally four- to eightfold relative to parental strains [235]. Single-step resistance selection rates for vancomycin, teicoplanin, and telavancin are low [131]. The poor performance of oritavancin in agar assays [31], due to its slow diffusion and binding to agar, has rendered the interpretation of experiments to investigate single-step resistance selection to oritavancin difficult [31].

Isolates with reduced susceptibility resulting from exposure to vancomycin and teicoplanin during therapy in humans have been reported [25, 154, 225]. Similarly, isolates with reduced susceptibility have been isolated in a rat model after exposure to vancomycin and teicoplanin [254]. In telavancin and oritavancin clinical studies, isolates with reduced susceptibility to the respective test drugs relative to baseline isolates have not yet been encountered [81, 231].

9.6 Bacterial Resistance Mechanisms

At least one mechanism of action of glycopeptides and lipoglycopeptides is binding of D-alanyl-D-alanine (D-Ala-D-Ala) termini of intermediates during peptidoglycan biosynthesis thereby inhibiting transglycosylation and transpeptidation [67]. In enterococci, resistance to vancomycin and teicoplanin results from synthesis of modified peptidoglycan precursors that end in D-Ala-D-Lac as found in strains expressing *van*A, *van*B or *van*D cassettes, or in D-Ala-D-Ser as found in strains expressing *van*C, *van*E, *van*G or *van*L cassettes; these termini have reduced affinities for vancomycin and teicoplanin [193, 255, 260]. In addition, enterococcal strains expressing glycopeptide-resistance genes produce D-alanyl-D-alanine dipeptidase that eliminates residual wild-type D-Ala-D-Ala termini of the peptidoglycan precursors, thereby resulting in vastly decreased effectiveness of vancomycin [192]. The organization, structure and features of the enterococcal glycopeptide resistance genes and the effects of their expression have been reviewed by Depardieu et al. [67].

Widespread use of vancomycin and teicoplanin in the treatment of infections caused by methicillin-resistant *S. aureus* has resulted in the emergence of isolates with reduced susceptibility to these glycopeptides. Current CLSI breakpoints for vancomycin are: susceptible, ≤2 μg/ml; intermediate, 4–8 μg/ml; resistant, ≥16 μg/ml [52]. Two types of strains with reduced susceptibility to glycopeptides have been described.

The first are glycopeptide-intermediate strains which are characterized by a thickened and a poorly cross-linked cell wall that accumulates D-Ala-D-Ala termini [59, 60]. It is thought that the thickened cell wall and accumulated termini sequester glycopeptides at the periphery thereby precluding the drugs from reaching their target [58–61, 124]. Strains that show heterogeneous intermediate resistance to vancomycin (hVISA) have been reported and are thought to be precursors to VISA strains [210]. While the phenotypic characteristics that may be associated with the VISA and hVISA phenotypes such as thickened cell walls, reduced peptidoglycan cross-linking and increased numbers of D-Ala-D-Ala residues have been clearly

defined, the genetic basis of the phenotypes is only beginning to emerge. Recent reports have linked the *vra*RS and *graRS* two-component systems to the VISA/hVISA phenotype [61, 110, 168, 174]. In one case, mutations in the response regulator, *graR*, were associated with the conversion from hVISA to VISA, but they had no impact on vancomycin susceptible strains [174]. Other reports link the sensor protein, *graS*, with increased vancomycin MIC in a vancomycin-susceptible strain [61, 110]. Although mutations in these two-component regulatory genes have been identified and implicated in the hVISA/VISA phenotype, they were not universally found in all strains that show the hVISA/VISA phenotype [61, 110], suggesting that mutations in other regions of the genome may lead to the hVISA/VISA phenotype.

The second type of *S. aureus* strains with reduced susceptibility to glycopeptides are the vancomycin-resistant *S. aureus* (VRSA). These are characterized by carriage and expression of the *van*A cassette, resulting in synthesis of peptidoglycan precursors that terminate in D-Ala-D-Lac with concomitant resistance to vancomycin and teicoplanin [193]. In almost all the VRSA isolates characterized, the *van*A cassette has been shown to have been transferred from a vancomycin-resistant Enterococcus [193]. To date, only 12 VRSA isolates have been described – ten from the US, seven of which were in the state of Michigan [171], and one each from India and Iran [1, 213].

9.7 Adsorbtion Distribution Metabolism and Excretion

9.7.1 ADME of Vancomycin and Teicoplanin

Vancomycin and teicoplanin have been used to treat infections for many years and their absorption, distribution, metabolism, and excretion (ADME) properties have been well characterized. The main pharmacological properties of these two glycopeptides and of the new lipoglycopeptides are summarized in Table 9.2. All five antimicrobial agents are poorly bioavailable and require parenteral administration.

Vancomycin has a relatively short half-life (4–8 h) [250], which requires a more frequent dose regimen – twice daily or continuous infusion. It binds moderately to serum proteins (10–55%) [227, 251]. The recommended dose for vancomycin generates a low AUC:MIC ratio [209] and it is necessary to monitor serum levels to optimize dosages [211, 212]. For systemic exposure, vancomycin is administered intravenously and does not distribute well in tissues; it has a low cellular accumulation [251]. Vancomycin is excreted via the kidneys in urine and as a result, vancomycin doses should be adjusted in case of renal insufficiency [251]. Vancomycin is not absorbed orally and as a result, has been found to have utility against *C. difficile* colitis [259, 268], being the only treatment approved by the US Food and Drug Administration (FDA) [91], with approval as a capsule formulation (Vancocin®) for this indication.

Table 9.2 Pharmacology properties of the glyco- and new lipoglyco-peptides molecules

Parameters	Vancomycin [250]	Teicoplanin [251, 262]	Telavancin [224]	Oritavancin [203]	Dalbavancin [141]
Dose (mg/kg)	15	6	10	3[a]	15
C_{max} (µg/ml)	20–50	43	87.5	25.9	312
Volume of distribution (l/Kg)	0.3	0.9–1.6	0.115	0.10[b]	0.11
Calculated AUC (µg*h/ml)	260	550 (mg*h*l)	858	133	27,103 (mg*h*l)
Half-life (h)	4–8	83–168	7.5	394	149
Clearance (l/h/Kg)	0.058	0.011	11.8 ml/h/Kg	0.584 (l/h)	0.04 (l/h)
% protein binding	10–55	90	90–93	86–90	93–98

[a]The clinical dose of oritavancin for the new clinical Phase 3 study will be 1,200 mg

[b]Total body volume of distribution of oritavancin is 110 l [203]

Teicoplanin has a higher degree of serum protein binding than vancomycin (90–93% versus 10–55%, respectively) [130]. This contributes to its longer half-life compared to vancomycin [262]. The longer half-life of teicoplanin allows a once-daily administration. Teicoplanin is also differentiated from vancomycin by its higher volume of distribution which indicates increased drug penetration into the tissues and organs. Penetration of teicoplanin into fat and cerebrospinal fluid is limited but it is known to penetrate well into bone tissue [74]. Three percent of the dose of teicoplanin is metabolized by the body [262]. Teicoplanin is mainly excreted via urine and consequently, the dosage of patients with renal impairment should be adjusted [80, 269].

9.7.2 ADME of New Lipoglycopeptides

Investigational (dalbavancin, oritavancin) or recently approved (telavancin) lipoglycopeptides were developed with a focus to improve the PK-PD characteristics of previously used glycopeptides. In this perspective, the longer half-life of these new drugs is one of the most interesting features since it allows a once-a-day dosing or in some cases, allows for an infrequent or even single dose regimen. Dalbavancin, oritavancin and telavancin need to be administered intravenously to achieve systemic exposure since they are poorly bioavailable when administered orally. These three lipoglycopeptides have a higher percentage protein binding than vancomycin. It is to be noted that the C_{max} in blood as well as the area under the plasma concentration curve (AUC) are important PK parameters for the lipoglycopeptides as AUC/MIC is a key parameter to predict efficacy, and Cmax/MIC is likely to be important as well.

9.7.3 Dalbavancin

In healthy subjects, the levels of dalbavancin in plasma showed dose-related increases following administration of single and multiple intravenous doses. At steady state, after a 15 mg/kg intravenous dose, the dalbavancin median C_{max} in blood is 312 μg/ml ranging from 292 to 371 μg/ml and the median AUC is 27,103 μg•h/L ranging between 22,937 and 27,299 μg•h/L [141]. Dalbavancin's pharmacokinetic profile is characterized by a prolonged long half-life. Mean half-life ranges from 149 to 300 h in human subjects [70, 71, 141]. The long half-life associated with dalbavancin administration supports once-weekly dosing for infections with dalbavancin-susceptible organisms [70, 71]. Dalbavancin serum protein binding is high (93–98%) [113, 252].

Dalbavancin distributes well in the body and the mean penetration of dalbavancin into skin blister fluid was 59.6% with mean concentrations after 7 days of 46.5 μg/ml in the plasma and 30.3 μg/ml in skin blister fluid [177]. A higher concentration of dalbavancin in the periosteum, articular cartilage, epiphyseal disk, and bone marrow than in blood has been reported 14 days after a single dose [226].

In humans, dalbavancin is excreted in urine with approximately 25–45% of the administered dose excreted unchanged [41, 71, 141]. In rats, after 70 days, 44.2% and 22.3% of the radioactivity-labeled dalbavancin was recovered in the urine and feces, respectively [41].

9.8 Oritavancin

Pharmacokinetic (PK) studies have shown that oritavancin displays linear PKs for weight-based or fixed dose ranges [203]. A population pharmacokinetic study revealed that a single 200 mg dose of oritavancin generates a median C_{max} of 25.9 (10.9–131) μg/ml and a median AUC_{0-24h} of 133 (42.2–618) μg•h/ml [137]. It remains in the body for an extended duration, with a half-life of 394 h which is longer than those of vancomycin and telavancin. Its long half-life allows for a once-a-day administration, and it has been suggested that it could be potentially used in an infrequent or even a single dose regimen [250]. The PK study suggested that dose modification may be necessary in patients weighing >110 kg but not for elderly patients [201].

Oritavancin is approximately 85% bound to serum from humans, mice, rats, and dogs [17] is extensively distributed in tissues [28], with a total body volume of distribution estimated at approximately 110 l [203], and it accumulates in lysosomes of macrophages, fibroblasts, and other cell lines [253]. Oritavancin distribution in skin blister fluid was assessed in healthy subjects by comparing a daily dose of 200 mg for 3 days to a single 800 mg dose [86]. While the mean peak concentration of oritavancin was approximately 8- to 11-fold higher in plasma than in blister fluid, the mean peak concentration in blister fluid remains approximately 50- to 100-fold above the MIC_{90} of oritavancin against *S. aureus* [16].

In a murine pneumonia model, oritavancin levels in lung ELF were sufficient to kill *S. pneumoniae* (in part owing to the exquisite susceptibility of the test strain; MIC = 0.001 μg/ml) [138] but a population PK model suggested that caution should be taken with regard to potential use of oritavancin for treatment of *S. aureus* pneumonia [29], perhaps at least in part due to the observation of modestly reduced activity of oritavancin in vitro in the presence of lung surfactant preparation [137]. In cell cultures, oritavancin has been found to accumulate in lysosomes of phagocytic and non-phagocytic cells [250] and in alveolar macrophages of healthy volunteers [202]. Because *S. aureus* and enterococci may reside within these cells, and since oritavancin demonstrates potent activity against intracellular staphylococci and enterococci [175], this observation raises the possibility that oritavancin could offer a therapeutic benefit to eradicate these microorganisms if they persist in these compartments [195].

Oritavancin is excreted in feces and urine and no metabolites have been detected. Less than 5% and 1% of administered drug were recovered in the urine and feces, respectively, after 7 days [28].

9.9 Telavancin

Telavancin exhibits linear, predictable pharmacokinetics. A single 10 mg/kg intravenous dose resulted in a C_{max} of 87.5 µg/ml and with a $AUC_{0\text{-}\infty}$ of 858 µg•h/ml [224]. The half-life of 7.5 h supports the once-daily dosing [270]. The drug is more extensively protein-bound than vancomycin (90–93% versus 10–55%, respectively) [224]. The mean penetration of telavancin into blister fluid was 40% [233], and the median AUC in lung epithelial lining fluid (ELF) is approximately 75% of the free AUC in plasma [144]. Telavancin is not affected by surfactant [99]. Telavancin is cleared primarily by renal excretion with 60–70% of the dose excreted unchanged in urine [46, 263]. A dosage adjustment is recommended for patients with renal impairment [270].

9.10 In Vivo Activity and Pharmacodynamics

9.10.1 Animal Models

Glyco- and lipoglycopeptides have been tested for efficacy in a variety of animal models against several pathogens. Studies describing the in vivo efficacy of these antimicrobials are listed in Table 9.3, and the ED_{50}s are included when specified by the authors.

It should be highlighted that teicoplanin showed reasonable efficacy in the implant related osteomyelitis models when formulated as biodegradable particles for treatment of bone-infected implants [184, 249, 266]; however, no efficacy of teicoplanin was observed when it was administered systemically in a rabbit osteomyelitis model [178]. Telavancin showed robust efficacy in a murine model of *S. aureus* pneumonia, supporting the potential clinical utility of telavancin in the treatment of MSSA pneumonia [104]. With their long half-lives, both dalbavancin and oritavancin have been confirmed to have good potential to be used in infrequent dosing regimens. In fact, the two drugs provided substantially more prolonged efficacy than vancomycin in the rat granuloma pouch model of *S. aureus* infection when administered as single doses [112, 136]. Oritavancin also has been shown to have strong activity against VRE in the ascending pylonephritis model in mice [34]. Finally, oritavancin showed unrivalled single-dose efficacy in a murine model of *B. anthracis* spore inhalation anthrax [107] and was also highly active in the hamster model of *C. difficile* infection [139].

9.11 Pharmacodynamics

As the first members of the family, vancomycin and teicoplanin have been used as controls and comparators in numerous experiments. The most important parameter to predict efficacy for vancomycin is its AUC/MIC [100, 165, 166]; however,

Table 9.3 Animal models in which glycopeptides and lipoglycopeptides have been tested for in vivo efficacy

		ED50 (mg/kg)				
Animal model	Microorganism	Vancomycin	Teicoplanin	Telavancin	Oritavancin	Dalbavancin
Mouse systemic/bacteremia infection	*S. aureus*	1.12 [39]	0.20 [39]	[105, 198]	0.15 [140]	0.08 [39]
	S.pneumoniae	0.79 [39, 128]	0.40 [39, 128]		0.18 [129]	0.56 [39]
	S. epidermidis	7.07 [39]	10.71 [39]			0.38 [39]
	E. faecali		0.51 [39]			1.53 [39]
Mouse pneumonia	*S. aureus*	[104]		[104]		
	S. pneumoniae				2.8 [56,137]	
Mouse inhalation anthrax	*B. anthracis*				1.2 [107]	[127]
Neutropenic mouse thigh infection	*S. aureus*	10.6 [103]	[191]	2.5 [103]	0.95 [35, 56]	37 [9]
	S. pneumoniae				[56]	0.91 [9]
Mouse subcutaneous infection	*S. aureus*	2.8 [103]		0.9 [103]		
Hamster *C. difficile* infection	*C. difficile*	[111, 139]			[139]	
Rat tissue cage	*S.aureus*		[218]			
Rat lobar pneumonia	*S. pneumoniae*					[39]
Rat haematogenous pneumonia	*S. aureus*				[185]	
Rat central venous catheter	*S. aureus*				[207]	
	E. faecium				[208]	
Granuloma pouch infection	*S.aureus*	[112, 136]			[136]	[112]
Rat endocarditis	*S. aureus*	[39]	[39]		[264]	[39]
	S. epidermidis	[39]	[39]			[39]
Tissue cage	*S. aureus*		[158]			
Mouse subcutaneous device infection	*S. aureus*	[265]	[19]		[265]	[19]
Rabbit subcutaneous device infection	*S. aureus*	[64]		[64]		[63]
Rabbit endocarditis	*S. aureus,*	[116, 148]	[18]	[148, 164]	[116]	[135]
	E. faecalis				[134, 216]	
Rabbit meningitis	*PSSP/PRSP*	[232]		[232]	[38, 90]	[232]
Osteomyelitis			[114, 249]	[267]		

Table 9.4 Pharmacodynamic breakpoint for glycopeptides and lipoglycopeptides [250]

Breakpoint	Vancomycin	Teicoplanin	Oritavancin	Telavancin	Dalbavancin
Free 24 h AUC/MIC Target	180[d]	nd	186–275[a,b]	3[a,c]	100–300[a]

nd not determined
[a] For *S. aureus* in the thigh infection model
[b] calculated 86% protein binding
[c] Authors considered 94–96% mouse plasma protein binding
[d] Free AUC:MIC value based on 55% protein binding if the total drug AUC:MIC value is 400

Cmax/MIC has also been found to play an important role [130]. In a mouse peritonitis model, Knudsen and colleagues found that the time the free fraction of the drug is above the MIC and C_{max} are the best parameters to predict the efficacy of teicoplanin [234]. The Infectious Diseases Society of America, the American Society of Health-System Pharmacists, and the Society of Infectious Diseases Pharmacists have recently updated guidelines for the use of vancomycin. The guideline recommends that the AUC/MIC ratio is the most useful pharmacodynamic parameter to predict vancomycin effectiveness and suggested a target ratio of 400 or greater to eradicate *S. aureus* [228].

Telavancin, dalbavancin, and oritavancin all display concentration-dependent activity that is best characterized by AUC/MIC and Cmax/MIC ratios [29, 35, 71, 103, 138, 195, 250, 270]. Pharmacodynamic breakpoints for clinical doses are compared in Table 9.4.

In the widely used neutropenic-mouse thigh infection, Andes and Craig showed that both the $AUC_{0–24}$: MIC and the C_{max}/MIC parameters correlated well with dalbavancin in vivo activity against *S. pneumoniae* ($r^2=78\%$ and 77%, respectively) and *S. aureus* ($r^2=90\%$ and 57%, respectively) [9]. The authors proposed that the free $AUC_{0–24}$/MIC target associated with efficacy against *S. aureus* was in the range of 100–300. Also in the neutropenic-mouse thigh infection model, in a dose fractionation study, it was demonstrated that oritavancin C_{max}/MIC was the PK-PD index that correlated the best with bactericidal activity against *S. aureus* [35]. Both $AUC_{0–24}$/MIC and the C_{max}/MIC parameters were found to best correlate with oritavancin in vivo efficacy against *S. pneumoniae* in a mouse lung infection model [138]. By simulating a human equivalent dose of oritavancin in the murine thigh infection model, the average 24 h AUC/MIC ratios corresponding to net bacterial stasis, 1-, 2- and 3-Log_{10} CFU reduction from baseline of *S. aureus*, were 1,330, 1,503, 1,694 and 1,967, respectively, at 48 h post-infection [182]. Studies simulating the human equivalent exposure also proposed that front-loading of oritavancin resulted in a faster and more sustained decline in CFU compared to an equivalent total dose fractionated over time [182]. This finding has been recently confirmed in a phase 2 study [75] as described in the Clinical Experience section below.

Pharmacokinetic-pharmacodynamic studies of telavancin in the neutropenic-mouse thigh infection model demonstrated that the 24 h AUC/MIC ratio was the best predictor of efficacy and that telavancin produced dose-dependent bacterial reduction in infected thighs [103]. From these experiments, it was proposed that the $AUC_{0–24}$/MIC ratio (free drug) of telavancin required for stasis is three.

9.12 Clinical Experience

9.12.1 Vancomycin and Teicoplanin

Members of the family of glycopeptide antibiotics have been in human use for more than 50 years. Two glycopeptides, vancomycin and teicoplanin, are widely used today. Vancomycin has been approved for human use in the United States since 1958 and has become an important agent for the treatment of antibiotic-resistant Gram-positive bacteria around the world [100, 165, 166]. Teicoplanin was first isolated in 1983 from an *Actinoplanes* culture and first authorized for human use in Europe in 1989. Teicoplanin has a serum half-life that allows once a day dosing, an advantage over vancomycin's twice a day dosing regimen. Teicoplanin has not been approved for use in the United States.

These two glycopeptides are used as systemically administered agents for the treatment of methicillin-resistant *S. aureus*. Teicoplanin can be administered both intravenously and intramuscularly while vancomycin is used intravenously only. Both also have utility as oral, non-absorbed, agents for the treatment of *C. difficile* colitis [65], although teicoplanin is not available commercially in an oral formulation. In a recent review and meta-analysis, vancomycin and teicoplanin showed similar effectiveness and some safety advantages were uncovered favoring teicoplanin [234].

Though very widely used, both of these glycopeptides have limitations (see reviews by Stevens [228]). Emerging resistance is being seen to both in enterococci and staphylococci (VRE, VISA, hVISA, VRSA) [55]. The slow bactericidal activity of these agents is considered a weakness and "MIC creep" (proposed gradual incremental decreases in susceptibility) may contribute to decreasing utility [68, 215].

To overcome the limitations of the widely used vancomycin and teicoplanin efforts have been successful in advancing dalbavancin, oritavancin, and telavancin into the clinic and in one case (telavancin) to initial approval.

9.13 New Lipopglycopeptide Clinical Efficacy and Safety

9.13.1 Dalbavancin

Dalbavancin has advanced into Phase 3 clinical testing for the complicated skin and skin structure indication (cSSSI). Taking advantage of the substantial serum half-life of the molecule, dalbavancin at a 1 g dose on day 1 and a 500 mg dose on day 8 was compared to 600 mg twice a day of linezolid, each for 14 days (Table 9.5). Both the clinical efficacy result and the microbiological result at the test of cure were similar [113]. In this study, there were 434 clinically-evaluable patients in the dalbavancin arm and 226 in the linezolid arm. 51% of the pathogens in the microbiologically evaluable group were MRSA. The treatment emergent adverse event percentages were favorable toward the dalbavancin arm, but not statistically significant.

Table 9.5 Clinical studies of dalbavancin, oritavancin, and telavancin

Phase 2	Ref.	Dose/duration	CE % success TOC	ME % success TOC	% MRSA	% AE
Dalbavancin	[221]	1,100 mg or 1,000 + 500 mg on day 8 or standard of care open label	61.5% 94.1% 76.2% (~20 CE per arm)	92 58 71	38 43 14 32 overall	
Dalbavancin CRBI	[196]	1 g + 500 mg on day 8 versus vancomycin 1 g BID 14 days	87% 50% 18–24 days after last dose	96 79 (~25/arm)	19 32	100 91
Telavancin	[229]	7.5 mg/kg QD or 1 g vanco BID or 2 g nafcillin or oxacillin QID	92% 96% (~84 CE per arm) 7–14 day after last dose	80 82	43	56 60
Telavancin	[230]	10 mg/kg QD or 1 g vancomycin BID/ antistaphylococcal β-lactam	82% 85% (~100 CE per arm) 7–14 days after last dose	97 93 (~60//arm)	29	56 57
Oritavancin	[75]	1,200 mg single dose, 800 mg + 400 mg day 5, 200 mg QD 3–7 days	81.5%, 77.5% 72.4%	79 81 69	51	56, 61, 61
Phase 3						
Dalbavancin	[113]	1,000 + 500 day 8 versus linezolid 600 BID 14 days	88.9% 91.2% 14 days after last dose	89.5 87.5	51	25 32
	[48, 49]	1,000 + 500 versus cefazolin/cephalexin uncomplicated	89.1% 89.1%			
	[96]	1,000 + 500 versus vancomycin for suspected mrsa	89.9% 86.7%			
Telavancin cSSSI	[231]	10 mg/kg QD versus 1 g vancomycin BID	88% 87% two parallel trials pooled ~745 treated with each agent	90 87	47	79 72
Telavancin HAP	[204, 205, 206]	10 mg/kg QD versus 1 g vancomycin BID 7–21 days	82.7% 80.9% N = 658	85 76 N = 164	54	82 81
Oritavancin	[258]	1.5 or 3.0 mg/kg 3–7 days versus 15 mg/kg van or 2 g cephalexin BID for 10–14 days				
	[92, 102, 167]	200/300 versus 15 mg/kg vancomycin or 2 g cephalexin BID	78.5% 75.9% pooled data	74.1 71.7	25	54 62 $p < 0.01$

In Phase 3 studies of uncomplicated and of cSSSI, a regimen of dalbavancin at 1 g on day 1 and 500 mg on day 8 was compared to either cefazolin/cephalexin (in the case of non MRSA infections) or vancomycin where MRSA was suspected [48, 96]. Clinical responses were similar in both studies. An open label Phase 2 study using dalbavancin as a single dose showed the antibiotic to likely be more effective when the second dose of 500 mg was added to the initial one gram dose (clinical efficacy at test of cure 61.5% for single dose, 94.2% for two doses) [221]. This is in contrast to the oritavancin Phase 2 results that show enhanced, although not statistically significantly improved, activity for a single 1,200 mg dose over split dosing [75]. Dalbavancin has also been examined in a Phase 2 study for bacteremia and the results were promising [196]. All of these primary dalbavancin data have recently been reviewed [20, 48].

9.13.2 Oritavancin

Oritavancin is currently in Phase 3 clinical development for cSSSI. Two Phase 3 efficacy studies of cSSSI have been completed, ARRD and ARRI. ARRD [258] was a Phase 3 study comparing either once daily oritavancin to twice-daily vancomycin with follow-on oral cephalexin, or in the case of confirmed MSSA, oral cephalexin alone [258]. Oritavancin met the primary clinical endpoint with a now controversial 15% non-inferiority margin. The second Phase 3 study ARRI was powered to a 10% design and successfully met that endpoint [92]. Pooled data from the two trials showed oritavancin to compare slightly favorably to the control arms for both the clinically evaluable and microbiologically evaluable populations [102]. When these trials were complete in the early portion of the last decade, the number of MRSA patients was high at ~20%, but this number now seems low in comparison to other recent studies (see Table 9.5). The FDA has requested more data on oritavancin's activity against MRSA. The sponsor is currently redesigning trials in cooperation with the FDA. Recently, a Phase 2 study was done comparing oritavancin at 200 mg once a day to a single dose of oritavancin 1,200 mg in cSSSI [75]. The activity seen in that study and the convenience of a single dose for cure, if demonstrated to be effective in well-controlled Phase 3 studies, may distinguish oritavancin. The existing safety profile of oritavancin is favorable versus control for treatment emergent adverse events ($p<0.001$) [102, 167].

9.13.3 Telavancin

Telavancin was approved for complicated skin and skin structure infections in September of 2009 [84]. In two parallel trials, 10 mg/kg QD of telavancin was compared to 1 g of vancomycin twice a day (dose adjustment allowed for serum levels for both drugs) [231]. The duration of treatment was 7–14 days. Telavancin demonstrated

non-inferiority to vancomycin using the endpoint of clinical efficacy at test of cure (7–14 days after the last dose of study medication). The microbiologically evaluable patients also showed a similar response, reinforcing the clinical endpoint with microbiological eradication of baseline pathogen(s). MRSA was isolated from 47% of the microbiologically evaluable patients and telavancin cure rates for MRSA were equally high as for MSSA. These Phase 3 data were supported by similar findings in two Phase 2 studies [229, 230].

Telavancin has shown several safety signals and these are reflected in the black-box warning on the FDA label [85]. These include warnings for fetal risk, nephrotoxicity, renal monitoring, QTc prolongation caution as well as more class-related warnings for infusion-related reactions and *C.difficile*-associated disease. Telavancin interferes with some laboratory coagulation tests including prothrombin time, international normalization ratio, and activated partial thrombiplastin time tests.

A New Drug Application (NDA) has been submitted for telavancin for the treatment of nosocomial pneumonia (NP). Recent data presented for this indication appear promising [204–206].

9.14 Regulatory Status of Recent Lipoglycopeptides

9.14.1 Dalbavancin

In December 2004, Vicuron Pharmaceuticals, which was acquired by Pfizer in September 2005, filed an NDA with the FDA for dalbavancin use in the treatment of cSSSI (see Pfizer's 2005 10-K submission to the Securities and Exchanges Commission [SEC] at [194]).

In July 2007, Pfizer submitted its marketing authorization application (MAA) for dalbavancin to the European Medicines Agency (EMEA) (Pfizer 2007 10-K; [194]); however, the application was withdrawn in September 2008, along with all other dalbavancin applications. In December 2009, Durata Therapeutics acquired Vicuron Pharmaceuticals from Pfizer. Durata is focused primarily on clinical development of dalbavancin; however, as of March 25, 2010, no clinical studies with dalbavancin were active in the U.S.

9.15 Oritavancin

Targanta Therapeutics (now a wholly-owned subsidiary of The Medicines Company) assumed sponsorship of the oritavancin IND in February 2006 and in February 2008 submitted the oritavancin NDA for treatment of cSSSI to the FDA. In November 2008, the safety and efficacy of oritavancin was discussed at a meeting of the Anti-infectives Drugs Advisory Committee (AIDAC). Whereas the Committee voted

11–6 that the larger Phase 3 study independently provided evidence of the effectiveness of oritavancin for cSSSI; the smaller Phase 3 study was not perceived to provide such evidence and as a result the Committee narrowly (by a vote of 8 to 10) rejected the adequacy of the overall data [82]. In its Complete Response letter just prior to the PDUFA date of December 8, 2009, the FDA indicated a requirement for Targanta to conduct an additional Phase 3 study including a sufficient number of patients with MRSA infections with which to demonstrate efficacy of oritavancin in patients with cSSSI [235]. The FDA further suggested that the clinical study evaluate the effect of oritavancin on macrophage function and monitor for the potential for subsequent infections that could possibly be related to macrophage dysfunction due to the long terminal half-life of oritavancin despite the absence of clinically relevant safety findings related to the long residence time of oritavancin or to its sequestration in macrophages. The FDA noted a number of safety findings including the higher rate of study discontinuations for lack of efficacy among oritavancin-treated patients, the greater number of oritavancin-treated patients who died or had a serious adverse event of sepsis, septic shock and related events, and more oritavancin-treated patients who experienced adverse events of osteomyelitis and other sepsis; however, the percentages of patients with these events were comparable between both vancomycin and oritavancin treatment groups owing to the 2(oritavancin):1(vancomycin) patient randomization ratio in both Phase 3 studies [81].

The demonstration that a single dose of oritavancin was as effective as once-daily and infrequent doses of oritavancin in a Phase 2 study of cSSSI [75], which was anticipated from earlier nonclinical studies, has prompted The Medicines Company to reconsider its Phase 3 design to maximize the PK and PD advantages of oritavancin. Specifically, since oritavancin activity is optimized with pooled rather than fractionated doses, and since unlike vancomycin, oritavancin's activity is concentration-dependent, a Phase 3 study design that compares the safety and efficacy of a 1,200 mg single dose of oritavancin to twice-daily vancomycin may be pursued.

The oritavancin MAA for once-daily treatment of cSSTI was submitted to the EMEA in May 2008, shortly after the NDA was submitted to the FDA. The MAA was withdrawn in August 2009 [78] on the basis of the Agency's provisional decision that the evidence was insufficient to support approval of the once-daily dosing regimen dossier.

9.16 Telavancin

Telavancin received fast-track designation from the FDA in March 2005 for the treatment of NP and cSSSI [236]. The telavancin NDA for cSSSI was submitted to the FDA in December 2006. On the basis of the overall safety and efficacy of telavancin, the AIDAC recommended approval of telavancin for cSSSI in November 2008 [83]. The FDA issued a Complete Response letter in February 2009 which included requirements for (1) a risk evaluation and mitigation strategy (REMS) to

prevent unintended telavancin use in pregnant women and in women of childbearing potential owing to potential teratogenicity risks posed to the fetus, (2) a pregnancy registry to evaluate telavancin safety in pregnant women and their offspring, and (3) data on patients with certain renal risk factors from both the cSSSI and NP studies.

On September 11, 2009, 33 months after the NDA submission, the FDA approved telavancin for the once-daily treatment of adults with cSSSI caused by susceptible isolates of Gram-positive bacteria [84]; telavancin was also approved in Canada on September 29, 2009. This was a landmark decision in that it represented the first approval of a lipoglycopeptide antibiotic in the U.S. and the second member of this class since the approval of teicoplanin in Europe two decades ago. Broth microdilution breakpoints for telavancin susceptibility of the indicated organisms were granted by the FDA as follows: MSSA and MRSA: MIC ≤ 1 μg/ml; *S. pyogenes*, *S. agalactiae*, and *S. anginosus* group: MIC ≤ 0.12 μg/ml; vancomycin-susceptible *E. faecalis*: MIC ≤ 1 μg/ml [85] (disk diffusion interpretive criteria for telavancin are also available). No telavancin-intermediate or -resistant breakpoints have been set for any organism; however, Theravance must conduct a 5-year prospective study to determine if decreased susceptibility to telavancin is occurring in the target population of bacteria. As a postmarketing commitment, Theravance must also determine whether the effect of renal function on telavancin antibacterial activity may explain its decreased efficacy in renally-impaired patients. Telavancin was launched in the U.S. on November 5, 2009 [239].

In January 2009 Theravance submitted its telavancin NDA for treatment of NP to the FDA [237]. On the PDUFA date for this indication, November 26, 2009, Theravance received a Complete Response letter from the FDA [238] requesting additional data and analyses using all-cause mortality as the primary efficacy endpoint and additional support for pooling of data between the two pivotal studies. These additional requirements will extend the FDA's review of telavancin for NP into 2010.

The telavancin MAA for complicated skin and soft tissue infections (cSSTI) was filed with the EMEA in May 2007 and subsequently withdrawn in October 2008 [77], owing to outstanding unresolved issues and insufficient data to support a positive benefit/risk balance [237]. Astellas has since strengthened its dossier with substantial clinical data from its NP studies and in October 2009, the telavancin MAA was re-submitted to the EMEA for treatment of both cSSTI and NP, including ventilator-associated pneumonia.

9.17 Summary

The glycopeptide family has been a life-saving class of antibiotics since the 1950s. Bacterial resistance to the class was slow to develop at least in part due to the unique mechanism of action. Dalbavancin, oritavancin and telavancin appear to show efficacy in complicated skin and skin structure infections. To date none have shown superior efficacy to their study comparators. There remain regulatory

hurdles for both dalbavancin and oritavancin before approval and the toxicity profiles of telavancin may be important to note as this efficacious compound is deployed in clinical practice. If approved, single dose oritavancin or the infrequent dose dalbavancin may offer unique convenience advantages for treatment of cSSSI. Further clinical investigations are needed to understand the range of other clinical indications that may be accessible to these lipoglycopeptides. None of these agents are currently applying for EMEA approval for cSSTI.

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Part III
The Rise of Antibiotic Resistance/ Resistance Mechanisms to Major Classes

Chapter 10
Efflux-Mediated Antimicrobial Resistance

Keith Poole

10.1 Introduction

Efflux, or the energy-dependent export or exclusion of antimicrobials from bacterial cells was first reported in the early 1980s and is now recognized as an increasingly important determinant of resistance in bacterial pathogens [275, 276]. Bacterial efflux systems capable of accommodating antimicrobials generally fall into five classes: (1) the major facilitator (MF) superfamily, (2) the ATP-binding cassette (ABC) family, (3) the resistance-nodulation-division (RND) family, (4) the small multidrug resistance (SMR) family [a member of the much larger drug/metabolite transporter (DMT) superfamily] and (5) the multidrug and toxic compound extrusion (MATE) family [289]. These can be single or multicomponent transporters that act at the cytoplasmic membrane of Gram-positive (Fig. 10.1) or Gram-negative (Fig. 10.2a) bacteria or more complex multicomponent systems that span the entirety of the Gram-negative cell envelope (i.e., cytoplasmic membrane periplasm and outer membrane) (Fig. 10.2b). Examples of the latter include the RND family pumps that occur almost, but not quite, exclusively in Gram-negative organisms and typically operate as part of a tripartite system that includes a periplasmic membrane fusion protein (MFP) and an outer membrane protein [now called outer membrane factor (OMF)] (Fig. 10.2b) [271]. A similar organization is also reported (infrequently) for ABC family pumps (e.g., the MacAB-TolC macrolide exporter) [157] (Fig. 10.2b). Drug efflux systems can be drug-/class-specific as for the original tetracycline-exporting Tet pump and more the more recently described Mef exporters of macrolides or capable of accommodating a range of chemically-distinct antimicrobials as for the chromosomally-encoded NorA-like MF transporters prevalent in Gram-positive

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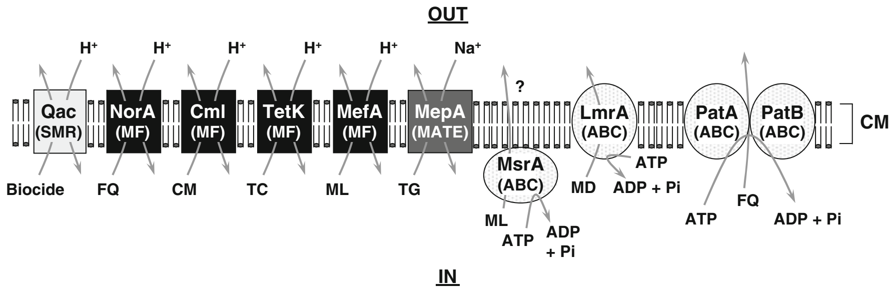

Fig. 10.1 Schematic of representative drug exporting systems in Gram-positive bacteria, highlighting the different families of pumps involved in resistance. *CM* chloramphenicol, *FQ* fluoroquinolone, *MD* multidrug, *ML* macrolides, *TC* tetracycline; *TG* tigecycline. While NorA and PatAB are, strictly speaking, multidrug transporters, they export only FQs (and biocides) as clinically relevant agents and, so, are highlighted here as a MF and ABC family efflux determinants, respectively of FQ resistance

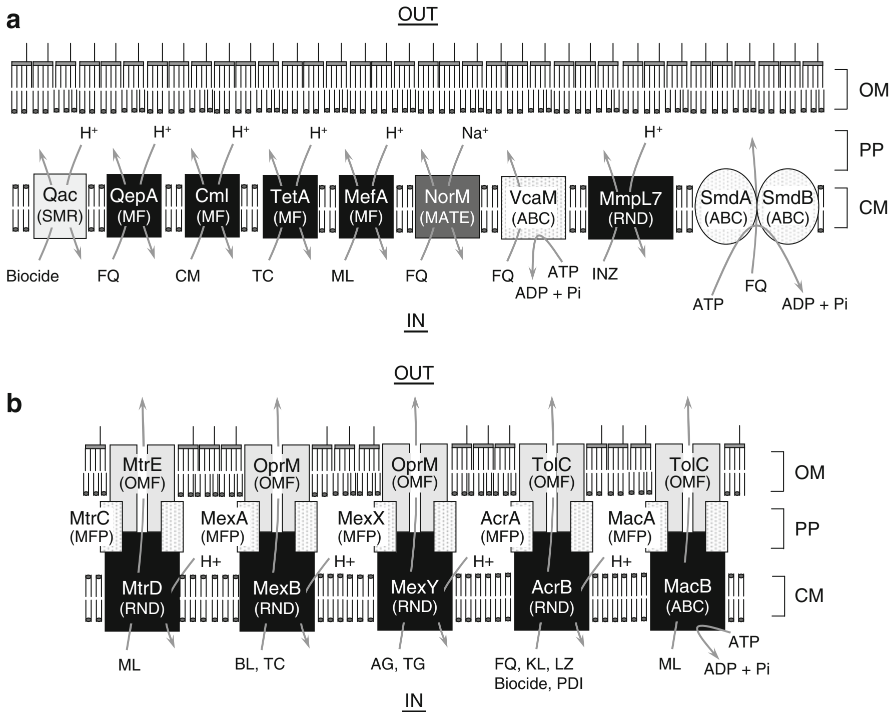

Fig. 10.2 Schematic of representative drug exporting systems in Gram-negative bacteria, highlighting the different families of pumps involved in resistance. (**a**) Pumps localized solely to the cytoplasmic membrane; (**b**) Pumps that span the cell envelope. *AG* aminoglycoside, *BL* β-lactams, *CM* chloramphenicol, *FQ* fluoroquinolone, *KL* ketolide, *LZ* linezolid, *ML* macrolides, *PDI* peptide deformylase inhibitor, *TC* tetracycline. While many of the indicated efflux system are multidrug exporters they are highlighted here for their contribution to resistance to specific agents because their expression in mutants is reportedly selected by those agents, they have been shown to contribute to resistance to the indicated agent in clinical isolates or the indicated agent is the only clinically-relevant antimicrobial exported by the efflux system

bacteria or RND transporters of Gram-negative bacteria [275]. The genes for agent-specific efflux mechanisms typically occur on mobile genetic elements (transposons, integrons, plasmids) whose acquisition from other organisms drives resistance [275]. In contrast, multidrug efflux systems are almost invariably encoded by endogenous, chromosomal genes that are expressed either constitutively (where they contribute to intrinsic resistance) or following mutation (where they contribute to acquired resistance) [275]. Chromosomal efflux genes can also be expressed naturally during certain growth states (e.g., in biofilms [198, 389]) or in response to specific growth conditions (e.g., the presence of bile normally found in the mammalian gut [18, 178, 239, 286]), with the immediate environment thus impacting an organism's intrinsic susceptibility to antimicrobials. Members of all but the ABC family (whose members hydrolyze ATP to drive drug efflux) function as secondary transporters, catalyzing drug-ion (H^+ or Na^+) antiport (Figs. 10.1 and 10.2).

10.2 Antimicrobial Transporters of Gram-Positive Bacteria

Efflux-mediated antimicrobial resistance in Gram-positive bacteria tends to be agent-/class-specific (e.g., chloramphenicol, tetracycline, macrolide-lincosamide-ketolide-streptogramin [MLKS] group) and mediated by MF (and occasionally ABC) family exporters (Table 10.1). Multidrug transporters in these organisms also tend to be noted for a contribution to resistance to a single agent, fluoroquinolones (FQs) (e.g., Nor pumps in *Staphylococcus* and related MF family pumps in other organisms) (Table 10.1). A large number of efflux mechanisms of resistance to the MLKS group of antibiotics have been identified in Gram-positive bacteria [82, 275, 305], typically of the MF and ABC families of drug exporters (Table 10.1). Still, efflux is a uncommon mechanism of resistance to MLKS agents in these organisms [196, 300], with the exception of the *mef*(A) and *mef*(E) efflux determinants of macrolide-specific (14- and 15-membered macrolides only) resistance (a.k.a. M resistance phenotype). Although identified in a number of Gram-positive organisms (Table 10.1), the *mef* determinants are major contributors to macrolide resistance in *Streptococcus* spp. only [93, 374] and, indeed, are implicated in the increasing incidence of macrolide resistance being seen worldwide in streptococci [73]. Efflux determinants of resistance to chloramphenicol are less frequently found in Gram-positive vs. Gram-negative bacteria [275], and while efflux is a common mechanism of tetracycline resistance in Gram-positive bacteria, there is less diversity in the determinants found in these organisms with only 4 of >20 known bacterial *tet* determinants identified in Gram-positive bacteria [304] (Table 10.1). A well-characterized ABC family transporter that accommodates a broad range of clinically-relevant antimicrobials [268], LmrA, has been described in the non-pathogen *Lactobacillus lactis* and serves as a model of bacterial ABC type multidrug transporters [194]. The first MATE-family antibiotic exporter to be described in Gram-positive bacteria, MepA, has recently been identified in *Staphylococcus aureus* as a determinant of tigecycline [216] and FQ [146] resistance in this organism.

Table 10.1 Antimicrobial efflux systems in Gram-positive bacteria

Antimicrobial	Efflux system[a]	Pump family	Gene location	Organism(s)
Chloramphenicol				
	Cml, Cmlv, Cmr, Cmx, CmrA	MF	Plasmid, chromosome	*Streptomyces* spp., *Corynebacterium* spp., *Rhodococcus* spp.
	LmrA	ABC	Chromosome	*Lactobacillus lactis*
Macrolides, lincosamides, streptogramins, ketolides				
Macrolides	Mef(A)	MF	Chromosome	*Streptococcus* spp., *Corynebacterium* spp., *Enterococcus* spp., *Micrococcus* spp., *Staphylococcus* spp.
	Mef(E)	MF	Chromosome	*Streptococcus* spp.
	Mef(I)[b]	MF	Chromosome	*S. pnemoniae*
	Mef(B)[c]	MF	Chromosome	*Streptococcus agalactiae*
	Mef(G)[c]	MF	Chromosome	*S. agalactiae*
Macrolides, type B streptogramins	Msr(A)	ABC	Plasmid	*Staphylococcus* spp., *Streptococcus* spp[d]., *Enterococcus* spp.[d], *Corynebacterium* spp[d].
	Msr(C)	ABC	Chromosome	*Enterococcus faecium*
Macrolides, ketolides	Msr(D)	ABC	Chromosome	*S. pneumoniae*
Macrolides, lincosamides, type A streptogramins	MdeA	MF	Chromosome	*S. aureus*, *Staphylococcus hemolyticus*, *Bacillus cereus*, *Baillus subtillis*
Type A streptogramins	Vga(A/B)	ABC	Plasmid	*S. aureus*, *Streptococcus* spp[d]., *Enterococcus* spp.[d]
Lincosamides, streptogramins	Lsa	ABC	Chromosome	*Enterococcus faecalis*
Clindamycin	Lsa(B)	ABC	Plasmid	*Staphylococus sciuri*
Lincosamides	LmrB	MF	Chromosome?	*B. subtilis*
Lincosamides	LmrB	MF	Chromosome	*Corynebacterium glutamicum*
Erythromycin	Cme	MF	Chromosome	*Clostridium difficile*
Macrolides, lincosamides, streptogramins	LmrA	ABC	Chromosome	*L. lactis*

Fluoroquinolones				
	NorA	MF	Chromosome	*S. aureus*
	NorB	MF	Chromosome	*S. aureus*
	NorC[e]	MF	Chromosome	*S. aureus*
	PmrA	MF	Chromosome	*S. pneumoniae*
	PatAB[f]	ABC	Chromosome	*S. pneumoniae*
	EmeA	MF	Chromosome	*E. faecalis*
	EfrAB	ABC	Chromosome	*E. faecalis*
	Lde	MF	Chromosome	*Listeria monocytogenes*
	?[g]	?[g]	Chromosome	*Bacillus anthracis*
	Bmr	MF	Chromosome	*B. subtilis*
	Bmr3	MF	Chromosome	*B. subtilis*
	Blt	MF	Chromosome	*B. subtilis*
	LmrA	ABC	Chromosome	*L. lactis*
Tetracyclines				
	Tet(K), Tet(L)	MF	Plasmid	Gram-positive bacteria
	Tet(33)	MF	Plasmid	*Corynebacterium*
	Tet(38)	MF	Chromosome	*S. aureus*
	LmrA	ABC	Chromosome	*L. lactis*
β-lactams				
	LmrA	ABC	Chromosome	*L. lactis*
Aminoglycosides				
	LmrA	ABC	Chromosome	*L. lactis*

[a] Except where indicated the listed efflux systems and/or their distribution are described and referenced in recent review articles [275, 276]
[b] [65]
[c] [38]
[d] [305]
[e] [353]
[f] First identified as SP2073-2075, the ABC genes were later dubbed *patAB* by another group [99, 206]
[g] Efflux of FQs was observed in *B. anthracis* although the system responsible was not identified

10.3 Antimicrobial Transporters of the Mycobacteria

MF and ABC type exporters are well-represented amongst antimicrobial efflux mechanisms identified in the mycobacteria with single examples only of SMR and RND type pumps reported to date in these organisms (Table 10.2). The vast majority

Table 10.2 Antimicrobial efflux systems in mycobacteria

Antimicrobial(s)	Efflux system	Pump family	Organism(s)	Reference[a]
Chloramphenicol				
	EfpA	MF	*M. smegmatis*	[176]
	Bcg0231/Rv0194	ABC	*M. bovis*, *M. tuberculosis*	[76]
	DrrAB	ABC	*M. tuberculosis*	[58]
β-lactams				
	Bcg0231/Rv0194	ABC	*M. bovis*, *M. tuberculosis*	[76]
Macrolides				
	Mmr	SMR	*M. smegmatis*	
	DrrAB	ABC	*M. tuberculosis*	[58]
Tetracyclines				
	Tet (K)/Tet(L)	MF	*M. fortuitum*	[250]
	Tet(V)	MF	*M. smegmatis*	[77]
	Tap/Rv1258	MF	*M. tuberculosis*, *M. fortuitum*	
	P55/Rv1410	MF	*M. tuberculosis*, *M. bovis*	
	Rv2333c/Stp	MF	*M. tuberculosis*	[293]
	Bcg0231/Rv0194	ABC	*M. bovis*, *M. tuberculosis*	[76]
	DrrAB	ABC	*M. tuberculosis*	[58]
Fluoroquinolones				
	LfrA	MF	*M. smegmatis*	
	EfpA	MF	*M. smegmatis*	
	Rv1634	MF	*M. tuberculosis*	
	Rv1258c	MF	*M. tuberculosis*	
	DrrAB	ABC	*M. tuberculosis*	[58]
	Rv2686c-v2687c-Rv2688c	ABC	*M. tuberculosis*	
Aminoglycosides				
	Tap/Rv1258	MF	*M. tuberculosis*	
	Rv2333c/Stp	MF	*M. tuberculosis*	[293]
	Bcg0231/Rv0194	ABC	*M. bovis*, *M. tuberculosis*	[76]
	P55/Rv1410	MF	*M. tuberculosis*, *M. bovis*	
	DrrAB	ABC	*M. tuberculosis*	[58]
Isoniazid				
	EfpA (MDR)	MF	*M. smegmatis*	[176]
	MmpL7	RND	*M. smegmatis*	[254]
Metronidazole		MF		
	MSMEG_5046[b]	MF	*M. smegmatis*	[156]

[a] Except where indicated the listed efflux systems are described and referenced in recent review articles [78, 271, 275]

[b] This putative efflux system is upregulated in a metronidazole-resistant mutant although a contribution to metronidazole resistance has not been demonstrated

of mycobacterial drug efflux systems are described as multidrug transporters, although they tend to contribute to resistance to a limited range of clinically relevant agents (e.g., tetracycline, aminoglycosides, FQs) (Table 10.2). Efflux systems contributing to resistance to first line antimycobacterial agents such as isoniazid and ethambutol [176, 254] as well as β-lactams [76] have also been described recently. Most if not all of these efflux systems have been characterized using surrogate hosts expressing the cloned genes and, as such, evidence supporting a contribution to antimicrobial resistance in lab-selected or clinical isolates is generally lacking [78]. Indeed, most data supporting efflux as a contributor to antimicrobial resistance in the mycobacteria is indirect (drug minimum inhibitory concentrations [MICs] increase in the presence of efflux pump inhibitors [EPIs] [78, 91, 337] and/or efflux genes are induced by antimicrobials [78, 142, 329]). Isoniazid-resistant strains of *Mycobacterium tuberculosis* have been isolated in vitro, for example, where resistance is compromised by the EPI reserpine, although efflux was not directly assessed and no resistance determinant was identified [110, 362]. In one study, an efflux-related gene induced by isoniazid and ethambutol and linked to tolerance to these agents, *iniA*, was identified, although it was unclear if its product is an efflux component or it simply impacts expression of an isoniazid- and ethambutol-accommodating efflux system [66]. An MF family *M. tuberculosis* pump, Rv1258c, is inducible by rifampicin and isoniazid [142, 329] although a contribution to rifampicin or isoniazid resistance was not examined and, indeed, studies of the cloned gene suggest a connection to FQ, tetracycline and aminoglycoside resistance (Table 10.2).

10.4 Antimicrobial Transporters of Gram-Negative Bacteria

10.4.1 MF Superfamily

The most common MF superfamily pumps found in Gram-negative bacteria are the tetracycline and chloramphenicol specific transporters that were the first efflux mechanisms of resistance to be described in bacteria [33, 57, 275, 321]. These mostly plasmid-encoded systems are widespread in Gram-negative bacteria and the major determinants of tetracycline and chloramphenicol resistance in these organisms [33, 275, 304, 321]. The MF family *mef*(A) locus historically implicated in efflux-mediated macrolide resistance in Gram-positive bacteria [303] has been described in a number of Gram-negative bacteria [302, 305], including 13 genera of human commensals [245] as well as *Neisseria* [68, 69] and *Bacteroides* [302] spp. A *mef* gene has also been reported in clinical strains of *Acinetobacter junii* and *Neisseria gonorrhoeae*, and shown to be mobilizeable to other Gram-negative where it promoted reduced susceptibility to erythromycin [195]. A chromosomal *mef(A)*-like gene, *cme*, has been reported in *Clostridium difficile* where it contributes to erythromycin resistance [168]. Chromosomally encoded MF family pumps capable of accommodating multiple antimicrobials are increasingly being described

Table 10.3 MF family pumps in Gram-negative bacteria

Antimicrobial(s)	Efflux system[a]	Organism(s)	Gene location
Chloramphenicol			
	Cml, CmlA, CmlB	*P. aeruginosa, E. aerogenes, K. pneumoniae, S. enterica* serovar Typhimurium	Mostly plasmid; some chromosome
	MdfA	*E. coli*	Chromosome
	KdeA[b]	*K. pneumoniae*	Chromosome
Chloramphenicol, florfenicol			
	Flo, FloR, pp-Flo	*E. coli*, *K. pneumoniae*, *V. cholerae*, *S. enterica* serovar Typhimurium	Plasmid, chromosome
Macrolides			
	Mef(A)	Numerous Gram-negative bacteria	Chromosome
	MdfA	*E. coli*	Chromosome
Tetracyclines			
	Tet(A), Tet(B), Tet(C), Tet(D), Tet(E), Tet(G), Tet(H), Tet(J), Tet(L)[c], Tet(Y), Tet(Z), Tet(30), Tet(39)[d], TetA(41), TetA(P)[e]	Numerous Gram-negative bacteria	Plasmid
	MdfA	*E. coli*	Chromosome
Fluoroquinolones			
	QepA[f]	*E. coli*	Plasmid
	QepA2[g]	*E. coli*	Plasmid
	Fsr[h]	*E. coli*	Chromosome
	MdfA	*E. coli*	Chromosome
	SmfY[i]	*S. marcescens*	Chromosome
	KdeA[b]	*K. pneumoniae*	Chromosome

[a] Except where indicated the listed efflux systems are described and referenced in recent review articles [271, 275]

[b] [267]

[c] *tet*(L) recently reported in *Mannheimia* and *Pasteurella* spp. [153] and *Actinobacillus pleuropneumoniae* [21]

[d] *tet*(39) recently reported in *S. marcescens* [350]

[e] *tet*(P) recently reported in *H. pylori* [171]

[f] [264, 377]

[g] [41]

[h] [95]

[i] [327]

(Table 10.3) although these tend to promote very moderate increases in antibiotic MICs and have not been linked to clinical resistance. Despite being defined as multidrug pumps, however, these pumps are typically highlighted for their accommodation of FQ antibiotics (Table 10.3). A plasmid, possibly transposon-borne gene, *qepA*, encoding an MF family exporter linked to reduced susceptibility to

Table 10.4 SMR family antimicrobial systems in Gram-negative bacteria

Pump[a]	Organism	Antimicrobial(s)
QacE	*K. pneumoniae*, *P. aeruginosa*	QAC
QacF	*E. aerogenes*, *E. cloacae*, *P. aeruginosa*[b]	QAC
QacG	*P. aeruginosa*, *A. salmonicida*	QAC
QacH	*Salmonella* spp.[c], *V. cholerae*[d]	QAC
EmrE	*P. aeruginosa*[e]	Aminoglycosides
SsmE	*S. marcescens*[f]	Fluoroquinolones, chlorhexidine

[a] Except where indicated the listed efflux systems and/or their distribution are described and referenced in recent review articles [271, 275]

[b] *qacF* recently identified in *P. aeruginosa* [317]

[c] *qacG* recently reported in *Salmonella* spp. [10]

[d] *qacG* recently reported in *V. cholerae* [44]

[e] Two- to four-fold decrease in MICs for neomycin, kanamycin and gentamicin was observed in an *emrE* knockout strain [173]

[f] The cloned gene promoted a 2- to 4-fold increase in MICs for ciprofloxacin, norfloxacin and chlorhexidine when expressed in a Δ*acrAB E. coli* strain [223]

hydrophilic quinolones has been described in clinical *Escherichia coli* isolates from Japan [377] and Belgium [264]. Though reportedly uncommon as a determinant of FQ resistance [378], *qepA* shows high co-prevalence with the *rmtB* gene encoding a 16 S rRNA methyl transferase that is responsible for high-level pan-aminoglycoside resistance in Gram-negative pathogens [83] (in a study of porcine isolates it was present in 28 of 48 *rmtB*-carrying *E. coli* [184]). A variant of this efflux gene, *qepA2*, unlinked to *rmtB* but also plasmid-borne and promoting resistance to hydrophilic quinolones has very recently been described in a clinical *E. coli* isolate [41].

10.4.2 SMR Family

The original Smr family efflux system representative identified variously as Smr, QacC, QacD or Ebr was a plasmid-encoded multidrug resistance determinant found in clinical staphylococci isolates [255]. Highlighted for its contribution to resistance to quaternary ammonium compounds (e.g., the biocide, benzalkonium chloride) Smr was thus clinically noteworthy solely for its contribution to reduced biocide susceptibility [183]. While Smr is unique to Gram-positive bacteria, additional Smr family QAC-resistance determinants have been described in Gram-negative bacteria, including QacE, QacF, QacG and QacH (Table 10.4). The corresponding *qac* genes typically reside on plasmids, as part of integron elements, and are thus mobile [275]. Still, they do not appear to be significant determinants of biocide resistance, being limited in their distribution amongst Gram-negative pathogens (Table 10.4) and not reportedly linked to changes in biocide MICs in any clinical isolates studied to date. A few examples of chromosomal Smr family multidrug exporters have been reported, including the *Serratia marcescens* SsmE [223] pump that provides a modest contribution to FQ resistance (2- to 4-fold change in MIC with vs. without the pump) and the *Pseudomonas aeruginosa* EmrE pump [271] implicated in aminoglycoside resistance (two- to fourfold decrease in MICs in an *emrE* null mutant).

Table 10.5 ABC family antimicrobial efflux systems in Gram-negative bacteria

Organism	Pump	MIC (μg/ml) for NOR (–/+ Pump)[a]	Fold change[b]	Reference(s)
C. hathewayi	CmpAB	1/4 (0.5/1.5)[c]	3-4	[291]
S. marcescens	SmdAB	0.016/0.125[d]	8	[214]
V. cholerae	VcaM	0.015/.125[d]	8	[130]
S. enterica (Typhimurium)	MdtK	0.016/.13[e]	8	[241]
E. coli	AcrAB[f]	0.025/0.20[g]	8	[240]

[a] MIC for norfloxacin (NOR) in the absence/presence of the relevant ABC family pump
[b] Fold change in NOR MIC without/with the indicated ABC family pump
[c] Data are for an *E. coli* strain (or *C. hathewayi* strain in parentheses) without/with a plasmid expressing the indicated ABC family pump
[d] Data are for a Δ*acrAB E. coli* strain without/with a plasmid expressing the indicated ABC family pump
[e] Data are for a Δ*acrAB S. enterica* strain without/with a plasmid expressing the indicated ABC family pump
[f] AcrAB-TolC is not a MATE family pump but is included here as an example of a known fluoroquinolone resistance determinant to highlight the possible significance of ABC family exporters as determinants of fluoroquinolone resistance
[g] Control showing the impact of the plasmid-encoded RND family AcrAB exporter on NOR MICs of the Δ*acrAB E. coli* strain

10.4.3 ABC Family

ABC family drug transporters, while common in mammalian systems [328], are comparatively rare in bacteria and tend to be better characterized in Gram-positive bacteria [194]. Typically chromosome-encoded, these pumps can be single or multicomponent (Fig. 10.1) and tend to make modest contributions to antimicrobial resistance [194]. The first ABC family pump reported in Gram-negative bacteria, MacABC, is a macrolide-specific exporter first reported in *E. coli* [157] but is also present in *N. gonorrhoeae* [308] and *Salmonella enterica* serovar Typhimurium [241]. The *msr(A)* determinant of macrolide and streptogramin A resistance in Gram-positive bacteria has been identified in *Pseudomonas* [246], although its significance vis-à-vis antimicrobial resistance is unclear. Most recently described ABC transporters capable of accommodating antimicrobials are multidrug transporters identified in *S. marcescens*, *Vibrio cholerae*, *S. enterica* and *Clostridium hathewayi* and most often highlighted for their contributions to FQ resistance (Table 10.5). Such contributions to resistance are, however, modest (3- to 8-fold increase in MIC) and are typically revealed using pump-deficient surrogate organisms expressing plasmid-encoded ABC pump genes (Table 10.5). Thus, while these have potential to contribute to resistance, they are unlikely to be primary determinants of resistance and there are, as yet, no reports of these as contributors/determinants of resistance in clinical isolates or, indeed, in vitro-selected FQ-resistant mutants. An ABC-MFP-OMF transporter (PA1875-77) has, however, very recently been identified as a determinant of biofilm-specific resistance to aminoglycosides and ciprofloxacin in *P. aeruginosa* [389].

Table 10.6 MATE family antimicrobial efflux systems in Gram-negative bacteria

Organism	Pump[a]	MIC (μg/ml) for NOR (−/+ Pump)[b]	Fold change[c]
A. baumannii	AbeM	0.03/1[d]	32
Bacteroides thetaiotaomicron	BexA	32/128[e]	4
C. difficile	CdeA	0.03/0.25[d]	8
Erwinia amylovora	NorM	0.02/0.10[d]	5
E. coli	NorE (YdhE)	0.06/0.38[d]	6
H. influenzae	HmrM	0.015/0.06[e]	4
N. gonorrhoeae, *N. meningitidis*	NorM	0.00002/0.00032[e]	16
P. aeruginosa	PmpM	0.03/0.12[d]	4
S. enterica serovar Typhimurium	MdtK[f]	0.016/0.13[d]	8
V. cholerae	VcmA[g]	0.015/0.5[d]	32
V. parahemolyticus	NorM	0.03/0.24[e]	8
E. coli	AcrAB[h]	0.025/0.20[i]	8

[a] Except where indicated the listed efflux systems are described and referenced in recent review articles [271, 275]

[b] MIC for norfloxacin (NOR) in the absence/presence of the relevant MATE family pump

[c] Fold change in NOR MIC without/with the indicated MATE family pump.

[d] Data are for a Δ*acrAB E. coli* strain without/with a plasmid expressing the relevant MATE family pump

[e] Data are for the indicated organism without/with the chromosomal gene encoding the corresponding MATE family pump

[f] [241]

[g] Additional norfloxacin resistance MATE family pumps from *V. cholerae* have been reported (VcmB, VcmD, VcmH and VcmN) with the cloned genes (in Δ*acrAB E. coli*) providing a 4-fold increase in Nor MIC for all except VcmH (8-fold increase) [17]

[h] AcrAB-TolC is not a MATE family pump but is included here as an example of a known fluoroquinolone resistance determinant to highlight the possible significance of MATE family exporters as determinants of fluoroquinolone resistance

[i] Control showing the impact of the plasmid-encoded RND family AcrAB exporter on NOR MICs of the Δ*acrAB E. coli* strain

10.4.4 MATE Family

The last of the efflux families to be identified and characterized, a number of the chromosomally-encoded, single-component MATE family multidrug transporters have now been described in a variety of Gram-negative bacteria where they are invariably highlighted for a contribution to FQ resistance (4- to 32-fold increase in norfloxacin resistance with vs. without the cloned gene; Table 10.6). Where studied, MATE family pumps have been shown to be drug-Na^+ antiporters that use a Na^+ rather than H^+ gradient to promote drug export [193, 226, 376]. As with the Gram-negative ABC family drug transporters, however, there are no reports to date of in vitro or in vivo isolated resistant strains where resistance is attributable to a MATE family pump.

10.4.5 RND Family

10.4.5.1 Contributions to Antimicrobial Resistance

Efflux systems of the RND family are widespread in Gram-negative bacteria and accommodate the broadest range of clinically relevant antimicrobials, typically encompassing multiple classes of antimicrobials (Table 10.7). These pumps are generally responsible for the lack of Gram-negative activity of typically Gram-positive agents (macrolides, oxazolidinones) [140, 261, 262, 275, 320] as well as the newer ketolides and glycylcyclines [79, 151, 152, 207, 260, 361] (Table 10.7); their accommodation of even experimental agents [80, 86, 175, 221, 306] makes them formidable barriers to new drug development [273]. Significantly, RND type efflux systems are the most common multidrug efflux determinants of antimicrobial resistance in clinical Gram-negative isolates, implicated in resistance to aminoglycosides, FQs, β-lactams, macrolides and glycycyclines (Table 10.8).

MLSK Antimicrobials

Not surprisingly, given the broad substrate specificity of this family, many RND type multidrug exporters of Gram-negative bacteria accommodate macrolides [49, 56, 211, 302] and, where tested, lincosamides [140]. Erythromycin-resistant strains where resistance is reversed by the efflux inhibitor Phe-Arg-β-naphthylamide (PAβN) have been reported in clinical *Campylobacter* spp., suggestive of an efflux mechanism of resistance [165, 202, 203], and the CmeABC RND-type pumps of *Campylobacter jejuni* and *Campylobacter coli* have been shown to contribute to acquired macrolide resistance in lab and clinical isolates [182, 257]. CmeABC-mediated efflux seems to explain instances of low-level macrolide resistance in these organisms [182], with high-level resistance attributable to mutations in the 23 S rRNA gene, often in conjunction with CmeABC overexpression [103]. Synergy between CmeABC and ribosomal protein mutations has also been seen in macrolide-resistant *Campylobacter jejuni* and *Campylobacter coli* [35]. Loss of AcrAB-TolC in *E. coli* had a modest (4-fold) impact on ketolide (telithromycin) resistance although treatment of *E. coli* or *Enterobacter aerogenes* with the efflux inhibitor PAβN had a marked impact in ketolide (and macrolide) resistance (128- to 512-fold) indicating that additional, presumed efflux mechanism(s) of macrolide and, especially ketolide resistance occur in these enteric organisms [56]. Expression of the RND family MtrCDE multidrug efflux system of *N. gonorrhoeae* has been reported in clinical isolates displaying reduced susceptibility to azithromycin and/or erythromycin [385, 386] indicating that this multidrug transporter can be a determinant of acquired macrolide resistance in *Neisseria*. Studies on macrolide resistance in *Haemophilus influenzae* also implicates this organism's three-component RND family multidrug transporter, AcrAB-TolC, as a co-determinant of intrinsic and acquired macrolide resistance [262], including high-level macrolide resistance [263].

Table 10.7 RND-family multidrug efflux systems in Gram-negative bacteria

Organism	Pump[a]	Antimicrobial substrates[b]	Reference(s)
Acinetobacter spp.	AdeABC	BL, CM, FQ, ML, TC, TG[c,d]	
	AdeDE	BL, FQ, ML, TC, TG[c]	
	AdeIJK	CM, BL, FQ, ML, TC	[75]
	AdeXYZ	?[e]	[60]
Aeromonas hydrophila	AheABC[f]	BL, ML, TC	[120]
Aggregatibacter (Actinobacillus) actinomycetemcomitans	TdeA[f]	CM, ML	[70]
B. fragilis	BmeAB3	BL, PX	[357]
	BmeABC5	MZ	[288]
Borrelia burgdorferi	BseABC	BL, ML, TC	[31]
Burkholderia cenocepacia	CeoAB-OpcM	CM, FQ	
	BCAS0765[g]	AG, FQ	[109]
Burkholderia pseudomallei	AmrAB-OprA	AG, ML	
	BpeAB-OprB	AG, ML	
	BpeEF-OprC	CM	[164]
Campylobacter coli	CmeABC	FQ, KL, ML	[34, 35, 100, 114, 379]
Campylobacter jejuni	CmeABC	CM, FQ, ML, TC, KL[h]	[100, 103, 179, 379]
	CmeDEF	BL, PX	[4]
Citrobacter freundii	AcrAB	CM, FQ, LZ[i]	[316, 320]
Enterobacter aerogenes	AcrAB-TolC	CM, FQ, ML, TC, LZ[j], KL[k]	
	EefABC	CM, ML, TC	[209a]
Enterobacter cloacae	AcrAB-TolC	BL, FQ, TC, TG[l], LZ[m]	[261]
Escherichia coli K12	AcrAB-TolC	BL, CM, FQ, ML, TC, TG[n], LZ[i], KL[k]	[48, 152, 225]
	AcrEF-TolC	BL, FQ, ML, TC, TG[o], LZ[p]	
	MdtABC-TolC	---[q]	
	YhiUV-TolC	---[q]	
	AcrAD-TolC	AG	
E. coli SMS-3-5	EefABC	FQ	[95]
Francisella tularensis	AcrAB-TolC	BL, TC	[19, 104]
	FtlC (tolC homologue)	AG, TC	[104]
Helicobacter pylori	HefABC	BL, ML, TC	[166]
Hemophilus influenzae	AcrAB-TolC	ML, DI[r]	
Klebsiella oxytoca	AcrAB	FQ	
Klebsiella pneumoniae	AcrAB-KocC	BL, FQ, ML, TC, TG[s]	[118, 177, 215]
Morganella morganii	AcrAB	FQ, ML, TC, TG[t]	[309]
Neisseria gonorrhoeae	MtrCDE	BL, FQ, ML	
Neisseria meningitidis	MtrCDE	PX	
Porphyromonas gingivalis	XepABC	---[q]	
Proteus mirabilis	AcrAB	CM, FQ, ML, TC, TG[u]	[313, 361]
Pseudomonas aeruginosa	MexAB-OprM	BL, CM, FQ, ML, TC, TG[v], OX[w]	
	MexCD-OprJ	BL, CM, FQ, ML, TC, TG[v]	
	MexEF-OprN	CM, FQ	
	MexXY/OprM	AG, BL, FQ, ML, TC, TG[v]	
	MexJK/OprM	FQ, ML, TC	
	MexHI-OpmD	FQ	[222]
	MexMN/OprM	CM	[222]
	MexPQ-OpmE	FQ, ML	[174]
	MexVW/OprM	CM, FQ, ML, TC	
Pseudomonas putida	ArpABC	BL, CM, ML, TC	
	MepABC	BL, ML, TC	
	TtgABC	BL, CM, TC	
	TtgGHI	BL	

(continued)

Table 10.7 (continued)

Organism	Pump[a]	Antimicrobial substrates[b]	Reference(s)
Pseudomonas syringae	MexAB-OprM	BL, CM, FQ, ML, TC	[341]
Ralstonea slonacearum	AcrAB	CM, BL	[29]
Salmonella enterica serovar Typhimurium	AcrAB-TolC	BL, CM, FQ, ML, TC	[241]
Salmonella enterica serovar Choleraesuis	AcrEF	FQ	[59]
	AcrAB	FQ	
Serratia marcescens	SdeAB-HasF	CM, FQ	[15, 163]
	SdeDEF	---[q]	[16]
	SdeXY	FQ, ML, TC	[50]
Shewanella oneidensis	MexEF	CM, TC	[108]
Stenotrophomonas maltophilia	SmeABC	AG, BL, FQ	[172]
	SmeDEF	FQ, ML, TC	
	SmeIJK	AG, TC	[71]
	SmeYZ	AG	[71]
V. cholerae	VexAB-TolC	ML, PX	[18, 20, 292]
V. parahaemolyticus	VmeAB-VpoC	BL, FQ, ML	[213]
	VexEF-TolC	ML	[292]
Yersinia pestis	AcrAB	CM, FQ, TC	[356]

[a] Except where indicated the listed efflux systems and/or their antimicrobial substrates are described and referenced in recent review articles [270, 275, 276]

[b] Representative antimicrobial substrates for the indicted efflux systems are highlighted: *AG* aminoglycosides, *BL* β-lactams, *CM* chloramphenicol, *DI* deformylase inhibitor, *FQ* fluoroquinolones, *KL* ketolides, *LZ* linezolid, *ML* macrolides, *MZ* metronidazole, *OX* oxazolidinones, *PX* polymyxins, *TC* tetracycline, *TG* tigecycline

[c] Deletion of *adeABC* or *adeIJK* in a clinical isolate increased tigecycline susceptibility 8- and 6-fold, respectively [75]

[d] Deletion of *adeABC* in a clinical isolate increased tigecycline susceptibility 8-fold [311]

[e] A contribution to antimicrobial resistance remains to be tested

[f] Inactivation of this TolC/OMF homologue rendered cells multidrug susceptible, suggesting that it might function as part of an efflux mechanism with hitherto unidentified RND and MFP components

[g] This RND homologue promoted multidrug resistance when cloned into AcrAB$^-$ *E. coli* suggesting that it functions as part of a MFP-RND-OMF efflux system

[h] Deletion of *cmeABC* in a clinical isolate increased telithromycin susceptibility 64-fold [34]

[i] Deletion of *acrAB* in wild type strains increased linezolid susceptibility 8- to 32-fold [140, 320]

[j] Increased resistance to this agent was observed in multidrug resistant isolate and was reduced 32-fold in a *tolC* mutant, suggestive of AcrAB-TolC involvement [320]

[k] Telithromycin MIC decreased 2-fold and 4-fold, respectively, in AcrAB-deficient derivatives of *E. aerogenes* and *E. coli* [56]

[l] Deletion of *acrAB* in a clinical isolate increased tigecycline susceptibility 16-fold [151]

[m] Deletion of *acrAB* in a clinical isolate increased linezolid susceptibility 32-fold [261]

[n] Deletion of *acrAB* in a clinical isolate increased tigecycline susceptibility 8-fold [125, 152]

[o] Overexpression of AcrEF in an AcrAB$^-$ strain increased tigecycline resistance 4-fold [125, 152]

[p] Overexpression of AcrEF in an AcrAB$^-$ strain increased linezolid resistance 32- to 64-fold [140, 320]

[q] No clinically-relevant antimicrobial substrates have been identified for this efflux system

[r] Deletion of *acrAB* in wild type and clinical isolates increased susceptibility to the deformylase inhibitor LBM415 4- and 64-fold, respectively [80]

[s] Deletion of *acrAB* in a clinical isolate increased tigecycline susceptibility 16-fold [310]

[t] Deletion of *acrAB* in a clinical isolate increased tigecycline susceptibility 128-fold [309]

[u] Deletion of *acrAB* in a clinical isolate increased tigecycline susceptibility 16-fold [361]

[v] The presence/absence of *mexXY*, *mexAB-oprM* or *mexCD-oprJ* had a 8- to 16-fold impact on tigecycline MICs [79]

[w] Intrinsic resistance of *P. aeruginosa* to linezolid and several experimental oxazolidinones (Pharmacia) was attributable to MexAB-OprM, where loss of this pump produced 2- to 32-fold increases in susceptibility (Li and Poole, 2003)

Table 10.8 RND family efflux systems of clinical significance[a]

Organism	Pump	Antimicrobial(s)[b]	Reference(s)
A. baumannii	AdeABC	Meropenem	[129]
		Cefepime	[28]
		Tigecycline	[28, 260, 311]
B. fragilis	BmeABC5	Metronidazole	[288]
C. jejuni	CmeABC	Macrolides, ketolides	[207]
E. aerogenes	AcrAB-TolC	Multidrug (including fluoroquinolones)	[281]
E. cloacae	AcrAB-TolC	Tigecycline	[151]
E. coli	AcrAB-TolC	Fluoroquinolones, tigecycline	[48, 152]
K. pneumoniae	AcrAB-TolC	Fluoroquinolones, tigecycline	[215, 310, 318]
N. meningitidis	MtrCDE	Fluoroquinolones	[90]
N. gonorrhoeae	MtrCDE	Macrolides	[385, 386]
P. aeruginosa	MexXY/OprM	Aminoglycosides	[132, 333, 363, 373]
		Cefepime	[127]
	MexAB-OprM	Fluoroquinolones	[85, 135, 137, 266]
		Meropenem	[87, 111, 204, 280, 364]
		Ticarcillin	[43, 334]
		Aztreonam	[290, 334]
	MexCD-OprJ	Fluoroquinolones	[121, 134, 135, 137, 139, 381]
	MexEF-OprN	Fluoroquinolones	[96, 137]
S. enterica	AcrAB-TolC	Fluoroquinolones	[13, 59] [14]
H. influenzae	AcrAB-TolC	Macrolides	[262]
M. morganii	AcrAB	Tigecycline	[309]

[a] Examples where efflux-mediated resistance has been demonstrated in clinical isolates of a given organism and attributed to a particular efflux system are highlighted
[b] Agents for which resistance in clinical isles has been linked to the indicated efflux system

A plasmid-encoded resistance determinant showing substantial similarity to the RND family MexCD-OprJ multidrug efflux system of *P. aeruginosa* and providing resistance to the macrolides erythromycin and roxithromycin has been reported in an environmental *Pseudomonas* spp., the first example of a naturally-occurring plasmid-encoded RND family multidrug transporter [348].

FQs

Efflux-mediated FQ resistance (where the selecting agent in vitro or in vivo was a FQ or a FQ resistance phenotype in particular was highlighted) has been reported in a number of Gram-negative pathogenic bacteria including *Aeromonas salmonicida*, *Campylobacter* spp., *Citrobacter freundii*, *Enterobacter* spp., *E. coli*, *Klebsiella* spp., *Morganella morgani*, *Proteus vulgaris*, *P. aeruginosa*, *Salmonella* spp., *S. marcescens*, *Shigella dysenteriae*, *Stenotrophomonas maltophilia*, *Vibrio fluvialis*, anaerobes such as *Bacteroides* spp., and, possibly, *N. gonorrhoeae* [37, 42, 52, 154, 275, 336, 338]. Although there are some indications of efflux contributing to FQ resistance in clinical isolates of *Acinetobacter baumannii* [358] and the AdeABC

pump of this organism is known to accommodate FQs and has been shown to be upregulated in clinical isolates resistance to FQs, it appears to be important in these only for resistance to non-FQs [124]. Where identified, efflux-mediated resistance to FQs is typically determined by three-component efflux systems of the RND family [1, 15, 51, 114, 225, 275, 320, 336, 379] (Table 10.7). Moreover, while RND family transporters accommodate a wide variety of clinically relevant antimicrobials and their production is, thus, associated with reduced susceptibility to multiple antibiotics, they are most frequently cited as determinants of FQ resistance [275] (Table 10.7). Still, not all RND family exporters accommodate and provide resistance to FQs (e.g., the AmrAB-OprA efflux system of *Burkholderia pseudomallei*) and some RND family transporters known to accommodate these agents have yet to be implicated as primary (selected for in vitro or in vivo by FQs) determinants of FQ resistance [e.g., RND family efflux systems in *A. baumannii*, *Burkholderia cepacia* (*cenocepacia*), *P. mirabilis*].

While target site mutations (i.e., in the so-called Quinolone Resistance Determining Region (QRDR) of type II topoisomerases) are frequently associated with resistance to FQs, particularly high-level resistance [122], clinical resistance to these agents is typically multifactorial, with many highly FQ-resistant isolates harbouring both efflux and target mutations [48, 51, 257, 258, 275, 313, 379]. Moreover, efflux appears not only to contribute to overall resistance, but it may also serve, during development/evolution of resistance in vivo, to protect cells long enough for target site mutations to develop [107]. Thus, even in the absence of evidence linking demonstrated efflux gene expression to resistance in a clinical isolate, it is possible that efflux played a role in the evolution of the observed resistance phenotype. In any case, studies showing that highly FQ-resistant target site mutants cannot be selected in vitro from efflux-deficient mutants of *P. aeruginosa* [191], *E. coli* [208], *S. enterica* serovar Typhimurium [301], and *Campylobacter* spp., [379] clearly highlight the significant contribution of efflux to high-level FQ resistance, as does the observation that loss of multidrug efflux [AcrAB in *E. coli* [208], *S. enterica* serovar Typhimurium [13], MexAB-OprM in *P. aeruginosa* [191], and CmeABC in *Campylobacter* [326]] undermines the resistance provided by target site mutations.

A plasmid-borne RND determinant in found in *E. coli* originally identified in a porcine *E. coli* isolate resistant to the swine growth enhancer olaquindox [116], *oqxAB* [242], promotes resistance to FQs in *E. coli* and is transferable to food-borne pathogens of the Enterobacteriaceae where it promotes reduced susceptibility to ciprofloxacin [117].

Aminoglycosides

Relatively few bacterial drug efflux systems are known to accommodate aminoglycosides (Table 10.7) with the AmrAB-OprA and BpeAB-OprB multidrug efflux systems of *B. pseudomallei* noteworthy for their contribution to this organism's intrinsic aminoglycoside resistance [275] and the AdeABC [205] and MexXY/OprM [274] multidrug efflux systems of *A. baumanni* and *P. aeruginosa*, respectively, implicated

in acquired aminoglycoside resistance. Only in *P. aeruginosa*, however, is efflux a significant determinant of aminoglycoside resistance, with numerous reports of impermeability-type pan-aminoglycoside resistance in clinical isolates [274] characterized by reduced drug accumulation that is now attributable to efflux via MexXY/OprM [63, 81, 119, 187, 333, 363, 373]. Interestingly, this efflux system appears to be the major determinant of aminoglycoside resistance in cystic fibrosis (CF) lung isolates of *P. aeruginosa* [119, 274], which contrasts with non-CF isolates where aminoglycoside-modifying enzymes are the primary determinants of resistance [119, 274]. Expression of the *mexXY* genes is inducible by aminoglycosides [126, 212], implicating MexXY/OprM-mediated aminoglycoside efflux in the long-known phenomenon of adaptive (i.e., inducible and transient) aminoglycoside resistance in *P. aeruginosa* [126]. Intriguingly, *mexXY* is inducible by a number of additional antimicrobials that, like aminoglycosides, target the ribosome (chloramphenicol, macrolides, tetracycline, tigecycline) [212, 228] and, indeed, it appears that MexXY recruitment by these agents is a response to ribosome disruption and not the antibiotics per se [138].

β-lactams

The most common mechanism of acquired resistance to β-lactam antimicrobials is β-lactam destruction by β-lactamase enzymes [272]. While many of the RND family multidrug efflux systems prevalent in Gram-negative bacteria do accommodate these agents [272, 275] (Table 10.7) and, indeed, the RND family Mex pumps of *P. aeruginosa* are, for some β-lactams, more effective determinants of resistance in vitro than this organism's chromosomally-encoded AmpC β-lactamase [210], there are few reports of efflux mechanisms contributing to β-lactam resistance in clinical or, indeed, in vitro-selected resistant strains. Efflux has, however, been linked to resistance to β-lactams [84], including carbapenems (doripenem, ertapenem, meropenem, and faropenem) [74, 97, 186, 290] in *P. aeruginosa*, with Mex-pump expressing strains showing reduced MICs to these agents [231]. MexAB-OprM, in particular, has been highlighted as a determinant of reduced susceptibility to meropenem in clinical isolates [105, 111, 280, 347, 364], although it is most effective as a mechanism of resistance in strains also showing loss of the outer membrane porin OprD that is the major portal for entry of carbapenems into *P. aeruginosa* [185]. MexAB-OprM has been implicated in resistance to the penicillin ticarcillin [25, 43] in clinical isolates and expression of various Mex efflux systems has been linked to ertapenem (and aztreonam) resistance, also in clinical strains [290], although direct evidence for an efflux contribution is lacking. MexAB-OprM has also been shown to be a determinant of reduced susceptibility to novel methylcarbapenems whose activity is not impacted by OprD loss [86]. Resistance to imipenem that characterizes *P. aeruginosa* strains overproducing the MexEF-OprN multidrug efflux system [269, 270, 279, 335] is not, however, explained by efflux but rather by the concomitant decline in the levels of OprD in such mutants [159, 243].

An efflux contribution to carbapenem resistance has also been noted in *Enterobacter cloacae*, although the pump was not identified [345], and meropenem resistance in clinical isolates of *S. maltophilia* correlates with elevated expression of the RND family SmeDEF multidrug efflux system [47] previously shown to accommodate β-lactams. Similarly, efflux has been implicated in carbapenem resistance in *A. baumannii* [128, 331], with this organism's AdeABC efflux mechanism a proposed contributor [129]; *adeABC* expression in multidrug resistance clinical isolates was shown, in one study, to correlate with cefepime resistance although a contribution to resistance was not directly examined [28]. Overexpression of the MtrCDE multidrug efflux system of *N. gonorrhoeae* has also been highlighted as an important contributor to the high-level penicillin resistance of certain clinical isolates of this organism [360]. The high-level ampicillin resistance in a so-called β-lactamase negative ampicillin-resistant (BLNAR) *H. influenzae* is apparently attributable, at least in part, to this organism's AcrAB-TolC efflux system [98, 147]. Finally, the MexXY multidrug efflux determinant of aminoglycoside resistance has been shown to contribute to cefepime resistance in clinical *P. aeruginosa* [119, 127].

Tetracyclines and Glycylcyclines

While many of the RND family multidrug resistance efflux systems of Gram-negative bacteria accommodate tetracyclines (Table 10.7), there are few reports of multidrug exporters as primary determinants of tetracycline resistance (i.e., selected by tetracyclines in vitro or in vivo). MexAB-OprM-overproducing multidrug-resistant isolates of *P. aeruginosa* [7, 113, 136] and SmeDEF-overproducing multidrug resistant *S. maltophilia* [6], respectively, can be selected with tetracycline in vitro. The CmeABC RND family efflux system of *C. jejuni* has also been shown to contribute to acquired tetracycline resistance in this organism [103]. Tetracycline has also been shown to positively influence expression of the *mexXY* genes encoding an RND family multidrug efflux system that, thus, contributes to intrinsic resistance to this agent in *P. aeruginosa* [212]. There are increasing reports, too, of efflux contributing to reduced tigecycline susceptibility in Gram-negative bacteria [148, 259, 260] with RND family pumps in *P. aeruginosa*, *P. mirabilis*, *M. morgannii*, *E. coli*, *E. cloacae* and *A. baumannii* linked to resistance in lab [79] or clinical [75, 125, 151, 152, 309–311, 361] isolates (Tables 10.7 and 10.8).

Biocides

A number of Gram-negative RND family multidrug transporters implicated in antibiotic resistance have been shown to contribute to resistance to biocides (antiseptics, disinfectants and preservatives), including QACs (e.g., benzalkonium chloride [BAK], cetrimide), chlorhexidine and triclosan that are commonly found in hospital disinfectants and/or commercially available at-home products (Table 10.9).

Table 10.9 RND family multidrug efflux systems that accommodate biocides

Pump	Organism	Biocide(s)[a]	Reference(s)
AcrAB-TolC	*S. enterica* (Typhimurium)	QAC, TRI	[11, 149, 150, 297, 370]
AcrAB-TolC	*E. coli*	QAC, TRI	[218, 224, 240]
BmeB3	*B. fragilis*	TRI	[285]
MexAB-OprM	*P. aeruginosa*	TRI	[322]
MexCD-OprJ	*P. aeruginosa*	TRI, CHX	[61, 94]
MexEF-OprN	*P. aeruginosa*	TRI	[61]
MexJK	*P. aeruginosa*	TRI	[323]
OqxAB	*E. coli*	BAC, CET, CHX, TRI	[117]
SdeXY	*S. marcescens*	BAC, CHX, TRI	[50]

[a]*BAC* benzalkonium chloride, *CET* cetrimide, *CHX* chlorhexidine, *QAC* quaternary ammonium compounds, *TRI* triclosan

Not surprisingly, then, biocides such as QACs [26, 149, 167] and triclosan [26, 27, 149, 294, 296, 370] have been shown to select for efflux mutants in *E. coli* and *S. enterica* that are multidrug-resistant. The AcrAB-TolC multidrug efflux system *S. enterica* serovar Typhimurium is linked to resistance to a variety of farm disinfectants [297], which have been shown to select for mutants of *S. enterica* serovar Typhimurium that hyperexpress the AcrAB-TolC multidrug efflux system [149, 150, 297] and are multidrug-resistant [149, 150]. Similarly, exposure of *Bacteroides fragilis* to benzene-derived active compounds of commonly used analgesics, antiseptics, and cleaning agents induces efflux pump expression and a multidrug resistance phenotype that is reversed by an efflux pump inhibitor [287]. Triclosan in particular readily selects multidrug-resistant mutants that over-express RND pumps in *P. aeruginosa* [62], *S. maltophilia* [315] and *S. enterica* [149, 370], and the MexAB-OprM pump is the primary determinant of this organism's innate insusceptibility to this biocide [62]. An association between reduced triclosan susceptibility and increased resistance to multiple antibiotics in human and animal *Campylobacter* spp., isolates [295] is also suggestive of the presence in *Campylobacter* spp., of an RND family multidrug exporter(s) that accommodates both triclosan and antibiotics. Still, the RND type pumps identified to date in *Campylobacter* have yet to be assessed for an ability to promote triclosan resistance. The RND family exporter, MexCD-OprJ, a determinant of FQ resistance in lab and clinical isolates of *P. aeruginosa* [139, 270] is inducible by BAK and chlorhexidine [227], but only promotes resistance to the latter biocide [94]. Reports of BAK- [26] and triclosan- [26, 27] adapted *E. coli* exhibiting a multidrug resistant phenotype typical of overproduction of an RND family multidrug transporter and showing reduced susceptibility to chlorhexidine also suggest that such pumps may also accommodate this biocide. Still, unlike triclosan and, possibly, QACs, chlorhexidine-adapted *E. coli* does not exhibit a multidrug resistant phenotype, suggesting that this biocide does not readily select for RND type drug exporter-producing mutant strains, at least in vitro [26]. Resistance to pine oil found in household cleaners has also been linked to the expression of RND family multidrug efflux systems with in vitro-isolated pine oil-resistant *E. coli* showing overproduction of the AcrAB-TolC efflux system [224].

The discovery that many RND pumps accommodate both biocides and antibiotics and that the former can select for multiple antibiotic resistance efflux mutants has sounded alarm bells, given the unregulated use of biocides in a plethora of household products [170, 217, 380]. In addition and in light of antibiotic use in food animal production spawning antibiotic-resistant bacteria that can be transmitted to humans, the widespread use of biocides for pathogen control in food production and in processing risks selecting for multidrug-resistant RND pump-producing strains whose contamination of the food chain could pose a risk to human health [365]. Still, there are as yet no reports of biocide selection of antibiotic resistant organisms outside the laboratory and it is rightly pointed out that RND pump-expressing mutants displaying reduced susceptibility to biocides fail to provide resistance to 'at-use' biocide concentrations and, so, would not be selected by biocides used at recommended concentrations [67, 371]. Nontheless, it should be recognized that household products that often needlessly contain biocides can be used 'improperly' by consumers and that diluted products and/or residues might allow for growth of RND pump-hyperexpressing strains that are concomitantly multidrug resistant.

10.4.5.2 Natural Function of RND Efflux Systems

Gram-negative bacteria often carry genes for multiple RND family multidrug efflux systems that accommodate many of the same antimicrobials (Table 10.7), but are generally regulated independently by dedicated regulatory elements [271]. With few exceptions, too, RND family multidrug efflux systems are not expressed in response to antimicrobials. These observations suggest that antimicrobial efflux and resistance is not the intended function of these systems, and this is supported by reports of RND family multidrug efflux systems contributing to virulence and in vivo survival, stress responses and bacterial cell-cell communication (i.e., quorum-sensing) [265, 275, 277]. Still, in many instances the intended function of these RND multidrug efflux systems is unclear and the environmental signals/circumstances that naturally trigger their expression remain largely unknown. RND pump-hyperexpession seen in most reported multidrug resistant strains, for example, typically results from mutation of regulatory genes [1, 36, 39, 80, 121, 187, 247, 318, 334, 367, 369], which provides no insights into function or the circumstances of efflux system recruitment in vivo. Given their significance vis-à-vis antimicrobial resistance, elucidating the 'natural' function of these efflux systems and the details of 'natural' efflux gene regulation, and the identification of the growth/environmental circumstances that promote efflux gene expression will be critical in permitting predictions of when and where in a clinical setting these efflux systems might be recruited in pathogenic bacteria and potentially compromise antimicrobial chemotherapy.

RND family pumps contribute to the virulence/pathogenesis of *V. cholerae* [20], *Francisella tularensis* [19, 104], *S. enterica* serovar Typhimurium [30] and *Borrelia burgdorferi* [31], and so their production is likely to be favoured during infection where they will promote antimicrobial resistance. In vivo survival of *N. gonnorhoeae* in animal infection models is linked to the MtrCDE RND family multidrug efflux

system of this organism [141, 366], with a direct correlation seen between in vivo fitness/survival and pump expression levels [367]. Similarly, Mex pump-producing *P. aeruginosa* mutants are readily recoverable from an animal model of pneumonia in the absence of antimicrobial selection [143] suggesting that the in vivo environment favours pump production. Many of the RND family multidrug pumps of gut bacteria [e.g., *S. enterica* serovar Typhimurium [239, 282], *C. jejuni* [178, 180, 181], *B. fragilis* [286], *Vibrio cholerae* [18], and *E. coli* [307]] are induced by bile and promote bile resistance and, hence, survival in the gut - again, this means that the gut environment will specifically recruit pumps in these organisms that will promote antimicrobial resistance in vivo. In this vein, a recent report highlights the ability of the major mucin protein found in human mucous to induce the CmeABC multidrug efflux system of *C. jejuni* [354].

In some instances, RND pumps appear to function as components of a bacterial stress response. The MexCD-OprJ efflux system of *P. aeruginosa* is, for example, inducible by a variety of membrane active agents and is implicated in this organism's envelope stress response [94]. Any encounter of membrane-active agents in vivo, then, could promote MexCD-OprJ-mediated antimicrobial resistance and/or select for MexCD-OprJ-hyperexpressing multidrug-resistant mutants. Similarly, the MexXY aminoglycoside-resistance determinant of this organism is induced in response to oxidative stress caused by a variety of co-called reactive oxygen species (ROS) (e.g., H_2O_2) [277]. This raises the specter of ROS promoting MexXY-mediated aminoglycoside resistance in vivo. Intriguingly, *P. aeruginosa* encounters substantial ROS in the lungs of cystic fibrosis (CF) patients [64], and efflux-mediated aminoglycoside resistance (involving MexXY) is disproportionately represented amongst CF vs. other clinical *P. aeruginosa* isolates [274]. Moreover, most CF *P. aeruginosa* isolates harbour mutations in the *mexZ* gene encoding a repressor of *mexXY* and, so, are likely to express this efflux system [119, 332]. Again, this is consistent with environmental circumstances at the site of infection (i.e., ROS) promoting/selecting for efflux gene expression and, so, antimicrobial resistance.

Beyond environmental factors impacting multidrug efflux gene expression, there is some concern that non-antimicrobial chemotherapeutic agents intended for other medical uses might influence efflux gene expression and, so, antimicrobial resistance in Gram-negative pathogens. There are reports, for example, of anti-inflammatory agents inducing efflux and a multidrug resistant phenotype in *E. coli* [106]. Similarly, the central nervous system depressant diazepam has been shown, at a typical adult dosage, to promote efflux-mediated multidrug resistance in *E. coli* and *Klebsiella pneumoniae* [349] and exposure of *B. fragilis* to benzene-derived active compounds of commonly used analgesics induces RND efflux pump expression and a multidrug resistant phenotype that is reversible by the EPI carbonylcyanide *m*-chlorophenylhydrazone (CCCP) [287]. It is likely that these compounds promote efflux gene expression as a result of their mimicking a natural inducer/signal and/or their creating physiological conditions in the bacterium that naturally favours pump recruitment. The elucidation of the natural signals/functions of these efflux systems might prove useful, in this instance, in permitting prediction of which chemotherapeutic agents are likely to influence multidrug efflux pump production and antimicrobial resistance in vivo.

10.4.5.3 Overcoming Efflux

Given that RND pumps are significant determinants of resistance to clinically-important antimicrobials in Gram-negative pathogens and are broadly-distributed in these organisms, they need to be actively considered in the design and development of new antimicrobials and in the use of existing agents [189, 197]. The observation that elimination of efflux by genetic means (i.e., deletion of the efflux genes) compromises resistance in various Gram-negative bacteria [192, 244, 301] provides support for efflux inhibition as a viable approach to dealing with efflux-mediated antimicrobial resistance. In countering efflux, agents may be designed to avoid efflux thereby retaining their activities, despite the presence of this resistance mechanism or to inhibit efflux pump activity, expression or assembly and thereby counter efflux directly [200, 278, 359]. In all but the first instance, inhibitors would be used in conjunction with antimicrobials, whose activities would thus be restored, despite their being substrates for efflux. The development of such combination therapy has its difficulties, however, because of the need for precision tailoring of pharmacokinetics of both agents (inhibitor and antibiotic) to achieve the desired activity. One solution to this problem is to create hybrid EPI-antimicrobial molecules and, indeed, a molecule obtained by coupling an MF pump EPI to the plant antimicrobial berberine has been described that is active against pump-overproducing Gram-positive bacteria [12]. Whether or not effective RND pump EPI-antimicrobial hybrids can be produced remains to be seen.

Avoiding Efflux

The strategy of developing agents that bypass efflux has had some success in Gram-positive bacteria, where newer FQs [89, 131, 133, 144, 278, 342] and ketolides [32, 388] have been developed that are less prone to export by the endogenous efflux systems of these organisms. Similarly, the effectiveness of glycylcyclines (e.g., tigecycline) stems from their not being substrates for the Tet pumps that are the major efflux determinants of tetracycline resistance in Bacteria [251]. Still, the broadly-specific RND family pumps of Gram-negative bacteria accommodate newer FQs [85, 233, 284, 391]; ketolides [22, 24, 34, 252] and tigecycline [75, 79, 151, 152, 309–311, 361] (Table 10.7). These pumps are also responsible for the general lack of activity of many of the newer Gram-positive agents (e.g., linezolid [140, 261, 320) (Table 10.7)] against Gram-negative pathogens and, indeed, compromise the activity of many experimental agents as well [175], including peptide deformylase inhibitors [80], a novel topoisomerase inhibitor [221] and oxazolidinones (Table 10.7). Given the broad distribution of these pumps in Gram-negative bacteria, their broad substrate specificity and the (often) presence of multiple RND pumps in a given organism, there is some debate as to whether it will be possible to develop antimicrobials that cannot be accommodated by RND family pumps. Still, cationic antimicrobials like the polymyxins B and E (a.k.a colistin), which are gaining favour in the treatment of multidrug resistant Gram-negative infections [92, 220, 298, 387],

appear to be poor RND pump substrates. Indeed, there are few reports of RND pumps linked to reduced polymyxin susceptibility [4, 20, 357] (Table 10.7) and in these cases the impact is typically modest (≤4-fold change in MIC with vs. without the pump). The MtrCDE RND pump of *Neisseria meningitidis* is, however, a significant contributor to the innate insusceptibility of this organism to polymyxins [355] and MexAB-OprM of *P. aeruginosa* is linked to colistin tolerance that develops in a sub-population of biofilm cells following colistin exposure in vitro [249]. A new anti-pseudomonal aminopyrazolium cephalosporin, FR264205, has been described that is active against efflux-positive *P. aeruginosa* [346] and, as such, may be a poor substrate for this organism's RND pumps. Similarly, the novel methylcarbapenem, tomopenem, appears to be a poor RND pump substrate, its activity being minimally impacted by RND pump overexpression in *P. aeruginosa* (≤2-fold decrease in MIC in MexAB-OprM-, MexCD-OprJ and MexEF-OprN-overproducing mutants) [158].

Efflux Inhibition

The concept of efflux inhibition as an approach to dealing with pump-mediated antimicrobial resistance is supported by observations that uncouplers like CCCP that dissipate the proton gradient across the cytoplasmic membrane are able to reverse RND pump-mediated resistance and/or drug export [15, 22, 118, 283, 320, 393]; however, uncouplers are not practical therapeutic agents, since they will also adversely impact mammalian systems. Targeting pump-mediated resistance through the use of EPIs has received a lot of attention recently, with many agents displaying activity against one of more RND family pumps reported in the literature (Table 10.10) (reviewed in [189, 200, 248, 276, 339, 359]). Most of these have been developed against the AcrAB-TolC pumps of the Enterobacteriacae (e.g., various quinoline agents) [199] or the Mex pumps of *P. aeruginosa* (e.g., PAβN [a.k.a. MC-207,110] and other peptidomimetics) [192, 299, 368] where they potentiate antibiotic against wild type and efflux-pump-overproducing mutants and, so, address intrinsic and acquired resistance. In most cases, activity has only been demonstrated against a limited number of pumps in a limited number of organisms, although PAβN appears to be generally active against RND family pumps [115, 190, 192, 207, 219, 248]. As such, this compound is now routinely used as an indicator of pump activity in Gram-negative bacteria (i.e., its ability to potentiate antimicrobial activity in a given organism is an indicator of an efflux contribution to resistance) [13, 40, 42, 55, 59, 112, 118, 161, 202, 207, 253, 256, 284, 312]. PAβN does not potentiate antimicrobial activity in BpeAB-OprB-expressing *B. pseudomallei* [45] or SmeDEF-expressing *S. maltophilia* [314], though this may reflect problems with outer membrane permeation or stability of this peptide EPI rather than a lack of activity on these RND pumps. PAβN was also reportedly unable to potentiate antimicrobial activity in clinical isolates of *S. marcescens* [319]. A number of plant-derived EPIs have also been described although these tend to be active against Gram-positive organisms [339] and, possibly, mycobacteria [169] but not Gram-negative bacteria [339].

Table 10.10 Efflux pump inhibitors active against Gram-negative bacteria

Inhibitor	Organism	Representative antibiotic(s) potentiated[a]	Reference(s)
Phe-Arg-β-Naph	*P. aeruginosa*	CAR[b], ERY[b,c] GEN[d], LVX[b], MER[b], NOR[e]	[105, 192, 219]
	A. baumannii	CLR, MOX, LVX	[253]
	E. cloacae	AZT, CLR, ERY, LIN, TIG	[261]
	C. freundii	CLR, LIN, LVX	[319]
	E. aerogenes	CLR, LIN, LVX	[319]
	K. pneumoniae	CLR, LIN, LVX	[319]
	E. coli	CLR, LIN, LVX	[155]
	C. jejuni	ERY, CEF	[207]
Pyridoquinoline	*E. aerogenes*	CIP, NOR	[53]
Alkylaminoquinolone	*E. aerogenes*	NOR, TET	[201]
Alkoxyquinoline	*E. aerogenes*	CAM, TET	[54]
Chloroquinolines	*E. aerogenes*	CAM, NOR, TET	[102]
Arylpiperazine [e.g., 1-(1-naphtylmethyl)-piperazine]	*E. coli*	CAM, CLR, LIN, LVX, OXA, TET	[23]
	E. coli	LIN, LVX	[155]
	C. freundii	LIN, LVX, OXA	[319]
	E. aerogenes	CAM, LIN, LVX, OXA, TET	[319]
	K. pneumoniae	CAM, LIN, LVX, TET	[319]
	A. baumannii	CLR, CFP, FQ, LIN, OXA	[253]
Phenothiazine	*B. pseudomallei*	AMG, LVX, MAC	[46]
Arylpiperidine	*E. coli*	LIN	[351]
Phenylpiperidine	*E. coli*	TET	[145]

[a] The antibiotics listed are intended to illustrate the range of agents potentiated by a given inhibitor and/or to highlight those agents most significantly potentiated by the inhibitors. *AMG* aminoglycosides, *AZT* aztreonam, *CAM* chloramphenicol, *CAR* carbenicillin, *CEF* cefotaxime, *CFP* cefepime, *CIP* ciprofloxacin, *CLR* clarithromycin, *ERY* erythromycin, *FQ* fluoroquinolones, *GEN* gentamicin, *LIN* linezolid, *LVX* lovofloxacin, *MAC* macrolides, *MER* meropenem, *OXA* oxacillin, *TET* tetracycline, *TIG* tigecycline

[b] Potentiation of the indicated agent was demonstrated in MexAB-OprM-expressing reference and clinical strains

[c] Potentiation of the indicated agent was demonstrated in MexCD-OprJ-expressing reference and clinical strains

[d] Potentiation of the indicated agent was demonstrated in MexXY-expressing reference and clinical strains

[e] Potentiation of the indicated agent was demonstrated in MexEF-OprN-expressing reference and clinical strains

While the mode of action of most EPIs is unknown, some (e.g., PAβN) are substrates for the RND pump and, so, act as competitive inhibitors [192]. In light of data suggesting that RND pumps possess multiple substrate-binding sites within a larger substrate-binding pocket [230], such inhibitors typically potentiate only a subset of antimicrobials (i.e., those sharing the same binding site as the EPI) [192]. Aryl-piperazine EPIs active against the *E. coli* AcrAB-TolC and AcrEF-TolC RND pumps [23] also appear to potentiate the activity of some but not all of the

antimicrobial substrates of these transporters suggesting that these molecules may also be competitive inhibitors. The concept of EPIs as pump substrates themselves has been used in the creation of FQ derivatives as pump inhibitors, the idea being to convert a known antimicrobial substrate for most/all pumps to a broad-spectrum inhibitor. An ofloxacin-based EPI has, for example, been developed that is active against Gram-positive MF and MATE pump-expressing organisms [101]. Whether this strategy will be effective in the case of RND pump-producing Gram-negative organisms remains to be seen. While a failure to potentiate all pump substrate antimicrobials might be seen as a disadvantage of this type of EPI, RND type multidrug pumps are usually relevant clinically for selected antimicrobials (e.g., FQs in Enterobacteriaceae and *P. aeruginosa*, macrolides in *H. influenzae*) such that competitive RND EPIs that potentiate only FQ and/or macrolide activity would still be useful. Moreover, it is expected that development of resistance to such EPIs would be difficult since pump mutations leading to inhibitor resistance (i.e., reduced inhibitor affinity) would also compromise binding and, so, efflux of and resistance to antibiotic substrates. Significantly, EPIs such as PAβN have been shown to reduce the frequency of emergence of erythromycin-resistant *C. jejuni* [207] and deletion of RND pumps of *P. aeruginosa* [191], *E. coli* [208], *S. enterica* serovar Typhimurium [301] and *Campylobacter* spp. [379] compromises selection of FQ resistant mutants suggesting that EPIs may not only potentiate antimicrobial activity but limit resistance development as well.

Efflux inhibitors might also act at sites distinct from those involved in antibiotic binding, but whose disruption impacts overall pump activity. Such non-competitive, allosteric inhibitors would be expected to potentiate the activity (i.e., impede efflux) of all pump antimicrobial substrates. A series of structurally diverse inhibitors with high selectivity towards the MexAB-OprM efflux pump from *P. aeruginosa* have been identified and shown to be non-competitive EPIs that negatively impact export of all MexAB-OprM antimicrobial substrates equally [232]. It was hypothesized that these EPIs bind not to substrate-binding sites on the pump but rather to site(s) that modulates pump activity (i.e., modulation sites). Mutants insensitive to these EPIs (i.e., recover drug export capability in the presence of the EPI) have been recovered and mapped to the *mexB* gene [278], consistent with these inhibitors targeting the MexB component. Various derivates have since been made with improved features [233–235, 382–384]. While the ability of a single inhibitor to affect multiple pump substrates seems advantageous, the fact that these are pump-specific (and that insensitive mutants can be isolated) limits their clinical utility.

Inhibition of Pump Assembly

Given that RND family pumps are multicomponent, with all components necessary for antimicrobial export, it is theoretically possible to inhibit pump function my interfering with pump assembly. One approach is to define regions of individual proteins involved in interactions with other proteins of the complex and to synthesize short peptides (so-called interface peptides) as mimics of these regions that

might then compete with and, so, displace the native proteins during assembly [330]. While this approach is hindered by the lack of a structure for the RND-MFP-OMF pump complex, available structures of individual pump components in *E. coli* [123, 160, 230, 324] and *P. aeruginosa* [2, 3] and information regarding regions of each involved in component-component interactions [88, 162, 188, 236–238, 340, 352] do provide a starting point. Alternatively, pump assembly inhibitors might be identified using an in vitro assay of protein-protein interaction and screening compound libraries for agents able to block this interaction and, so, likely to impede assembly. This approach was used successfully in identifying inhibitors of *E. coli* RNA polymerase assembly that were effective in inhibiting RNA polymerase activity [9]. Conveniently, sites of RND-MFP-OMF interaction apparently occur within the periplasm and, so, assembly-inhibitory compounds, peptides or peptidomimetics would only need to cross the outer membrane to be effective.

Other Approaches

The observation that the periplasmic entrance of the TolC OMF component of the *E. coli* AcrAB-TolC pump is the sole constriction of this outer membrane channel [160], that it is widely conserved amongst OMF components of RND-MFP-OMF efflux systems [2], and that TolC channel-forming activity can be inhibited by divalent and trivalent cations that target this region of TolC [8], suggests that targeting the OMF channel may also be a viable approach to inhibiting efflux. In theory, too, interfering with efflux gene expression will counter efflux-mediated antimicrobial resistance. Proof-of-principle of this was provided by the demonstration that antisense DNA analogues of *marA*, encoding a broadly-conserved activator of *acrAB-tolC* expression in the Enterobacteriaceae [5] were able to restore FQ activity against multidrug-resistant *E. coli* overexpressing AcrAB [5]. Apparently, these compounds blocked MarA production and, so, *acrAB* expression. An active ingredient from the herb *Andrographis paniculata*, andrographolide, has been shown to counter (modestly) antimicrobial resistance in both wild type and MexAB-OprM-overproducing strains, apparently by reducing *mexAB-oprM* gene expression [375]. Similarly, the macrolide azithromycoin has been shown to reduce *mexAB-oprM* expression and resistance to multiple antimicrobials in wild type *P. aeruginosa* [344]. Whether these compounds are acting on regularly elements to directly manipulate efflux gene expression or are impacting cell physiology such that they indirectly influence this is unclear. Finally, a novel antimicrobial approach that usurps rather than blocks efflux has been described recently [392]. In this case, pump substrates have been modified to contain a metal chelator moiety, the idea being that the pump substrate-chelator conjugates enter cells, bind metals and are then actively exported, thereby upsetting intracellular metal homeostasis. While these compounds have demonstrable antimicrobial activity against Gram-positive organisms (e.g., *Bacillus subtilis*) [392], it is far from clear that this approach will work in Gram-negative bacteria given evidence that RND pumps capture their substrates in the periplasm [229, 325] possibly even before they enter the cytosol.

EPIs and Diagnostics

Given the significance of efflux vis-à-vis antimicrobial resistance, there is obvious value from a diagnostic as well as epidemiological standpoint in being able to rapidly identify efflux determinants when they are contributing to antimicrobial resistance in clinical isolates. The efflux status of an organism can, for example, be assessed using quantitative real-time RT-PCR of known efflux genes [48, 105, 204] or via DNA microarray-based genotyping of efflux mutations [372]. Still, since efflux gene expression often fails to correlate with resistance [132, 333] these are unreliable indictors of efflux as active determinants of resistance. A plate-based fluorescence assay of ethidium bromide accumulation has been described and may be useful as a general indicator of efflux activity in bacteria [209]. EPIs can be effective indicators of an efflux contribution to resistance, with broad-spectrum inhibitors such as PAβN useful as general indicators of efflux and pump-specific EPIs (e.g., the MexAB-OprM-specific inhibitors discussed above) useful in identifying specific efflux mechanisms. Still, it is unclear whether it will be practical (or possible) to develop specific inhibitors for all clinically-relevant efflux mechanisms in a given Gram-negative pathogen. Combining a broad-spectrum inhibitor with an antimicrobial uniquely exported by a given RND pump (e.g., carbapenem for MexAB-OprM and aminoglycosides for MexXY-OprM) is one way around this problem since potentiation of the activity of such ‘reporter’ antimicrobials will confirm that the corresponding efflux system is present and active. This approach has been validated in a study of various RND pump-producing mutants of *P. aeruginosa*, where pump expression as predicted by antimicrobial potentiation was, for the most part, backed up by RT-PCR evidence [219].

10.5 Conclusions and Future Perspectives

Efflux as a mechanism of resistance, drug-specific and multidrug, is well established in the antimicrobial literature, although clearly all the players, real and potential, have yet to be identified. There are, for example, many reports of efflux activity independent of known/identified efflux systems and a somewhat recent study of the reservoir of resistance determinants present in soil-dwelling bacteria is suggestive of novel, possibly efflux mechanisms of resistance to agents for which efflux is not yet identified as a resistance mechanism [72]. Conversely, the clinical significance of many known efflux determinants has yet to be demonstrated - studies that rely on cloned genes and in vitro strain construction in identifying efflux determinants of resistance serve only to identify potential resistance mechanisms. In any case, as efflux mechanisms are identified and characterized, there needs to be an effective means of monitoring them in clinical populations so as to better assess their prevalence and, thus, significance as resistance determinants in clinical strains. Gram-negative RND pumps are, perhaps, most worrisome owing to their multiplicity in given organisms, their broad distribution amongst human pathogens and their broad substrate-specificity,

which compromises the effectiveness of many existing antimicrobial agents and likely limits the possibilities for developing effective new agents. As such, therapeutic approaches that incorporate efflux inhibiton need to be seriously considered, at least for those organisms and those agents where RND pumps are primary determinants of resistance. Given their broad substrate specificity, too, RND pumps have the potential to adversely impact whole cell screens of compound libraries – new and potentially useful agents may well be missed if they are pump substrates, and will not be available for use if/when anti-efflux strategies become practical and/or necessary. Using pump-deficient strains in such screens is one way to address this. Finally, the general lack of information as regards regulation of RND pump genes and the environmental signals that might influence pump gene expression in vivo is also problematic. With little or no knowledge of environmental circumstances that naturally promote efflux gene expression and efflux-mediated antimicrobial resistance or the possible adverse consequences of other therapeutics stimulating efflux gene expression, the potential for exacerbation of the problem of efflux-mediated antimicrobial resistance is very real. In addition, since in vivo conditions may well influence efflux gene expression and antimicrobial resistance in ways that are not reflected in in vitro tests, environmental impacts on efflux gene expression may provide one explanation for in vitro susceptibility testing not always providing an accurate or useful measure of in vivo susceptibility [343].

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Chapter 11
Structural Mechanisms of β-Lactam Antibiotic Resistance in Penicillin-Binding Proteins

Robert A. Nicholas and Christopher Davies

11.1 History

The discovery of penicillin is one of the most fortuitous events in modern medicine. Sir Alexander Fleming described in 1929 that staphylococcal bacteria in an agar dish lysed in the area surrounding a contaminating mold (*Penicillium notatum*), and he correctly surmised that the fungus was secreting a bactericidal substance, which he called penicillin [1]. Although this observation was instrumental in the future development of the antibiotic, it wasn't until the 1940s, when Ernst Chain and Howard Florey were able to isolate enough of the compound for human testing, that the full power of the antibiotic was realized. In 1945, Giuseppe Brotzu isolated a fungus that produced cephalosporin C, and the discovery of an easy method to produce large amounts of 6-aminopenicillanic acid and 7-aminocephalosporonic acid, which are the core structures of penicillin and cephalosporin antibiotics (Fig. 11.1), led to the synthesis of hundreds of different β-lactam antibiotics, many of which are still in use today. These antibiotics share a common β-lactam ring structure, but they differ in both the second ring fused to the β-lactam ring and substitutions at two positions. These substitutions determine both the spectrum of action of the antibiotics and their pharmacokinetic properties (see Chap. 3). Based on these structures, it is clear that the minimal structural unit with antimicrobial activity is the β-lactam ring structure and an acidic moiety.

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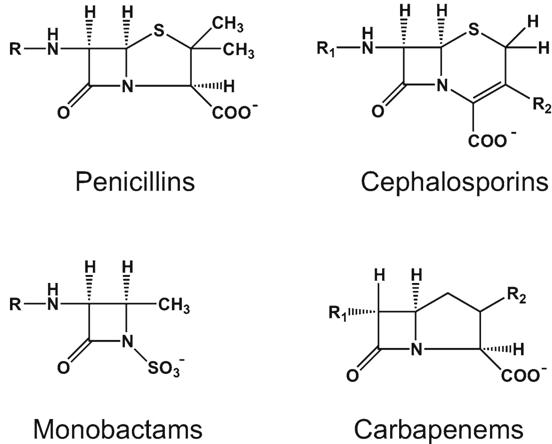


Fig. 11.1 Structure of the four main classes of β-lactam antibiotics

11.2 Peptidoglycan Structure and Biosynthesis

Much of the work in the 1950s and 1960s was focused on determining the mechanism of action of penicillin. It was known early on that penicillin affected the integrity of the bacterial cell wall, which surrounds the bacteria and prevents lysis under osmotic stress. A concerted focus to understand its structure and biosynthesis revealed that the cell wall is composed of both linear glycan strands containing a repeating disaccharide (*N*-acetylglucosamine-β-1,4-*N*-acetylmuramic acid; GlcNAc-MurNAc) and a peptide chain attached to the carboxylate from MurNAc (thus the name "peptidoglycan" was adopted). In mature peptidoglycan, the peptide chains are cross-linked to one another, which confers strength to the bacterial cell wall (Fig. 11.2). The building block of peptidoglycan, a disaccharide pentapeptide, is synthesized in both the cytoplasm and the inner leaflet of the cytoplasmic membrane and is then translocated to the outer leaflet, where it is incorporated into nascent peptidoglycan strands. It was clear from early studies that penicillin interfered only with the final stage of peptidoglycan biosynthesis, the cross-linking of the peptide chains.

The peptide strands of peptidoglycan vary between Gram-positive and Gram-negative bacteria, but the presence of a free NH_2 group in the side chain of the third amino acid and the D-Ala-D-Ala at the C-terminus of the peptide are invariant. For example, in *Staphylococcus aureus* peptidoglycan, the third amino acid is L-Lys, which is then modified by the addition of five glycine residues onto the ε-NH_2 group; however, this modification retains a terminal NH_2 group while increasing its distance from the peptide chain. In contrast, peptidoglycan from *E. coli*, *N. gonorrhoeae*, and most other Gram-negative organisms contains *meso*-diaminopimelic acid (*m*-DAP), a derivative of lysine, at the third position of the peptide chain, and no further amino acid additions are observed (Fig. 11.2).

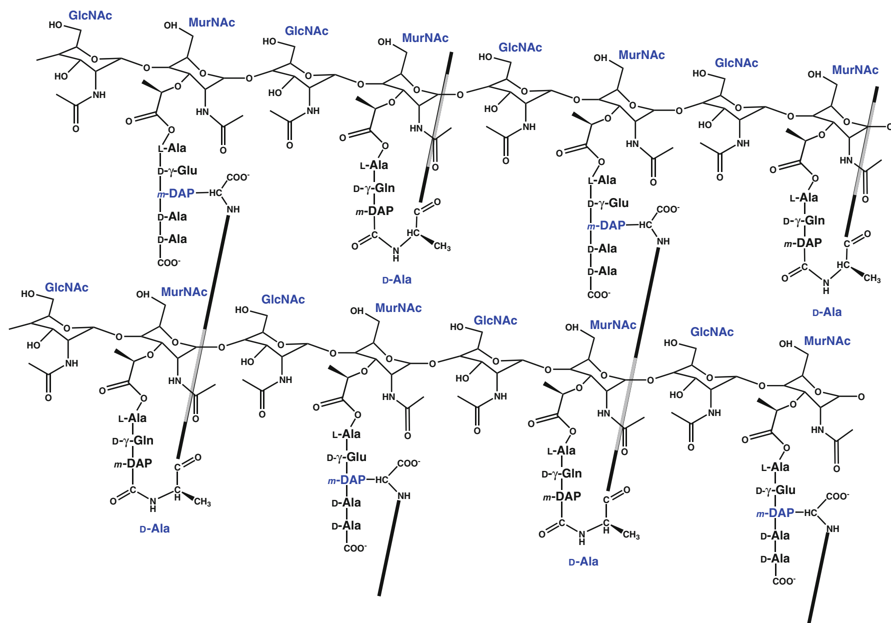


Fig. 11.2 Peptidoglycan structure in Gram-negative bacteria. Peptidoglycan consists of disaccharide polymers comprised of repeating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) moieties. Pentapeptides of L-Ala-D-γ-Glu-*meso*-diaminopimelic acid (*m*-DAP)-D-Ala-D-Ala are attached to the carboxylate from the MurNAc sugar residues, and these peptides are cross-linked to other peptides via a *m*-DAP transpeptide linkage to the penultimate D-Ala of a peptide chain from an adjacent glycan strand

During normal synthesis, a nucleophilic Ser residue in peptidoglycan-synthesizing enzymes reacts with the D-Ala-D-Ala C-terminus of the peptide chain, forming a covalent acyl-enzyme complex with the penultimate D-Ala residue and releasing the ultimate D-Ala. The acyl-enzyme complex can react with the NH_2 group from the third amino acid (either Gly-NH_2 or m-DAP-NH_2) of another peptide chain to form a peptide cross-link (transpeptidation) or with H_2O to release the peptide (carboxypeptidation) (Fig. 11.3). Penicillin exerts its lethal effect because it is a structural mimic of the acyl-D-Ala-D-Ala C-terminus of the peptide chain [2, 3]. It reacts with the same Ser nucleophile that reacts with peptide [4, 5], but once the acyl-enzyme complex is formed, it is very stable and the enzyme is essentially irreversibly inactivated (Fig. 11.3). For this reason, the peptidoglycan-synthesizing enzymes are known collectively as penicillin-binding proteins or PBPs. β-lactamases, which are structurally and mechanistically related to PBPs, have evolved an efficient hydrolysis mechanism for the β-lactam-enzyme complex, thereby inactivating the antibiotic and providing an effective means of resistance to β-lactams (Fig. 11.3: see below and Chap. 12).

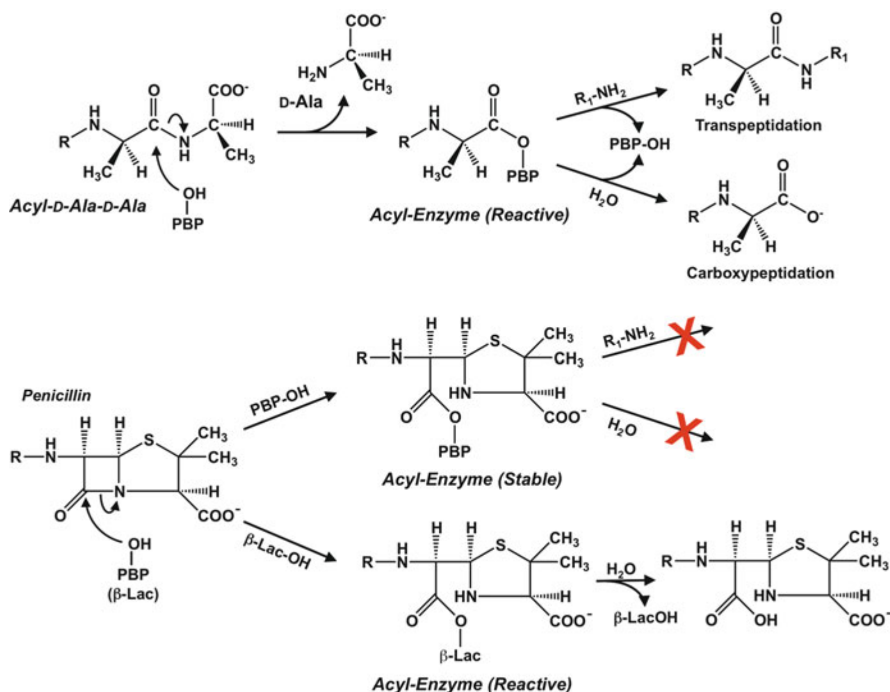


Fig. 11.3 Mechanism of action of β-lactam antibiotics. Shown at the *top* is the normal reaction catalyzed by PBPs. PBPs attack the amide bond between the two D-Ala residues of a peptide chain, resulting in an acyl-enzyme covalent complex. This complex rapidly reacts with either an NH_2 group from m-DAP to form a cross-link (transpeptidation) or H_2O to release the peptide (carboxypeptidation). Penicillin and other β-lactam antibiotics are substrate analogs, and react with PBPs to form an penicilloyl-enzyme complex, which, in contrast to the PBP-peptide complex, is stable, thereby blocking both hydrolysis and aminolysis. β-lactamases, which are structurally related to LMM PBPs [94, 95], react in the same manner as PBPs, but the β-lactamases have evolved a highly efficient hydrolysis mechanism that rapidly inactivates the β-lactam

11.3 PBPs and Their Roles in Cell Morphology and Viability

Because PBPs form a stable complex with β-lactam antibiotics, PBPs are readily identified by incubating cell membranes with either a radioactive (e.g., [^{14}C] penicillin G; [6, 7]) or fluorescent (e.g., BOCILLIN FL; [8]) β-lactam antibiotic, followed by SDS-PAGE and detection; these PBPs are named in order of decreasing migration by SDS-PAGE (Fig. 11.4). In *E. coli*, the organism that has been best studied, eight PBPs can be observed, while in *N. gonorrhoeae*, only three PBPs are observed; moreover, *E. coli* has two PBPs (PBPs 1C and 6b) encoded in the genome that are not readily detectable by this method, while *N. gonorrhoeae* has one such PBP (PBP 4). Because different organisms have different numbers of PBPs, PBP 2 (or any other numbered PBP) from one organism is not necessarily the homologue

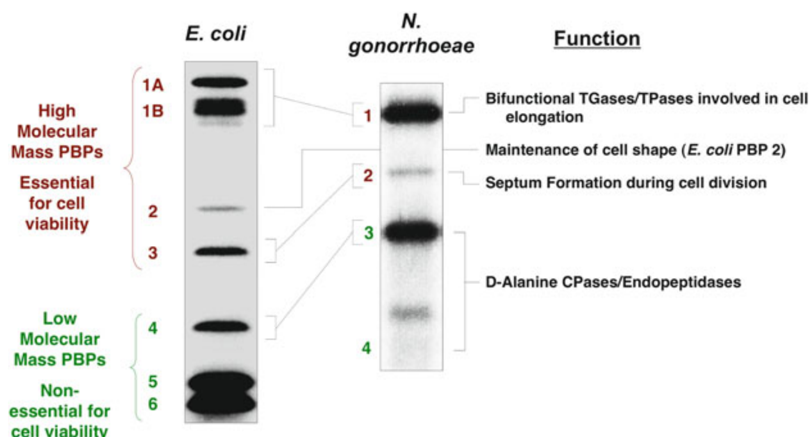


Fig. 11.4 PBPs from *E. coli* and *N. gonorrhoeae*. PBPs from the indicated bacterial species were identified by incubating membranes with labeled β-lactams followed by SDS-polyacrylamide gels and imaging. The PBPs can be separated into two groups, the high-molecular-mass (HMM) PBPs, which are essential for viability and catalyze TPase activity, and the low-molecular-mass (LMM) PBPs, which are non-essential for viability and catalyze CPase and/or endopeptidase activity. The expression of PBP 4 in *N. gonorrhoeae* is only inferred based on genomic sequences and cannot be detected by this method; however, deletion of both PBP 3 and 4, but not either one alone, results in marked defects in cell morphology [19, 20]. PBP 7 is not shown in the gel for E. coli PBPs, although it can be detected by SDS-PAGE

of PBP 2 from another. These PBPs can be classified into two main groups: the high-molecular-mass (HMM) PBPs, which are essential transpeptidases (TPases) involved in peptidoglycan cross-linking, and the low-molecular-mass (LMM) PBPs, which are non-essential carboxypeptidases (CPases) and/or endopeptidases [9] (Fig. 11.4). The HMM PBPs can be further subdivided into two classes: Class A and Class B. Class A PBPs are bifunctional enzymes that harbor both transglycosylase (TGase) and TPase domains, which catalyze polymerization of glycan strands and cross-linking of peptide chains, respectively. These PBPs synthesize a major portion of the cell wall peptidoglycan during elongation of a daughter cell into one that is ready to divide again. Class B PBPs have only a TPase domain and an additional domain that is hypothesized to target the PBP to its location during cell wall synthesis [10]. Class B PBPs are involved in determination of cell shape (for rod-shaped bacteria such as *E. coli*) or in septation during cell division (all bacteria). Class B PBPs are often the major targets of β-lactam antibiotics due to their higher rate of inactivation with these compounds relative to Class A PBPs. There have been suggestions and at least some data supportive of the idea that PBPs form multi-protein complexes with other enzymes involved in the synthesis or breakdown of peptidoglycan [11–14], including the idea of a "divisome" that functions during cell separation [15]; however, whether these are de facto complexes or are simply the co-localization of enzymes remains an open question.

All PBPs and β-lactamases (the exception being metallo-enzymes, which utilize a different mechanism) contain a structurally conserved penicillin-binding domain

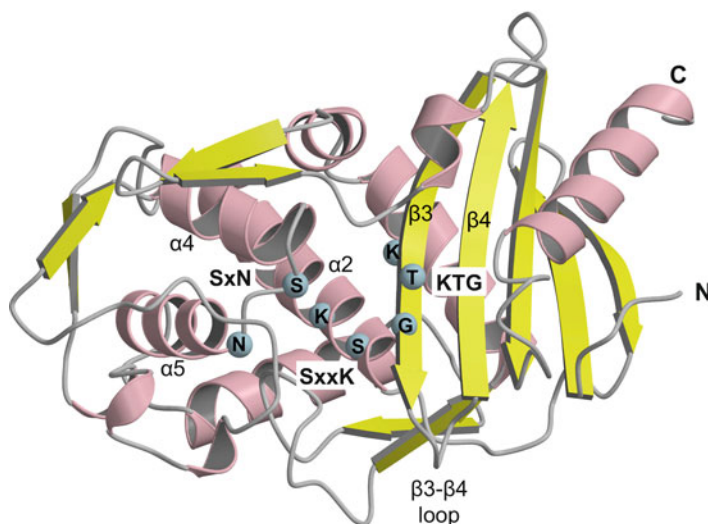
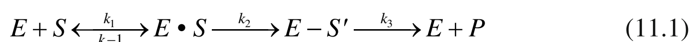


Fig. 11.5 Structure of a typical penicillin-binding domain conserved in all serine-based PBPs and β-lactamases. The structure of the penicillin-binding domain of EcPBP 5 [96] is shown as a representative example. The structure is displayed in *ribbon* format with α helices colored *pink* and β strands colored *yellow*. The structure comprises two sub-domains: one predominantly α helical (on the *left*) and one containing a five-stranded antiparallel β sheet (on the *right*). The Cα carbons of the residues that comprise the three active site motifs are shown as *blue* spheres

consisting of two sub-domains: a set of five antiparallel β-strands packed on both sides by α-helices and a cluster of α-helices (Fig. 11.5). The active sites of these enzymes invariably contain a conserved set of three sequence motifs. These include the SxxK tetrad, which contains the active site Ser nucleophile, the SxN triad, and the KTG triad (Fig. 11.5). As discussed below, these amino acids form a dense network of hydrogen bonds, which helps to activate the Ser nucleophile and promote acylation by the peptide substrate or β-lactam antibiotic.

11.4 Kinetics and Catalytic Mechanism of PBPs

The kinetic scheme for the interaction of a PBP with a β-lactam antibiotic is defined by Eq. 11.1, where *E•S* is the Non-Covalent



$$E + S \underset{k-1}{\overset{k_1}{\longleftrightarrow}} E \bullet S \xrightarrow{k_2} E - S' \xrightarrow{k_3} E + P \qquad (11.1)$$

Michaelis complex between the PBP and β-lactam antibiotic or peptide substrate, *E-S′* is the covalent acyl-enzyme complex, and *P* is the released and hydrolyzed product [16]. The constant k_2/K_S, in which $K_S=(k_{-1}+k_2)/k_1$, describes the initial rate

of formation of covalent β-lactam antibiotic-PBP conjugates ($E-S'$) at sub-saturating concentrations of β-lactam antibiotics. In essentially all PBPs, $k_2 << k_{-1}$ and thus K_S represents the Michaelis constant for non-covalent binding of β-lactam antibiotics to the PBP. For PBPs, the deacylation rate constant, k_3, is almost always very slow relative to the other rates, so that acylated $E-S'$ accumulates. Prolonged occupation of the active site that denies access to the natural substrate is the basis for the lethal action of these antibiotics. For β-lactamases, k_3 is quite rapid and the enzyme catalyzes the efficient hydrolysis of the β-lactam, rendering it inactive.

Different PBPs, even from the same species, can have markedly different rates of acylation with a particular β-lactam antibiotic. For example, *N. gonorrhoeae* has four PBPs, denoted PBPs 1–4. In vitro acylation rate constants of penicillin G for the four purified PBPs are 4,000, 75,000, 200,000, and 30,000 M^{-1} s^{-1} for PBPs 1–4, respectively [17–20]. Superimposition of active site residues in the crystal structures of two unrelated PBPs demonstrates a high degree of overlap to the point that one active site looks very much like any other, and the structural basis for high rates of acylation is not obvious. This observation suggests that the sphere of amino acids around the catalytic center plays an important role in defining the acylation rates by β-lactam antibiotics. In fact, it is these amino acids, and not those within the conserved motifs of the active site, that are often altered in PBPs from resistant bacteria; this will be discussed in further detail below.

The catalytic mechanism of PBPs has been studied extensively, and although great strides have been made, consensus has yet to emerge. To assist, analogies have been drawn from multiple structural and biochemical studies of β-lactamases, which are evolutionarily and mechanistically related to PBPs; however, it is noteworthy that after all the intense investigations of β-lactamases, the mechanism of acylation remains unresolved [21–23]. The peptidoglycan substrate for PBPs is an extensive and complicated polymer, and a challenge for enzymologists is to develop in vitro assays of activity. To date, there is no straightforward assay for transpeptidation with HMM PBPs. A thioester depsipeptide substrate has been used as a reporter of TPase activity with HMM PBPs (both Class A and Class B), but this assay relies on the labile thioester linkage in the substrate for activity and requires HPLC to determine the levels of transpeptidation [24]. A linked TGase/TPase assay also has been developed for analysis of Class A PBPs [25, 26]. This assay utilizes radiolabeled lipid II substrate (C55 lipid-P-P-GlcNAc-MurNAc-pentapeptide), isolated PG, and purified Class A PBPs. TGase activity is quantified by measuring the amounts of radioactivity attached to PG, and TPase activity is determined by HPLC. Because both of these assays are rather cumbersome and low throughput, most of the focus of the field has been directed toward LMM PBPs that function as CPases, because these can be assayed using minimal peptide substrates such as N,N′-diacetyl-L-Lys-D-Ala-D-Ala [27–29]. One such CPase is EcPBP 5, a non-essential PBP from *E. coli*, and this enzyme has been exploited as a model system to probe the catalytic mechanism of PBPs.

For the acylation step in PBPs, it is generally accepted that the nucleophilicity of the active-site serine residue present on the SxxK motif (Ser44 in EcPBP 5) is enhanced by the lysine of the same motif (Lys47), and thus a mechanism in which Lys47 acts as a general base to abstract the proton from the serine hydroxyl is

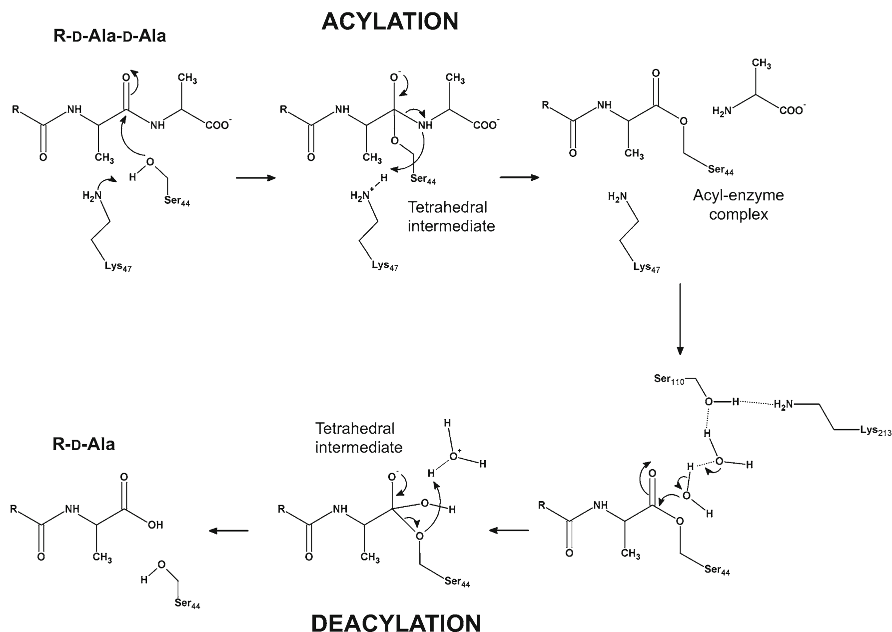


Fig. 11.6 A putative catalytic mechanism for carboxypeptidation catalyzed by *E. coli* PBP 5. The reaction comprises two steps: acylation and deacylation. During acylation, Ser44, which is polarized by Lys47, undergoes nucleophilic attack on the penultimate D-Ala of the peptide substrate, leading to loss of the terminal D-Ala and formation of a short-lived covalent ester intermediate. During deacylation, the catalytic water is activated by Ser110, which in turn is polarized by linkage to Lys213 from the KTG motif, through a bridging water molecule and hydrolyzes the ester linkage. For transpeptidases, the nucleophile for deacylation is the amino group of the *meso*-diaminopimelic acid (*m*-DAP) moiety of an adjacent peptide and the result is a peptide cross-link (see also Figs. 11.2 and 11.3)

envisioned (Fig. 11.6) [30]. This means that in the ground state, the amino group of the Lys side chain must exist in the neutral, unprotonated state, and hence its pKa must be shifted considerably towards neutrality. The very dense hydrogen network in the active site (Fig. 11.7) may permit this otherwise unfavorable shift in pKa. The pKa value of 8.2 on the ascending limb of the pH profile of EcPBP 5 has been attributed to Lys47 of the SxxK motif [29]. Once activated, Ser44 attacks the carbonyl of the penultimate D-Ala (or the equivalent carbonyl of β-lactams), leading to a tetrahedral transition state that breaks down to form a covalent intermediate. The negative charge of the oxygen in the tetrahedral intermediate is stabilized by an oxyanion hole comprising the amide nitrogen of the serine nucleophile (Ser 44), and the amide nitrogen of the residue that immediately follows the KTG motif in β3 (His216 in EcPBP 5) (Fig. 11.6). The fate of the abstracted proton remains an unresolved issue, but its most likely destination is the amino group of the D-Ala leaving group.

The mechanism of deacylation in PBPs remains less clear, and yet this is crucial information, because it is the blockage of this step with β-lactams that lies at the heart of their bactericidal activity. There are two schools of thought regarding the

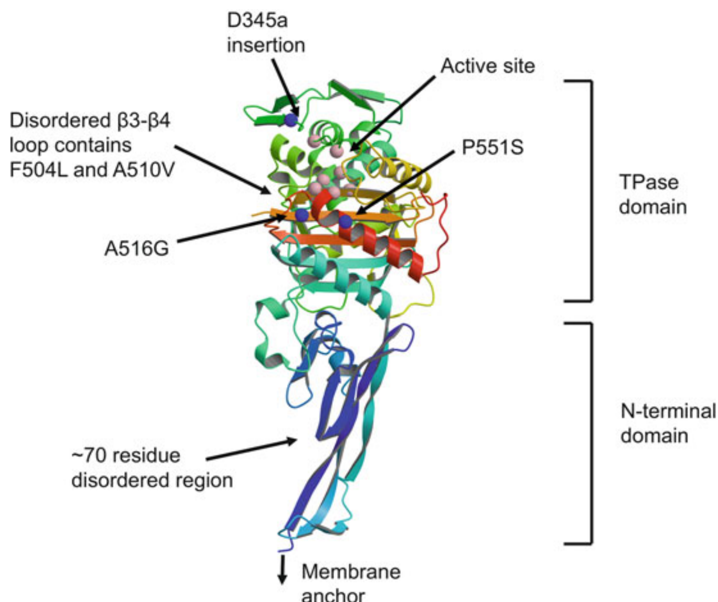


Fig. 11.7 The active site of a representative PBP using *E. coli* PBP 5 as an example. The three conserved active site motifs that are the hallmark of all serine-based PBPs and β-lactamases are shown. The SxxK motif, which contains the serine nucleophile, Ser44, is colored *blue*, the SxN motif is colored *green* and the KTG motif is shown in *orange*. The *blue spheres* near Ser44 and just past the KTG motif are the amide nitrogens that form the oxyanion hole of *E. coli* PBP 5

mechanism of deacylation: The first posits a symmetrical mechanism in which the lysine of the SxxK motif activates the incoming amino group of an adjacent peptide (in the case of TPases) or water molecule (in the case of CPases). Evidence in favor of this mechanism is the proposed participation of Lys47 (of EcPBP 5) in the descending limb of the pH profile, as well as molecular dynamics simulations [31]. The second theory, which we favor, holds that the Ser110 of the SxN motif is primarily responsible for deacylation by activating the incoming amino group or water via a polarization network or proton relay involving Lys213 and a second water molecule that bridges the incoming hydrolytic water and the serine hydroxyl [30]. This mechanism is supported by the proximity of Ser110 and bridging water molecule to the presumed position of the hydrolytic water, which corresponds to the O3 oxygen of the boronic acid inhibitor established by the crystal structure of PBP 5 in complex with a boronic acid mimic of the tetrahedral intermediate for deacylation [30]. Similar mechanisms have been proposed for both the *Streptomyces* R61 and *Actinomadura* R39 DD-peptidases, with the exception that the Ser residue in the SxN motif (or Tyr in the R61 DD-peptidase) activates the water molecule directly [32, 33]. Although it appears less than optimally positioned in the crystal structure of EcPBP5, a role for the lysine of the SxxK motif in the deacylation mechanism of PBPs cannot be ruled out at this stage [31, 33].

Whilst debate over the mechanistic details continues, the establishment of the position of the hydrolytic water in the active site of PBP 5 has illuminated how

deacylation is blocked in a complex of PBP with a β-lactam and not a peptide. It had long been assumed that the continued presence of the thiazolidine ring after acylation (see Fig. 11.3) in some way blocked the incoming water molecule (or amino group for TPases) [34, 35], and this was confirmed by recent structures of EcPBP 5 in complex with β-lactams [36]. Superimposition of three such acylated structures showed that the ring nitrogen of the β-lactam blocks the trajectory of the incoming hydrolytic water molecule toward the covalent bond of the acylated complex. Such blockage does not occur for peptide complexes because this region of the β-lactam corresponds to the terminal D-Ala, the leaving group for acylation. Hence, it is the ring structure of β-lactams that is critical for their success as inhibitors of PBPs [34].

11.5 Factors that Determine the Antimicrobial Activity of a β-Lactam Antibiotic

There are multiple factors that contribute to the efficacy of a particular β-lactam antibiotic against a specific organism. The first and arguably most important is that the β-lactam antibiotic must have a high rate of acylation for at least one essential PBP (e.g., PBPs 1A, 1B, 2 or 3 in *E. coli* and PBPs 1 or 2 in *N. gonorrhoeae*). Inhibition of even a single essential PBP is sufficient to render a β-lactam antibiotic potentially effective in treating infections caused by that organism. When a β-lactam antibiotic targets only one essential PBP, the MIC of that antibiotic is inversely proportional to its k_2/K_S constant for that PBP, such that the higher the k_2/K_S constant, the lower the MIC of the antibiotic for that organism. When a β-lactam targets multiple essential PBPs, the PBP that is inhibited by the lowest concentration of antibiotic (i.e., the one with the highest k_2/K_S) is considered the lethal target.

An additional factor is permeation of the antibiotic through porin channels in the outer membrane of Gram-negative bacteria. These porin channels are large, water-filled pores through which the antibiotics must diffuse to reach the PBPs in the periplasm. Most porins show ion selectivity, with many porins being slightly more anion selective. Another selectivity filter is the degree of hydrophobicity, in which hydrophilic compounds permeate through *E. coli* porins more readily than hydrophobic compounds [37]. For example, penicillin G is not particularly effective against most Gram-negative bacteria, in part because it does not diffuse through porins as readily as the more hydrophilic (and more effective) antibiotic, ampicillin (ampicillin differs from penicillin by a single amino group in the phenylacetyl R group). A notable exception to this "rule" is *N. gonorrhoeae*, where penicillin is very effective in treating infections caused by susceptible wild type strains (see Chapter 26 for more information on porin-mediated diffusion).

The final factor that determines the effectiveness of an antibiotic is whether the organism has acquired resistance to the antibiotic. The primary mechanisms of resistance to β-lactam antibiotics employed by pathogenic bacteria include production of β-lactamases, which hydrolyze β-lactam antibiotics and render them

inactive (discussed in more detail in Chap. 12), decreased influx/increased efflux, which in Gram-negative bacteria prevents the antibiotics from reaching their targets (discussed in Chapters 10 and 26), and alterations in essential PBPs, which lowers their rates of acylation by the β-lactam antibiotic (this chapter). As will be discussed below, *N. gonorrhoeae* utilizes all three of these mechanisms to become resistant to penicillin G.

PBP-mediated resistance mechanisms-PBP-mediated resistance is a complex phenomenon. There are two primary mechanisms employed by pathogenic organisms: (1) acquisition of a new PBP with a low rate of acylation with β-lactam antibiotics (observed only in methicillin-resistant *Staphylococcus aureus* or MRSA) or (2) recombination of genes encoding essential PBPs with the corresponding genes from commensal species to generate "mosaic" alleles that encode PBPs with lower rates of acylation by β-lactam antibiotics. The latter phenomenon occurs in pathogens that undergo high rates of homologous recombination (i.e., *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Enterococcus*, and *N. gonorrhoeae)*.

MRSA strains are characterized by the presence of a new Class B PBP, called PBP 2a, which has very low acylation rates with essentially all β-lactam antibiotics [38]. Although methicillin-sensitive *S. aureus* strains lacking PBP 2a have several essential PBPs, inactivation of any of which leads to cell death, after its acquisition, PBP 2a becomes the only TPase that is essential for cell survival. Since β-lactam antibiotics are poor inhibitors of this enzyme, MRSA strains can no longer be treated effectively with these antibiotics. In addition, because PBP 2a does not contain a TGase domain, the TGase activity of a Class A PBP must also be required for growth. The *mecA* gene encoding PBP 2a is present on a genomic element called the Staphylococcal Cassette Chromosome *mec*, and this element appears to be transmissible between Staphylococcal strains. Recent genomic studies have suggested that the *mecA* gene is derived from the animal-related Staphylococcus species, *Staphylococcus fleurettii* [39].

An entirely different mechanism for PBP-mediated resistance is observed in *S. pneumoniae*, *H. influenzae*, and *N. gonorrhoeae*. These species undergo interspecies recombination in their PBP genes with those from commensal species to generate new alleles that encode PBPs with lower rates of acylation by the antibiotic normally used to treat infections. These new alleles encode PBPs with anywhere between 5 and 90 amino acid changes compared to the wild-type alleles. Because of the requirement of >80% DNA sequence identity for homologous recombination to occur with reasonable efficiency, the new genes are essentially chimeras of two closely related species homologues, which, following recombination, results in a decrease in the rate of acylation.

In some species (e.g., *S. pneumoniae)*, alterations occur in multiple essential PBPs, leading to relatively high levels of resistance [40, 41]. When multiple PBPs are altered, transformation of a sensitive recipient strain with DNA from a resistant donor strain occurs in a defined order. Thus, the first PBP to be replaced by an altered form in the recipient strain is the PBP with the highest acylation rate for the antibiotic being tested, since this PBP is the lethal target for that antibiotic; altering

other PBPs does not increase resistance. Once the acylation rate of the target PBP has been lowered, a new target (another essential PBP) often emerges that now has the highest rate of acylation. Since this PBP now sets the new MIC of the organism, it must be altered to increase resistance further (PBP-mediated resistance in *S. pneumoniae* is discussed in more detail in Chap. 18).

11.6 Antibiotic Resistance in Neisseria Gonorrhoeae

The discussion above provides an overview of resistance mechanisms, but in the section that follows, we will use the example of β-lactam antibiotic resistance in *N. gonorrhoeae* to illustrate in more depth many of the resistance mechanisms at play. *N. gonorrhoeae*, the etiological agent of the sexually transmitted infection, gonorrhea, is responsible for over 62 million infections per year worldwide. Infections are characterized by a mucopurulent discharge, and while symptoms are consistently observed in males, obvious symptoms can be absent in females, which likely increases the transmission of the disease. If left untreated, gonococcal infections can lead to pelvic inflammatory disease and ectopic pregnancy in females, and disseminated infections in both sexes. Because of the lack of an effective immune response, antibiotics are the mainstay of treatment for gonococcal infections.

Unlike most Gram-negative bacteria, wild-type strains of *N. gonorrhoeae* are exquisitely sensitive to penicillin G, with minimum inhibitory concentrations (MICs) of 0.01 μg/ml. Infections caused by *N. gonorrhoeae* were treated with penicillin G for nearly 40 years, but the MICs of the antibiotic kept increasing until in 1986, and the emergence of penicillin-resistant strains necessitated the switch to other antibiotics. During this time, resistance to tetracycline, chloramphenicol, and spectinomycin also developed. Fluoroquinolones saw increased usage as anti-gonococcal agents beginning in 1990, but by 1995, resistance had already emerged in Asia. In 2007, resistance was so widespread in many parts of the United States, that these antibiotics were removed entirely from the list of recommended agents [42], leaving only the expanded-spectrum cephalosporins, ceftriaxone and cefixime, as antimicrobial agents recommended by the Centers for Disease Control (CDC). The rapid emergence of resistance to the fluoroquinolones (and other antibiotics) highlights the difficulty in treating gonococcal infections, and recent susceptibility trends suggest that it is only a matter of time before the expanded-spectrum cephalosporins become obsolete (see below).

11.7 Resistance to Penicillin G in *N. gonorrhoeae*

Although penicillin G is no longer used to treat *N. gonorrhoeae*, the lessons learned from identifying and understanding the molecular mechanisms underlying penicillin resistance employed by this organism may help avoid a similar loss of future

Table 11.1 Resistance determinants involved in chromosomally mediated β-lactam resistance in *N. gonorrhoeae*

Resistance determinant	Protein encoded/mechanism
penA	PBP 2/altered forms that have a decreased acylation rate with β-lactam antibiotics
mtrR	---/promoter mutation that increases transcription of the MtrC-MtrD-MtrE efflux pump that pumps hydrophobic agents and antibiotics out of the cell
penB	$PorB_{1b}$/mutations in $PorB_{1b}$ porin at positions G120 and A121 in loop 3, which folds into the barrel and constricts the pore; requires the presence of the *mtrR* mutation for phenotype
ponA	PBP 1/altered form that has a decreased affinity for β-lactam antibiotics
Factor X	---/unknown determinant(s) that is(are) required for high-level (MIC ≥ 2 μg/ml) penicillin resistance

antimicrobials used to treat gonococcal infections. Resistance to penicillin G (defined as an MIC ≥ 2.0 μg/ml) can be due to plasmid-mediated production of a β-lactamase or chromosomally mediated acquisition of resistance determinants. The most recent Gonococcal Isolate Surveillance Project (GISP) survey from the CDC, which has monitored the susceptibility of gonococcal isolates since 1986, reveals that 1% of current isolates express a β-lactamase, while nearly 12% utilize chromosomally mediated resistance mechanisms [43]. Thus, although penicillin G has not been used to treat *N. gonorrhoeae* for nearly 35 years, a significant portion of strains have retained their resistance to the antibiotic. Plasmid-mediated β-lactamase resistance emerged in the Far East and England, in which strains of *N. gonorrhoeae* harbored a plasmid containing the Tn2 transposable element, encoding a TEM-like β-lactamase. This plasmid, which is spread by conjugation, was likely obtained from *Haemophilus parainfluenzae* by conjugation [44, 45], highlighting the interspecies transfer of resistance genes that is commonly seen in disease-causing organisms. Although this plasmid confers high-level resistance to penicillin G (MIC > 50 μg/ml), the β-lactamase it encodes does not hydrolyze the expanded-spectrum cephalosporins.

Compared to plasmid-mediated resistance, chromosomally mediated resistance mechanisms are much more complex and multi-faceted. Penicillin resistance can be transferred in the laboratory from a penicillin-resistant clinical isolate to a penicillin-susceptible strain in a step-wise manner by DNA uptake and homologous recombination [46, 47]. At least five resistance genes/loci are required for high-level resistance: two are altered alleles of gonococcal PBPs (*penA* and *ponA*), two are mutant genes whose gene products regulate antibiotic permeability (i.e., influx and efflux (*penB* and *mtrR*, respectively), and there is a fifth determinant whose identity is unknown (Table 11.1) [17, 48–50]. These determinants are transferred in a defined order such that many of them increase resistance only when at least some of the other determinants are already present. Each step results in an increase in MIC

between two- to eightfold, but the accumulated changes increase the MIC of penicillin G 400- to 800-fold.

The first step in resistance is acquisition of an altered *penA* allele encoding mutant forms of PBP 2, the lethal target for penicillin in *N. gonorrhoeae* [48, 51]. These altered alleles were generated by interspecies recombination between *N. gonorrhoeae* and an unknown commensal *Neisseria* species [52–54]. Unlike PBP 2× from penicillin-resistant *S. pneumoniae*, which can harbor >80 mutations [55], PBP 2 variants from penicillin-resistant, but cephalosporin-susceptible, *N. gonorrhoeae* have an unusual amino acid insertion and between four and eight mutations in the C-terminal region of the protein that together decrease the k_2/K_S constant for penicillin G by ~16-fold and increase the MIC ~ 6-fold (see below for more details on these mutations) [18, 52].

The next step in the transfer of resistance is the *mtrR* locus, which most often is a mutation in the promoter for the gene encoding the MtrC-MtrD-MtrE efflux pump [56, 57]. This mutation both disrupts the binding site for the MtrR repressor and increases transcription by RNA polymerase, resulting in a ~6-fold increase in transcription of the *mtrCDE* operon and a 12-fold increase in MtrC-MtrD-MtrE efflux pump expression (unpublished results). Less common are mutations in the repressor itself, which results in a more modest increase in transcription of the *mtrCDE* operon [58]. MtrC-MtrD-MtrE is a tripartite efflux pump that is a member of the Resistance-Nodulation-Cell Division (RND) family of efflux pumps [59]. MtrD is the cytoplasmic membrane-located efflux pump (structurally related to *E. coli* AcrB), while MtrC is a periplasmic bridging protein and MtrE is an outer membrane porin-like channel. The pump requires a proton-motive force for activity, and it has broad substrate specificity, effluxing hydrophobic agents, detergents, and antibiotics [59]. The *mtrR* promoter mutation increases the MIC of penicillin only twofold, but its activity is required for high-level penicillin resistance [17, 60, 61].

The third step in resistance is called *penB*, which encodes mutations in one of the two *porB* alleles ($porB_{1b}$) present in *N. gonorrhoeae* (only one *porB* allele is expressed at any one time). These mutations map to a putative α-helix in the constriction loop that folds into the barrel of the porin and constricts the pore. Wild type residues G120 and A121 (G101 and A102 of the mature sequence) are most often mutated to either G120K/A120X or G120D/A121D in *penB* resistance alleles [62, 63]. Interestingly, *penB* mutations are silent in the absence of an *mtrR* mutation, suggesting that overexpression of the efflux pump "activates" the porin mutations, thereby resulting in a decrease in the influx of antibiotics [61, 64]. Strains that have acquired all three mutations have an ~80-fold increase in the MIC of penicillin.

Despite repeated attempts by multiple labs and investigators, the final steps of resistance in *N. gonorrhoeae* have not been identified by transformation; this has stymied a more detailed investigation into the mechanisms of high-level penicillin resistance [50, 65]. Because PBP 1, which is encoded by the *ponA* gene, is also an essential PBP Ropp et al., [66] cloned the *ponA* gene from wild type and several high-level resistant strains and showed that PBP 1 from the latter strains invariably contained a single amino acid mutation (L421P) that lowers the k_2/K_S of penicillin G by three- to fourfold. Transformation of a strain containing the first three resistance

determinants with the altered *ponA* gene, however, did not increase resistance over the parental strain. In contrast, reversion of the altered *ponA* gene back to wild type in several high-level penicillin-resistant strains lowered the MIC of penicillin by twofold [17]. The most straightforward explanation for these results is that the altered *ponA* allele is involved in resistance, but it requires an additional determinant(s), which we call Factor X (Table 11.1), to increase resistance.

11.8 Genetic Mechanisms of Decreased Susceptibility to Expanded-Spectrum Cephalosporins in *N. gonorrhoeae*

The expanded-spectrum cephalosporins ceftriaxone and cefixime have extremely low MICs (0.0006 and 0.0012 μg/ml, respectively) for wild type strains of *N. gonorrhoeae* [67]. In the last decade, however, the MICs of both antibiotics have increased markedly, with MICs for the least susceptible strains (called cephalosporin-intermediate-level resistant or $Ceph^I$ strains) that are 200- to 400-fold higher than for wild-type strains. Despite this increase, widespread occurrence of treatment failures has yet to be documented, although, at the current pace, these antibiotics have only a few years remaining as effective anti-gonococcal agents.

The primary difference between penicillin-resistant and $Ceph^I$ strains is the presence of mosaic *penA* alleles in the $Ceph^I$ strains that harbor up to 60 amino acid alterations relative to wild-type compared to the four to eight mutations in *penA* genes from penicillin-resistant strains [68]. These mosaic alleles arose via interspecies recombination with *penA* alleles from commensal and other pathogenic *Neisseria* species such as *N. perflava*, *N. sicca*, *N. cinerea*, *N. flavescens*, and *N. meningitidis* [68] and encode PBP 2 variants with acylation rates for ceftriaxone and cefixime that are 150- and 200-fold lower than wild type, respectively [69].

In addition to the mosaic *penA* gene, geographically and temporally distinct $Ceph^I$ strains also contain the *mtrR*, *penB*, and *ponA* resistance determinants [70, 71]. Transformation of a wild type strain with a mosaic *penA* allele from a $Ceph^I$ strain increases the MIC of ceftriaxone 20-fold (to 0.012 μg/ml) and the MIC of cefixime 100-fold (to 0.128 μg/ml) [64]. While markedly increased, the MICs for the transformed strain do not equal those for the donor $Ceph^I$ strain, suggesting that the other resistance determinants play a role in resistance. This hypothesis is consistent with the results from transformation of FA6140, a high-level, penicillin-resistant (but cephalosporin-susceptible) isolate containing all of the known resistance determinants, with the mosaic *penA* allele from a $Ceph^I$ strain. The MICs of ceftriaxone and cefixime for these transformants increased to levels that were the same or even higher than those of the donor strain [64].

Unlike high-level penicillin-resistant strains, reversion of the altered *ponA* gene to the wild-type allele in $Ceph^I$ strains does not decrease resistance to either ceftriaxone or cefixime, even though these strains very often contain an altered *ponA* gene [64]. Because the altered *ponA* gene has no influence on the MICs of expanded-spectrum cephalosporins for $Ceph^I$ strains, these strains likely did not arise independently, but

instead from transfer of mosaic *penA* alleles into existing high-level, penicillin-resistant isolates (for which an altered *ponA* gene is important; [17]) that remain prevalent in the community.

11.9 Structural and Biochemical Analysis of PBP 2 Mutations in Penicillin-Resistant Strains of *N. gonorrhoeae*

As discussed above, a common mechanism utilized by many bacteria is to evolve mutated variants of essential PBPs. These variants contain mutations that lower the rate of acylation by β-lactam antibiotics without apparently compromising the natural TPase function of these enzymes. This is an intriguing phenomenon, because β-lactam antibiotics are analogues of the D-Ala-D-Ala terminus of the peptidyl substrate, and react with the same serine nucleophile as the peptide substrate [5, 72]. Hence, any given mutation must lower the rate of acylation for only one of two molecules that binds at the active site. How bacteria achieve this molecular feat is the subject of intense investigation.

PBP 2 from penicillin-resistant strains of *N. gonorrhoeae* contains 4–8 amino acid substitutions [52] that together lower the rate of acylation of penicillin by 16-fold [18]. Amongst these, the most common mutation is an insertion of an aspartate at position 345 (Asp345a) that alone lowers the rate of acylation by penicillin G by sixfold [18, 73]. Although a structure of PBP 2 containing this mutation is not yet available, the crystal structure of wild-type PBP 2 (i.e., from the penicillin-susceptible strain FA19) shows that the Asp345a insertion is located on a loop that lies above the active site in close proximity to the SxN motif (Fig. 11.8). There is an intimate network of hydrogen bonds involving Asp346, Ser363 (the X of the SxN motif), and two amide nitrogens at the N-terminal end of helix α5 (Fig. 11.9) that presumably is altered or disrupted by the Asp345a insertion. This likely impacts the function of the SxN motif, which, as mentioned above, functions directly in the deacylation step of the reaction [30, 32] and probably also plays a role in acylation [74].

One possible mechanism by which the Asp345a insertion lowers the rate of acylation is that disruption of the hydrogen-bonding network leads to disordering of the β2c-β2d loop and increased exposure of the SxN motif. Such a mechanism, however, is considered unlikely because such a major alteration in the protein immediately adjacent to the active site would likely have a negative impact on the binding and reactivity with peptide, thus compromising TPase function as well as β-lactam binding activity. This is also borne out by the requirement of an aspartate at the insertion site, because insertion of any other codon generates a PBP 2 gene that cannot transform *N. gonorrhoeae* to higher penicillin resistance (i.e., TPase function of PBP 2 in vivo is lost [75]); hence, a very specific role for the side chain carboxylate of Asp345a is implicated. Since the adjacent residue at position 346 is also an aspartate, it is possible that Asp345a replaces Asp346 and therefore mediates similar hydrogen bonds; this modified arrangement must discriminate against acylation by penicillin, while retaining reactivity with the peptide substrate.

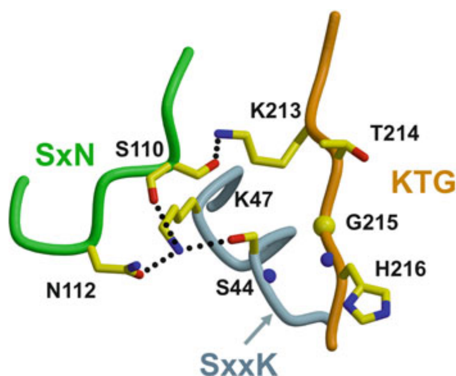


Fig. 11.8 The crystal structure of wild-type *N. gonorrhoeae* PBP 2 and locations of mutations important for penicillin resistance. The structure is shown in *ribbon* format and color-ramped from *blue* at the N-terminus to red at the C-terminus. Each mutation is marked by a *blue sphere* corresponding to the Cα atom of that residue, except for F504L and A510V, because these lie within the β3-β4 disordered loop. The Cα positions of the residues that comprise the active site motifs are shown as *pink spheres*. The N-terminal membrane anchor (missing in this structure) is located at the *bottom* of the protein, with the active site near the *top*

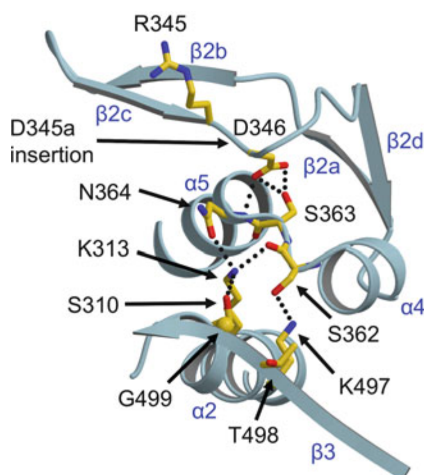


Fig. 11.9 The structure of PBP 2 from a wild-type strain of *N. gonorrhoeae* in the vicinity of the Asp345a insertion that is associated with penicillin resistance. The site of the insertion is on a β-hairpin extension (β2a-β2b-β2c-β2d) that in this view lies above the active site. The protein fold is colored *blue* and individual residues are colored *yellow*. Potential hydrogen bonds are shown as *dashed lines*. Note how Asp346, which is immediately adjacent to the insertion site, mediates several hydrogen bonds with the SxN motif of the active site

The apparent coincidence of Asp346 adjacent to Asp345a creates something of a quandary because it is not possible to know whether the insertion is after position 345 (345a) or after 346 (346a); the outcome in terms of sequence is identical. Saturation mutagenesis of position 346a showed that there also is an absolute functional

requirement for an aspartate at this position (Tomberg, et al., submitted). Therefore, in order to generate resistance to penicillin without abrogating TPase activity, *both* aspartates are required. A structure of PBP 2 containing the Asp345a insertion remains a highly desirable goal in order to elucidate the structural consequences of this fascinating mechanism of resistance.

Interestingly, a connection between the β2c-β2d loop and the SxN motif also appears to be important in other PBPs. PBP 2X, for instance, contains a very similar hydrogen bond between Ser396 (the x of the SxN motif) and Asp373. In fact, the presence of a Ser at the x position of the SxN motif corresponds with an Asp in the β2c-β2d loop in nearly 300 sequences of Class B PBPs, suggesting that the connection is highly conserved. The importance of this interaction is also highlighted by mutagenesis experiments. In NgPBP 2, mutation of either Asp346 or Ser363 to Ala decreases k_2/K_S by ~fivefold, about the same as the Asp345a insertion; however, in contrast to the *penA* gene with an Asp345a insertion, neither of the constructs with point mutations in Asp346 or Ser363 were capable of transforming a recipient strain to decreased penicillin susceptibility. This result suggests that the two mutations render the protein functionally inactive as a TPase (Tomberg, et al., submitted).

The other mutations in PBP 2 associated with penicillin resistance are all substitutions that cluster toward the C-terminal end of the protein. In PBP 2 from the penicillin-resistant strain FA6140, there are four such mutations. PBP 2 containing these four mutations, but not the Asp345a insertion, exhibits a sixfold lowering of the rate of acylation by penicillin G [18], demonstrating that these mutations are not simply neutral changes but do contribute to resistance. A crystal structure of this construct (6140CT) has been solved [18]. Two of the four substitutions (F504L and A510V) are not visible in the electron density because they lie on a disordered loop connecting β3-β4 (Fig. 11.10). The same loop is also disordered in wild-type PBP 2 [18]. Reference to published structures of acylated PBPs (e.g., PBP 2X [76]) suggests that any substitutions in this region could impact the recognition of the R1 side chain of penicillin G. Prior to this loop, there is a slight shift of β3 towards the active site in the crystal structure of 6140CT. This is potentially significant because the KTG active site motif, as well as a portion of the oxyanion hole (see above), resides on β3, and thus small shifts could alter acylation rates substantially. Another mutation (A516G) is located on β4, but when mutated alone in PBP 2, the acylation kinetics do not change dramatically and there is minimal impact on structure. In contrast, the P551S substitution, which is located close to the active site on helix α11, by itself lowers the rate of acylation by threefold, and, when combined with the F504L mutation, the acylation rate is decreased to nearly the same as when all four mutations are present. In the structure of wild-type PBP 2, Pro551 is located at the mid-point of α11 where the helix is markedly kinked. At the N-terminal end of the helix lays a tyrosine residue (Tyr544), whose side chain projects toward the active site and, in a model of the complex with penicillin G, is close to the carboxylate group of the β-lactam. Surprisingly, substitution of Pro551 for Ser does not alter the architecture of this helix and the position of Tyr544 is also unaltered.

Hence, the prevailing picture from the structural studies of *N. gonorrhoeae* PBP 2 is that, with the exception of the Asp345a insertion (whose effect is unknown), the

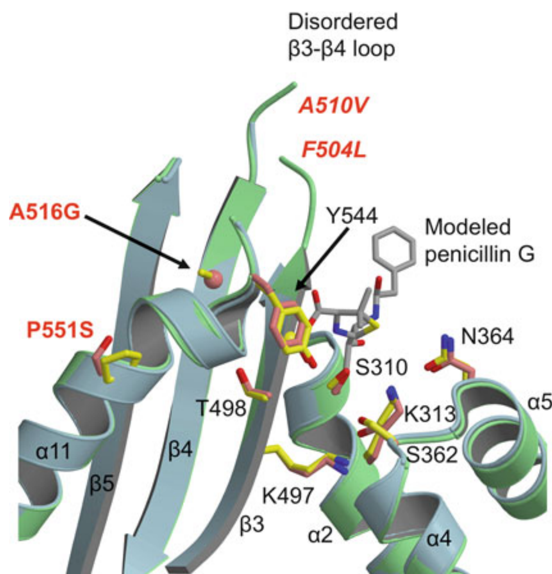


Fig. 11.10 Superimposition of wild-type and 6140CT PBP 2 structures showing a lack of structural differences in mutated forms of PBP 2 associated with penicillin resistance. Both structures are shown in *ribbon* format in which wild-type PBP 2 is colored *blue* with *orange* bonds and PBP 2 6140CT is colored *green* with *yellow* bonds. The four mutation sites are labeled *red*; note that two of these (F504L and A510V) lie within the β3-β4 disordered loop. Residues of the three active site motifs are also shown along with a molecule of penicillin G (*grey* bonds) that was docked into the structure by superimposition with the corresponding acylated structure of EcPBP 5 [36]

mutations associated with penicillin resistance do not alter the architecture of the protein significantly. Aside from a slight shift in β3, there is almost no difference between the two variants of PBP 2, and the architectures of the active sites are essentially identical. At first blush, this rather surprising finding is seemingly at odds with the kinetic data. However, since both peptide substrate and β-lactam bind in the active site and react with the same serine nucleophile, there would be a strong evolutionary constraint against any mutations that also impaired TPase activity, and loss of TPase function is more likely to occur if mutations induce large conformational changes in the protein architecture. Thus, a more subtle mechanism in which small alterations to the architecture of the protein lower reactivity with β-lactams without compromising reactivity with peptide appears entirely logical.

Some structural changes, however, must occur in the protein to explain the altered kinetics of the "resistant" forms of PBP 2. Indeed, measurements of the thermal stabilities of wild-type PBP 2 and its variant from 6140 using circular dichroism show that the T_m for the mutated form is 5°C lower than for the wild-type enzyme [18], which is tangible evidence, at least, that the protein has been altered in some way due to the mutations. One possible explanation is that, rather than inducing a structural change, the mutations may alter the dynamic equilibrium between distinct

states of protein, one of which is more readily acylated by penicillin than the other. Only one such state is observed in the crystal structure, because the latter is an average of conformations that define these states or because one of the states was excluded during crystal packing. There has been a suggestion that dynamics may play a role in antibiotic resistance in a β-lactamase [77] and further studies to address this question directly are warranted.

The apparently subtle mechanism of penicillin resistance associated with mutations in *N. gonorrhoeae* PBP 2 contrasts with similar investigations of other PBPs where large structural changes have been posited. For example, there is a considerable body of literature on structural alterations of PBP 2X from *S. pneumoniae*. In contrast to PBP 2 from *N. gonorrhoeae* FA6140, which contains only five alterations, PBP 2X from penicillin-resistant strains of *S. pneumoniae* contains upwards of 90 mutations [78, 79]. This makes the task of separating mutations that have a direct effect on penicillin reactivity from neutral changes very challenging and perhaps unsurprisingly, a clear picture of the mechanism underlying penicillin resistance mediated by PBP 2X has yet to emerge. One major difference between the structure of PBP 2X derived from a penicillin-susceptible strain and the penicillin-resistant strain Sp328 is disordering and displacement of approximately 30 residues immediately prior to the SxN motif of the active site [80]. Increased susceptibility of this loop to proteolytic digestion in PBP 2X from penicillin-resistant strain 5204 provided corroborating evidence of this flexibility in solution [81]. Interestingly, this loop is equivalent to the loop in NgPBP 2 that carries the Asp345a insertion (described above) and the equivalent loop in PBP5fm from penicillin-resistant strains of *E. faecium* contains a serine insertion [82, 83]. The dramatic change in the structure of PBP 2X would be expected to affect TPase activity (see below) and, consistent with this, peptidoglycan isolated from penicillin-resistant strains of *S. pneumoniae* shows an increase in the proportion of branched stem peptides [84, 85], suggesting that the mutations alter the substrate specificity of PBP 2X. Although the structure of peptidoglycan from penicillin-resistant strains of *N. gonorrhoeae* has not been examined in detail, only relatively small changes were observed in penicillin-resistant strains of *N. meningitidis* [86].

PBP 2X from penicillin-resistant strains of *S. pneumoniae* also contain mutations that may distort α2, the helix that contains the serine nucleophile. Two such mutations, T338A and M339F, lie between Ser337 and Lys340 of the SxxK motif, and it has been proposed that the loss of the Thr338 hydroxyl displaces a functional water molecule [87]. Curiously, a comparison of NgPBP 2 from penicillin-susceptible strains and SaPBP 2a from MRSA shows the opposite situation. Wild-type NgPBP 2 has an alanine at position 311 (equivalent to 338 in SpPBP 2X), whereas SaPBP 2a (from MRSA) contains a threonine. So either these are neutral changes in reality or the individual PBPs have evolved to have optimal activity with one of the two amino acids, and subsequent mutation to the other results in decreased acylation with β-lactam antibiotics.

Structural studies of other PBPs have also implicated the importance of β3 and the β3-β4 loop that immediately follows. In PBP 2× from the pneumococcal strain Sp5259, mutation of Gln552 to Glu is associated with a shift and twist in β3 [88].

Similarly, remodeling of β3 to create a conformational barrier against acylation of SaPBP 2a is also posited as the mechanism underlying methicillin resistance of *S. aureus* [89]. Furthermore, whilst the β3-β4 loop exhibits flexibility in both PBP 1a and PBP 2b derived from the pneumococcal penicillin-resistant strain 5204, it is ordered in each enzyme derived from the penicillin-susceptible strain R6 [90–92].

11.10 Structural and Biochemical Analysis of PBP 2 Mutations in Ceph^I Strains

Given the diminishing effectiveness of cefixime and ceftriaxone as frontline anti-gonococcal agents, it is imperative to understand resistance to cephalosporins. As noted above, we have found that the primary difference between Pen^R and Ceph^I strains is the presence of a mosaic *penA* allele in the latter strains [64]. The *penA* gene from the Ceph^I strain 35/02 (called *penA35*) contains 58 mutations compared to *penA* from FA19 [70] and so distinguishing mutations that contribute to resistance from neutral changes is a major challenge. By a divide-and-conquer approach, involving cloning of segments (or modules) of *penA35* into the *penA* gene from FA19 and measuring MICs of the resulting strains, we determined that two regions of the TPase domain of PBP 2 are primarily responsible for resistance to cefixime and ceftriaxone [69]. One region encompasses the SxxK active site motif and the other the C-terminal end of helix α11, which, as described above, is the location of the P551S mutation that confers decreased acylation with penicillin G [18]. These two regions include three mutations (G545S, I312M, and V316T) that previously were implicated in cephalosporin resistance [93]. In addition, we also identified N512Y, which is located on the disordered loop connecting β3 and β4 that follows the KTG active-site motif. This loop also contains the F504L and A510V mutations found in penicillin-resistant strains [18].

Several important observations arose from this work. First, incorporating the three Ceph^I mutations (G545S, I312M, and V316T) into the *penA* gene from FA19 does not significantly increase MICs (< 3.5 fold) for penicillin, cefixime, and ceftriaxone and yet, when the reverse mutations are made in *penA35*, the MICs are reduced to near wild-type (FA19) levels. Hence, the ability of these mutations to confer resistance depends on other mutations in *penA35*, which were hitherto considered to be neutral. This epistatic mechanism conveys an unforeseen level of complexity in how these mutations work at the molecular level. Secondly, some mutations impact acylation rates by penicillin, cefixime, and ceftriaxone very differently, which provides insight into the specificity of resistance with respect to individual antibiotics. For example, whereas the N512Y mutation decreases acylation by ceftriaxone and cefixime, it has no effect on acylation rates of penicillin. Finally, as one predictor of what may happen in the future, when an A501V mutation that occurs in non-mosaic *penA* alleles associated with decreased susceptibility to expanded-spectrum cephalosporins was introduced into *penA35* and transformed into the Pen^R strain FA6140, the MICs for ceftriaxone and cefixime increased to

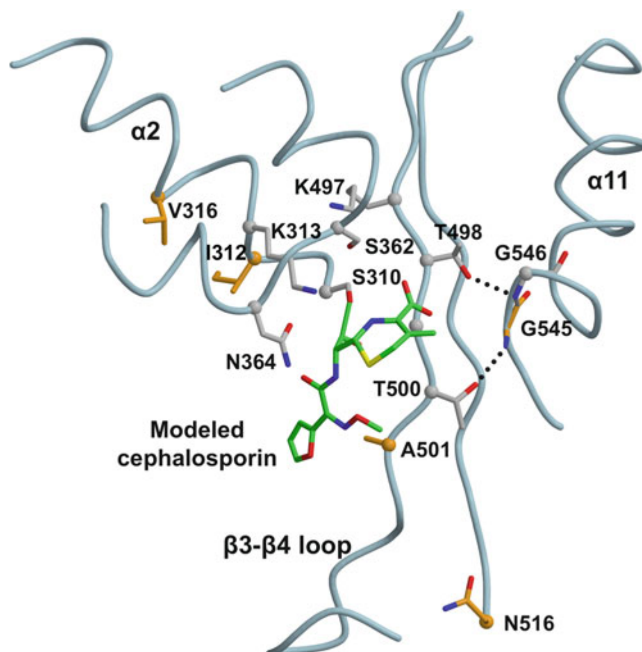


Fig. 11.11 Mutations associated with Ceph[I] resistance mapped onto the structure of *N. gonorrhoeae* penicillin-binding protein 2. The structure shown is that of PBP 2 from the penicillin-resistant strain cdc84 (unpublished) because more residues of the β3-β4 loop are visible in this structure. Residues that are mutated in *penA* from Ceph[I] strains of *N. gonorrhoeae* are colored with *orange* bonds, whereas active site residues have *grey* bonds. Hydrogen bonds involving Gly545 and Gly546 with Thr498 and Thr500 of the KTG motif are shown as *dashed lines*. In *green* is the cephalosporin cefuroxime, which was docked into the structure of NgPBP 2 by superimposition with the structure of SpPBP 2× [76]

above their respective breakpoints (≥0.25 μg/ml). Hence, if this mutation were to appear in mosaic *penA* alleles, treatment failures with these cephalosporins are likely to become more commonplace, amply illustrating the precariousness of the situation we now face with *N. gonorrhoeae*.

Inspection of the crystal structure of PBP 2 provides insight into how these mutations might impact the rate of acylation by cephalosporins. G545S is the most important mutation for cephalosporin resistance in mosaic penA alleles [69]. The main chain amides of Gly545 and Gly546 are each within hydrogen-bonding distance of the side chain hydroxyls of Thr498 and Thr500, respectively, located within or adjacent to the KTG motif (Fig. 11.11). The main chain amide of Thr500 is predicted to form part of the oxyanion hole that stabilizes the transition state, so one effect of the G545S mutation might be to lower the level of acylation by compromising the geometry of the transition state/tetrahedral intermediate. Alternatively, the hydroxyl side chains of the equivalent residues in PBP 2X (Ser548 and Thr550)

interact with the β-lactam carboxylate in a covalent complex with cefuroxime [76], and thus alteration of these contacts also may compromise the rate of acylation with β-lactams. Ile312 and Val316, which are located on the same α2 helix that harbors Ser310 and Lys313 of the SxxK motif, pack into a hydrophobic pocket, and thus mutation to larger (I312M) or more hydrophilic (V316T) side chains might alter the position of the SxxK motif and decrease acylation rates. A similar argument has been made for the M339F mutation in *S. pneumoniae* PBP 2X that lowers penicillin acylation rates [87]. Lastly, superimposition of the cefuroxime-bound structure of PBP 2X with NgPBP 2 revealed that Ala501 would be very close to the R1 substituents of ceftriaxone and cefixime, suggesting that a steric clash of these groups with Val501 is responsible for the increase in the MIC (Fig. 11.11). It remains unclear why the three mutations, G545S, I312M, and V316T, increase the MIC only marginally when incorporated into the wild-type sequence; a comparison of the structures of wild type PBP 2 and PBP $2^{35/02}$, once obtained, will likely be required to fully understand the role of the epistatic mutations in PBP $2^{35/02}$.

11.11 What Have We Learned Thus Far?

Can any lessons concerning the molecular mechanism of resistance to β-lactams be drawn from these structural and biochemical investigations of PBPs? The emerging picture is the absence of a common mechanism of resistance that applies to all PBPs; it appears that each PBP has evolved a distinct strategy to evade acylation by β-lactams. Such strategies range from major remodeling of the protein, as seen in PBP 2X, to very subtle changes that cannot be detected by standard structural approaches, as in the case of NgPBP 2. One commonality, however, is in the location of mutations. Rather than occurring in the center of the active site or even in active site residues, these mutations tend to localize to the surrounding shell of residues. Some appear positioned to impact the precise architecture of the transition state, whereas others may alter interactions with the R1 or R2 groups of β-lactams (e.g., mutations on the β3-β4 loop). In particular, the latter strategy may allow the mutations to target groups specific to β-lactams with less risk of compromising the natural TPase activity of the enzyme.

Another common theme is a tendency toward increased flexibility in mutated forms of PBPs, as evidenced by the disordering of a loop in PBP 2X from Sp5204 [80] and the lowered thermal stability of NgPBP 2 [18]. Increased flexibility may create an entropic penalty against formation of the acyl-enzyme intermediate, which would disproportionately affect β-lactams because presumably there are more contacts between enzyme and peptidoglycan compared to a β-lactam, allowing the PBP to overcome such a barrier when it reacts with its natural substrate. This is also related to the mechanism proposed for SpPBP 2a, in which the requirement for conformational change to occur during acylation by methicillin creates an energetic barrier. Again, the larger number of contacts with peptidoglycan may facilitate the induced-fit mechanism, whereas methicillin is less able to do so. Finally, hints are

emerging that protein dynamics may contribute to antibiotic resistance in PBPs and more detailed kinetic and structural investigations are needed to determine whether this is a general phenomenon or is specific for a given PBP.

11.12 What Does the Future Hold?

β-lactam antibiotics remain highly useful compounds, but their future effectiveness is imperiled due to the continued evolution of resistance in many pathogenic organisms normally treated with these compounds. *N. gonorrhoeae* is considered the "poster child" of pathogens, because this species readily evolves a variety of different and individually complex mechanisms to evade the lethal action of antibiotics. Whether we can contain such bacteria or whether they break free and re-emerge as resistant pathogens of high social and economic cost depends on our ability to understand these mechanisms and use this knowledge to drive the development of replacement antimicrobials. The looming threat of cephalosporin resistance in *N. gonorrhoeae* necessitates urgency in these efforts.

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Chapter 12
Evolution of β-Lactamases: Past, Present, and Future

Karen Bush

12.1 Introduction

β-Lactamases are enzymes that have been responsible for a preponderance of resistance to β-lactams throughout the history of use of these antibiotics. Resistance to the β-lactam antibiotics is often directly related to β-lactamase production, especially among the Gram-negative bacteria. These enzymes inactivate β-lactams by the addition of a water molecule across the common β-lactam bond in this antibiotic family. Specific biochemical profiles or structural characteristics vary for each enzyme within the β-lactamase families, resulting in distinctive profiles.

Abraham and Chain published the first description of a β-lactamase in 1940 [2], describing an enzyme with penicillin-hydrolyzing activity that was identified from a strain of *Bacillus coli*, now known as *Escherichia coli*. However, the immediate clinical effects of β-lactamases were seen in the treatment of staphylococcal infections; as penicillin began to be used to treat infections caused by Gram-positive bacteria, penicillin-resistant staphylococci were soon observed [54]. We now realize that the Gram-positive penicillinases are different from the enzymes that emerged from the Gram-negative bacteria. However, all β-lactamases have the property of destroying the antibacterial effects of penicillin, the only β-lactam antibiotic in clinical use in the 1940s.

In this chapter, the evolutionary path of β-lactamases will be discussed, relating the appearance of new β-lactamase families to the introduction of new β-lactams. The current prevalence of major β-lactamase families will be presented. Based on our historical experience, predictions will be made as to the future of these enzymes and the kinds of organisms that may be expected to be major contributors to our next series of β-lactam resistance mechanisms.

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12.2 Classification of β-Lactamases

The earliest groupings of β-lactamases were based on functional characteristics and phenotypic behavior of the producing organisms [101,109]. In 1980, two molecular classes were proposed, classes A and B, based on the differentiating structural properties of three enzymes with serine at the active site and one metallo-β-lactamase (MBL) [5]. Class C [50] cephalosporinases and class D penicillinases [46] were later added to the first two molecular classes. Today β-lactamases are classified either according to their specificity profile for substrate hydrolysis and inhibition properties (functional classification), or according to homologies in their amino acid sequences (molecular classification).

In the late 1980s, Bush made a first attempt to correlate these two classifications on the basis of limited sequence data [14,15]. By the mid-1990s, considerably more sequence data for the β-lactamases became available, and a more extensive correlation of structure and function was provided by Bush, Jacoby, and Medeiros [20]. The 1995 classification was recently updated with new subgroupings of β-lactamases that had been identified in the ensuing 15 years [19]. The various classifications are summarized in Table 12.1 in which the most important functional groupings are correlated with molecular classifications for the most important β-lactamases in today's clinical practice. Due to the current ease and low cost of gene sequencing, it has become facile for investigators to obtain amino acid sequences, so that molecular classifications often exist before full functional profiles are available. Thus, the molecular classes of β-lactamases are more frequently referred to in the literature than the functional groups.

12.2.1 Classification Characteristics

β-Lactamases can hydrolyze β-lactam antibiotics using one of two biochemical reaction mechanisms. Most β-lactamases have an active site serine residue that becomes acylated during reaction with the β-lactam ring; following the addition of water, the hydrolyzed β-lactam is microbiologically inactive. These serine enzymes include molecular classes A, C, and D, or functional groups 1 and 2. The metallo-β-lactamases (MBLs known also as class B or functional group 3 β-lactamases), a smaller group of β-lactam-hydrolyzing enzymes, contain either one or two zinc ions at their active site to assist with catalysis [93]. At one time the MBLs were the only clinically important β-lactamases known to hydrolyze carbapenems, and could generally be recognized by their ability to confer resistance to this class of β-lactam [16]. However, an important subgroup of serine β-lactamases has recently emerged with carbapenem-hydrolyzing activity [55].

Serine β-lactamases and MBLs are now most easily differentiated based on their inhibition profiles. Serine enzymes may be inhibited by clavulanic acid, sulbactam, and/or tazobactam with varying potencies, whereas the MBLs are not affected by the commercially available β-lactamase inhibitors. Instead, MBLs at this time are

Table 12.1 Correlation of functional and molecular classifications of bacterial β-lactamases

Bush–Jacoby group[a]	Molecular class	Distinctive substrate(s)	Inhibited by[b] CA	Inhibited by[b] EDTA	Clinically important enzymes
1	C	Cephalosporins	No	No	AmpC, ACT-1, CMY-2, MIR-1
1e	C	Cephalosporins	No	No	GC1, CMY-37
2a	A	Penicillins	Yes	No	PC1
2b	A	Penicillins, early cephalosporins	Yes	No	TEM-1, TEM-2, SHV-1
2be	A	Extended-spectrum cephalosporins, monobactams	Yes	No	TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1
2br	A	Penicillins	No	No	TEM-30 (IRT-2) SHV-10
2ber	A	Extended-spectrum cephalosporins, monobactams	No	No	TEM-50 (CMT-1)
2c	A	Carbenicillin	Yes	No	PSE-1, CARB-3
2ce	A	Carbenicillin, cefepime	Yes	No	RTG-4
2d	D	Cloxacillin	Variable	No	OXA-1, OXA-10
2de	D	Extended-spectrum cephalosporins	Variable	No	OXA-11, OXA-15
2df	D	Carbapenems	Variable	No	OXA-23, OXA-48
2e	A	Extended-spectrum cephalosporins	Yes	No	CepA
2f	A	Carbapenems	Variable	No	KPC-2, SME-1
3a	B (Subclass B1) B (Subclass B3)	All β-lactams except aztreonam	No	Yes	IMP-1, VIM-1, CcrA, L1, GOB-1, FEZ-1
3b	B (Subclass B2)	Carbapenems	No	Yes	CphA, Sfh-1

[a]Modified from Bush and Jacoby [19]

[b]Clavulanic acid (CA) or ethylenediaminetetraacetic acid (EDTA)

inhibited only by metal ion chelators such as *o*-phenanthroline, dipicolinic acid, or EDTA, inhibitors that are useful as biochemical tools but that have no clinical utility [20,104].

12.2.2 Functional Groups

In each of the functional groups, subgroups of β-lactamases have been defined, based on hydrolysis preferences for specific penicillins, cephalosporins, monobactams, and carbapenems (Table 12.1). The serine β-lactamases collectively can hydrolyze every β-lactam antibiotic, but individually have distinctive hydrolysis profiles. Metallo-β-lactamases generally hydrolyze all β-lactams with the exception of the monobactams (e.g., aztreonam), although catalytic efficiencies can vary considerably among β-lactam classes. MBLS are almost always found in organisms that also produce a second, or third, β-lactamase, so that the producing bacteria are often resistant to all β-lactams [93].

12.2.2.1 Group 1 Cephalosporinases

Group 1 cephalosporinases were initially identified in Gram-negative bacteria as species-specific chromosomal enzymes. Although there is considerable variability in amino acid sequences among these β-lactamases, many of them are known as AmpC cephalosporinases with very similar phenotypes. AmpC enzymes can hydrolyze most cephalosporins to some extent, but may not confer β-lactam resistance to any agents except the early cephalosporins when the enzyme is present at basal levels. However, organisms with elevated levels of AmpC usually become resistant to most cephalosporins and penicillins.

During the past 20 years, plasmid-encoded bla_{ampC} genes related to chromosomal *ampC* genes from organisms such as *Aeromonas* and *Citrobacter* have been identified [47]. These genes usually appear on transferable elements, usually as part of an integron [98] that can be shuttled among Gram-negative bacteria. When produced at high levels, these enzymes confer resistance to most penicillins and cephalosporins, including the cephamycins, and can also mediate carbapenem resistance if the producing organism has a porin defect [11,49]. They are poorly inhibited by the current β-lactamase inhibitors, especially when large quantities of enzymes are present.

12.2.2.2 Group 2 β-Lactamases

Group 2 enzymes include the staphylococcal penicillinases and the common plasmid-encoded TEM-1 and SHV-1 “broad-spectrum” enzymes that hydrolyze penicillins and early cephalosporins. These enzymes are inhibited by clavulanic acid, tazobactam, and, to a lesser extent, by sulbactam [20]. Group 2 β-lactamases also include carbenicillin and oxacillin-hydrolyzing enzymes, enzymes with lesser clinical importance today due to the decreased use of these penicillins to treat infections

caused by Gram-negative pathogens, although subgroups of the oxacillin-hydrolyzing β-lactamases have emerged with expanded hydrolytic capabilities. Among the Group 2 enzymes are “inhibitor-resistant” enzymes, variants of the common TEM-1 or SHV-1 enzymes that have decreased binding of the β-lactamase inhibitors. The clinical impact tends to be low, as many of the producing isolates remain susceptible to cephalosporins and carbapenems [4]; however, their importance is emphasized when an inhibitor-resistant TEM is produced in an organism with a second β-lactamase that can hydrolyze both cephalosporins and carbapenems [12].

Two important Group 2 enzyme families include the extended-spectrum β-lactamases (ESBLs, group 2be and 2de) and the serine carbapenemases (groups 2f and 2df). ESBLs can hydrolyze extended-spectrum cephalosporins (e.g., cefepime, cefotaxime, ceftazidime, and ceftriaxone) and aztreonam, as well as penicillins and earlier cephalosporins. The serine carbapenemases demonstrate not only an ESBL profile, but are also associated with carbapenem resistance due to their ability to hydrolyze carbapenems [93]. The OXA-related carbapenemases are being recognized more frequently in multidrug-resistant *Acinetobacter* spp. that are also resistant to carbapenems [45]. The genes encoding these OXA-related enzymes may be carried on multidrug-resistant plasmids that confer resistance to almost all classes of antibiotics [124].

12.2.2.3 Group 3 Metallo-β-lactamases

MBLs were first recognized as chromosomal enzymes that were identified specifically in each producing species [14]. Bacteria such as Gram-positive *Bacillus* spp., anaerobic *Bacteroides* spp., and the non-fermentative Gram-negative *Stenotrophomonas maltophilia* all produced a unique MBL, together with another penicillinase or cephalosporinase, that was a concern only for that species. In the early 1990s, however, a plasmid-encoded MBL was identified [125], followed by the emergence of two dominant MBL families, the IMP family and the VIM family [93]. Today these enzymes have become important as a source for carbapenem resistance in Asia and southern Europe, especially in non-fermentative bacteria, but have not yet become established in the United States. Unlike the ESBL proliferation, no country has seen a predominance of MBLs compared to other β-lactamase families, perhaps due to the duplication of non-carbapenem hydrolysis by other β-lactamases in the same organism.

12.3 Emergence of Clinically Important β-Lactamases

12.3.1 β-Lactamase Evolution

Serine β-lactamases have a long evolutionary history, with an origin estimated to be at least 2 billion years ago [41]. Class C cephalosporinases are presumed to have diverged from a common ancestor earlier than class A or class D enzymes [40], with

close phylogenetic relationships for enzymes within the same species [47]. Although the dogma has been that plasmid-encoded β-lactamases are recent evolutionary events, calculations based on phylogenetic analyses have determined that they appeared millions of years ago [41]. Thus, these enzymes have been present in commensal and environmental Gram-negative bacteria during the full development of β-lactam antibiotics.

To date, more than 900 β-lactamases with unique structures have been described [19,48], with the number of identifiable enzymes almost doubling every 5 years since 1989 when ESBLS began to emerge (Fig. 12.1). The antibiotic era certainly has exerted selective pressure on a well-established family of enzymes, resulting in the selection of variants that could hydrolyze the latest cephalosporin or carbapenem. However, the identification of this plethora of β-lactamases is due to a large extent to technological advances in gene sequencing that allow facile structure determinations for any enzyme thought to play a role in β-lactam resistance. It is also due to an unwise decision on the part of the β-lactamase community that determined in the mid-1990s to name every enzyme variant with a new name, even for those enzymes with single amino acid substitutions resulting in no observable functional modifications. However, there are clear examples where β-lactamase-mediated resistance can be readily traced to the introduction and use of β-lactam antibiotics.

12.3.2 *Gram-Positive Resistance*

Penicillin G was introduced into clinical use during World War II, primarily as a drug to treat streptococcal infections. Following its introduction, some staphylococci were soon observed to exhibit reduced sensitivity to penicillin due to an inactivating

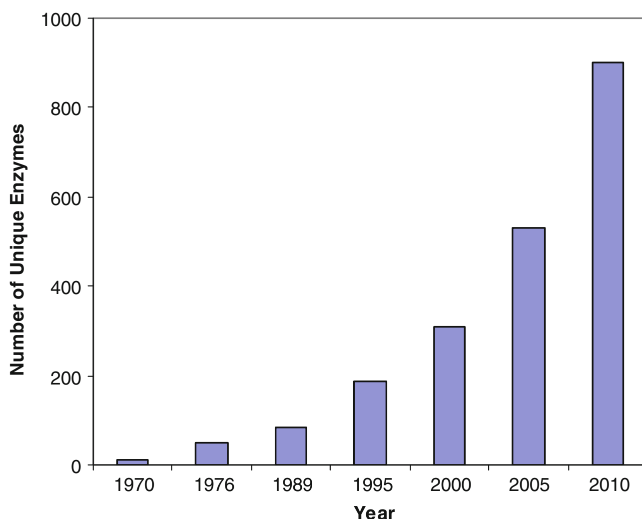

Fig. 12.1 Increase in numbers of unique β-lactamases from 1970 to 2010

material [54], even though the drug was primarily being used to treat infections caused by bacteria that even today do not produce β-lactamases. In the early 1940s, records from a single British hospital show that penicillinase production in the staphylococci increased from less than 8% to approximately 60% within 5 years [53,68]. These data indicate that the penicillinase gene was already in the environment, in commensal organisms that were not the intended target of penicillin therapy. By the mid-1980s, the selective pressure of penicillin and other β-lactam antibiotics was associated with penicillinase production in 80–90% of *Staphylococcus aureus* isolates [52].

β-Lactamase production has been sporadic among the enterococci and virtually nonexistent in the streptococci. Murray and Mederski-Samaroj were the first to identify the penicillinase activity in a strain of *Enterococcus faecalis* in 1983 [74]. After the producing gene was sequenced, they found only three silent nucleotide differences between the enterococcal gene and the gene that encoded the Type A penicillinase from *S. aureus* [137], resulting in identical amino acid sequences. Differences between the two included the fact that enterococcal penicillinases were produced constitutively as intracellular enzymes, whereas staphylococci release most of their β-lactamases into the extracellular medium [137]. The enterococcal enzymes also were produced at much lower levels and could be induced only minimally, in contrast to the penicillinases from the staphylococci that could be induced to high levels of activity.

Over time, β-lactamases have not become a predominant resistance determinant in β-lactam-resistant enterococci, with less than 0.1% prevalence reported in more recent *E. faecalis* isolates [75]. In 2009, a study of 235 *E. faecium* isolates showed that 48% of the isolates were ampicillin-resistant, but not due to β-lactamase production [1], consistent with previous studies of 78% penicillin-resistance in β-lactamase-negative *E. faecium* strains isolated in 1988–1989 [39]. The absence of β-lactamases is most likely due to the production of low-affinity penicillin-binding proteins (PBPs) that confer resistance to most β-lactam antibiotics in both *E. faecalis* and *E. faecium* [22,39,80]. This may also partially explain the fact that no β-lactamases have ever been reported among the streptococci. In penicillin-resistant *S. pneumoniae*, multiple resistance mechanisms resulting in β-lactam resistance have been described, none of which include β-lactamase production. Penicillin-resistant streptococci may arise as a result of PBP mutations, mosaic PBPs, acquisition of low-affinity PBPs from another source, or missense mutations in the coding region of the peptidoglycan GlcNAc deacetylase gene [23,116]. Thus, among the Gram-positive aerobic bacteria, only the staphylococci have developed a set of clinically relevant β-lactamases.

12.3.3 Gram-Negative Resistance

β-Lactamase-mediated resistance to β-lactams in Gram-negative bacteria can be closely correlated with the introduction of multiple variations of these agents into clinical usage. It is likely that many of the responsible enzymes were already present

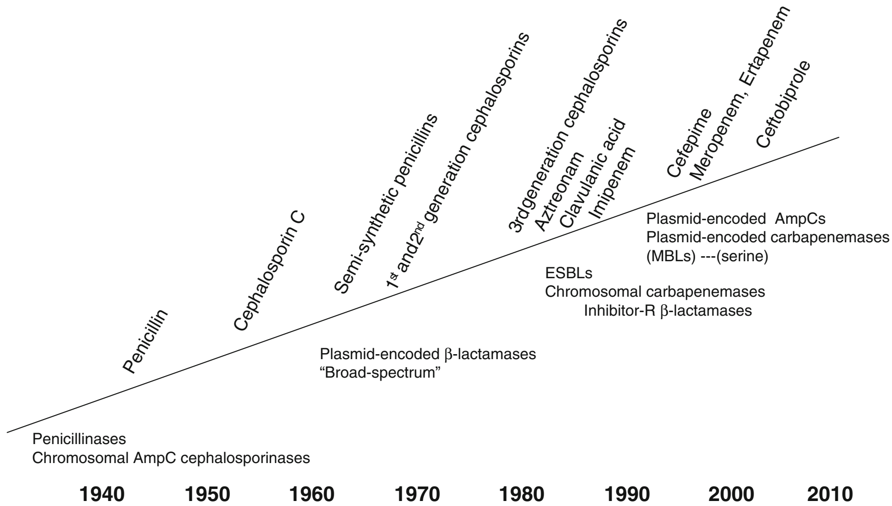

Fig. 12.2 Alignment of the introduction of new β-lactams into clinical practice and the description of new β-lactamases

in environmental species on transferable elements that began to appear in clinical isolates from patients treated with a penicillin or cephalosporin. A timeline is shown in Fig. 12.2, highlighting the introduction of major β-lactams and the later appearance of a related inactivating enzyme.

In the early antibiotic era, Gram-negative bacteria causing serious infections were generally treated with antibacterial agents other than penicillins, with the exception of gonococci that were initially exquisitely sensitive to the action of benzylpenicillin [117]. However, lower sensitivity to penicillin in these pathogens was noted as early as 1955, although no mechanism for resistance was provided. It is noteworthy that TEM (or RTEM) penicillinase was the first important plasmid-encoded β-lactamase identified in Gram-negative bacteria, in *E. coli*, and *Salmonella* isolates from 1962 and 1963 [28]. A decade later, the plasmid-encoded TEM-1 β-lactamase was identified in *Neisseria gonorrhoeae* isolates worldwide [7], a major blow to the clinical treatment of this easily transferred infectious agent. However, based on the early reports in the 1960s of decreasing sensitivity to penicillin in *Neisseria* isolates, it is probable that the enzyme had been transferred at a much earlier date but had not found a highly receptive host.

Additional plasmidic β-lactamases in Gram-negative bacteria were soon identified, with the ability to hydrolyze the semisynthetic penicillins and cephalosporins that had been developed to evade staphylococcal penicillinases [101,114]. A parade of new cephalosporins also served to select for AmpC cephalosporinase-producing Enterobacteriaceae, especially those with constitutive AmpC production leading to resistance to most cephalosporins and penicillins [51]. However, cephalosporinase

production was species-specific, whereas the plasmid-encoded β-lactamases were perceived to be a greater problem due to their promiscuity with respect to host. Thus, after the identification of the plasmid-borne TEM-1 enzyme in the gonococci, many pharmaceutical companies targeted this enzyme in efforts to develop β-lactamase-stable β-lactams or to develop penicillin combinations with a β-lactamase inhibitor that would inactivate TEM-1, in addition to the staphylococcal penicillinases [18], resulting in the successful inhibitor combinations of amoxicillin-clavulanic acid, ampicillin-sulbactam, and piperacillin-tazobactam.

For a few short years, it appeared that the infectious disease community was well-served with the appearance of the third generation cephalosporins (cefotaxime, ceftazidime, and ceftriaxone), the monobactams (aztreonam and carumonam), the carbapenems represented by imipenem, and the β-lactamase inhibitor combinations. All of these provided effective antimicrobial activity capable of treating infections caused by pathogens with the common TEM-1 and SHV-1 group 2be β-lactamases as well as the penicillinases from *S. aureus* [56,58,78,115]. Unfortunately, the introduction of these new agents stimulated the identification of new β-lactamases with previously unrecognized hydrolytic abilities.

β-Lactamases with extended-spectrum hydrolytic activities were frequently carried on various mobile elements and readily transferred among the Enterobacteriaceae. ESBLs were identified worldwide with the ability to hydrolyze the new cephalosporins and monobactams, only a few years after the introduction of these agents [88]. The prevalence of ESBL-producing enzymes has now become so great that the use of agents such as ceftazidime and cefotaxime has been sharply curtailed, or eliminated, in some parts of the world [132]. However, the number of ESBLs continues to rise as demonstrated in Fig. 12.1, where the rapid increases in the number of unique β-lactamase sequences since 1989 are driven by the prolific identification of new ESBL variants [19].

In many health-care institutions where ESBLs became endemic, carbapenems have been widely used [96,132] because of their stability to most serine β-lactamases [93]. This allowed for the emergence of β-lactamase-mediated carbapenem resistance. Serine carbapenemases were first identified in a few *Enterobacter* and *Serratia* clinical isolates [97,130], and later in fish isolates from US rivers [8], demonstrating an environmental source for these enzymes. When the carbapenemases began to appear on plasmids, they became a much more deadly threat [63]. Both MBLs and the serine carbapenemases can be transferred in combination with resistance determinants for a variety of antibiotic classes, resulting in multidrug-resistant pathogens with little left on the cupboard as treatment options. Although the class A serine carbapenemases tend to be found in the Enterobacteriaceae, both MBLs and the OXA family of serine carbapenemases are major threats in non-fermentative bacteria [93]. The genes encoding these latter enzyme families also are transferred together with other resistance factors, resulting in multidrug resistance. In many cases, the only treatment options are colistin or polymyxin B, both of which are viewed as agents of last resort with controversial liabilities such as low efficacy or risk of toxicity [87].

12.4 Status of Currently Important β-Lactamases

12.4.1 Serine β-Lactamases

Enterobacteriaceae. Among the Enterobacteriaceae the serine β-lactamases of molecular classes A and C (functional groups 1 and 2) are the most prevalent β-lactamases. Most Enterobacteriaceae, with the notable exceptions of the Klebsiellae and some *E. coli* isolates, produce a chromosomal AmpC-type cephalosporinase that can be upregulated from a (low) basal level of production, or may be produced at a high level in the derepressed state [47]. Because organisms producing large amounts of AmpC cephalosporinases can become resistant to most β-lactams, the recent emergence of plasmid-encoded AmpC genes appearing with high copy numbers has made this an increasing problem, especially when the mobile elements are transferred into porin-defective species [11].

Multidrug-resistant Enterobacteriaceae producing group 2 plasmid-encoded β-lactamases in addition to the chromosomal AmpC cephalosporinases have been increasing globally for many decades. By the late 1970s and early 1980s, the most common plasmid-encoded β-lactamases reported from Gram-negative isolates were the TEM-1, TEM-2, or SHV-1 broad-spectrum β-lactamases and the OXA-1 oxacillinase [68]. This provided a rich background for selection of the first ESBLs, identified as TEM and SHV variants with point mutations in the β-lactamase genes. These early ESBLs generally had only one or two amino acid substitutions from their parent enzyme, resulting in an active site that could accommodate the bulkier side chains of the cephalosporins and monobactams introduced after 1980 [88]. Because of the prevalence of these enzymes in the environment and the variety of β-lactams that have been used as selecting agents, it is not surprising that today over 175 TEM, 125 SHV, and 160 OXA variants have been identified with unique amino acid sequences [48].

Serine carbapenemases began to be identified in the mid-1980s in single clinical isolates, but have now become entrenched in some geographical areas with clonal dissemination of strains of multidrug-resistant Enterobacteriaceae [59,91,108]. Today Gram-negative pathogens producing ESBLs or serine carbapenemases represent major challenges for the continued use of β-lactam antibiotics. Both families of enzymes in the same strain have been reported in Buenos Aires [85] and the metropolitan New York area [13]. The genes encoding these enzymes are readily transmitted among species, in addition to additional resistance determinants for other antibiotic classes that can be added to gene cassettes that include β-lactamase genes, resulting in the pan-resistant strains that are emerging worldwide [106].

Non-fermenters. P. aeruginosa and *Acinetobacter* spp. are the major representatives of non-fermentative bacteria found in current clinical practice, although *S. maltophilia* and *Burkholderia* spp. may also be found in infections such as pneumonia [66] and cystic fibrosis [24]. *P. aeruginosa* and *Acinetobacter* spp. in particular carry the genes encoding group 1 cephalosporinases, and frequently acquire additional resistance determinants to cause multidrug resistance. Regulation of porins or efflux mechanisms

occurs frequently in *P. aeruginosa* strains that also contain an upregulated chromosomal AmpC cephalosporinase [44]. Although *S. maltophilia* strains produce a chromosomal MBL [70], carbapenem resistance in the other non-fermentative bacteria may occur by the acquisition of either serine or metallo-carbapenemases. However, strains of *Acinetobacter* spp. are particularly plagued by production of carbapenemases from the OXA family [124]. The genes encoding these enzymes may be found either in the chromosome or they may also be acquired on mobile elements, for example, as with the OXA-23 and OXA-58 enzymes [69].

12.4.2 Most Frequently Identified Serine β-Lactamases in Recent Isolates

Families of serine β-lactamases that are considered to be of most importance in current clinical setting are listed in Table 12.2. Included in this compilation is a listing of the geographical locations in which these enzymes have been recently reported. The largest numbers of β-lactamases have been reported as ESBLs, primarily in the TEM, SHV, and CTX-M families. Among the carbapenemases, the OXA and KPC families appear most frequently. This reflects the high usage of cephalosporins and carbapenems worldwide.

Individual enzymes are listed in Table 12.3 if they were reported to be present with at least a frequency of 4% for a set of data. These compilations reveal some striking observations. Although over unique 175 TEM β-lactamases have been sequenced [48], none of these enzymes currently appears as a dominant ESBL anywhere in the world (Table 12.3). This is in contrast to the late 1980s and 1990s, when TEM-derived variants were the most frequent cause of resistance to the extended-spectrum β-lactams among the Enterobacteriaceae [10,17]. However, the previously common TEM-10, TEM-12, and TEM-26 enzymes have almost disappeared from the US surveillance studies (Table 12.3) [10,17]. SHV variants that are ESBLS are still being identified at a few centers, with surveys through 2002 from hospitals in the Eastern United States reporting the most prominent ESBLs to be the SHV-7 and SHV-12 enzymes [17]. More recently, SHV enzymes including SHV-2 and SHV-11 have been identified mainly in the United States [32,61,111,133]. However, of the 127 SHV variants that have been sequenced [48], only four enzymes are predominant in surveillance reports (Table 12.3).

The CTX-M family of β-lactamases has become the most prominent set of ESBLs globally. These enzymes were initially identified in single isolates and emerged in epidemic strains only in the past decade. As seen in Table 12.3, at least 50% of the ESBLs belong to the CTX-M family in China [62,133,136], India [33], the Philippines, Belgium [38], France [35,60], the United Kingdom [128], Kuwait [34], Chicago [84], Pittsburgh [111], Texas [61], and regions of South America [95,99]. Even more impressive is the replacement of most other ESBLs by the CTX-M enzymes as reported from hospitals in China [133], the Philippines [118], Spain [81], and Texas [61], where at least 95% of the extended-spectrum enzymes

Table 12.2 Geographical distribution of major families of serine β-lactamases

Enzyme family	Major functional subgroups (number)[a]	Most prevalent enzymes	Geographical location	Reference
TEM	2b, 2be, 2br (175)			[19]
	2b (12)	TEM-1, TEM-2	Global	[10, 17, 68]
	2be (78)	TEM-10, TEM-12, TEM-26	USA	[10, 17]
	2br (36)	TEM-30, TEM-103	France, New York	[4, 12]
SHV	2b, 2be, 2br (127)		Global	[19]
	2b (30)	SHV-1, SHV-11		[19, 32]
	2be (37)	SHV-2	Texas	[61]
		SHV-5	China, Philippines, USA	[61, 118, 133]
		SHV-7	USA	[31]
		SHV-12	Tunisia, China, USA	[31, 32, 61, 111, 133]
CTX-M	2be (91)			[19]
		CTX-M-1	France, Venezuela	[35, 60, 99]
		CTX-M-2	France, Buenos Aires, USA	[35, 84, 95]
		CTX-M-3	Belfast, China	[30, 62, 133]
		CTX-M-8[b]	Texas, Chicago	[61, 84]
		CTX-M-14	France, China, Chicago	[35, 60, 62, 133, 136]
		CTX-M-15	UK, Belfast, Belgium, France, Spain, Tunisia, Kuwait, China, India, Philippines, USA	[31, 33, 34, 35, 38, 60, 61, 62, 67, 81, 84, 105, 111, 118, 121, 128, 136]
		CTX-M-16[b]	Texas	[61]
		CTX-M-24	China	[133]
		CTX-M-55	Philippines	[118]
KPC	2f (9)	KPC-2	USA, Puerto Rico, Israel, Brazil, Colombia, China, Greece, Norway, Trinidad	[29, 32, 103, 108]
		KPC-3	USA, Puerto Rico, Israel, Canada, Sweden	
SME	2f (3)	SME-2	USA	[29]
		SME-3	USA	
OXA	2d, 2de, 2df (159)			[19]
	2d (5)	OXA-1,OXA-10	Global	[19]
	2df (48)	OXA-23 (ARI-1)	Global	[93, 124]
		OXA-58-type	Global	[93, 124]

[a]Number of unique amino acid sequences recorded according to the Web site “ß-Lactamase Classification and Amino Acid Sequences for TEM, SHV and OXA Extended-Spectrum and Inhibitor Resistant Enzymes” [48]. The numbers for each subgroup may not equal the sum for the entire family, as not all enzymes have been described with full functional characterization

[b]Not reported after 2005

were CTX-M β-lactamases. The CTX-M enzymes differ from the TEM and SHV ESBL families in which only a limited number of mutations are seen for each variant compared to the parent TEM-1 or SHV-1 enzymes [48]. Greater variation is seen among the 91 CTX-M enzymes that can be divided into five major groups, with amino acid homologies as low as 79% among these groups [9]. Each group, however, comprises a set of enzymes with at least 98% amino acid identity.

As shown in Table 12.3, specific CTX-M enzymes have become dominant throughout the world, partially as a result of clonal dissemination of strains. CTX-M-1 and CTX-M-2 have been dominant in South America where the CTX-M family was reported as early as 1989 [99]. Today the most widespread enzymes are CTX-M-3, found in Belfast [30], CTX-M-14 which has been dominant in China [62,133,136], and CTX-M-15 which is becoming the most common ESBL worldwide (Table 12.3) [31,33–35,38,60,61,81,84,105,111,118,121,128,136].

The CTX-M enzymes were very slow to become established in the United States. In 2003, nine isolates from five states were reported to produce a CTX-M-like ESBL during a US hospital surveillance study for ESBLs conducted in 2001–2002 [73]. No other CTX-M enzymes were reported from the United States until 2007, when a San Antonio, Texas clinical microbiology laboratory revisited their ESBL-producing Enterobacteriaceae isolates from 2000 to 2006 (mostly isolates from 2003 to 2006) [61]. After 2003, CTX-M-15 became the predominant ESBL, with 18 of 19 CTX-M ESBLS identified as CTX-M-15 in mid-2006. No TEM-derived ESBLs were identified after 2003. Similar reports are now available from Chicago [85] and Pittsburgh [111].

Subgroup 2f β-lactamases are serine carbapenemases that were first recognized as chromosomal enzymes in single isolates of British or US strains of Enterobacteriaceae [97,130]. The SME-2 and SME-3 chromosomally produced carbapenemases, variants of the first British SME-1 enzyme from *S. marcescens*, still occasionally are reported in carbapenem-resistant *Serratia marcescens* isolates [29]. However, even more threatening are the KPC plasmid-encoded serine carbapenemases, first reported in the late 1990s in *K. pneumoniae* isolates [93,131]. These enzymes are now a major problem in many geographical areas, including the United States [26,29,85], Israel [77], and Greece [36,91], resulting in both clonal and nonclonal outbreaks. A recent CDC study of the KPC-producing *K. pneumoniae* isolates from the United States and Israel extending through 2008 in their culture collection showed that 70% of the strains were of sequence type ST258 [55]. Either KPC-2 or KPC-3 could be produced by strains with this sequence type; the producing genes were often found on unique plasmids.

KPC-2 is a serine carbapenemase with the same sequence as the first reported KPC. Note that the KPC-1 sequence was subsequently updated to be identical to KPC-2 as the result of an initial sequencing error [131]. KPC-2 has been the most frequently reported serine carbapenemase to date (Table 12.2); it has been identified in many outbreaks of carbapenem-resistant infections caused by Enterobacteriaceae in various parts of the United States [13,29,112], Israel [76], and Greece [36,91]. KPC-2 has also been found in smaller numbers in *K. pneumoniae* and other

Table 12.3 Geographical prevalence of ESBLs occurring in at least 4% of the isolates

Location	Date of survey	Population of isolates	Enzyme family (n)	Major enzymes (n)	% of Total family[a]	Reference
Africa						
Tunisia	1999–2005	ESBL-producing *K. pneumoniae*	ESBLs (101)	CTX-M-15 (43) SHV-12 (58)	42% (ESBLs) 57% (ESBLs)	[31]
Asia						
China	NA	ESBL-producing *E. coli* and *K. pneumoniae*	All ESBLs (416) CTX-M family (399) SHV family (17)	CTX-M-3 (70) CTX-M-14 (271) CTX-M-24 (35) [CTX-M-3 with CTX-M-14] (<30)	96% (ESBLs) 17% (CTX-Ms) 65% (CTX-Ms) 8% (CTX-Ms) <7% (CTX-Ms) 4% (ESBLs)	[133]
China	NA	Enterobacteriaceae	All isolates (425) All ESBLs (142) CTX-M family (109)	CTX-M-3 (32) CTX-M-14 (52) CTX-M-15 (19)	33% 77% (ESBLs) 29% (CTX-Ms) 48% (CTX-Ms) 17% (CTX-Ms)	[62]
Southern China	2007–2008	ESBL-producing *E. coli* and *K. pneumoniae*	All ESBLs (361) CTX-M family (243)	CTX-M-14 CTX-M-15	67.3% (ESBLs) 28–35% (CTX-Ms) 22–26% (CTX-Ms)	[136]
India		Cephalosporin-resistant *E. coli* or *K. pneumoniae*	All ESBLs (130) CTX-M family (95)	CTX-M-15 (95)	73% (ESBLs) 100% (CTX-Ms)	[33]
Manila, Philippines	2007	ESBL-positive Enterobacteriaceae	All ESBLs (39) CTX-M family (37)	Full sequence (28): CTX-M-15 (24) CTX-M-55 (4) SHV-12 (5%)	95% (ESBLs) 86% (CTX-Ms) 14% (CTX-Ms) 5% (ESBLs)	[118]
Europe						
Belgium	2008	ESBL-positive Enterobacteriaceae (91 hospitals)	All ESBLs (402) CTX-M family (221)	CTX-M-15 “most frequent”	55% (ESBLs)	[38]

France	2004	ESBL-producing *E. coli* (4 hospitals)	All ESBLs (112) CTX-M family (65)	CTX-M-1 CTX-M-14 CTX-M-15	58% (ESBLs) 20% (CTX-Ms) 20% (CTX-Ms) 57% (CTX-Ms)	[60]
France	2008	ESBL-positive Enterobacteriaceae	All ESBLs (182) CTX-M family (102)	CTX-M-1 CTX-M-2 CTX-M-14 CTX-M-15	56% (ESBLs) 28% (CTX-Ms) 5% (CTX-Ms) 21% (CTX-Ms) 36% (CTX-Ms)	[35]
Spain	NA	*K. pneumoniae* isolates highly cephalosporin-resistant (5 centers)	All ESBLs (162) CTX-M family (162)	CTX-M-15 (162)	100% (ESBLs) 100% (CTX-Ms)	[81]
England	2004	Cephalosporin-resistant *E. coli* or *K. pneumoniae*	All ESBLs (605) CTX-M family (482) Non-CTX-M ESBL (113)	NA	80% (ESBLs) 19% (ESBLs)	[63a]
Belfast, Northern Ireland	2004–2006	*E. coli* from gut flora (long-term care facilities)	All ESBLs (119) CTX-M family (NA)[b]	CTX-M-15 (58)	41% (all isolates) 49% (ESBLs)	[105]
Belfast, Northern Ireland	2004–2006	CTX-M – positive *E. coli* from gut flora (long-term care facilities)	CTX-M family (54)	CTX-M-3 (44) CTX-M-15 (10)	81% (CTX-Ms) 19% (CTX-Ms)	[30]
UK	2003	CTX-M-producing *E. coli*	CTX-M family (291)	CTX-M-15-like (279)	96% (CTX-Ms)	[128]
North America						
Chicago	2008 (1 month)	ESBL-producing *E. coli*	All ESBLs (29) CTX-M family (26)	CTX-M-2 or −8 (2) CTX-M-14 (2) CTX-M-15 (22)	90% (ESBLs) 8% (CTX-Ms) 8% (CTX-Ms) 85% (CTX-Ms)	[84]
Philadelphia	2007	Extended-spectrum cephalosporin-resistant Gram-negative rods	All ESBL isolates (143) CTX-M family (15)		10% (ESBLs)	[67]

(continued)

Table 12.3 (continued)

Location	Date of survey	Population of isolates	Enzyme family (n)	Major enzymes (n)	% of Total family[a]	Reference
Pittsburgh	2007–2008	ESBL-producing *E. coli*	All ESBLs (57) CTX-M family (46) SHV family (11)	CTX-M-15 (30) SHV-7 or −12 (11)	81% (ESBLs) 67% (CTX-Ms) 19% (ESBLs) 100% (SHVs)	[111]
Texas	2000–2006	Enterobacteriaceae	All ESBL isolates (88) CTX-M family (52)[c] SHV family (41) TEM family (2)	CTX-M-8 (4) CTX-M-15 (39) CTX-M-16 (7) SHV-2 (9) SHV-5 (10) SHV-12 (21)	59% (ESBLs) 8% (CTX-Ms) 75(CTX-Ms) 13%(CTX-Ms) 47% (ESBLs) 22% (SHVs) 24% (SHVs) 51% (SHVs) 2%	[61]
Texas	2006	Enterobacteriaceae	CTX-M family (19)	CTX-M-15 (18)	95% (CTX-Ms)	[61]
South America						
Buenos Aires	2002	ESBL-positive Enterobacteriaceae	NA	CTX-M-2	75% (ESBLs)	[95]
Venezuela	2006–2007	ESBL-positive Enterobacteriaceae	All ESBLs (97) CTX-M family (42)	CTX-M-1 CTX-M-2	43% (ESBLs) 91% (ESBL-*E. coli*) 57% (ESBL-*K. pneumoniae*)	[99]

[a]Family that is referenced is in parentheses
[b]Number isolated was not available

Enterobacteriaceae in Brazil [85], Colombia [123], China [126], Norway [108], and Trinidad [3]. The only other KPC carbapenemase that has appeared in multiple isolates is the KPC-3 enzyme, with KPC-3 producing strains identified in the United States [26,29,32,55,120], Israel (an outbreak clone identical to the major US clone) [77], and Sweden [108]. However, the KPC-3 enzyme is increasing in prominence, in at least some centers. In a 2006–2007 study from five hospitals in the Eastern United States, 42 KPC-producing *K. pneumoniae* isolates were associated with genes encoding the following β-lactamases: KPC-2 (59.5%), KPC-3 (40.5%), TEM-1 (90.5%), SHV-11 (95.2%), and SHV-12 (50.0%) [32]. From three to five β-lactamases were produced by these isolates, with a mean number of 3.5 enzymes per strain [32]. Recently KPC carbapenemases were also identified in isolates of *Acinetobacter* sp. from Puerto Rico [103].

12.4.3 Occurrence of Metallo-β-Lactamases in Recent Isolates

MBLs were first recognized in nonclinical bacterial strains, with many early biochemical and structural studies conducted on the MBL from *Bacillus cereus* [107]. In 1989, only four MBLs with a requirement for Zn^{2+} had been described in the literature, all appearing as chromosomal, species-specific enzymes [16]. However, the identification of a plasmid-encoded MBL in Japan in 1991, IMP-1, [125] introduced an era for the discovery of new MBLs. Genes encoding these acquired enzymes are often located in gene cassettes associated with plasmids or transposons that can be readily transferred among species [93]. The IMP family of MBLs emerged in Japan initially in a *P. aeruginosa* isolate [125], and later in various Japanese isolates of the Enterobacteriaceae family [43]. IMP variants then appeared in an Italian *Acinetobacter baumannii* isolate [100] and, concurrently, in other parts of Asia including China [42] and Taiwan [129]. Currently 26 IMP variants have been identified worldwide [48]. Of all the MBLs associated with clinical failures, the IMP family has remained most widespread in Japan, although outbreaks are still recorded in southern Italy [82].

Another prominent plasmid-encoded MBL family of VIM enzymes emerged in southern Europe, particularly in Greece [71] and Italy [65], as well as in countries as widespread as Australia [90] and South Korea [134]. Interestingly, plasmidic MBLs are not well established in North America, although two outbreaks of infections caused by *P. aeruginosa* producing VIM-2 and IMP-7-have been reported from Canada [37,89]. To date, only limited sightings of MBLs have been made in the United States, with two reports of VIM-2 producing-*P. aeruginosa* isolates, including an outbreak strain in Chicago where the β-lactamase gene was transferred on an integron [64]. Perhaps the North American reports are low because many of the isolates in which these enzymes appear have carbapenem MICs in the high susceptible range and are not flagged as resistant strains using the carbapenem breakpoints established in those countries. It is possible that these enzymes will eventually emerge worldwide, similarly to the CTX-M enzymes, where catalytically inefficient

enzymes conferred low levels of resistance that were not initially detected due to relatively high cephalosporin susceptibility breakpoints used for US testing.

12.5 Continued Evolution of Current β-Lactamase Families

As β-lactamase-interactive agents continue to be used to treat both community and nosocomial infections, we can only expect to see new β-lactamases emerge. ESBLs based on the families already described will almost assuredly evolve to allow for even broader spectrum activities. With the more frequent use of carbapenems to treat drug-resistant Gram-negative pathogens, new carbapenemases will be identified, either as new families of enzymes or as variants of known enzymes. These behaviors are anticipated due to the well-accepted descriptions of the plasticity of the β-lactamase structure [83]. When stable variants of catalytically efficient enzymes in the CTX-M and carbapenemase families are produced from high copy number genes, it is likely that clonal strains will become a global menace.

With the introduction of new β-lactamase inhibitors into clinical practice, additional inhibitor-resistant enzymes will evolve. The unanswered question, however, is how the amino acid modifications responsible for inhibitor resistance will affect the hydrolytic activity of the enzyme against common cephalosporins and carbapenems. This is even more intriguing when one considers the development of non-β-lactam-containing molecules as broad-spectrum β-lactamase inactivators, as seen with NXL-104 [113].

Introduction of the anti-MRSA β-lactams such as ceftobiprole and ceftaroline [21] brings another level of complexity to β-lactamase evolution. The staphylococcal penicillinases have not yet evolved to exhibit broad-spectrum activity, probably due to the highly effective resistance mechanism furnished by the low-affinity PBP2a. However, these new cephalosporins exhibit tight binding to PBP2a and show little differential activity against methicillin-susceptible and methicillin-resistant *S. aureus* [6]. It is possible that widespread use of these kinds of agents for the treatment of staphylococcal infections will place additional pressure on the penicillinases to evolve into extended-spectrum β-lactamases with potent cephalosporin-hydrolyzing activities.

Sources for new β-lactamases abound and are not confined to the hospital setting as the genes for these enzymes are readily transferred to and from environmental locations. Long-term health-care facilities have become prime incubation reservoirs, as patients are transferred in and out of hospital settings to high-density populations of residents who frequently have chronic health issues [122,128]. Environmental origins include common dirt sources from which many antibiotics, and concurrently, many antibiotic resistance determinants, have been isolated [127]. Serine carbapenemases have been identified from US rivers [8]. MBL-producing organisms have been isolated from urban sewage systems in Portugal (VIM-2) [94] and in Italy (IMP-22) [86], with associations of these enzymes to nosocomial sources. Wastewater treatment plants have become a common source for β-lactamase genes [92,110].

Recently, a novel class A β-lactamase was identified from a bacterium that originated from 1050 m below the surface of the Pacific Ocean [119], indicating untapped sources for these ubiquitous enzymes.

12.6 Notable Species Without Apparent β-Lactamase Production

Although β-lactamases have been described from most pathogenic bacteria, there are notable exceptions. As mentioned previously, the pneumococci have yet to produce a recognizable enzyme with measurable β-lactamase activity. *Helicobacter pylori* is another organism without a confirmed β-lactamase in any natural isolate. Both of these organisms have been exposed to penicillins therapeutically for many years, but have apparently found more efficient ways to develop resistance to β-lactam antibiotics, primarily through the production of low-affinity PBPs [23,57,102,116]. It is possible that low-level β-lactamase activity may be associated with some of these PBPs that exhibit slow deacylation rates to mimic the activity of a β-lactamase [25]. Additionally, other enzymes may assume the role of β-lactam hydrolysis. For example, in *H. pylori*, a cysteine-rich Protein A has been proposed to serve a β-lactamase function [72] with low hydrolysis rates of $<0.2\ s^{-1}$ for some penicillins and cephalosporins.

Other organisms that are reported to lack β-lactamase production are *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, and Planctomycetes. Interestingly, all these organisms lack the ability to synthesize murein, a component of the cell wall found in most pathogenic bacteria [27]. This observation supports the arguments from those who believe that the actual physiological role of β-lactamase is not to protect bacteria from the onslaught of β-lactam antibiotics, but to function in the regulation of murein and cell wall biosynthesis [68,79].

12.7 Concluding Comments

Antibiotics have always posed a threat to the existence of pathogenic bacteria, but the bacteria continue to outmaneuver the onslaught. Today, the β-lactam medicine cabinet is facing even more obstacles than ever before. New enzymes keep appearing with even broader hydrolyzing capabilities. Our predictive capabilities have not been very successful in anticipating the next family of β-lactamases. Moreover, multiple β-lactamase production is one of the most serious aspects of the current resistance profiles that will continue to plague infectious disease treatment options. Even though most class A/group 2 β-lactamases are sensitive to inhibition by one of the commercially available β-lactamase inhibitors, the inhibitor combinations have become useless for the treatment of infections caused by pathogens producing multiple β-lactamases. When the number of β-lactamase molecules exceeds the amount of

inhibitor on a stoichiometric basis, the inhibitor has no opportunity to inactivate sufficient amounts of enzyme to allow all for therapeutic efficacy. Carbapenems are also not the answer; as has been noted, overproduction of AmpC cephalosporinases combined with porin loss can render the producing organisms resistant to the carbapenems [11], not to mention the ready acquisition of a carbapenemase.

Although highly potent, broad-spectrum β-lactamase inhibitor combinations may play a role in the future, they will only select for more resistant β-lactamases, or the development of active efflux mechanisms, thereby conferring resistance to the newly introduced agents. It is possible that three-component combinations may be necessary if the plasmid-encoded MBLs become a prevalent component of our nosocomial pathogens. When these metalloenzymes are produced in tandem with large amounts of serine β-lactamases, it is unlikely that a single inhibitor will be able to inactivate all the enzymes effectively. As a result, the study of β-lactamases will remain a highly active area of investigation as we continue to try to understand the ecological implications of their continued evolution, and the implications of this on the development of new therapeutic approaches.

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Chapter 13
Inducible Resistance to Macrolide Antibiotics

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13.1 Ribosomes and Macrolides

The ribosome, one of the central cellular machines, is responsible for production of all the cellular polypeptides. The 70S ribosome is composed of two subunits, large (50S) and small (30S). Each subunit is built of ribosomal RNA (rRNA) and ribosomal proteins (r-proteins). rRNA, which accounts for two thirds of the ribosome weight, is its main structural and functional component. The main function of the small ribosomal subunit is deciphering genetic information by selecting aminoacyl-tRNAs whose anticodon is complementary to the mRNA codon in the ribosome decoding center. Catalysis of peptide bond formation and polymerization of amino acids into a polypeptide chain takes place in the catalytic peptidyl transferase center of the large ribosomal subunit. The nascent polypeptide leaves the peptidyl transferase center through the exit tunnel, which serves as a passage for the newly synthesized proteins on their way out of the ribosome (Fig. 13.1a, b). The tunnel starts at the peptidyl transferase center at the interface side of the subunit, spans the body of the subunit, and 'opens' at its exterior ('solvent') side. The nascent peptide exit tunnel is approximately 100Å in length and on average is fairly narrow (ca. 15Å in width) [44]. The walls of the exit tunnel are built primarily of rRNA.

The ribosome emerges as one of the best and evolutionary preferred antibiotic targets. As a result, a great variety of natural antibiotics inhibit cell growth by interfering with the various functions of the large or small ribosomal subunits. Many of these protein synthesis inhibitors were developed into clinically useful medicines.

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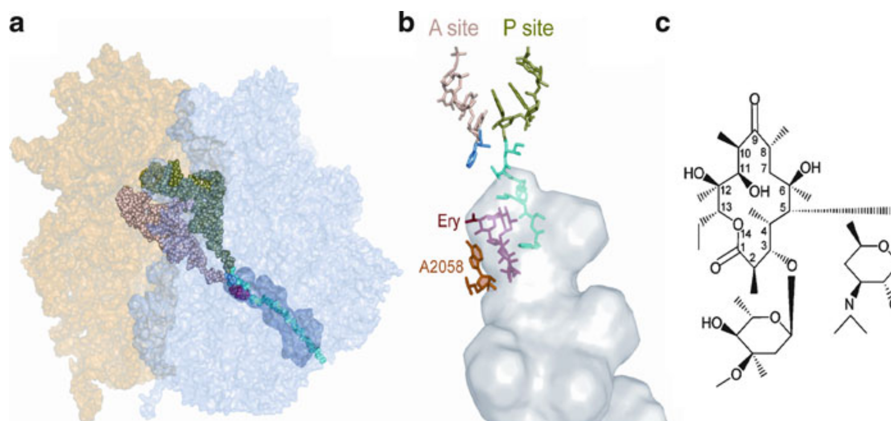

Fig. 13.1 Nascent peptide and macrolide antibiotic in the exit tunnel of the ribosome. (**a**) 70S ribosome is shown as a translucent surface with the small subunit shown in *yellow* and large subunit in *violet*. The contour of the exit tunnel is shown as a *gray shadow*. A model 30 amino acid long nascent peptide spanning the tunnel is color *cyan*. The nascent peptide esterifies tRNA (*green*) positioned in the ribosomal P site. The A site-bound aminoacyl tRNA is color *beige* with its aminoacyl moiety highlighted in *blue*. The macrolide antibiotic (erythromycin) bound in tunnel is *red*. (**b**) A close-up view of erythromycin (*red*) in the exit tunnel (*gray*). Aminoacyl- and peptidyl-tRNAs are colored as in (a). Peptidyl-tRNA carries a model six amino acid-long nascent peptide. The nucleotide residue A2058 of 23S rRNA which participates in drug binding and is targeted by Erm-type methyltransferase enzymes is shown in *orange*. (**c**) Chemical structure of a 14-member ring macrolide antibiotic erythromycin

Macrolides are among the most successful drugs that affect functions of the ribosome. Macrolides are built of a macrolactone ring (containing 12–16 atoms) decorated with several sugar residues and, sometimes, other side chains (Fig. 13.1c). These drugs bind to the large ribosomal subunit in the nascent peptide exit tunnel, at its constriction, located approximately 25Å away from the peptidyl transferase center [9]. Due to its location in the nascent peptide exit tunnel, this site is accessible to drug binding when the ribosome is vacant or when the nascent peptide is very short but is occluded when a long nascent peptide is present in the exit tunnel.

When macrolides bind to the ribosome, the macrolactone lays flat against the tunnel wall, due largely to hydrophobic interactions between the hydrophobic side of the ring and the rRNA residues that form the tunnel (Fig. 13.1b). The appendages protrude up (towards the peptidyl transferase center) or down (towards the constriction) and are involved in hydrophobic as well as in hydrogen bonding interactions with the rRNA residues. Some of these interactions contribute significantly to the drug's binding energy. Perturbing them (for example, by mono- or dimethylation of A2058 by Erm-type methyltransferase enzymes) dramatically reduces the affinity of macrolides for the ribosome and results in high levels of resistance.

It is generally believed that macrolides interfere with protein synthesis by inhibiting progression of the nascent polypeptide though the ribosome exit tunnel. When the growing polypeptide reaches the site of antibiotic binding, its growth is obstructed by the drug [1, 6]. An attempt by the ribosome to 'push' the nascent peptide down the tunnel results in dissociation of peptidyl-tRNA from the ribosome [25, 35].

Similar to most other antibiotics, the clinical value of macrolides has been curbed, due to development of resistance. The main resistance mechanisms include modification of rRNA in the drug-binding site by Erm-type methyltransferase enzymes, rRNA mutations, efflux of the drugs by Mef-type transporters, and chemical modification of the drugs. Significant progress has been achieved in understanding how these resistance mechanisms operate and how they protect cells from the inhibitory action of the drug. Several excellent reviews document our progress in understanding these mechanisms of macrolide resistance from medical and molecular standpoints [20, 28, 64]. However, one of the most important aspects of the resistance mechanism, the regulation of expression of the resistance genes, is understood only poorly. Many, possibly most, of the macrolide resistance genes are tightly controlled. They are repressed when there are no macrolides around but are activated when the cell ‘senses’ the presence of macrolide antibiotics. Understanding the molecular mechanisms that control expression of macrolide resistance genes can not only lead to better regimens of the use of macrolide antibiotics, but may provide new venues for the development of superior drugs that would not trigger expression of resistance.

In this chapter, we will attempt to summarize the current knowledge of molecular mechanisms that underlie regulation of expression of macrolide resistance genes.

13.2 Inducibility Reduces the Cost of Fitness of Resistance

Antibiotics are a hazard for bacteria. They are perilous for hospital pathogens treated with the drugs, treacherous for bacteria in their natural habitats with antibiotic producers lurking around, and can be detrimental for the producers themselves. The obvious evolutionary response to the existence of antibiotics is the development of antibiotic resistance. However, resistance comes at a cost.

The redundancy of rRNA genes in many bacterial species makes it difficult for an organism to develop resistance to protein synthesis inhibitors via target site mutations. A much more common mechanism of preventing the drug from binding to the ribosome is by chemical modification of rRNA residues in the drug binding site. Such posttranscriptional modification of specific rRNA residues by Erm-type methyltransferase enzymes is one of the key mechanisms of acquired resistance to many clinically relevant ribosomal antibiotics, including macrolides [20, 48, 63]. However, since macrolides act upon a functionally important site in the ribosome, even small alterations in the structure of the site may negatively influence ribosome function.

An alternative to drug target modification is preventing the drug from accumulating in the cell. The simplest solution is the active efflux of the drug. Although seemingly benign, such resistance mechanisms are not without their intrinsic flaws. The over-expressed low-specificity multidrug efflux transporters can pump out along with the hazardous antibiotics some useful nutrients and metabolites thereby upsetting the biochemical balance of the cell. Even the highly specific drug-dedicated pumps are not cheap. Continuous pumping of the drug out of the cell, which is required to

counterbalance the in-flow of antibiotic from the media, is an energy-hungry process. Therefore, running such a pump at a high capacity can dramatically increase the energy bill of the cell.

The resistance enzymes that inactivate the drugs by altering their chemical structure are also not toll-free. Production of these enzymes could be biochemically expensive and may produce metabolites with off-target inhibitory action.

To make things worse, the acquired genes that encode resistance proteins are usually foreign to the cell. Because of that the encoded polypeptides may misbehave in the well-organized and evolutionarily optimized cellular environment and may not properly obey the rules of cooperation with the 'indigenous' molecular inhabitants of the cell milieu. Containing the possibly rowdy behavior of these newcomers requires additional investment of energetic and biochemical resources of the cell.

Although any resistance mechanism needs to be paid for, the price is not an issue when the mere survival of the cell is at stake. Therefore, the cell should not hesitate to express the resistance trait when antibiotic is present. The question is what do you do with the resistance arsenal when there is no obvious antibiotic threat? Here nature came up with a wonderfully elegant solution. Many of the resistance mechanisms mentioned above are dormant when no antibiotic is present. Only when the first scouting molecules of the drug appear is expression of the inducible resistance genes activated so that the cell can rapidly build up its drug-resistance capacity. Because of that, inducible drug-resistance is much less expensive for the cell than constitutively expressed resistance.

13.3 Key Principles of the Control of Expression of Inducible Macrolide Resistance Genes

Inducible expression of the resistance genes requires operation of a fast response mechanism that can sense with high sensitivity and precision the presence of an antibiotic, and then activate the production of the resistance enzyme. Like most other protein synthesis inhibitors, macrolide antibiotics have been evolutionary optimized to bind to and modulate the activity of the ribosome, the key component of the gene expression apparatus. Therefore, the ribosome is perfectly suited to play the roles of both the sensor and the responder to the presence of a macrolide antibiotic.

Although the regulation of only several macrolide resistance genes, mostly from the *erm* family, has been studied in detail [16, 18, 19, 37, 60, 64], the basic principles that emerged likely apply to a broader array of resistance genes. In the next few paragraphs, we will outline these principles.

The inducible antibiotic-resistance genes are usually present on a bi-cistronic mRNA where the resistance gene is preceded by a regulatory (sensor) open reading frame (ORF) (Fig. 13.2) encoding a so-called leader peptide. (In the following sections, in the figures and tables, we will indicate the leader ORF by an index 'L' – for 'leader'). In the absence of the drug, expression of the resistance gene is attenuated either because of a premature transcription termination (transcriptional attenuation)

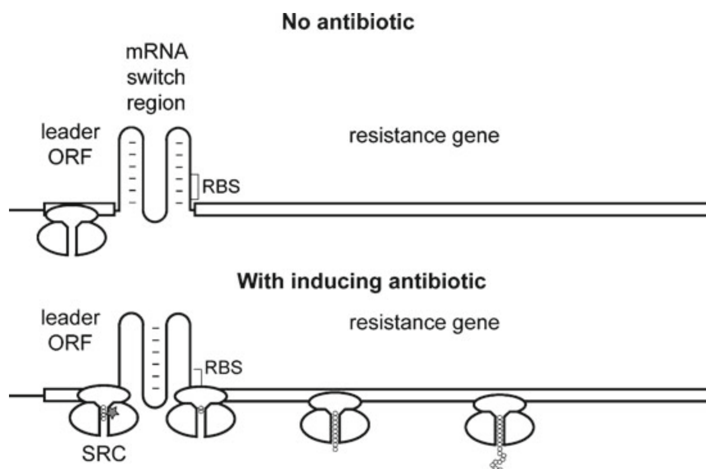

Fig. 13.2 The general scheme of regulation of expression of inducible antibiotic resistance genes (a translational attenuation scenario). In absence of the inducing antibiotic, the leader ORF is constantly translated, but the resistance gene is not because its ribosome binding site (RBS) is sequestered in the mRNA secondary structure. In the presence of an inducing antibiotic, a stalled ribosome complex (SRC) is formed at the leader ORF in a drug- and nascent peptide-dependent manner. The ribosome stalling at the leader ORF results in remodeling of the switch region in mRNA. The RBS of the resistance gene is liberated and translation of the resistance gene is activated. In the case of transcriptional attenuation (not shown), the structure of the switch region in the non-induced state leads to a premature termination of transcription, preventing RNA polymerase from transcribing the resistance gene. The SRC formation and remodeling of the switch region prevents premature termination of transcription resulting in activation of expression of the resistance gene

or because the ribosome binding site of the resistance gene is sequestered in the mRNA secondary structure preventing initiation of translation (translational attenuation). At the same time, the regulatory ORF is constitutively transcribed and translated. (It is generally assumed that the complete polypeptides encoded in the regulatory ORFs do not have any specific function in the cell and are rapidly degraded after they are released from the ribosome).

At low concentrations of an inducing antibiotic, a fraction of ribosomes in the cell is associated with the drug. Progression of such drug-bound ribosomes along the regulatory ORF is impeded in a specific and peculiar way. When the first few amino acids of the leader peptide are polymerized, the drug bound ribosome stalls. Such stalling critically depends on the length and sequence of the nascent peptide in the exit tunnel and occurs at a specific site of the leader ORF, usually at the eighth, ninth, or tenth codon. Formation of the stalled ribosome complex (SRC) alters the mRNA conformation relieving the transcriptional or translational attenuation. Since induction of resistance occurs when the intracellular concentrations of macrolides are still low (10- to 1,000-fold below MIC) [66], the fraction of drug-free ribosomes in the cytoplasm is large enough to afford efficient expression of the resistance gene and rapid onset of resistance.

13.4 Molecular Interactions that Control Ribosome Stalling at the ermCL Regulatory ORF

The general principles of induction of macrolide resistance genes have been initially discovered and described for the *S. aureus ermC* gene in a series of elegant papers from Weisblum and Dubnau laboratories (reviewed in [8, 64]). Molecular details of the operation of the central component of the induction mechanism – the ribosome stalling – have been worked in further detail more recently [60]. In this section, we will have a closer look at inducible expression of *ermC,* which likely sets the main theme for regulation of expression of other inducible macrolide resistance genes.

Expression of *ermC* is controlled by a leader ORF *ermCL* that encodes a 19-amino acid long peptide (Fig. 13.3) and is translationally attenuated in the absence of the drug. The translation initiation site of *ermC* is sequestered in the secondary structure formed by elements 3 and 4 of the intergenic ('switch') region of *ermCL-ermC* mRNA.

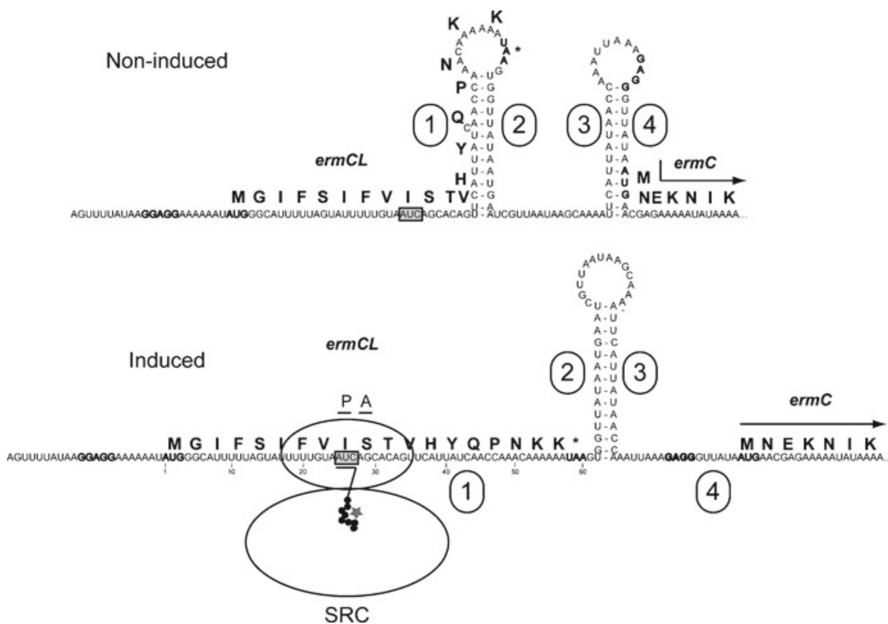

Fig. 13.3 Control of *ermC* expression by translational attenuation. Shine-Dalgarno sequences and the initiator codons of *ermCL* and *ermC* are highlighted in *bold*. In the non-induced state, *ermCL* is constitutively translated whereas translation of *ermC* is precluded because its translation initiation site is sequestered in the secondary structure of mRNA in the non-induced conformation. In the presence of erythromycin or other inducing antibiotics, the stalled ribosome complex (SRC) that forms at the ninth (Ile) codon of *ermCL* disrupts one or two base pairs at the bottom of the hairpin structure 1–2. This triggers remodeling of the mRNA switch region into the 'induced' conformation in which translation initiation site of *ermC* is accessible. The *ermCL* codon in P site of the SRC is boxed and shaded. *ermCL* codons 9 and 10 occupy P and A sites respectively

Pairing of the segments 3 and 4 is favored because segment 2, which can compete for pairing with segment 3, is engaged in the formation of the 1–2 structure in the uninduced state.

In the presence of an inducing macrolide (for example, erythromycin), the ribosome stalls when the ninth (Ile) codon of the *ermCL* ORF enters the ribosomal P site [60]. The stalled ribosome destabilizes helix 1–2 and, as a result, promotes formation of an alternative structure of the switch region in which the translation initiation site of *ermC* is liberated (Fig. 13.3b). Mutational analysis and biochemical structural studies [10, 12, 30, 31] provide convincing evidence that induction of *ermC* expression relies on such a conformational switch from the 1–2/3–4 structure to the 2–3 configuration of the switch region. However, the energy-based structure prediction algorithms favor the 2–3 pairing over the 1–2/3–4 scheme. Thus the non-induced fold of the switch region might represent the kinetically favored but thermodynamically unstable transient mRNA conformation, which is formed simply because segment 1 is transcribed prior to segment 3. Since the intrinsic helicase activity of the ribosome operates at a distance of eight nucleotides downstream from the P site codon [55], the stalled ribosome can destabilize nothing more than just the very first base pair of the 1–2 helix. Such a small change will be not enough to significantly change the equilibrium distribution between the alternative structures of the switch region, but it is likely sufficient to increase the isomerization rate of a less stable non-induced fold into a more energetically-favorable induced conformation. Since segment 3 is complementary to both segment 2 and segment 4, the conformational switch does not require complete unwinding of both helices, but can proceed via strand invasion mechanism [22].

One of the most unexpected findings of the original studies of inducible *ermC* was realization that not only the drug, but also the sequence of the leader peptide encoded in the *ermCL* ORF are critical for the ribosome stalling and *ermC* induction. Genetic and biochemical studies verified the identity and location of the control elements in the ErmCL peptide [41, 56, 60, 66]. In the stalled ribosome complex (SRC), the $tRNA^{Ile}$, located in P site of the ribosome, carries a nine amino acid-long nascent peptide with the sequence fMet-G-I-F-S-I-F-V-I. Four C-terminal amino acids (IFVI) have been shown to be critical for the formation of the SRC because replacement of any of these residues with alanine prevents drug-dependent stalling [32, 60]. A number of other amino acid substitutions at these positions prevent ribosome stalling required for erythromycin-dependent induction of *ermC* [32]. Since the critical sequence is located at the C-terminus of the nascent peptide in the SRC, the sensory elements of the ribosome that recognize and respond to the nascent peptide-stalling signal must be positioned in the segment of the exit tunnel, which is proximal to the peptidyl transferase center.

The inhibitory action of erythromycin on translation is mediated by drug-induced peptidyl-tRNA drop-off [26, 27, 34, 57]. The drug promotes active dissociation of peptidyl-tRNA from the ribosome when the nascent peptide reaches the size of six to eight amino acids [57]. Thus only a fraction of the drug-bound ribosomes that initiate *ermCL* translation are able to reach the ninth codon of the *ermCL* ORF and polymerize the critical IFVI sequence. This consideration explains why moving the

IFVI sequence away from the N-terminus of ErmCL alleviates induction – too few ribosomes manage to 'hold on' to the mRNA long enough to synthesize the critical sequence. Interestingly, placement of the critical IFVI sequence closer to the N-terminus of the ErmCL peptide also does not benefit induction [60], indicating that the nascent peptide has to reach a certain length to efficiently exert its stalling effect. Most likely, the N-terminus of the nascent peptide might engage some additional 'sensors' located in the tunnel farther away from the peptidyl transferase active site.

The nascent peptide-dependent induction of *ermC* is controlled by the presence of a macrolide antibiotic in the ribosome exit tunnel. The chemical structure of the drug may dramatically influence its ability to promote SRC formation. Thus, 14- and 15-member ring macrolides, which carry a cladinose sugar residue at the C3 position of the macrolactone ring, readily promote ribosome stalling and *ermC* expression. However, ketolides, which lack C3 cladinose, are unable to cause the ribosome to stall at the *ermC* leader ORF [60]. Required for the formation of a stable SRC at *ermCL*, is not only the presence of a C3-linked sugar, but also its precise structure. Replacement of the cladinose with the comparably bulky aromatic or aliphatic side chains, or even minor modification in the structure of the cladinose sugar dramatically reduces the efficiency of stalling [46, 69]. These observations suggest that cladinose is engaged in specific interactions either with the ribosome or the nascent peptide, which are important for ensuring the translation arrest. The exact structure of the macrolactone ring is also important. The 14-member ring clarithromycin and a 15-member ring azithromycin bind to the same ribosomal site and assume similar poses [50, 51, 59]. Both drugs carry C3-cladinose. Yet, while clarithromycin efficiently induces ribosome stalling at the ninth codon of *ermCL*, azithromycin favors stalling at codon 16 (Pro) (Vazquez-Laslop and Mankin unpublished). Although the shift in the site of stalling does not prevent azithromycin from being an efficient inducer of *ermC*, it underscores the importance of the drug structure for the SRC formation.

It is worth noting that although ketolides do not induce the ribosome stalling at the *ermC* regulatory ORF, experiments with an *ermC*-based reporter in *E. coli* cells suggest that ketolides are nevertheless capable of inducing expression of the resistance gene, albeit at a slower rate and at a narrower range of concentrations compared to erythromycin [2]. Ketolide-dependent induction of *ermC* in *E. coli* cells requires translation of the leader ORF but appears to operate through a mechanism principally different from that described for cladinose-containing macrolides. Such an 'alternative' mechanism may also contribute to the effect of some other inducers. The alternative mechanism of the *ermC* induction, not mediated by the ribosome stalling, might account for some reports of the activation of inducible *erm* genes by ketolides [43, 68].

Our understanding of the molecular mechanisms within the ribosome that govern recognition of the antibiotic and the critical IFVI sequence of the *ermCL* nascent peptide and subsequent switch from elongation mode to the arrest state is very rudimentary. Among a number of 23S rRNA residues in the exit tunnel which may potentially participate in monitoring the nascent peptide sequence, two nucleotide

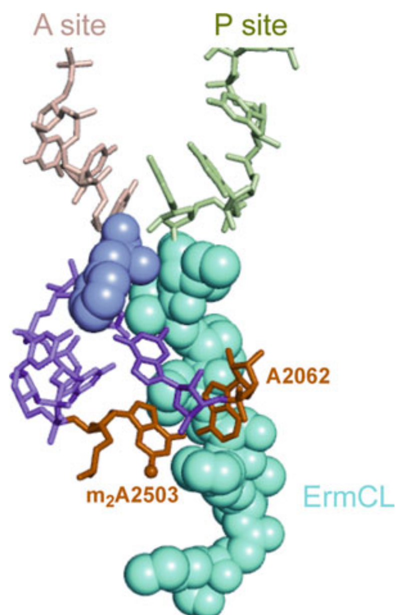

Fig. 13.4 The sensors of the ErmCL nascent peptide in the ribosome exit tunnel. The nine amino acid-long ErmCL nascent peptide (fMGIFSIFVI), shown in *cyan*, esterifies the P site-bound tRNA (*green*). The A site-bound aminoacyl-tRNA is shown in *beige* and its aminoacyl residue is shown in *blue*. The 23S rRNA nucleotides critical for sensing the ErmCL peptide, A2062 and m_2A2503 are shown *orange* (the posttranscriptionally-added C2-methyl group of m_2A2503 is shown as a *sphere*). The nucleotides neighboring A2062 and m_2A2503 which project into the A site of the peptidyl transferase center are highlighted in *magenta*

residues, A2062 and A2503, have been identified as critical for the SRC formation ([60] and Vazquez-Laslop, Mankin unpublished). Both of these highly conserved residues are located in the upper chamber of the nascent peptide exit tunnel and thus are positioned at the exactly right site to interact with the critical IFVI sequence of the ErmCL nascent peptide (Fig. 13.4). A2062 is highly flexible and is seen in different conformations in various crystallographic complexes of the large ribosomal subunit. In one of the conformations, it protrudes in the tunnel lumen [59] and in the other; it is rotated towards the tunnel wall and forms hydrogen bond interactions with A2503 [3]. This conformational flexibility of the A2602 base could be utilized in the operation of the stalling mechanism. A2503 is posttranscriptionally modified to m_2A – a signature of its functional importance in the ribosome [17, 58]. Mutations of either A2062 or A2503 prevent the ribosome from forming the stalled complex and inducing *ermC* expression [60]. The immediate neighbors of A2062 and A2503 in 23S rRNA, G2061, C2063, and U2504 project into the A site of the peptidyl transferase center and their precise orientation is likely critical for the formation of the peptidyl transferase active site. Therefore, it is conceivable that even a small shift in placement of A2062 or A2503 in response to the presence of the specific nascent peptide and inducing antibiotic in the ribosome exit tunnel could result in

disrupting the active conformation of the peptidyl transferase center. In agreement with this scenario, it has been established that the ribosome, stalled at the *ermCL* ORF, is unable to catalyze the formation of the peptide bond between the nonapeptidyl residue that esterifies tRNA in the ribosomal P site and the incoming aminoacyl-tRNA [60].

13.5 Regulation of Inducible Expression of Different erm Genes

Unraveling the regulation of *ermC* expression established the paradigm of activation of macrolide resistance genes in response to the presence of the inducing antibiotic. The main principle of this paradigm is antibiotic-induced nascent peptide-dependent stalling of the ribosome at the regulatory ORF, resulting in de-repression of the resistance gene. However, the implication of this key principle discovered in the studies of *ermC* regulation may vary substantially between different resistance genes. Currently, the regulation of only a handful of macrolide resistance genes has been investigated in some detail. In the next few sections, we will briefly summarize the variations of the main scheme of *ermC* regulation seen in several investigated macrolide resistance genes.

13.6 ermA

The gene *ermA* is found in *Staphylococci*, *Streptococci,* and several other Gram-positive organisms. The 5′ leader region of *ermA* (a. k. a. *ermTR*) gene is 211 bp long [40] (Fig. 13.5). It comprises two regulatory ORFs: the 5′-proximal ORF, *ermAL1*, encodes a 15 amino acid long peptide and the second ORF, *ermAL2*, codes for a 19 amino acid long peptide. The ribosome-binding site of *ermA* gene is sequestered in a hairpin structure, suggesting that *ermA* is under translation attenuation control (Fig. 13.5b). The hypothetical secondary structure of the *ermA* 5′ leader region is compatible with translation attenuation mechanism and indicates that stalling of the ribosome during translation of *ermAL2* may compel the mRNA to switch into a conformation conducive for the *ermA* expression.

The regulatory role of *ermAL2* ORF is reasonably well established. The similarity of peptides encoded in *ermAL2* and *ermCL* ORFs hints that, by analogy with the thoroughly investigated ermCL, the drug-dependent stalling of the ribosome will occur at the 9th codon of *ermAL2* ORF. This prediction is supported by direct experimental data [70]. The regulatory function of *ermAL2* is further supported by the observations that spontaneous deletions that encompass *ermAL2* lead to constitutive expression of *ermA* [40, 59, 52].

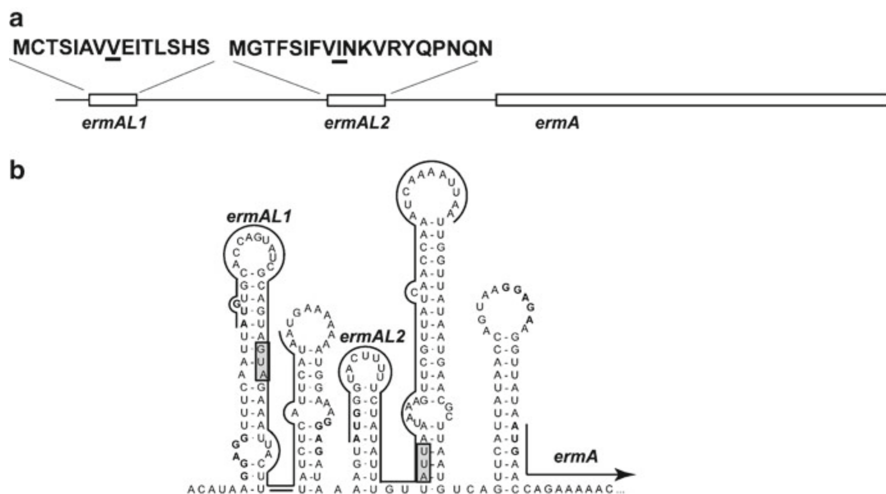

Fig. 13.5 Organization of the *ermA* operon and structure of the *ermA* regulatory region. (**a**) The location of two control leader ORFs (*ermAL1* and *ermAL2*) upstream of the *ermA* gene and the sequences of the encoded leader peptides [40]. The codon located in the P site of the stalled ribosome complex is *underlined*. (**b**) A hypothetical secondary structure of the *ermA* regulatory region in the non-induced state (Adapted from [5]). The initiator codons and Shine-Dalgarno sequences of *ermAL1*, *ermAL2* and *ermA* are shown in *bold* and the open reading frames are indicated by *solid lines*. The codons of the leader ORFs at which SRC is formed are *boxed* and *shaded*

Although the role of *ermAL1* in induction of *ermA* is less well documented, several facts point to its importance in control of *ermA* induction. Thus, a *S. aureus* strain carrying a 83 bp deletion that removed the entire *ermAL2* ORF but preserved *ermAL1* ORF remained inducible by macrolides [5]. Biochemical experiments demonstrated erythromycin-dependent stalling of the ribosome at the eighth codon of the *ermAL1* ORF (Ramu and Mankin unpublished). Such stalling may account for erythromycin-dependent stabilization of the *ermA* mRNA [49].

The interplay of the two regulatory ORFs in control of *ermA* induction is not clearly understood. Modeling of the mRNA secondary structure led to the proposal that the ribosome stalling at *ermAL1* activates translation of *ermAL2* whereas the SRC formation at *ermAL2* directly leads to translational de-repression of *ermA* [40]. Alternatively, stalling of the ribosome at *ermAL1* may control mRNA stability whereas formation of the stalled complex at *ermAL2* regulates translation of *ermA*. The presence of two regulatory ORFs may also affect the specificity of induction: while *ermAL2* is involved only in macrolide-controlled induction, translation of *ermAL1* appears to respond also to lincosamides [5, 56].

The structure, and thus, operation of the *ermA* regulatory region closely resembles those of the *ermG* operon – the other macrolide resistance gene with two clearly identifiable regulatory *ORFs* [47] (Table 13.2).

Table 13.1 Mode of action and inducibility of MLSB resistance genes

Gene name	Resistance mechanism	Organism	Resistance profile[a]	Inducibility	GenBank accession number	Reference – Pubmed ID
ermA	Target site modification	*Staphylococcus aureus*	MLS_B	Inducible	X03216	3004956
ermA	Target site modification	*Streptococcus pyogenes*	MLS_B	Inducible	AF002716	9527769
ermB	Target site modification	*Escherichia coli*	MLS_B	Constitutive	M19270	2832378
ermB	Target site modification	*Streptococcus sanguinis*	MLS_B	Inducible	K00551	6406429
ermB	Target site modification	*Enterococcus faecalis*	MLS_B	Inducible	M11180	2997130
ermB	Target site modification	*Lactobacillus reuteri*	MLS_B	Unknown	AF080450	10413663
ermB	Target site modification	*Staphylococcus intermedius*	MLS_B	Inducible[b]	AF299292	11230937
ermB	Target site modification	*Enterococcus faecalis*	MLS_B	Inducible	U86375	9791136
ermC	Target site modification	*Staphylococcus simulans*	MLS_B	Constitutive	AF019140	9742700
ermC	Target site modification	*Staphylococcus aureus*	MLS_B	Inducible	V01278	6279574
ermD	Target site modification	*Bacillus licheniformis*	MLS_B	Inducible	M77505	1713206
ermD	Target site modification	*Bacillus anthracis*	MLS_B	Inducible	L08389	8473865
ermD	Target site modification	*Bacillus licheniformis*	MLS_B	Inducible	M29832	6429477
ermE	Target site modification	*Saccharopolyspora erythraea*	MLS_B	Unknown	M11200	3934045 2998943
ermF	Target site modification	*Bacteroides fragilis*	MLS_B	Unknown	M17808	2820936
ermF	Target site modification	*Bacteroides fragilis*	MLS_B	Unknown	M62487	1905805
ermG	Target site modification	*Lysinibacillus sphaericus*	MLS_B	Inducible	M15332	3025178
ermG	Target site modification	*Bacteroides thetaiotaomicron*	MLS_B	Unknown[c]	L42817	8834912
ermH	Target site modification	*Streptomyces thermotolerans*	MLS_B	Inducible	M16503	3036668
ermN	Target site modification	*Streptomyces fradiae*	MLS_B	Constitutive	X97721	8973363 1995426
ermO	Target site modification	*Streptomyces ambofaciens*	MLS_B	Unknown	AJ223970	10517588
ermQ	Target site modification	*Clostridium perfringens*	MLS_B	Unknown	L22689	8067735
ermR	Target site modification	*Arthrobacter sp.*	M	Unknown	M11276	4043733
ermS	Target site modification	*Streptomyces fradiae*	MLS_B	Inducible	M19269	3127381

ermT	Target site modification	*Lactobacillus reuteri*	MLS_B	Constitutive	M64090	8171126
ermT	Target site modification	*Lactobacillus fermentum*	MLS_B	Constitutive	AJ488494	14597008
ermV	Target site modification	*Streptomyces viridochromogenes*	MLS_B	Inducible	U59450	6163765 9055987
ermW	Target site modification	*Micromonospora griseorubida*	MLS_B	Unknown	D14532	8163173
ermX	Target site modification	*Corynebacterium diphtheriae*	MLS_B	Inducible	M36726	11408212
ermY	Target site modification	*Staphylococcus aureus*	MLS_B	Inducible	AB014481	11751136
erm30	Target site modification	*Streptomyces venezuelae*	Unknown	Unknown	AF079138	9770448
erm31	Target site modification	*Streptomyces venezuelae*	Unknown	Unknown	AF079138	9770448
erm32	Target site modification	*Streptomyces fradiae*	M	Inducible	AJ009971	10348045
erm33	Target site modification	*Staphylococcus sciuri*	MLS_B	Inducible	AJ313523	12384375
erm34	Target site modification	*Bacillus clausii*	MLS_B	Unknown	AY234334	14711653
erm35	Target site modification	*Bacteroides coprosuis*	MLS_B	Unknown	AF319779	–
erm36	Target site modification	*Micrococcus luteus*	MLS_B	Inducible	AF462611	12177341
erm37	Target site modification	*Mycobacterium tuberculosis*	MLS_B	Inducible	Z74025 BX842578	9634230 16174779
erm38	Target site modification	*Mycobacterium smegmatis*	ML	Inducible	AY154657 CP000480	14506008 16127056
erm39	Target site modification	*Mycobacterium fortuitum*	ML	Inducible	AY487229	15590712
erm40	Target site modification	*Mycobacterium mageritense*	ML	Constitutive	AY570506	17005837
erm41	Target site modification	*Mycobacterium abscessus*	ML	Inducible	EU177504	19171799
msrA	Efflux	*Staphylococcus epidermidis*	MS_B	Inducible	X52085	2233255
msrC	Efflux	*Enterococcus faecium*	MS_B	Inducible	AY004350	11120975
msrD	Efflux	*Streptococcus pneumoniae*	M	Inducible	AF274302	11398110 16223938
oleB	Efflux	*Streptomyces antibioticus*	Oleandomycin	Unknown	L36601	7565095
oleC	Efflux	*Streptomyces antibioticus*	Oleandomycin	Unknown	L06249	8326867
srmB	Efflux	*Streptomyces ambofaciens*	Spiramycin	Inducible	X63451	1508047
tlrC	Efflux	*Streptomyces fradiae*	Tylosin	Inducible	M57437	1864505

(continued)

Table 13.1 (continued)

Gene name	Resistance mechanism	Organism	Resistance profile[a]	Inducibility	GenBank accession number	Reference – Pubmed ID
mefA	Efflux	*Streptococcus pneumoniae*	ML	Unknown	AF274302	11398110
mefB	Efflux	*Escherichia coli*	ML	Unknown	FJ196385	19131424
ereA	Drug modification	*Providencia stuartii*	M	Unknown	AF099140	12654734
ereA	Drug modification	*Escherichia coli*	M	Constitutive	AY183453	14506050
ereA	Drug modification	*Vibrio cholerae*	M	Unknown	AF512546	12183252
ereB	Drug modification	*Escherichia coli*	M	Constitutive	X03988	3523438
ereB	Drug modification	*Escherichia coli*	M	Constitutive	AB207867.1	2546492
ereB	Drug modification	*Escherichia coli*	M	Unknown	A15097	–
mphA	Drug modification	*Escherichia coli*	M	Inducible	D16251	8619599 10960087
mphB	Drug modification	*Escherichia coli*	M	Constitutive	D85892	8900063
mphC	Drug modification	*Staphylococcus aureus*	M	Unknown	AF167161	–
mphD	Drug modification	*Pseudomonas aeruginosa*	M	Unknown	AB048591	11184231

[a]Abbreviations key for Resistance profile: *M* macrolides, *L* lincosamides, S_B streptogramins B
[b]Both inducible and constitutive isolates of this strain were found
[c]Reported as "Likely to be constitutive"
[d]Though the encoded protein is similar to rRNA methyltransferases, methylation of rRNA was not demonstrated experimentally. The mechanism of resistance could involve drug modification

13.7 ermB

The *ermB* gene (a.k.a. *erm AM, ermAMR, ermBC, ermIP, ermZ* [48]) is often found in macrolide-resistant isolates of a number of Gram-positive and Gram-negative bacteria, including *Enterococci*, *Streptococci*, *Staphylococci*, *Clostridium*, *Enterobacter*, and others. It can be present on plasmids or transposons. In the pAM77 plasmid, *ermB* is preceded by a 317 nt leader region, which contains a regulatory ORF *ermBL* encoding a 36 amino acid-long leader peptide MLVFQMRNVDKTSTILKQTKN-SDYVDKYVRLIPTSD [13]. In the Tn917 transposon, the upstream-transcribed region of *ermB* is shorter and contains only 259 bp. In Tn917, a four base pair insertion in the *ermBL* coding sequence introduces a premature stop codon and truncates the encoded leader peptide to 27 amino acid residues [45, 54]. Variants of *ermBL* have been described, which carry an additional 12 bp insertion extending the leader peptide from 27 to 31 codons [36].

Induction of resistance is controlled by translation of *ermBL* and likely involves translational de-repression of *ermB* [13, 37]. Mutational analysis [37] and direct biochemical mapping (Ramu and Mankin unpublished) showed that erythromycin-induced ribosome stalling takes place when the tenth codon of *ermBL* enters the P site of the translating ribosome. Stalling induces a conformational switch in the mRNA, which results in liberation of the translation initiation site of *ermB* (Fig. 13.6) [37]. However, neither the exact structure nor stability of the switch region in any of the conformations nor the kinetics or structural pathways of inter-conformation transitions are known.

In addition to erythromycin, expression of *ermB* is induced by 16-member ring macrolides (tylosin, josamycin) as well as by lincosamides and streptogramins [21]. At the moment, it remains unclear whether all of these drugs induce ribosome stalling at *ermBL* and, if so, what is the site of the SRC formation. Interestingly, mutations in the *ermBL* region preceding the stalling site differentially affect induction by 14- and 16-member ring macrolides [36], suggesting that specific interactions between the nascent peptide, the drug, and the ribosome are critical for the SRC formation at the precise site in *ermBL*.

13.8 ermD

The *ermD* class of methyltransferases includes genes, which in various reports were dubbed *ermD*, *ermJ,* or *ermK* [48]. The *ermD*- type genes have been found in *Bacilli* and *Salmonella*. *ermD* was reported to be inducible by 14-member ring macrolides, erythromycin, and oleandomycin [7]. The *ermD* leader region is 354–357 bp long (depending on assignment of the transcription start site) and contains a leader ORF *ermDL* encoding a 14 amino acid-long peptide MTHSMRLRFPTLNQ (Fig. 13.7). Nonsense mutations in *ermDL* or deletion of the leader segments, which included the leader ORF, resulted in the lack of induction or in constitutive expression of the

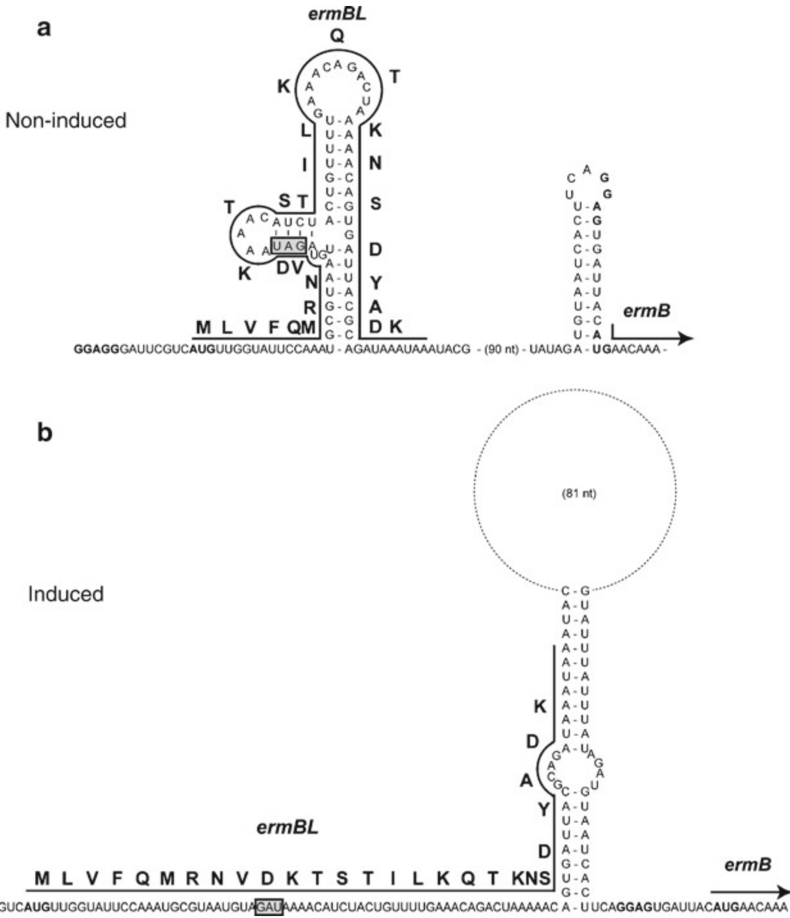

Fig. 13.6 The regulatory region of the *ermB* gene (Adapted from [37]). The hypothetical secondary structure of the regulatory region in non-induced (**a**) and induced (**b**) conformations was modeled on the basis of chemical probing of the mRNA structure both in vitro and in vivo [37]. The control leader ORF *ermBL* is indicated by *solid line*. The initiator codons and Shine-Dalgarno sequences of *ermBL* and *ermB* are shown in *bold* and the *ermBL* codon at which SRC is formed is *boxed* and *shaded*

methyltransferase, suggesting that translation of *ermDL* controls *ermD* expression [11, 18]. Existence of a second leader ORF *ermDL2* in the *ermD* leader was also proposed [14]. This putative ORF starts with a non-conventional UUG initiator codon and encodes a 13-amino acid long peptide MCMQSKRDQSVLF, which shows homology to a MNKYSKRDAIN peptide encoded in the *ermGL2* ORF of the *ermG* gene [39]. Involvement of this second putative leader ORF in regulation of *ermD* has not been explored.

It is not entirely clear whether *ermD* is controlled by translational or transcriptional attenuation [11, 14, 18]. An easily identifiable rho-independent transcription

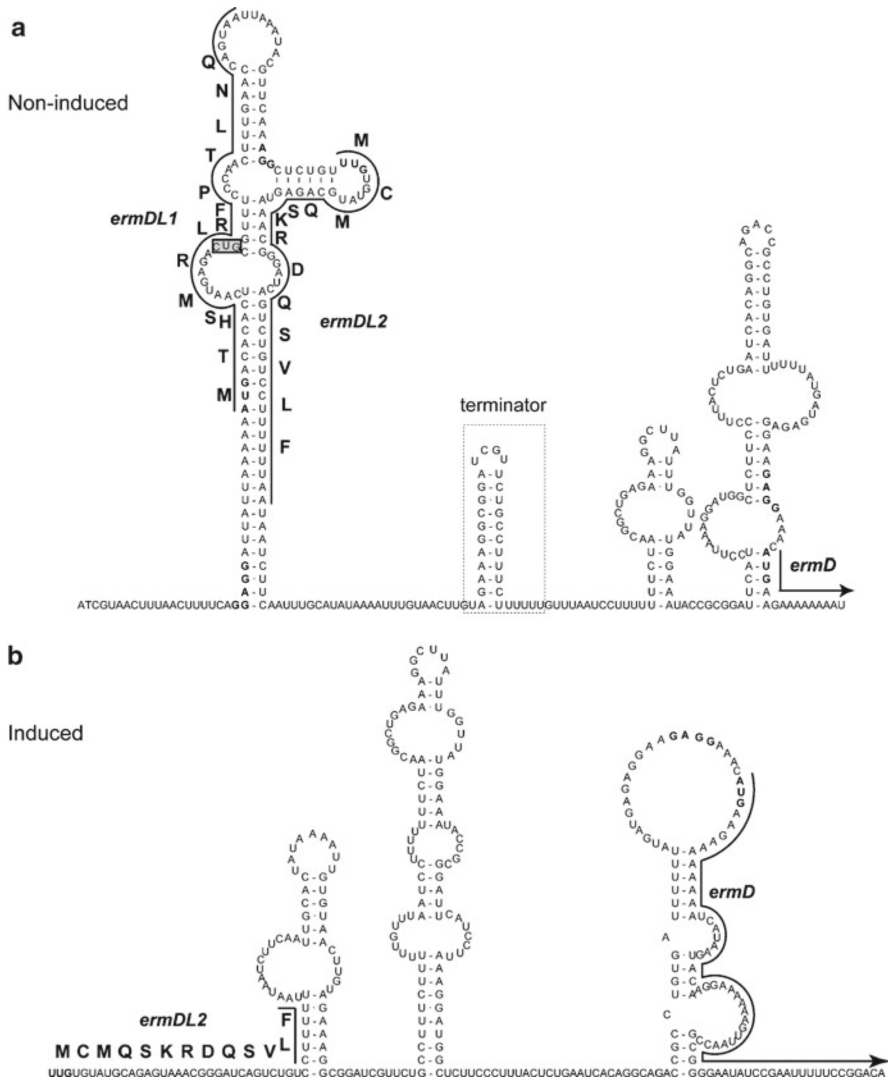

Fig. 13.7 Hypothetical secondary structure of the *ermD* regulatory region in (**a**) non-induced and (**b**) induced conformations (Adapted from [18]). A putative transcription terminator is boxed by a *dashed line*. The experimentally mapped ribosome stalling site in *ermDL1* is *boxed* and *shaded*. The second regulatory ORF, *ermDL2*, proposed by Hue, K.K. and Bechhofer, D. H. [14] is indicated

terminator is present in the *ermD* leader region and its function in attenuation of the *ermD* transcription has been experimentally confirmed [14, 18], arguing that transcription attenuation may be the main mechanism controlling inducible expression of *ermD* [18]. Stalling of the ribosome at the *ermDL* leader ORF has been predicted to disrupt the terminator structure, allowing RNA polymerase to proceed

with transcription of the downstream *ermD* message. However, there are conflicting reports whether the extent of transcription termination at this site depends on the presence of erythromycin [14, 18] and some of the mutational data and structure modeling attempts appear to better fit the translation attenuation scenario where the drug-induced ribosome stalling at *ermDL* frees the ribosome binding site of *ermD* [14, 19].

Genetic studies were carried out to define the segment of the *ermDL* ORF critical for stalling. Individually mutating codons 4–6 of *ermDL1* to a stop codon reduced erythromycin-dependent induction of *ermD* whereas a stop codon at position 7 resulted in a high level constitutive expression, leading to the hypothesis that ribosome stalls at the *ermDL1* ORF with the codon 6 (R) in the P site of the SRC [19]. Nevertheless, biochemical data showed that in fact it is codon 7 (L) of *ermDL1* that is located in the P site of the stalled ribosome (Ramu and Mankin unpublished). Interestingly, the location of the experimentally-defined programmed ribosome stalling site does not appear to be compatible with the predicted conformational switch of the attenuator region required for translational de-repression of *ermD* [18] suggesting that further analysis is required to characterize the exact mechanism of induction.

13.9 ermS

The methyltransferase *ermS* (originally called *ermSF* or *TlrA*) was identified in the tylosin producer, *Streptomyces fradiae* [15]. While *ermS* is expressed inducibly, there are at least three other tylosin resistance genes in *S. fradiae*, *tlrB, tlrC and tlrD,* which are expressed constitutively [16]. Constitutive expression of *tlrB* and *tlrD* results in N6 monomethylation of adenines at positions 748 and 2058, respectively [24, 67], whereas *ermS* introduces two methyl groups to N6 of A2058.

The ribosome-binding site of *ermS* is likely sequestered in an mRNA secondary structure, suggesting that the gene is regulated via translation attenuation (Fig. 13.8). Analysis of the 385 bp transcribed upstream region of *ermS* showed the presence of a short ORF *ermSL,* which encodes a 24 amino acid-long leader peptide MSMGIAARPPRAALLPPPSVPRSR [15]. Compatible with the translation attenuation model, putative ribosome stalling at *ermSL* has been predicted to alter mRNA conformation allowing translation of *ermS*. However, more recent studies indicated that transcription attenuation could be an important component of the induction mechanism [16].

Cloned *ermS* is induced by erythromycin but not by tylosin [15]. However, in the tylosin producer, *S. fradiae*, induction of *ermS* does occur in response to high concentrations of tylosin when A748 and A2508 in 23S rRNA are already methylated, due to the action of TlrB and TlrD methyltransferases [33]. Thus it is possible that only the ribosome mono-methylated at A748 and A2058 is capable of ‘proper’ tylosin-induced stalling at the *ermS* regulatory ORF.

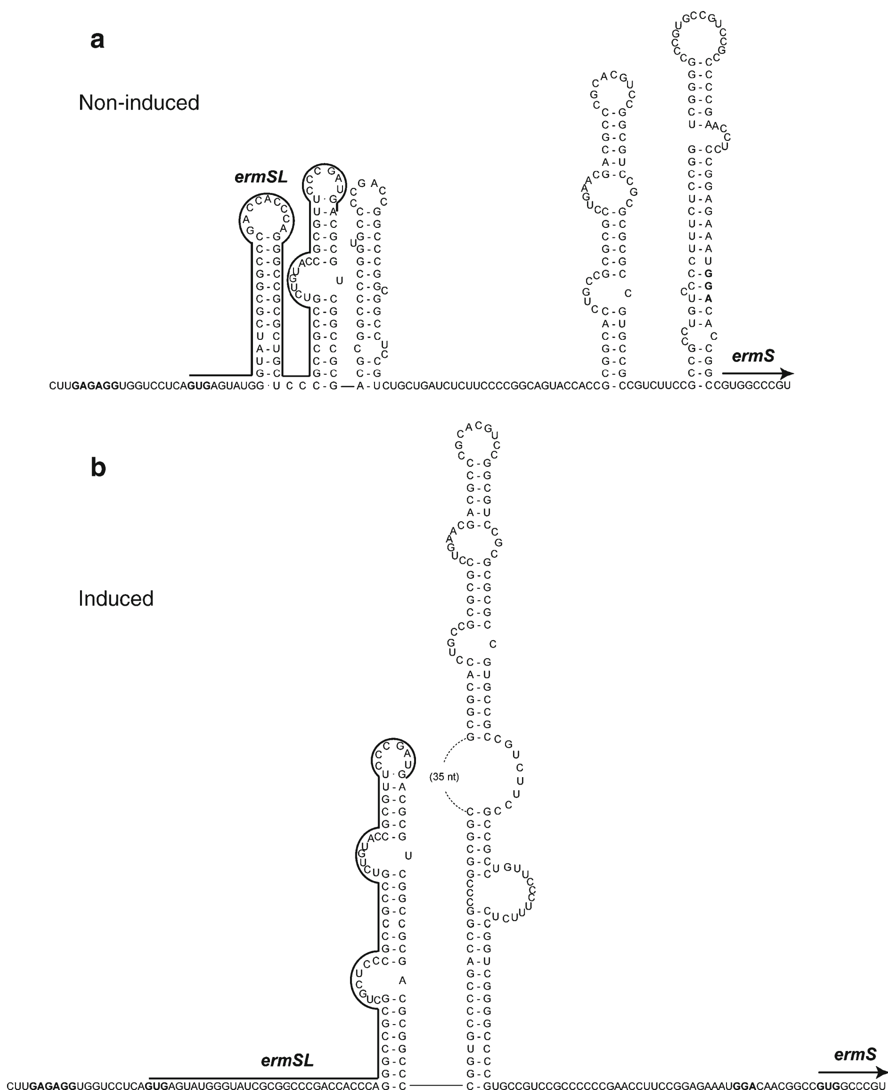

Fig. 13.8 Hypothetical secondary structure of the *ermS* regulatory region in (**a**) non-induced and (**b**) induced conformations (Adapted from [15]). The leader ORF *ermSL* is indicated by *solid line*

13.10 Inducibility of Other Macrolide Resistance Genes

Among a plethora of macrolide resistance genes, the genes *ermA, ermB, ermC, ermD*, and *ermS* represent the only few examples for which the operation of the induction mechanism has been examined at a molecular level at least in some detail. Although sporadic reports of inducibility of other macrolide resistance genes periodically

appeared [23, 29, 42, 43, 49], the mechanism and specificity of induction are usually left beyond the scope of the study.

In Table 13.1, we attempted to summarize the available data about inducibility of the known macrolide resistance genes. The table was composed using as a starting point the database of macrolide resistance genes maintained by M. Roberts [48] and expanded searching the database for homologs of the major macrolide resistance genes. The information about inducibility of the genes was extracted from the publications.

About half of the listed genes were reported to be inducible, clearly indicating that drug-dependent inducibility is a general trend of macrolide resistance. In spite of this trend, a considerable number of investigated genes showed a constitutive mode of expression. However, a seeming abundance of constitutive genes has to be treated cautiously. While inducibility of a resistance gene is usually viewed as its natural trait, the constitutive expression often results from 'artificial' selection. The antibiotic resistance profile conferred by a gene does not precisely match the spectrum of inducing antibiotics. Thus, dimethylation of A2058 by *erm*-type methyltransferase enzymes renders cells resistant to at least three classes of antibiotics, macrolides, lincosamides, and streptogramins B (the MLS_B resistance). Yet, usually only macrolides serve as inducers. Exposure of organisms carrying inducible *erm* genes to non-inducing antibiotics (lincosamides or streptogramins) rapidly selects for the constitutive versions of the resistance genes [65]. Transition from the inducible to constitutive mode of gene expression often results from point mutations, deletions, or insertions, which favor the 'induced' conformation of the mRNA switch region. Thus, although some of the genes listed in Table 13.1 might exhibit a constitutive phenotype, there is reason to think that in their original 'native' form they might have been inducible.

Table 13.1 presents clear evidence that a large fraction of macrolide resistance genes are either known or suspected to be inducible. If the mechanism of induction of these genes follows the paradigm discovered for *ermC* and several other investigated *erm* genes discussed above, then many inducible macrolide resistance genes are likely controlled by a regulatory leader ORF whose translation is sensitive to the presence of an inducing antibiotic. Conversely, the presence of a short translated ORF in an mRNA segment preceding a resistance gene points to the possibility of drug-mediated regulation of the gene. Indeed, short ORFs with possible regulatory functions have been identified upstream of several resistance genes (Table 13.2). Our more comprehensive survey of the 5′ transcribed regions of macrolide resistance genes showed that putative regulatory ORFs can be found in ca. 30% of the known macrolide resistance genes (Table 13.2). Many of the leaders ORFs are equipped with well-defined Shine-Dalgarno sequences, which help the ribosome recognize the translation initiation codon; the presence of a Shine-Dalgarno signature is a strong indication that the ORF is translated and thus plays role in regulation of the downstream resistance genes. However, even if the suspected regulatory ORF lacks a recognizable Shine-Dalgarno sequence, this does not rule out its possible role in control of expression of a downstream gene. There are many examples of actively translated genes in bacteria which lack Shine-Dalgarno sequences or whose initiator codon is located at the very 5′ end of the mRNA transcript [4, 38].

Table 13.2 Features of putative regulatory ORFs of MLSB resistance genes

Gene name	Inducibility	Annotated leader peptide[a]	Putative Shine-Dalgarno sequence	Leader peptide start codon	Peptide length (amino acid residues)	Distance between leader peptide ORF & resistance gene (bp)	Reference – Pubmed ID
ermA	Inducible	Yes (LP2)	AAGGAG	AUG	19	57	3004956
ermA	Inducible	Yes (LP2)	AAGGAGGU	AUG	19	57	9527769
ermA	Inducible	Yes (LP1)	UAAGGAGG	AUG	15	143	9527769
ermA	Inducible	Yes (LP1)	UAAGGAGGU	AUG	15	144	3004956
ermB	Constitutive	Yes	AAGGAGG	AUG	32	125	2832378
ermB	Inducible	Yes	AAGGAGG	AUG	36	92	6406429
ermB	Inducible	Yes	AAGGAGG	AUG	27	124	2997130 10413663
ermB	Inducible[b]	Yes	AAGGAGG	AUG	26	128	11230937
ermB	Inducible	Yes	AAGGAGG	AUG	27	124	9791136
ermC	Constitutive	Yes	UAAGGAGG	AUG	19	121	9742700
ermC	Inducible	Yes	UAAGGAGG	AUG	19	60	6279574
ermD	Inducible	Yes	AGGAGG	AUG	14	276	1713206 8473865 6429477
ermE	Unknown	No	AGGA	AUG	13	146	3934045 2998943
ermF	Unknown	No	GGUG	AUG	20	152	2820936
ermF	Unknown	No	AGGU	AUG	31	35	1905805
ermF	Unknown	No	–	GUG	14	94	1905805
ermG	Inducible	Yes (LP2)	UAAGGAGGU	AUG	19	56	3025178
ermG	Unknown[c]	LP1	–	AUG	8	122	8834912
ermG	Inducible	LP1	AGAGG	AUG	11	–	3025178
ermG	Unknown[c]	LP2	–	AUG	19	17	8834912

(continued)

Table 13.2 (continued)

Gene name	Inducibility	Annotated leader peptide[a]	Putative Shine-Dalgarno sequence	Leader peptide start codon	Peptide length (amino acid residues)	Distance between leader peptide ORF & resistance gene (bp)	Reference – Pubmed ID
ermN	Constitutive	No	GAGG	UUG	32	17	8973363 1995426
ermQ	Unknown	No	AGGAGG	AUG	14	80	8067735
ermS	Inducible	Yes	AGAGGUG	GUG	24	227	3127381
ermT	Constitutive	Yes	AAGGAG	AUG	19	85	8171126
ermT	Constitutive	Yes (truncated LP)	AAGGAGG	AUG	22	126	14597008
ermV	Inducible	Yes	–	AUG	38	102	6163765 9055987
ermW	Unknown	No	AGGA	AUG	39	187	8163173
ermX	Inducible	Yes	AAGU	UUG	21	80	11408212
ermY	Inducible	Yes	AAGGAGG	AUG	19	60	11751136
erm33	Inducible	No	AAGGAG	AUG	19	60	12384375
erm34	Unknown	Yes	GGAGG	AUG	13	232	14711653
erm36	Inducible	Yes	–	AUG	14	98	12177341
erm37	Inducible	No	GGAG	AUG	10	98	9634230 16174779
erm38	Inducible	Yes	–	AUG	19	62	14506008 16127056
erm39	Inducible	No	–	AUG	11	67	15590712
erm41	Inducible	No	GGAG	AUG	21	55	19171799
msrA	Inducible	Yes	AAGGAGG	AUG	8	231	2233255
msrC	Unknown	Yes	AAGGAGGU	AUG	15	226	11120975
msrD	Inducible	Yes	GGAGG	AUG	6	78	10952626 11398110 16223938

mefA	Unknown	No	AGGAGG	AUG	8	284	11398110
mefB	Unknown	No	–	AUG	6	168	19131424
ereA	Unknown	No	GGAG	AUG	15	62	12654734
ereA	Constitutive	No	GGAG	AUG	16	81	14506050
ereA	Unknown	No	–	GUG	10	33	12183252
ereA	Unknown	No	AAGG	AUG	19	83	12183252
ereB	Constitutive	No	GAGG	AUG	21	209	3523438, 2546492
mphA	Inducible	No	UAAGG	UUG	17	62	8619599, 10960087
mphB	Constitutive	No	GAGG	AUG	11	249	8900063
mphC	Unknown	No	AGGU	AUG	8	127	–

[a]"Yes" indicates that the leader peptide ORF is annotated in the GenBank entry or mentioned in the corresponding reference

[b]Both inducible and constitutive isolates of this strain were found

[c]Likely to be constitutive

Table 13.3 Putative leader peptides of MLSB resistance genes

Name	Leader peptide sequence	GenBank
IAVV peptides		
ErmAL1	MCTCIAVVDITLSHL	AF002716
ErmAL1	MCTSIAVVEITLSHS	X03216
Erm36L	MGSPSIAVTRFRRF	AF462611
IFVI peptides		
ErmAL2	MGMFSIFVIERFHYQPNQK	AF002716
ErmAL2	MGTFSIFVINKVRYQPNQN	X03216
ErmCL	MGIFSIFVISTVHYQPNKK	V01278
ErmGL2	MGLYSIFVIETVHYQPNEK	M15332
ErmTL	MGIFSIFVINTVHYQPNKK	M64090
ErmYL	MGNCSLFVINTVHYQPNEK	AB014481
Erm33L	MGIFSIFVINTVHYQPNKK	AJ313523
RLR peptides		
EreAL	MLRSRAVALKQSYAL	AF099140
Erm34L	MHFIRLRFLVLNK	AY234334
ErmDL	MTHSMRLRFPTLNQ	M29832
MefAL	MTASMRLR	AF274302
MsrAL	MTASMRLK	AB016613
MsrCL	MTASMKLRFELLNNN	AY004350
Erm39L	MSVTYIRLRIT	AY487229
ErmXL	MLISGTAFLRLRTNRKAFPTP	M36726
ErmQL	MIMNGGIASIRLRR	L22689
EreAL	MTPNNSFKPTPLRGAA	AY183453
ErmFL	MKTPTGLSGSISQRVRTLVK	M17808
ErmWL	MGFSFTGSAFIRLRTA	D14532
Miscellaneous peptides		
MefBL	MYLIFM	FJ196385
MsrDL	MYLIFM	AF274302
ErmGL1	MRIDDYCS	L42817
MphCL	MYQIKNGN	AF167161
EreAL	MSLVIGEAKV	AF512546
Erm37L	MRTAPEPWGW	BX842578
MphBL	MAKEALEVQGS	D85892
ErmGL1	MNKYSKRDAIN	M15332
ErmEL	MRVSVRVAACARC	M11200
ErmFL	MMLCCRLSFFLLSR	M62487
MphAL	MNKTKGCLIANFATVPD	D16251
Erm38L	MSITSMAAPVAAFIRPRTA	AY154657
EreAL	MQLTVKSFVRFACYASYRN	AF512546
ErmGL2	MNHEYVLFSKNINIRKEMQ	L42817
EreBL	MRIXRKTAYARPCALAEEGRX	A15097
EreBL	MRINRKTAYARPCALAEEGRG	AB207867
Erm41L	MMVLRRVRPTVATPVGLVSAH	EU177504
ErmSL	MSMGIAARPPRAALLPPPSVPRSR	M19269

(continued)

Table 13.3 (continued)

Name	Leader peptide sequence	GenBank
ErmBL	MLVFQMCNVDKTSTVLKQTKNSDYADK	U86375
ErmBL	MLVFQMRNVDKTSTVLKQTKNSDYADK	M11180
ErmTL	MRNVDKTSTVLKQTKNSDYADK	AJ488494
ErmBL	MLVFQMRNVDKTSTVLKQTKNSDLRR	AF299292
ErmBL	MLVFQIRNVDKTSTGLKQTKNSDYADK	AF080450
ErmBL	MLVFQMRNVDKTSTILKQTKNSDYVDKYVRLIPTSD	K00551
ErmBL	MLVFQMRYQMRYVDKTSTVLKQTKKSDYADK	M19270
ErmFL	MLSAFIFSSFSLIYRAKLLNLPLYNYKRISL	M62487
ErmNL	MARTLFAGRTELWAPAIEPPVKAATHTAVRRD	X97721
ErmVL	MAANNAITNSGLGRGCAHSVRMRRGPGALTGPGSHTAR	U59450

Sequence analysis of the established and hypothetical leader ORFs of the macrolide resistance genes shows that the encoded peptides fall into several sequence classes (Table 13.3) [47]. Since the functionally critical segment of the leader peptide is expected to be encoded in the mRNA segment prior to the site of the ribosome stalling, it is not surprising that the 5′ terminal segments of the leader ORFs show a higher degree of conservation compared to the 3′ proximal segment. Using in vitro biochemical techniques, we have accurately mapped the precise site of the ribosome stalling at several of the regulatory ORFs of macrolide resistance genes (Ramu and Mankin unpublished), which allow us to align a number of the sequences according to the site of the ribosome stalling. The most predominant and easily recognizable class of the regulatory peptides contains the IFVI sequence at the C terminus of the nascent peptide in the stalled ribosome complex (Table 13.3). The IFVI sequence was experimentally demonstrated to be critical for the SRC formation [32, 60]. In these peptides, which are encoded in the control regions of a number of *erm* genes, the ribosome is expected to stall at the ninth (Ile) codon. In all the peptides of this type, the C-terminal isoleucine of the IFVI sequence is positioned exactly nine positions away from the peptide's N-terminus. The precise placement of the ribosome stalling site in the ORFs encoding these peptides is corroborated by our finding that adding or removing codons prior to the stalling site in the *ermCL* ORF negatively affects the efficiency of stalling [60].

The IAVV peptides (previously designated as SIAV [47]) exhibit a certain degree of similarity to the IFVI peptides. Stalling occurs at the eighth codon of the leader ORF at the second Val codon of the IAVV sequence. At the leader ORF of the *erm36* gene, the ribosome stalls at the ninth codon after the sequence MGSPSIAVT is synthesized (Ramu and Mankin unpublished). The identity of four C-terminal amino acids (IAVV in the *ermAL1* peptide) is important for stalling. There is, however, an important difference between macrolide-induced ribosome stalling at the leader ORFs of the IFVI class compared to the IAVV class. The identity of the A site codon is not critical for the IFVI ORFs – irrespective of its nature, the stalled complex is efficiently formed. In contrast in IAVV ORFs, the identity of the A site codon and thus, the nature of the A site aminoacyl-tRNA affects the efficiency of

formation of the stalled complex (Ramu and Mankin unpublished). Thus, the key component of the induction mechanism, the drug- and nascent peptide-dependent ribosome stalling may operate under different rules when controlling expression of macrolide resistance genes.

A large group of the leader peptides carry an RLR motif or its variations. Experimental testing showed that the ribosome stalls at the seventh (Leu) codon of the RLR-type leader ORFs of ErmD, MsrC, and MsrSA genes. Alanine scanning indicated the importance of Thr_2, Met_5 and Leu_7 for stalling. Interestingly, the distance of the RLR motif from the N-terminus of the peptide does not appear to be conserved. In EreAL, the LR sequence is immediately adjacent to the initiator formyl-methionine, whereas in ErmXL it is ca. 10 amino acids away from the N-terminus. It is worth noting, however, that no obvious Shine-Dalgarno sequence precedes the initiator AUG codon of the proposed ErmXL ORF [53], but a hypothetical ribosome-binding site can be found in front of the Leu_9 UUG codon. Thus, it is possible that the actual regulatory peptide of *ermX* has the sequence MRLRTNR and resembles more closely the leader peptide of the *ereA* gene.

The site of the ribosome stalling at the leader peptide of the *ermB* gene was mapped to the Asp codon 10 of the *ermBL* gene. Interestingly, a point mutation that changes the ErmBL peptide N-terminal sequence from MLVFQM*R*NVDK to MLVFQM*C*NVDK shifts the site of stalling one codon towards the 5′ end of the gene (Ramu and Mankin unpublished). Apparently a small variation in the position of the stalled ribosome is compatible with the drug-dependent induction. It is unclear, however, why the change of a single amino acid residue in the nascent peptide alters the site of stalling.

A number of the resistance genes are preceded by ORFs which encode peptides without any obvious homology to the regulatory peptides of the investigated macrolide resistance genes. The possible involvement of these ORFs in regulation of expression of the respective resistance genes and the possibility of formation of the drug-dependent stalled translation complex still awaits experimental testing.

13.11 Concluding Remarks

Inducible expression of macrolide resistance genes evolved apparently in the course of evolution as a cost-efficient way of providing “on-demand” resistance. The ribosome plays the role of both the sensor of the presence of antibiotic in the medium and the responder because it modulates its function in the presence of the drug. The altered mode of translation of a regulatory ORF is used for translational or transcriptional activation of expression of the downstream resistance genes.

The details of the interaction of the ribosome with the inducing antibiotic and specific nascent peptide sequences, which direct formation of the stalled ribosome complex at the regulatory ORF are only starting to emerge. The leader peptides show a substantial variation in their structures, suggesting that they have been optimized to differentially respond to various inducing antibiotic agents. Furthermore,

it is likely that different stalling peptides push different 'buttons' in the ribosome exit tunnel in order to elicit formation of the stalled translation complex. Although our understanding of the interaction of the ribosome with the nascent peptides and antibiotics are still very rudimentary, unraveling molecular details of these interactions should pave the way for developing better drugs and for a better understanding of the fundamental aspects of the mechanisms of translation.

The inducing activity of the antibiotics can be clearly distinguished from their general inhibitory effect on translation. Using convenient reporter systems and assays can facilitate identifying inhibitory compounds that can slide under the radars of the antibiotic-monitoring systems of bacterial pathogens in order to efficiently inhibit protein synthesis in bacteria equipped with inducible resistance genes.

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Chapter 14
Fluoroquinolone Resistance: Mechanisms, Restrictive Dosing, and Anti-Mutant Screening Strategies for New Compounds

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14.1 Introduction: Overview of Quinolone Action

The fluoroquinolones are broad-spectrum antibacterial agents that are receiving increasing attention as resistance develops to other compounds. Examination of many new derivatives has improved our understanding of how the quinolones act and has led to the discovery of structures with improved activity. Indeed, several new quinolones have entered clinical practice recently. In the present chapter, we discuss mechanisms by which bacteria exhibit resistance, and we use that information to develop a framework for considering how resistance arises. That framework provides ways to restrict emergence of resistance. We then focus on screening strategies that can be introduced early in antimicrobial development programs to identify compounds that are least likely to select resistant mutants. We expect such strategies to apply to many antimicrobial-pathogen combinations. At the end of the chapter, we briefly discuss plasmid-borne fluoroquinolone resistance, since it poses a major threat to continued use of the compounds.

The prototype quinolone is nalidixic acid, an agent that exhibits modest activity against a few Gram-negative species. Addition of fluorine to the C-6 position, a piperazinyl ring to the C-7 position, and an exchange of a carbon for the nitrogen at position 8 produced norfloxacin, a compound with substantially improved activity and pharmacokinetic properties. Ciprofloxacin emerged from norfloxacin by replacement of the N-1 ethyl with a cyclopropyl group, which broadened the spectrum and improved lethal activity with non-growing cells. Compounds such as sparfloxacin,

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moxifloxacin, and gatifloxacin, are distinguished from ciprofloxacin by having a halogen or methoxy group attached to position C-8 (other substituents, particularly those attached to C-7, have important effects that currently require case-by-case consideration). The C-8 methoxy moiety is associated with improved activity, particularly against resistant mutants. We have suggested that nalidixic acid, norfloxacin, ciprofloxacin, and the C-8 methoxy/halogen fluoroquinolones represent four distinct groups [1]. Levofloxacin, one of the best-selling antimicrobials, evolved along a separate pathway. Two recent agents, garenoxacin and gemifloxacin, were also derived via lines distinct from the ciprofloxacin group. Garenoxacin is noteworthy because it lacks the C-6 fluorine characteristic of fluoroquinolones (see [2] for review).

The quinolones have as their targets two essential bacterial enzymes: DNA gyrase (topoisomerase II) [3] and DNA topoisomerase IV [4]. These two enzymes act by passing one region of duplex DNA through another [5, 6], and during that process the drugs trap a reaction intermediate containing quinolone, enzyme, and broken DNA. The resulting complexes interfere with the movement of replication and transcription complexes, thereby blocking bacterial growth. Since active DNA replication is not required for rapid quinolone-mediated lethality [7], the reversible blockage of replication caused by the drug-enzyme-DNA complexes is not responsible for rapid cell death.

Quinolone-mediated cell death occurs in two general ways (reviewed in [8, 9]; see Fig. 14.1): One involves protein-synthesis-dependent chromosome fragmentation; this pathway probably arises from an unidentified suicide factor induced by quinolone. It is likely that the factor(s) releases DNA ends, created by gyrase and topoisomerase IV action, from protein-mediated constraint. Anaerobic shock and chloramphenicol block this pathway [10]. Another pathway to cell death is observed with the newer fluoroquinolones; this pathway does not require ongoing protein synthesis or aerobic growth [10]. Since some fluoroquinolones destabilize drug-gyrase-DNA complexes *in vitro*, we postulated that protein-synthesis-independent cell death arises from the drugs prying apart the gyrase subunits and thereby fragmenting bacterial chromosomes [9].

Reactive oxygen species are likely to contribute to quinolone lethality occurring by the protein-synthesis-dependent pathway (Fig. 14.1). With *Escherichia coli,* the lethal activity of oxolinic acid, a first-generation quinolone, is completely blocked by either chloramphenicol, an inhibitor or protein synthesis, or by a combination of thiourea plus 2,2′-bipyridyl, which inhibits the accumulation of hydroxyl radical [11]. None of these treatments interfere with the lethal action of PD161144, a C-8-methoxy fluoroquinolone of the ciprofloxacin family [11]. Moxifloxacin exhibits an intermediate effect, which made it possible to determine whether chloramphenicol and thiourea plus 2,2′-bipyridyl are additive; they are not [11]. Thus, the chloramphenicol-sensitive event and the toxic action of hydroxyl radical appear to be in the same pathway, as depicted in Fig. 14.1. Since chromosome fragmentation is blocked by chloramphenicol but not by thiourea plus 2,2′-bipyridyl (X. Wang 2011, unpublished observation), we place fragmentation upstream from hydroxyl radical accumulation.

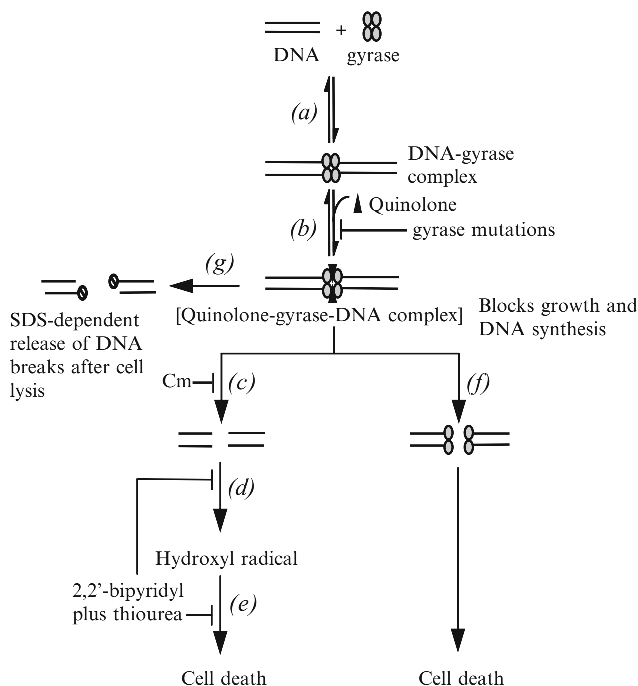

Fig. 14.1 Schematic representation of quinolone action. DNA and gyrase interact (**a**) to form a gyrase-DNA complex. Quinolones bind to the complex (**b**), forming a ternary complex in which the DNA is broken. These ternary complexes block DNA replication and bacterial growth. Gyrase resistance mutations block step **b**. In the lower left-hand portion of the scheme, an undefined activity releases DNA breaks from complexes and causes bacterial chromosomes to fragment (**c**). Chloramphenicol blocks this step. Fragmented chromosomes stimulate a cascade of reactive oxygen species leading to hydroxyl radical accumulation (**d**), which causes cell death (**e**). Hydroxyl radical accumulation is blocked by 2, 2′-bipyridyl and thiourea. A second lethal pathway, shown in (**f**), is proposed to derive from fluoroquinolone-mediated destabilization of the ternary complexes. The presence of broken DNA in ternary complexes is demonstrated (**g**) by the recovery of DNA fragments when lysates from quinolone-treated cells are incubated with sodium dodecyl sulfate (SDS)

Earlier work with norfloxacin, a fluoroquinolone that exhibits complex behavior with respect to the two pathways of cell death [10], indicated that quinolone lethality is enhanced by genetic alterations expected to increase peroxide concentration and suppressed by alterations expected to decrease it [12–14]. Moreover, norfloxacin causes hydroxyl radical to accumulate, and blocking hydroxyl radical accumulation lowers norfloxacin-mediated lethality [14]. These data fit the idea that reactive oxygen species contribute to quinolone-mediated cell death. Since removal of catalase-peroxidase exacerbates effects of reactive oxygen species [14], it is likely that small-molecule inhibitors of this enzyme may constitute a way to enhance quinolone lethality.

The two topoisomerase targets of the quinolones share many mechanistic properties; consequently, conclusions about drug action with one target can often be

extrapolated from work with the other. Quinolone-topoisomerase-DNA complexes also share features with cleaved complexes formed by anti-tumor agents, eukaryotic topoisomerase II, and DNA. Thus, inferences about the bacterial enzymes can sometimes be drawn from studies of their eukaryotic counterpart. Since differences between the two bacterial enzymes may reflect different chromosomal functions or locations [15, 16], quinolone-containing complexes with one target may be more cytotoxic than with the other. Such information potentially contributes to a rationale for choosing one compound over another for treatment of a particular pathogen.

14.2 General Features of Quinolone Resistance

14.2.1 Stepwise Accumulation of Resistance Mutations

Fluoroquinolone resistance is characterized by the gradual accumulation of mutations that lower intracellular drug concentration and/or the sensitivity of the target DNA topoisomerases. Most of the mutations reported to date are chromosomal. Repeated cycles of increasingly stringent fluoroquinolone challenge, punctuated by periodic outgrowth of pathogen populations, are expected to cause stepwise accumulation of mutations. The order in which target and nontarget alleles arise probably depends on the incremental increase in quinolone concentration. If the initial concentration is low, then non-target alleles will be selected, as seen with mycobacteria and *S. pneumoniae* [17–19]. If the initial concentration is moderately high, then target mutations are selected [17, 19–21], and small increases in concentration lead to the recovery of additional non-target alleles [21, 22].

The gradual accumulation of resistance causes surveillance studies to underestimate the emergence of resistance, since strains can contain resistance mutations and still be considered clinically susceptible. Those mutations increase the propensity for attaining additional resistance determinants by raising the upper limit of the selection window (discussed below). Eventually strains accumulate enough mutations for MIC to exceed the resistance breakpoint. When these resistant mutants disseminate, they can cause a rapid increase in the prevalence of resistance, as has been observed with *Staphylococcus aureus* [23, 24]. Consequently, resistance can appear to arise suddenly even though the early stages are intrinsically gradual.

14.2.2 Sources of Resistance

Understanding resistance requires identifying the sources of resistance. One is likely to be the clinical use of the compounds [25], since use correlates with resistance for other antibacterial agents [26, 27]. During the 1990s, fluoroquinolone usage increased, and for that reason alone we would expect the prevalence of resistance to have increased (in the United States fluoroquinolone prescriptions increased about

9% per year between 1990 and 1998; in 1998 they reached almost 13 million [28], and by 2001 they approached 30 million annually (G. Tillotson 2007, personal communication)). Another source is likely to involve agricultural use [29], since agricultural resistance can develop quickly. For example, within 2 years after the introduction of enrofloxacin in Denmark, ciprofloxacin resistance was found in *E. coli* that was isolated from cattle and in *Staphylococcus hyicus* that was obtained from pigs [30]. By 1998, 30% of the *S. aureus* isolates obtained from poultry in Denmark were ciprofloxacin-resistant [31]. Increased prevalence of resistance among isolates of *Salmonella* [32] and *Campylobacter* [33] is also attributed to agricultural use of fluoroquinolones. In 2005, the U.S. Food and Drug Administration won a court ruling that restricted some agricultural use of fluoroquinolones [34], but such use is still common in many parts of the world. A third source is environmental contamination by hospital and agricultural waste [35]. The quinolones are very stable, broad-spectrum agents that can select resistant mutants in many bacterial species. In tropical areas where malaria is prevalent, chloroquine use is likely to be a fourth source of quinolone resistance [36]. In principle, modifying human activity can alter each of these sources.

The source of resistance can also be considered from a molecular perspective. Errors in DNA replication and repair cause mutants to be present in bacterial populations containing more than 10^7 cells; consequently, fluoroquinolone treatment need only enrich the mutant subpopulation for resistance to develop. Error rate is increased by faulty replication proteins called mutators (for example, in *E. coli* the Pol III mutator *dnaQ-49* creates a deficiency in the proofreading activity of DNA polymerase). Mutators have been observed in a variety of bacterial species [37–40], generally being enriched by weak selective pressure [41].

14.2.3 Quinolone-Induced Quinolone Resistance

The quinolones are themselves mutagenic. One suggestion is that they induce mutations through the generation of free radicals, since the mutagenic effect of nalidixic acid, pipemidic acid, and norfloxacin is blocked by β-carotine [42], a scavenger of free radicals. Indeed, hydroxyl radical accumulation has been observed following quinolone treatment [11, 13]. Another idea is that quinolone-topoisomerase-DNA complexes are misrepaired. To address this hypothesis, quinolone mutagenicity has been examined by the Ames test [43–45]. In one study, the majority of mutations created were deletions [44], which would be consistent with misrepair of the ternary complexes. When the test was designed to reveal reversions, excision repair and error-prone repair appeared to be involved [44]. These data are consistent with the observation that quinolones induce the mutagenic SOS response [44, 46].

Induced mutations are readily observed when *E. coli* is applied to quinolone-containing agar, and then colonies are scored on a daily basis for about a week [47, 48]. As expected, the number of mutants recovered decreases dramatically in *lexA* Ind$^-$, *recA*$^-$, *recB*$^-$, or *recC*$^-$ mutants. In contrast, the frequency of spontaneous

mutants present in the absence of quinolone is affected little by blocking the SOS response with a *recA* mutation of *M. smegmatis* or by a *lexA* (Ind⁻) mutation of *E. coli* (X. Zhao 2011, unpublished observations). Since the frequency at which mutants are induced is sensitive to quinolone structure [49], the agar-plate assay is a simple test that can be applied early in drug discovery to identify compounds unlikely to allow emergence of induced resistance.

14.2.4 Cross-Resistance

In general, mutations that confer resistance to one quinolone also lower susceptibility to other members of the class [50]. Nevertheless, cross-resistance among quinolones can be low with organisms that contain two targets (gyrase and DNA topoisomerase IV), if the primary target differs for the two compounds. Such is the case with *S. pneumoniae*. Some derivatives such as ciprofloxacin and levofloxacin have topoisomerase IV as the primary target, while others, such as sparfloxacin, moxifloxacin, and gatifloxacin, have gyrase as the preferred target. Consequently, a ciprofloxacin-resistant mutation may have little effect on the gatifloxacin MIC and vice versa [17, 51]. Cross-resistance among quinolones can also be affected by some GyrA amino acid substitutions at the N-terminal end of α-helix-4 (position 81 in *E. coli)*. These mutations raise MIC more for fluoroquinolones that contain a C-7 ring than for quinolones that lack the ring [52].

Cross-resistance to other antibacterials generally arises through efflux mutations and plasmid-borne genes such as *aac (6')-lb-cr*, both of which are discussed below. In addition, chloroquine treatment of malaria is likely to lead to the emergence of ciprofloxacin-resistant *E. coli* [36], as mentioned above. In this case, chloroquine appears to bind to gyrase and DNA topoisomerase IV, since resistant mutants have amino acid substitutions that are typical of resistance selected by the quinolones.

14.3 Molecular Basis of Fluoroquinolone Resistance

14.3.1 Permeability-Based Resistance

Gram-negative bacteria have two cell envelope membranes (reviewed in [53]): In enterobacteria, the outer membrane is itself composed of two layers, an outer surface of lipopolysaccharide and an inner phospholipid bilayer. Underlying the phospholipid is a peptidoglycan network and then the plasma membrane. Proteins called porins serve as channels for certain hydrophilic molecules, and decreased quinolone susceptibility can correlate with a deficiency in particular porin proteins (OmpF in *E. coli* and D2 in *P. aeruginosa* [54, 55]). Uptake of hydrophobic molecules is poorly understood. Presumably the highly charged lipopolysaccharide serves as a permeability barrier to hydrophobic quinolones; consequently, alterations in that layer can also affect susceptibility [55, 56].

In *E. coli*, membrane-associated quinolone resistance sometimes arises when resistance to chloramphenicol or tetracycline is selected. This phenomenon is explained in part by an effect of the multiple antibiotic resistance (Mar) system [57, 58] influencing the expression of the OmpF porin. Mutation of *marA* increases expression of *micF*, a gene encoding an RNA that post-transcriptionally decreases *ompF* mRNA. Therefore, upregulation of *marA* reduces the amount of OmpF [57]. The influence of *marA* on quinolone uptake is probably not limited to OmpF, since the abundance of other proteins is also changed. Moreover, susceptibility of *marA* mutants is lower than seen when *ompF* is simply deleted, and deletion of *micF* in a *marA* mutant fails to restore wild-type susceptibility [58]. Thus, a complex regulatory network exists for excluding particular compounds from the cell. As pointed out below, the *mar* system also influences efflux.

14.3.2 Efflux-Based Fluoroquinolone Resistance

Efflux-based resistance to fluoroquinolones arises from constitutive expression of native efflux transporters and up-regulation of genes involved in normal transport-mediated bacterial processes [59, 60]. These processes include efflux systems that remove metabolic end products and signaling molecules from cells. In addition, efflux systems pump out a variety of antimicrobials and other noxious materials. Decades of effort to improve quinolone-class antibacterials have revealed that many compounds are substrates for one or more bacterial efflux pump. Five general types of efflux system have been identified: ATP-binding cassette (ABC) superfamily, major facilitator (MF) superfamily, resistance-nodulation-division (RND) family, multi-drug and toxic compound extrusion (MATE) family, and small multidrug resistance (SMR) family. The most clinically relevant efflux systems have been reviewed for both Gram-negative and Gram-positive bacteria [61].

Gram-negative bacteria possess genes for multiple members of each of the five efflux gene families (the *E. coli* genome contains genes for at least 37 efflux transporters [62]). Virtually every type of bacterium expresses one or more efflux pump that recognizes and exports some, if not many of the quinolones. In addition to recognizing quinolone-class structures, efflux pumps of Gram-negative organisms generally have broad substrate specificity. For example, MFS-type pumps, represented by the EmrAB and MdfA efflux systems of *E. coli,* export a variety of antibiotics in addition to nalidixic acid and several fluoroquinolones [63, 64]. Since fluoroquinolones are structurally diverse, generalities regarding activity with a given organism or particular type of efflux pump are limited, and surprises occasionally emerge. For example, gatifloxacin inhibits some efflux pumps in *P. aeruginosa*, which allows gatifloxacin and ciprofloxacin to act synergistically with efflux-expressing, ciprofloxacin-resistant strains [65].

The AcrAB-TolC system of *E. coli* is among the better characterized RND systems (reviewed in [66]). In addition to recognizing many fluoroquinolones, this system exports a variety of agents including tetracycline, β-lactams, chloramphenicol, erythromycin, rifampicin, disinfectants, dyes, and organic solvents. The TolC protein,

which is anchored in the outer membrane, forms a long channel that spans both the outer membrane and the periplasmic space. AcrA is a lipoprotein located in the periplasm; the inner membrane protein, AcrB, is a proton-motive-force transporter of the RND type. Strains deficient in the AcrAB proteins are hypersusceptible to many quinolones [67]; conversely, fluoroquinolone-resistant efflux mutants often overproduce the periplasmic protein AcrA [68]. Such mutants exhibit decreased accumulation of ciprofloxacin and ethidium bromide; they also display decreased susceptibility to tetracycline, ampicillin, and chloramphenicol. Deletion or inactivation of proteins that repress expression of *acrAB* (AcrR and AcrS) reduces fluoroquinolone susceptibility [69, 70].

Efflux can be a significant contributor to fluoroquinolone resistance; one of the best examples is seen with *Pseudomonas aeruginosa* [71]. In this organism, at least six RND-type efflux transporters are associated with fluoroquinolone efflux [72, 73]. Three have been extensively studied: (1) MexAB-OprM (regulated by *nalB* (*mexR*)), (2) MecCD-OprJ (regulated by *nfxB*), and (3) MexEF-OprN (regulated by *nfxC* (*mexT*)). High-level clinical resistance of *P. aeruginosa* to fluoroquinolones has been attributed largely to efflux pump over-expression [74, 75]; however, resistant mutants also contain resistance mutations in genes encoding gyrase and DNA topoisomerase IV [76]. *In vitro*, gyrase mutants are recovered when fluoroquinolone concentrations are high enough to suppress efflux-mediated growth [77]. Consequently, gyrase mutants must be considered when adjusting dosing regimens to restrict the emergence of resistance [77]. Since quinolones select bacterial strains expressing high levels of efflux systems that also remove members of other antimicrobial classes from cells, treatment of *P. aeruginosa* with fluoroquinolones can facilitate the emergence of multi-drug resistance [78].

Pump systems also afford significant levels of fluoroquinolone efflux in Gram-positive bacteria, where members of the MF and SMR families are most often identified. The *S. aureus* NorA pump system, which exports hydrophilic fluoroquinolones, was one of the earliest recognized to impact clinical utility of quinolones [79–85]. NorA, along with NorB and NorC, is regulated by the global regulator *mgrA* [86, 87]. Examples have also been found in which MATE family pump systems contribute to loss of susceptibility. One of these genes, *mepA*, is controlled by the MepR repressor [88, 89]. These regulatory genes are potential sites of resistance alleles; in *S. pneumoniae,* the major NorA-type pump is called PmrA [90]. This pump recognizes older fluoroquinolones such as ciprofloxacin and norfloxacin, but it is less effective with newer agents that have bulky groups attached to the C-7 position [91]. Variants of NorA-type efflux in Gram-positive bacteria are frequently defined by the ability of reserpine, a pump inhibitor, to lower fluoroquinolone MIC; however, the modest increase in susceptibility associated with reserpine treatment fails to account for the contribution of efflux to the emergence of mutation-based resistance in *S. pneumoniae* [92–95]. It appears that PmrA effects are supplemented by over-expression of the ABC transport proteins PatA and PatB, which can be fluoroquinolone-induced [96, 97].

Several reports suggest that the ability to efflux fluoroquinolones is associated with a propensity to acquire additional fluoroquinolone resistance determinants

[98, 99]. With efflux-deficient strains or treatment with an inhibitor of efflux, the recovery of mutants decreases, sometimes by orders of magnitude [98, 100–103]. Since mutant recovery for many species depends strongly on fluoroquinolone concentration [17, 19, 104], comparison of mutation frequency among strains that differ in susceptibility is not straightforward. For example, the efflux inhibitor reserpine probably increases the intracellular concentration of quinolone, which would shift the mutant selection window (described in a subsequent section) with respect to extracellular drug concentration. That shift would make it appear that mutation frequency had changed with respect to external drug concentration even though no intrinsic change may have occurred. Such is likely to have been the case with an *S. aureus* study, since mutation frequency and the inhibitory dose decline in parallel [102]. For analysis of mutation frequency, strains need to be compared at fluoroquinolone concentrations that have comparable inhibitory effects on growth (MIC or a multiple of MIC). Recovery of mutants over a wide concentration range provides a mutant selection profile that facilitates comparisons [19].

One strategy for restricting the selection of efflux mutants is to use fluoroquinolones that are not preferentially pumped from cells [91, 95, 105, 106]. Indeed, many newer fluoroquinolones having C-8 substituents and C-7 groups that are more hydrophobic, such as moxifloxacin, are not recognized by many of the systems that efflux ciprofloxacin and norfloxacin. Another strategy is to supplement quinolone treatment with pump inhibitors such as MC207,110, a compound that showed early promise with fluoroquinolones against *P. aeruginosa* [100]. Unfortunately, therapeutic efforts using pump inhibitors have been plagued by the inability to block multiple pump systems and by toxicity due to inhibition of mammalian transporters [107–109]. A third strategy is to treat with fluoroquinolone concentrations that are high enough to prevent the growth of efflux mutants. Regardless of the approach taken, neutralizing the effect of efflux systems will be critical for restricting the emergence of high-level resistance.

14.3.3 Topoisomerase-Protecting Proteins

In 1998 a strain of *Klebsiella pneumoniae* was described that exhibited plasmid-borne resistance to fluoroquinolones and 13 other agents [110]. The fluoroquinolone resistance is due to a protein named Qnr, an abbreviation for quinolone resistance. This protein was renamed QnrA when a variety of related proteins were discovered (other types are QnrB [111] and QnrS [112]). QnrA is a 218-amino-acid protein that belongs to a large protein family characterized by pentapeptide repeats [113]. Every fifth amino acid of these repeats is either leucine or phenylalanine [114], which suggests that the proteins may be involved in protein-protein interactions. The pentapeptide protein family includes roughly 500 members that display a wide variety of properties [115].

Insight into how the gyrase-protecting pentapeptide proteins might act came from structural analysis of MfpA [116]. This protein, which is responsible for

Table 14.1 Effect of Qnr on fluoroquinolone susceptibility

Fluoroquinolone	Transconjugant MIC_{90} with *qnr*[a]	Recipient MIC lacking *qnr*[a]	Ratio
AM-1121	0.5	0.008	63
Bayy3118	0.125	0.004	31
Ciprofloxacin	1	0.008	125
Garenoxacin	2	0.008	250
Gatifloxacin	0.5	0.008	63
Gemifloxacin	1	0.004	250
Levofloxacin	0.5	0.015	33
Moxifloxacin	1	0.03	33
Nalidixic acid	32	4	8
Premafloxacin	0.25	0.03	8
Sitafloxacin	0.125	0.008	16
Sparfloxacin	1	0.008	125

[a]MIC in µg/ml for *Escherichia coli* receiving *qnr*-containing plasmids from 17 clinical isolates of *E. coli* and *Klebsiella pneumoniae* [121]

low-level fluoroquinolone resistance in *Mycobacterium smegmatis* [117], was identified from analysis of a plasmid-borne genomic library in which the *mfpA* gene increased ciprofloxacin MIC by 4-fold and sparfloxacin MIC by 8-fold. An *M. tuberculosis* homologue (67% amino acid sequence identity with the *M. smegmatis* protein) was expressed in *E. coli*, purified, and crystallized [116]. Determination of the three-dimensional structure of MfpA revealed that the MfpA dimer has size, shape, and electrostatic similarity to B-form DNA [116]. This DNA mimic appears to bind gyrase, thereby interfering with quinolone binding to gyrase-DNA complexes [116].

The Qnr proteins lower quinolone binding to DNA complexes formed with gyrase or topoisomerase IV by binding the GyrA and GyrB subunits of gyrase [118] and the ParC and ParE subunits of topoisomerase IV [119]. Binding of Qnr to gyrase, which appears to be specific rather than a general protein-binding property, does not require quinolone, DNA, or ATP [118]. Qnr also reverses quinolone-mediated inhibition of the supercoiling activity of gyrase [118]. Even a 1,000-fold excess of ciprofloxacin over Qnr fails to overcome the Qnr-gyrase interaction; consequently, Qnr is likely to act by altering the DNA-binding properties of gyrase rather than by competitive binding to a quinolone interaction site [118].

While the MIC of the *qnr*-containing strains is often below the resistance breakpoint, the effect of *qnr* is substantial, sometimes raising MIC by 100 fold [120, 121]. The effect varies among quinolones, as indicated by examination of a set of quinolones with transconjugants generated from clinical isolates of *E. coli* and *K. pneumoniae* (Table 14.1). Since quinolone resistance arises in a stepwise fashion, reduced susceptibility due to the presence of *qnr* is expected to be an important factor in the emergence of resistance, either by adding to the effect of an existing resistance allele to render a strain clinically resistant or by serving as an early step in the pathway to resistance. Indeed, increased MPC has been reported with *qnr*- containing bacteria (MPC, section 14.4) [122].

14.3.4 Quinolone-Modifying Enzymes

In 2006, another form of low-level quinolone resistance was discovered when two *qnr*-containing-plasmids were found to confer different levels of protection from quinolones [123]. A mutant library prepared by insertion into the more protective plasmid was screened for insertions that lowered MIC to the level attributed to the presence of *qnr*. Insertions mapping within or immediately upstream of *aac(6')-Ib* were associated with increased quinolone susceptibility [123]. This gene encodes an aminoglycoside acetyltransferase known to confer resistance to tobramycin, kanamycin, and amikacin [124]. Two codon changes, Trp-102 to Arg and Asp-179 to Tyr, confer a ciprofloxacin-resistant phenotype [125]. The variant enzyme was named Aac(6′)-Ib-cr to indicate its effect on ciprofloxacin, which is to place an acetyl substituent on the unsubstituted nitrogen of the C-7 piperazinyl ring [123]. The enzyme also lowers susceptibility to norfloxacin, which has the same C-7 ring as ciprofloxacin. However, it has no effect on quinolones, such as enrofloxacin, pefloxacin, levofloxacin, and gemifloxacin, that lack an unsubstituted piperazinyl nitrogen [125].

The presence of *aac (6')-Ib-cr* increases recovery of resistant mutants. Such a result is consistent with the *aac (6')-lb-cr* gene shifting MIC of mutant subpopulations to higher values, perhaps increasing MPC. Such an observation has important implications for emergence of resistance, because quinolone concentrations during treatment are more likely to be inside the mutant selection window (Sect. 14.4) when *aac (6')-Ib-cr* and *qnr* are present.

14.3.5 Topoisomerase-Based Resistance

Gyrase and topoisomerase IV are the intracellular targets of the quinolones, and mutations in *gyrA, gyrB, parC*, and *parE* confer resistance. For both the *gyrA* and *parC* genes, resistance mutations map within a narrow stretch of nucleotides termed the quinolone-resistance-determining region (QRDR), which in *E. coli gyrA* encodes amino acids 51 through 106 [126–129]. Target changes that lower susceptibility the most correspond to alterations of amino acids 83 and 87 in *E. coli* GyrA. A similar region exists in ParC.

Mutations mapping in *gyrB* and *parE*, the genes encoding the energy-transducing portion of type II DNA topoisomerases, generally reduce susceptibility by only low or moderate amounts. A conformational change in gyrase normally occurs during formation of ternary complexes [130], and a model has been proposed that would place the GyrB resistance regions near the GyrA recognition helix [131]. This model is now supported by a crystal structure of a complex between DNA and yeast topoisomerase II [132].

A variety of target-based resistance alleles have been recovered. For example, with mycobacteria at least 12 different *gyrA* and nine different *gyrB* mutations can be obtained when cells are selected for growth on fluoroquinolone-containing agar [19]. These mutations confer many different levels of protection that vary from

compound to compound [133]. For organisms that contain topoisomerase IV, a variety of *parC* and *parE* mutations provide protection [20, 105, 134–137]. In principle, such organisms could contain many different combinations of gyrase and topoisomerase IV alterations that would provide many different levels of protection from quinolone action.

14.4 Mutant Selection Window Hypothesis

14.4.1 Description of the Window

In the 1990s, Baquero [138, 139] suggested that a dangerous concentration range exists in which specific mutant types are most frequently selected. We defined the boundaries of the range experimentally when we noticed that the recovery of mycobacterial mutants from agar plates displays a characteristic response to fluoroquinolone concentration (Fig. 14.2a). At very low concentrations, the drug has no effect on colony formation until MIC is approached; then colony recovery drops sharply as susceptible growth is blocked. At higher concentrations, a broad plateau is observed as a variety of resistant mutant colonies grow. Eventually a high concentration is reached at which colony recovery drops sharply a second time. The second drop occurs at the MIC of the least susceptible first-step mutant subpopulation [104]. This value is called the mutant prevention concentration (MPC), because it severely limits the recovery of mutants (at concentrations above the MPC bacterial growth requires the acquisition of two or more concurrent resistance mutations, which is a rare event). At low drug concentrations (slightly below MIC), selection pressure is greatly diminished and mutants do not selectively amplify. The concentration range in which resistant colonies selectively grow is called the mutant selection window. The lower boundary of the window is approximated by MIC, the upper by MPC. These concepts, which were derived from agar-plate experiments, can be applied to pharmacokinetic profiles as illustrated in Fig. 14.2b.

Fluoroquinolone concentration (position in the window) is a key variable in determining which resistance allele is selected. For example, with *M. smegmatis* low concentrations of fluoroquinolone (slightly above MIC for 99% of the population) select nongyrase mutations [19], of which about 60% exhibit cross-resistance to ampicillin and chloramphenicol [19]. As fluoroquinolone concentration is increased, the non-gyrase variants decline in frequency, and *gyrA* mutants become increasingly prevalent. At very high fluoroquinolone concentration, colonies arise only from the GyrA variant that has the highest MIC. Eventually a concentration is reached (MPC) at which no mutant can be obtained even when 10^{11} cells are plated.

The pattern is slightly different for *M. tuberculosis*. For this organism, most of the low-level mutants are GyrB variants [19], with nongyrase mutants being difficult to obtain (GyrB variants are not readily found with *M. smegmatis*). At higher quinolone concentrations, a variety of GyrA variants are recovered, as with *M. smegmatis*, and the fraction of GyrB variants falls significantly [19].

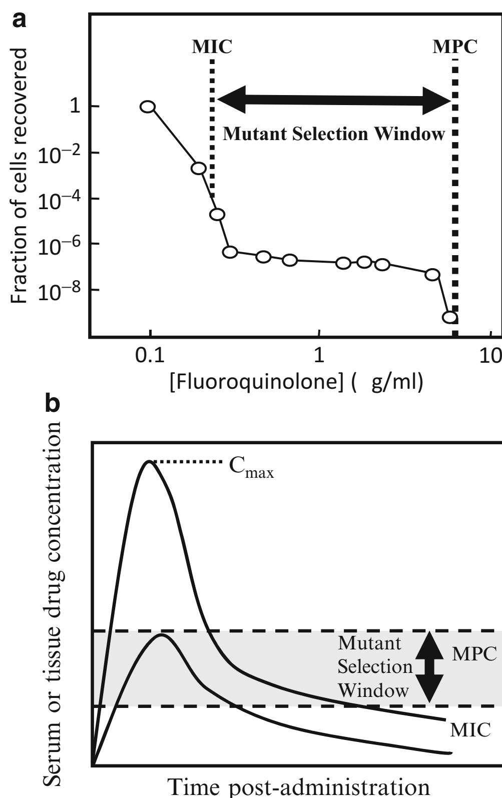

Fig. 14.2 Mutant selection window. Panel **A**. Population analysis of *Mycobacterium smegmatis* for fluoroquinolone-resistant mutants. Aliquots of a growing culture were applied to agar plates containing the indicated concentration of PD160788. Following incubation, colonies were counted, and their number was expressed as a fraction of the inoculum applied to the agar. MIC and MPC are indicated by *dotted lines*. Data taken from [19]. Panel **B**. Relationship between drug pharmacokinetics and mutant selection window. Values of MIC and MPC, determined from data similar to those shown in Panel **A**, are placed on pharmacokinetic data obtained with animal or human studies using a particular dose. In the panel, two hypothetical pharmacokinetic curves are shown, one that exceeds the window boundaries and one that falls inside the window. Each antimicrobial-pathogen combination exhibits a unique relationship between the pharmacokinetic curve and the selection window

The presence of a broad plateau in mutant recovery, which is most clearly seen when only one intracellular target (gyrase) is present [104], can also be observed with two-target situations if target susceptibility differs sufficiently. An example is seen when *S. aureus* is treated with norfloxacin [140], a fluoroquinolone that strongly prefers topoisomerase IV to gyrase. The plateau is reduced, sometimes to an inflection point [104], when the compound attacks the two targets more equally, as is the case when *S. aureus* is treated with ciprofloxacin [140]. *S. pneumoniae* has also been characterized with respect to recovery of mutants and fluoroquinolone concentration. As with *M. smegmatis*, non-topoisomerase mutants are obtained by challenge

with low fluoroquinolone concentrations [18]. Increasing the drug concentration shifts mutant recovery to either GyrA or ParC variants, depending on the particular fluoroquinolone being examined. Thus, the characteristics of the mutant selection window vary among bacterial and fluoroquinolone species; however, the selection window is a general property of antimicrobials (for survey see [141]).

14.4.2 Experimental Support for the Selection Window Hypothesis

Since the mutant selection window is defined with static drug concentrations, either using agar plates [104] or using large volumes of liquid medium [142], it was important to determine how well the static boundaries apply when drug concentrations fluctuate. Measurements with *in vitro* dynamic models show that the window can be observed with fluctuating antimicrobial concentrations for fluoroquinolones, vancomycin, and daptomycin [143–152]. It is also readily seen in rabbits infected with *S. aureus* and treated with levofloxacin [153]; in both cases, static data fit well with dynamic measurements.

The selection window hypothesis differs qualitatively from the coventional idea in which the danger zone for selection of resistant mutants lies below MIC [154] rather than between the MIC and MPC. The two ideas make different predictions about the emergence of resistance. According to the conventional view, eradication of the susceptible population will suppress acquisition of resistance ("Dead bugs don't mutate" [155]). In contrast, the selection window hypothesis maintains that resistance can emerge even when the susceptible population is eliminated. *In vitro* and animal studies described above support the window hypothesis, as does a small clinical trial [156]. In the clinical study, newly hospitalized tuberculosis patients were screened for nasal colonization by *S. aureus* and then treated for tuberculosis using a protocol in which rifampicin was the only agent that was active with *S. aureus*. After several weeks, patients were again sampled for nasal colonization. In 92% of the cases, *S. aureus* colonization was removed; 8% of the colonizing isolates became rifampicin resistant. DNA analyses indicated that the resistant isolates evolved from the original, susceptible cells rather than from dissemination of resistant strains in the hospital. Thus, resistant mutants are recovered even when susceptible bacterial populations drop below the detection limit.

14.4.3 Lethal Action and Resistant Mutant Selection

Bacteriostatic and bactericidal events associated with fluoroquinolone treatment are physiologically distinct. They are also distinct in their effects on resistance. Restricting resistance by keeping concentrations above the mutant selection window

is based on blocking mutant growth. Lethal action is an added effect that directly reduces pathogen numbers. That should help shorten treatment times, which in turn should reduce costs, toxic side effects, and the chance that new resistance will develop. Removal of susceptible cells should also increase the probability that host defense systems will eliminate resistant mutants.

Lethal action has additional importance for fluoroquinolones that have gyrase as their primary target because resistance is genetically recessive. A recessive resistance mutation is not phenotypically expressed until the resistant, mutant protein has replaced the sensitive, wild-type one. Until that time, the mutants will be killed. Thus compounds that are more lethal will be better at restricting the selection of newly formed resistant mutants. When topoisomerase IV is the main target, resistance is codominant [15]; consequently, resistance would be expressed soon after the mutation occurred. In this situation, lethal action would not have as great an effect as when resistance is recessive. Recessive-dominance considerations may partly explain why the frequency for obtaining target mutants of *S. pneumoniae* is 1,000 times higher for fluoroquinolones whose primary target is topoisomerase IV rather than gyrase [17, 51].

Direct killing of resistant mutants is a separate issue. Some fluoroquinolones, such as the C-8-methoxy derivatives, avidly kill more resistant mutants than their C-8-H derivatives [17, 157–161]. This mutant-active feature could contribute to the C-8-methoxy compounds being better at restricting the selection of resistant mutants [17, 157, 160, 162]. Enhanced killing of resistant mutants should also contribute to the ability of a compound to control a larger bacterial population. For example, if the mutation frequency for resistance is 10^{-8}, then a population of 10^{9} cells would contain on average 10 mutants. A compound that kills more than 99% of the mutant cells during an overnight treatment would reduce the number of mutants present from 10 to fewer than 1. The same result could be achieved with 10^{10} cells if 99.9% of the mutants were killed. In contrast, a compound that kills 99% of the susceptible cells, but not mutants, would allow all of the mutants to persist and perhaps reproduce. That would enrich the mutant fraction of the population. Thus, simply improving lethality against susceptible microbes may not suppress the development of resistance if the mutants are allowed to replicate, as might be the case in immunocompromized hosts. Indeed, it may actually speed the enrichment process by killing susceptible cells.

14.4.4 Pharmacodynamics and the Selection Window

Some of the complexities of lethal action can be bypassed by empirical PK/PD considerations, since they take into account both bacteriostatic and bactericidal activity. For antimicrobials, the efficacy of a compound is commonly related to two parameters, its potency against a particular pathogen, usually measured as MIC, and the concentration achieved at the site of infection. For fluoroquinolones, the two parameters are conventionally combined by dividing the area under the time-concentration

curve in a 24-h period (AUC_{24}) by MIC. This pharmacodynamic index (AUC_{24}/MIC) correlates empirically with favorable patient and microbiological outcome with some infections [163, 164]. To extend this idea to restricting emergence of resistance, MIC is replaced with MPC (the MIC of the least susceptible mutant subpopulation). Thus, a value of AUC_{24}/MPC can be determined experimentally to define the upper boundary of the mutant selection window. That value takes into account lethal activity of fluoroquinolones with resistant mutants [165, 166]; consequently, treatment should not require maintenance of fluoroquinolone concentrations above the MPC throughout therapy, as would be the case for bacteriostatic agents. Experimentally, restricted amplification of resistant mutant subpopulations requires fluoroquinolone concentrations to be above MPC for only 20% of the dosing interval when *S. aureus* is treated with levofloxacin [153].

Ambrose and Drusano [154, 167, 168] developed a general approach for relating dose to patient outcome through measurements of AUC_{24}/MIC. The idea can be used to evaluate particular doses for their ability to restrict the emergence of resistance [166]. Briefly, an animal model of infection is used to determine a target value of AUC_{24}/MPC at which no resistance emerges. The ability of a given dose to reach the target with a human population is then estimated by (1) determining AUC_{24} for the given dose using a patient population, (2) determining pathogen MPC for the compound using isolates from the patient population to be treated, and (3) mathematically combining the population AUC_{24} and pathogen population MPC. The output is the fraction of the patient population that will reach the pharmacodynamic target using a particular dose. Widespread use of this method requires additional measurements of pathogen population MPC [77, 169, 170].

14.4.5 Combination Therapy

When the concentration of a compound cannot be maintained above the selection window (above MPC for bacteriostatic agents or an empirically determined value of AUC_{24}/MPC for "concentration-dependent killers"), severely restricting the development of resistance probably requires the use of combinations of agents having different intracellular targets. With combination therapy, pharmacokinetics are likely to be important because mismatches can allow the occurrence of periods equivalent to monotherapy [171, 172]. A clinical experiment using HIV-1-positive tuberculosis patients [173] illustrates this point. After 2 months of standard therapy, patients were divided into two groups:one received a combination of isoniazid and rifampicin, and the second received a combination of isoniazid and rifapentine, a long-lived derivative of rifampicin. In the isoniazid-rifampicin group, drug concentration fell below MIC later for isoniazid than for rifampicin. Thus, no period equivalent to rifampicin monotherapy was expected to occur. In the other group, the concentration of rifapentine dropped below MIC long after that of isoniazid. In this case a period equivalent to rifapentine monotherapy was extensive. Mutants resistant to rifampicin/rifapentine were obtained from patients only in the second group.

We suggested that efforts should be made to superimpose pharmacokinetic profiles so that no agent in a combination therapy will be above its MIC while the concentration of the other agents is below [172]. Newly developed fluoroquinolones tend to have long half-lives (> 8 h). Consequently, a compound such as moxifloxacin will exhibit poor pharmacokinetic overlap with conventional anti-tuberculosis agents. After each dose, a period equivalent to moxifloxacin monotherapy is expected, and we predict that widespread use of moxifloxacin for tuberculosis will lead to widespread resistance.

14.5 Screening New Compounds

14.5.1 Anti-Mutant Activity

The selection window hypothesis predicts that emergence of resistance will be severely restricted by agents that have a very narrow window opening (i.e., MIC = MPC). Compounds can be screened for such activity by measuring MIC with a set of resistant mutants and then seeking derivatives for which the ratio of MIC_{mutant} to $MIC_{wildtype}$ approaches unity. As a test of this idea, we examined a series of 8-methoxy-quinazoline-2,4-diones with *E. coli* mutants and found structural changes that lowered the ratio to approximately 1 [174]. The most active compound by this criterion had a narrow selection window when recovery of resistant mutants from a wild-type population was examined at various 2,4-dione concentrations. This measurement is important, because in principle the initial screen with existing gyrase mutants could lead to compounds that simply have a non-gyrase target and perhaps a very wide window. Since the initial screen uses data normalized to wild-type measurements, differences among compounds associated with uptake and efflux do not confound the interpretation of anti-mutant activity (differences in uptake and efflux appear with screens seeking low absolute values of MIC). Since isogenic sets of fluoroquinolone-resistant mutants are available with a variety of pathogens, the anti-mutant testing strategy can be readily implemented.

14.5.2 Dual Targeting

Many bacteria contain two fluoroquinolone targets, gyrase and topoisomerase IV. That raises the possibility that new quinolones can be developed having equal effects on both enzymes, thereby requiring a cell to acquire two concurrent mutations to be resistant [175–178]. MIC would equal MPC, and the mutant selection window would be closed. Several of the new fluoroquinolones (moxifloxacin, sitafloxacin, gemifloxacin, and clinafloxacin) approach this condition with *S. pneumoniae* as judged by (1) very low mutation frequencies [162], (2) recovery of both *gyrA* and

parC resistance mutations from the same bacterial population [162], and (3) a diminished plateau (inflection point) in plots of mutant recovery versus fluoroquinolone concentration [17]. Moreover, neither *gyrA* nor *parC* single mutations lower susceptibility appreciably, while double mutants are highly resistant [17].

14.5.3 Suppression of Induced Mutants

In Sect. 14.2.3, we mentioned that the induction of resistant mutants can be detected by plating bacterial cells on drug-containing agar and then measuring colony accumulation over time. Since induced mutants derive from a susceptible bacterial population, the size of the initial inoculum and the ability of the compound to kill susceptible cells influence mutant recovery. Restricting the induction of mutants depends on fluoroquinolone structure [49]. A key observation for long-term mutant induction studies is that fluoroquinolones tend to be stable in agar for many days [49]; even *M. tuberculosis* can be used for screening of this type (M. Malik 2011, unpublished observations).

14.6 Plasmid-Mediated Resistance

Plasmid-borne resistance poses a serious threat to fluoroquinolone efficacy for two reasons. First, plasmids can carry resistance to multiple antibiotics, which allows fluoroquinolone resistance to be selected by use of other antimicrobial classes and *vice versa*. Second, plasmids can introduce resistance factors into a bacterial population at a much higher frequency than occurs with spontaneous mutations [179–181]. Higher frequency means that mutant subpopulations will be larger, which in turn increases the probability that the overall population will acquire cells with two resistance mutations. For those and the mutants cells, MIC would exceed the bulk population MPC, and the mutants will be selectively enriched even by treatment that keeps drug concentrations above the MPC of the bulk population. Consequently, resistance is expected to emerge more rapidly in bacterial populations containing plasmid-borne resistance genes than in populations lacking them.

Three forms of plasmid-mediated quinolone resistance have been observed: The first and best studied involves Qnr [110]. As pointed out in a previous section, Qnr interferes with quinolone binding to gyrase and topoisomerase IV. The second type expresses the quinolone-acetylating Aac (6′)-Ib-cr enzyme (discussed above) that inactivates compounds such as ciprofloxacin. The third involves an efflux pump encoded by *qepA* [182]. Of the three, Qnr appears to have the most activity, increasing MIC up to 250 fold (Table 14.1) (QepA increases MIC by 10 fold [183] and Aac (6′)-Ib-cr by 4 fold [123]).

Often plasmids that have a QnrA determinant also carry genes that confer resistance to other anti-bacterials, such as aminoglycosides, β-lactams, chloramphenicol, and sulfonamide [184]. The presence of multiple antibiotic resistance genes on the

Table 14.2 Prevalence of plasmids with *qnr* or *aac (6')-lb-cr* in bacterial strains with reduced susceptibility to ciprofloxacin[a]

Country	Year	Bacterial species	Prevalence of *qnr* plasmid	Prevalence of *aac (6')-lb-cr* plasmid	Reference
China	2008	*C. freundii*	43%(17/40)	28% (11/40)	[189]
		E. cloacae	63% (27/43)	9% (4/43)	[189]
		E. coli	5% (5/105)	13% (14/105)	[189]
		K. pneumoniae	50% (38/77)	21% (16/77)	[189]
Korea	2005	*C. freundi*	60% (39/65)		[192]
		E. cloacae	57% (51/89)		[192]
		E. aerogenes	19% (5/26)		[192]
		S. marcescens	2% (2/110)		[192]
USA	1999-2004	*Enterobacter*		7.5% (12/160)	[202]
		E. coli		32% (15/47)	[202]
		K. pneumoniae		16% (17/106)	[202]
		Enterobacter	31% (50/160)		[197]
		E. coli	4% (2/47)		[197]
		K. pneumoniae	20% (21/106)		[197]

[a]All strains had MIC for ciprofloxacin that was greater than 0.25 μg/ml. Additional surveillance data can be found in references [182, 187, 188, 193, 198]

same plasmid explains the frequent multidrug-resistant phenotype of Qnr-positive enterobacterial isolates. The fluoroquinolone-resistance plasmids, which have a broad host range, are conjugative plasmids that also carry integrons and transposons [184]. Indeed, the *aac (6')-Ib-cr* gene is part of an integron cassette [123, 185], suggesting that it could readily move among plasmids.

Qnr-expressing plasmids are widely distributed with respect to both geography and bacterial species [184]. For example, these plasmids have been isolated in Bangladesh [186], China [187–189], France [190], Germany [191], Korea [192], Japan [112], Taiwan [193], Thailand [194], Turkey [195], United Kingdom [196], and the United States [197, 198]. The plasmids have been obtained from a variety of enterobacteria: *Citrobacter freundii, C. koseri, Enterobacter aerogenes, E. amnigenus, E. cloacae, E. sakazakii, Escherichia coli, Klebsiella oxytoca, K. pneumoniae, Providencia stuartii, Salmonella enterica, Serratia marcescens, Shigella dysenteriae,* and *S. flexneri* [192, 198–200]. Although the geographical distribution of *aac (6')-Ib-cr* has not been studied as thoroughly, isolates have been recovered from China [123, 189], France [201], the United States [202], and Uruguay [203]. As with *qnr*, the *aac (6')-lb-cr*-containing plasmids are found in a variety of Enterobacteriaceae including *C. freundii, E. cloacae*, *E. coli,* and *K. pneumoniae* [189, 203]. The prevalence of *qnr* and *aac (6')-lb-cr* can be substantial (Table 14.2).

The most recently discovered type of plasmid-mediated quinolone resistance involves the QepA efflux pump, which was first found in 2006 in a clinical isolate of *E. coli* from Japan [182]. MIC for hydrophilic fluoroquinolones, such as norfloxacin and ciprofloxacin, increases by 10 fold compared with plasmid-free

counterparts. When *qepA*-containing cells are treated with efflux pump inhibitors, a 10-fold decrease in susceptibility to norfloxacin and ciprofloxacin is observed [183]. Analysis of *qepA*-containing plasmids revealed a class 1 integron integrase and a high percentage (72%) of GC nucleotides [183]. So far, the prevalence of QepA-mediated resistance in humans is low (0.3% among *E. coli* isolates collected from 140 Japanese hospitals between 2002 and 2006 [182]; 0.8% of ESBL-producing enterobacterial isolates collected in France during 2007 [204]). However, QepA was found in half of 48 *rmtB*-positive *E. coli* isolates from pig feces collected from 2005 to 2006 in China (*rmtB* encodes a 16 S rRNA methyltransferase) [205]. The significance of the association with *rmtB* is unclear.

14.7 Concluding Remarks

Several aspects of quinolone biology will make resistance a serious problem for developing new quinolones and quinolone-like compounds. One is cross-resistance between new and old derivatives. With some pathogens such as *S. aureus*, resistance is already widespread, especially among hospital isolates. With others, such as *M. tuberculosis*, incorporation of moxifloxacin into standard regimens is likely to create widespread fluoroquinolone resistance. In principle, the issue of cross-resistance can be bypassed by seeking agents that are unaffected by existing resistance, as illustrated by studies of *E. coli* with 2,4-diones [174]. Another aspect is the multitude of resistance mechanisms, because they allow the gradual accumulation of diverse mutant subpopulations. Fortunately, many of these processes confer only low levels of protection that can be bypassed by keeping drug concentrations above the mutant selection window. A third consideration is the double-edged sword of broad-spectrum activity. That allows empiric therapy to be effective, but it also results in major disruptions to patient microbiomes and creates a risk of future resistance. The fourth, and most serious issue, is plasmid-borne resistance. Even though current plasmid-borne genes may not by themselves confer clinical resistance, they facilitate the acquisition of other resistance factors. One hope is that small molecules will be found that target plasmid-containing cells [206].

We conclude by noting that many aspects of fluoroquinolone resistance apply to other antimicrobials. For example, anti-mutant and dual-targeting screens are not limited to fluoroquinolones, nor are the population analyses that are needed as controls. Indeed, the selection window hypothesis is likely to apply broadly, since it has been measured for a variety of compounds and microbial species. Thus, the selection window idea can help us judge new compounds for the ability to restrict emergence of resistance.

X-ray crystallography of ternary complexes is discussed in Malik, M. et al. (2011) Antimicrob. Agents Chemother. 55: 2335-2343 and references therein

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Chapter 15
Glycopeptide Resistance

Bruno Périchon and Patrice Courvalin

15.1 Background

Glycopeptides were made available for clinical use 50 years ago. Among them, vancomycin and teicoplanin are the two glycopeptides that are used clinically. Vancomycin, the first glycopeptide which was developed in the 1950s, is a tricyclic glycopeptide antibiotic synthesized by *Amycolatopsis oriental is,* and teicoplanin, described approximately 20 years later and initially referred to as teichomycin, is a complex of five closely-related glycopeptide antibiotics produced by *Actinoplanes teichomyceticus*. Introduction of β-lactams that are active against staphylococci initially limited the interest for this class of drugs. However, the emergence of methicillin-resistant *Staphylococcus aureus* in the late 1960s is one of the factors responsible for the increased use of vancomycin in clinical practice. Currently, glycopeptides are often used as a last resort in the treatment of infection that is due to multi-drug-resistant Gram-positive bacteria such as staphylococci and enterococci.

15.2 Mode of Action of Glycopeptides

Glycopeptides inhibit peptidoglycan synthesis by binding to the D-alanyl-D-alanine (D-Ala-D-Ala) C-terminal residues of the muramyl pentapeptide of the peptidoglycan precursors that are substrates of transglycosylases and transpeptidases (Fig. 15.1). Stability of the complex between vancomycin with N-acyl-D-Ala-D-ala termini is provided by five hydrogen bonds (Fig. 15.2a). The large glycopeptide molecules prevent

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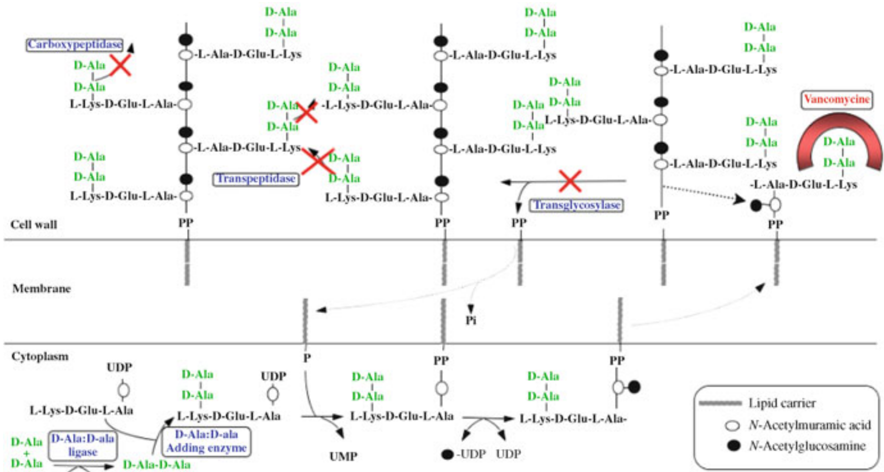

Fig. 15.1 Schematic representation of peptidoglycan synthesis and mode of action of vancomycin. Binding of vancomycin to the D-alanyl-D-alanine C-terminal residues of the late peptidoglycan precursors blocks the final steps of the biosynthesis of peptidoglycan (From [16])

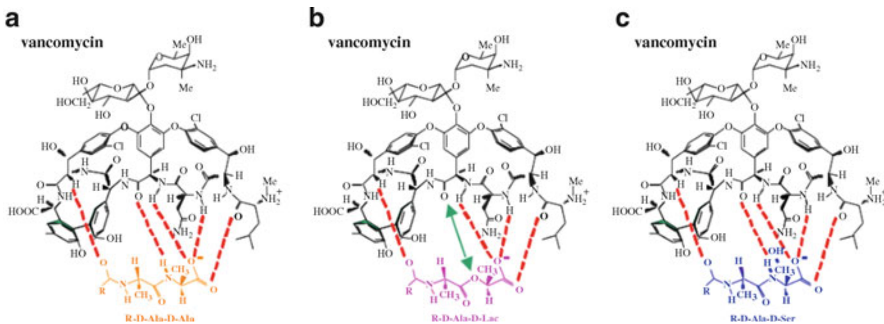

Fig. 15.2 Interactions between vancomycin and the (**a**) N-Acetyl-D-Ala-D-Ala, (**b**) N-Acetyl D-Ala-D-Lac, and (**c**) N-Acetyl-D-Ala-D-Ser. Hydrogen bonds are indicated by dotted lines. With the D-Ala-D-Lac depsipeptide, a central hydrogen bond is missing due to substitution of a NH group by an oxygen. Double green arrow indicates a repulsion between the two oxygens. With the D-Ala-D-Ser pentapeptide, replacement of a CH_3 group by a CH_2OH group is responsible for conformational changes

the action of the transglycosylases and transpeptidases, respectively responsible for the extent of the peptidoglycan backbone and of the cross-linking of the growing chain to the cell wall (Fig. 15.1) [96]. Inhibition of the transglycosylation and transpeptidation steps of peptidoglycan synthesis weakens the peptidoglycan layers. Impermeability of the outer membrane is responsible for the insensitivity of Gram-negative bacteria to this class of antibiotics.

15.3 Mechanism of Resistance

Resistance to glycopeptides was initially reported in enterococci in 1988 [72, 113], approximately 30 years after the introduction of this antimicrobial agent into clinical practice. Classification of glycopeptide resistance is based on the primary sequence of the structural gene for the resistance ligase. To date, eight types of resistance (VanA, VanB, VanC, VanD, VanE, VanF, VanG, and VanL) have been described. All are due to a specific operon (*vanA*, *B*, *C*, *D*, *E*, *F*, *G*, and *L*) responsible for (1) synthesis of a new target (precursors ending in D-lactate (D-Lac) for VanA, B, D, and F-types or D-serine (D-Ser) for VanC, E, G, and L-types) and (2) elimination of the normal precursors ending in D-Ala and synthesized by the host Ddl ligase [14, 100]. Synthesis of the depsipeptide precursors ending in D-Ala-D-Lac is responsible for the absence of a critical hydrogen bond between vancomycin and the target, for repulsion between two oxygens, and for alteration of the three-dimensional structure of the target (Fig. 15.2b) whereas resistance due to the replacement of D-Ala-D-Ala by D-Ala-D-Ser does not affect the hydrogen bonds but results from the increased bulk of the hydroxymethyl group of serine relative to the methyl group of alanine (Fig. 15.2c). The affinity of vancomycin for precursors terminating in D-Ala-D-Lac and D-Ala-D-Ser is, respectively, approximately 1000- and 6-fold lower than for precursors terminating in D-Ala-D-Ala [21, 28]. Some of the enzymes implicated in the D-Ala-D-Ser resistance pathway are different from those involved in the D-Ala-D-Lac ending precursors synthesis, suggesting the existence of, at least, two routes of evolution [98].

Glycopeptide resistance can be acquired (only present in certain strains of the species) (VanA, B, D, E, F, G, L) or intrinsic (present in all the strains of the species) (VanC).

15.4 Glycopeptide Resistance in Enterococci

Enterococci are part of the gastrointestinal tract of humans and animals. Most of the enterococcal infections are caused by *E. faecalis* and *E. faecium*. These two species exhibit intrinsic low level resistance to several classes of antibiotics (such as lincosamides, aminoglycosides, trimethoprim-sulfamethoxazole, β-lactams) and have also acquired high level resistance to β-lactams, fluoroquinolones, macrolides-lincosamides-streptogramins, aminoglycosides, chloramphenicol, fusidic acid, tetracycline, rifampin, and glycopeptides [52]. Although vancomycin-resistant enterococci (VRE) have disseminated throughout the world, geographical repartition of VRE isolates is not homogeneous. In 2008 in Europe, the rates of vancomycin resistance varied from 0% to 32% and from 0% to 5% for *E. faecium* and *E. faecalis*, respectively (data from the European Antimicrobial Resistance Surveillance system, www.rivm.nl/earss/database/). In the USA, a large surveillance study demonstrated that 2% of *E. faecalis* and 60% of *E. faecium* implicated in nosocomial bloodstream infections were resistant to vancomycin [123].

15.4.1 Acquired Resistance Due to Modified Peptidoglycan Precursors Ending in D-Ala-D-Lac

15.4.1.1 VanA-Type

VanA, which is the most common type of glycopeptide resistance in enterococci, was the first described [72, 113] and has been the most extensively studied. The VanA-type is characterized by an inducible high level of resistance to both vancomycin and teicoplanin. The *vanA* gene cluster, carried by the transposon Tn*1546* [15] or related elements [61], is usually located on self-transferable plasmids [59, 72, 73] or on the bacterial chromosome as part of larger conjugative elements [61]. It has been reported in several species of *Enterococcus* (Table 15.1).

Tn*1546* is composed of genes that are (1) responsible for the movements of the element (ORF1, a transposase, and ORF2, a resolvase) and (2) involved in regulation and expression of glycopeptide resistance (*vanR*, *vanS*, *vanH*, *vanA*, *vanX*, *vanY*, and *vanZ*) (Fig. 15.3a). The *vanH*, *vanA*, and *vanX* genes code for proteins that are necessary for expression of resistance (Fig. 15.3b). The VanH dehydrogenase converts pyruvate to D-Lac [14], the VanA ligase synthesizes the depsipeptide D-Ala-D-Lac, which is incorporated into the peptidoglycan precursors in place of D-Ala-D-Ala [28], and the VanX D,D-dipeptidase hydrolyses the dipeptide D-Ala-D-Ala formed by the host chromosomal D-Ala:D-Ala ligase (Ddl) (Fig. 15.3b) [99, 125]. The VanX activity allows reduction of the level of peptidoglycan precursors ending in D-Ala-D-Ala. VanY is a membrane-bound penicillin-insensitive D, D-carboxypeptidase that cleaves the D-Ala C-terminal residue of the pentapeptide precursors synthesized from the D-Ala-D-Ala dipeptide that has escaped VanX hydrolysis [16]. Vancomycin has no affinity for the resulting tetrapeptide precursors. The *vanZ* gene confers low-level resistance to teicoplanin by an unknown mechanism [11]. The genes of the *vanA* gene cluster are expressed only in the presence of glycopeptides in the medium. This regulation is mediated by a two-component regulatory system composed of a sensor kinase (VanS) and a response regulator (VanR). The *vanRS* genes are located upstream from the resistance genes (Fig. 15.3a).

The heterogeneity of Tn*1546*-like transposons results from mutations and deletions, but is mainly due to acquisition of insertion sequences that have transposed outside of the regions implicated in glycopeptide resistance [84, 121].

15.4.1.2 VanB-Type

Strains with the VanB phenotype are resistant to vancomycin but remain susceptible to teicoplanin, the latter antibiotic not being an inducer for expression of glycopeptide resistance (Table 15.1).

Resistance of VanB-type is due to the *vanB* operon that is also responsible for production of peptidoglycan precursors ending in the depsipeptide D-Ala-D-Lac. Its organization is similar to that of *vanA* (Fig. 15.4). The *vanB* cluster is composed of

Table 15.1 Van-type glycopeptide resistance

Resistance	Acquired						Intrinsic	
Type	VanA	VanB	VanD	VanE	VanG	VanL	VanC	
MIC (mg/L)								
Vancomycin	64–1000	4–1000	64–128	8–32	8–16	8–16	2–32	>1,000
Teicoplanin	16–512	0.5–1	4–64	0.5	0.5	0.5	0.5–1	>256
Expression	Inducible	Inducible	Constitutive	Constitutive Inducible	Inducible	Inducible	Constitutive Inducible	Constitutive
Location	Plasmid Chromosome	Plasmid Chromosome	Chromosome	Chromosome	Chromosome	Chromosome	Chromosome	
Species	*E. faecium* *E. faecalis* *E. gallinarum* *E. casseliflavus* *E. avium* *E. durans* *E. mundtii* *E. raffinosus* *S. gallolyticus* *B. circulans* *A. haemolyticum* *O. turbata*	*E. faecium* *E. faecalis* *S. bovis* *S. gallolyticus*	*E. faecium* *E. faecalis* *E. gallinarum* *E. raffinosus* *Ruminococcus* sp.	*E. faecalis*	*E. faecium* *E. faecalis* *C. difficile* *Ruminococcus* sp.	*E. faecalis*	*E. gallinarum* *E. casseliflavus* *E. flavescens*	*Leuconostoc* *Lactococcus* *Pediococcus*
Modified target	D-Ala-D-Lac	D-Ala-D-Lac	D-Ala-D-Lac	D-Ala-D-Ser	D-Ala-D-Ser	D-Ala-D-Ser	D-Ala-D-Ser	D-Ala-D-Lac

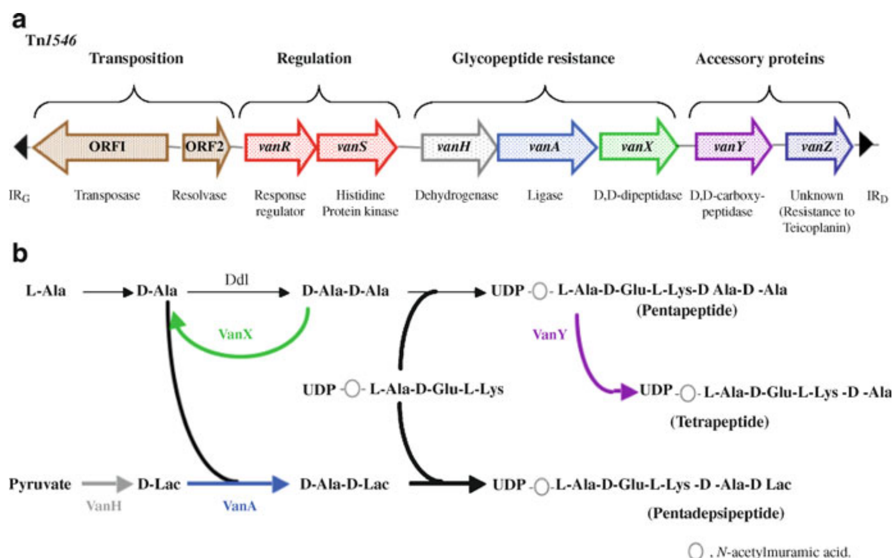

Fig. 15.3 Mechanism of resistance of the VanA-type. (**a**) Organization of Tn*1546*. IR_L and IR_R, Inverted repeat, left and right. Arrows indicate coding sequences and direction of transcription. (**b**) Schematic representation of the synthesis of peptidoglycan precursors in a VanA-type resistant strain after induction with glycopeptides. Ddl, D-Ala:D-Ala ligase

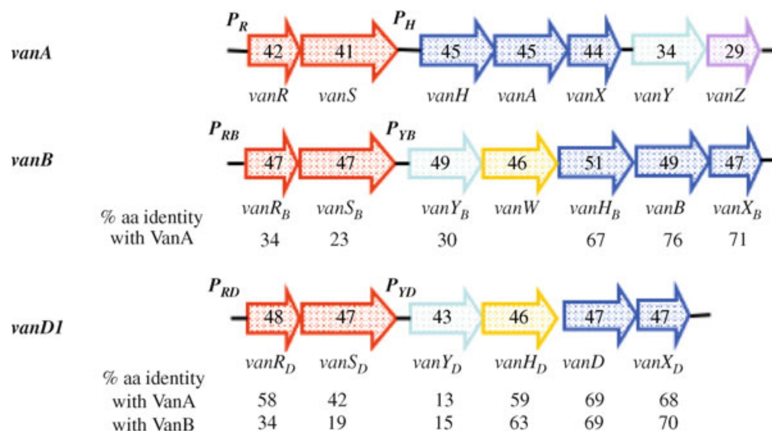

Fig. 15.4 Comparison of the *vanA, vanB, and vanD1* operons. *Arrows* indicate coding sequences and direction of transcription. The percentage of amino acid (aa) identity between the deduced proteins is indicated under the *arrows*. The guanosine plus cytosine content (% GC) is indicated in the *arrows*

resistance genes ($vanH_B$-*vanB*-$vanX_B$), of the accessory $vanY_B$ gene, and of regulatory genes ($vanR_B$-$vanS_B$) [44]. The function of *vanW* is unknown.

The $VanR_B$-VanSB system displays only limited sequence identity with VanR-VanS (34% and 23%, respectively) [16]. The *vanB* gene cluster is generally carried by large (from 90 to 250 kb) conjugative elements that are transferable from chromosome to

chromosome [95]. These elements contain transposons such as Tn*1547* found in a 250-kb genetic element [94] or Tn*5382* [30, 33]. It is of interest to note that, in the 250-kb chromosomal element, Tn*1547* is delineated by two insertion sequences [94] allowing dissemination of resistance by vertical and horizontal transfer. The *vanB* element can also be located on plasmids (Table 15.1) [101, 124]. Dissemination of VanB-type resistance could also result from the spread of the *vanB* gene cluster carried by two-related Tn*916*-like conjugative transposons, Tn*5382* (27 kb) [30] and Tn*1549* (34 kb) [50]. Tn*1549* contains 30 open reading frames organized in three functional regions implicated in (1) excision-integration, (2) vancomycin resistance, and (3) conjugative transfer. Interestingly, analysis of the base composition indicated that the origin of the left end of the transposon is different from that of the two other functional regions.

Three subtypes, *vanB1*, *vanB2*, and *vanB3* [34, 53, 88] of the *vanB* operon can be distinguished on the basis of specific nucleotide sequences in the $vanS_B$-$vanY_B$ intergenic region. There is no correlation between *vanB* subtype and the level of vancomycin resistance.

15.4.1.3 VanD-Type

VanD-type strains present moderate levels of resistance to vancomycin and teicoplanin. As in VanA- and VanB-type strains, VanD resistance is due to synthesis of peptidoglycan precursors that end in D-Ala-D-Lac [39, 92]. This resistance has been mainly detected in *E. faecium*, but also in *E. faecalis*, *Enterococcus gallinarum* [25], *Enterococcus raffinosus* [112], *Enterococcus avium* [37], and in the anaerobic Gram-positive *Ruminococcus* sp. [41]. Although the biochemical mechanism of resistance is similar to those of VanA and VanB, VanD-type resistance displays some peculiarities such as the following: it is constitutively expressed despite the presence of a $\mathrm{VanR_D}$/$\mathrm{VanS_D}$ system and is not transferable by conjugation to other enterococci [39, 89, 92]. Furthermore, the $\mathrm{VanY_D}$ D,D-carboxypeptidase activity is inhibited by benzylpenicillin [31, 38, 39, 89] whereas VanY and $\mathrm{VanY_B}$ activities are penicillin insensitive. The $\mathrm{VanX_D}$ D,D-dipeptidase activity is low in VanD-type strains despite the presence of a putatively functional protein [39]. The organization of the *vanD* operon, which is exclusively chromosomally located, is similar to those of *vanA* and *vanB* (Fig. 15.4) [31, 39, 83, 92]. No genes homologous to *vanZ* from the *vanA* operon or *vanW* from the *vanB* operon are present in the *vanD* cluster. In all the VanD-type strains described, except one, the susceptible pathway is not functional, due to an inactive Ddl host ligase resulting from various mutations in the *ddl* gene. The resistance pathway is constitutively expressed, due to mutations in the $vanS_D$ or $vanR_D$ genes that are responsible for synthesis of an inactive $\mathrm{VanS_D}$ or $\mathrm{VanR_D}$ proteins [31, 37–39, 89]. Thus, the VanD strains rely on the constitutive expression of the resistance pathway to grow in the absence of vancomycin. Surprisingly, in spite of synthesis of peptidoglycan precursors ending essentially in D-Ala-D-Lac, the level of resistance to teicoplanin remains low (Table 15.1). The *vanD* operon can be divided into two main subtypes, *vanD-1* and *vanD-4*, on the basis of sequence differences in VanD and $\mathrm{VanY_D}$ [37].

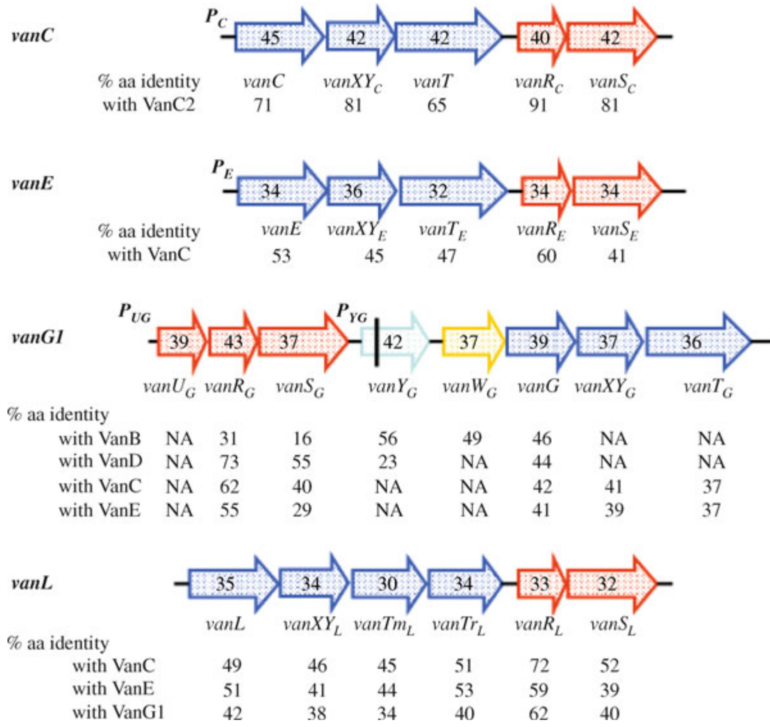

Fig. 15.5 Comparison of the *vanC, vanE, vanG1,* and *vanL* operons. *Arrows* indicate coding sequences and direction of transcription. The percentage of amino acid (aa) identity between the deduced proteins is indicated under the *arrows*. The guanosine plus cytosine content (% GC) is indicated in the arrows. The vertical bar in $vanY_G$ indicates a frameshift mutation leading to a predicted truncated protein. *NA* not applicable

15.4.2 Acquired Resistance Due to Modified Peptidoglycan Precursors Ending in D-Ala-D-Ser

15.4.2.1 VanE-Type

VanE-type resistance has been detected in *E. faecalis* [1, 23, 45, 116]. The resistant strains are characterized by an inducible low-level of resistance to vancomycin only not transferable by conjugation. They synthesize late peptidoglycan precursors ending in D-Ala-D-Ser [45]. The organization of the *vanE* operon is identical to that of *vanC,* which will be described in the "intrinsic resistance" section of this chapter (Fig. 15.5). Three proteins are required for resistance: VanE, a D-Ala:D-Ser ligase, $VanXY_E$, a D,D-peptidase, and $VanT_E$, a serine racemase [2]. Two genes coding

for a two-component regulatory system are located downstream from *vanT*$_E$ [2]. The five genes are co-transcribed from a P_E promoter located upstream from *vanE*. Although the VanS sensor is likely to be inactive due to the presence of a stop codon in the 5′ portion of the gene, expression of vancomycin resistance is inducible in VanE prototype strain BM4405 [2], suggesting the existence of a probable cross-talk with another regulatory system of the host.

15.4.2.2 VanG-Type

Acquired VanG-type resistance is characterized by a low-level of resistance to vancomycin, due to inducible production of modified precursors ending in D-Ala-D-Ser, and susceptibility to teicoplanin [35, 78]. VanG-type was initially detected in *E. faecalis*, but the *vanG* gene cluster was also detected in *Clostridium difficile* [106] and in *Ruminococcus* sp [41]. Study of the *vanG* cluster, which is composed of eight chromosomal genes, revealed that its organization differed from that of the other *van* operons (Fig. 15.5) [35, 78]. The mutated *vanY*$_G$ gene encodes a truncated D,D-carboxypeptidase most likely inactive; *vanW*$_G$ (49% identity with *vanW*$_B$) encodes a protein of unknown function; the three resistance genes, *vanG*, *vanXY*$_G$, and *vanT*$_G$ code for a D-Ala:D-Ser ligase, a bi-functional D,D-peptidase, and a serine racemase, respectively. A putative three-component regulatory system, composed of *VanR*$_G$, *vanS*$_G$, and of *vanU*$_{G,}$, which encodes a predicted transcriptional activator, is located upstream from the resistance genes. Transfer of VanG resistance is obtained at low frequency to *E. faecalis* and is associated with the movement from chromosome to chromosome of large genetic elements of *ca.* 240 kb [35]. The flanking regions of the *vanG* gene cluster revealed a structure typical of conjugative transposons [35].

A *vanG2* gene cluster, which has a structure that differs from that of *vanG*, was detected in *E. faecalis* [24] (Fig. 15.6). The proteins deduced from *vanG2* share between 74% and 96% identity to those encoded by the *vanG* operon. The location of the element containing the *vanG2* gene cluster was found to be different from that of the element harboring the *vanG* operon [24]. The *vanG*-like cluster detected in *C. difficile* 630 differs slightly from that of *E. faecalis* (Fig. 15.6).

15.4.2.3 VanL-Type

Recently, a new letter was added to the so-called "*van* alphabet". In 2008, the *vanL* gene cluster was detected in an *E. faecalis,* exhibiting low-level resistance to vancomycin [26]. It is likely that this cluster is located in the chromosome, and the resistance appears to be not transferable. The *vanL* cluster is similar to that of *vanC* and *vanE*. However, as opposed to $VanT_C$, $VanT_E$, and $VanT_G$, the $VanT_L$ serine racemase, is encoded by two separate genes, which are *vanTm*$_L$ (membrane binding domain) and *vanTr*$_L$ (racemase activity) (Fig. 15.5) [26].

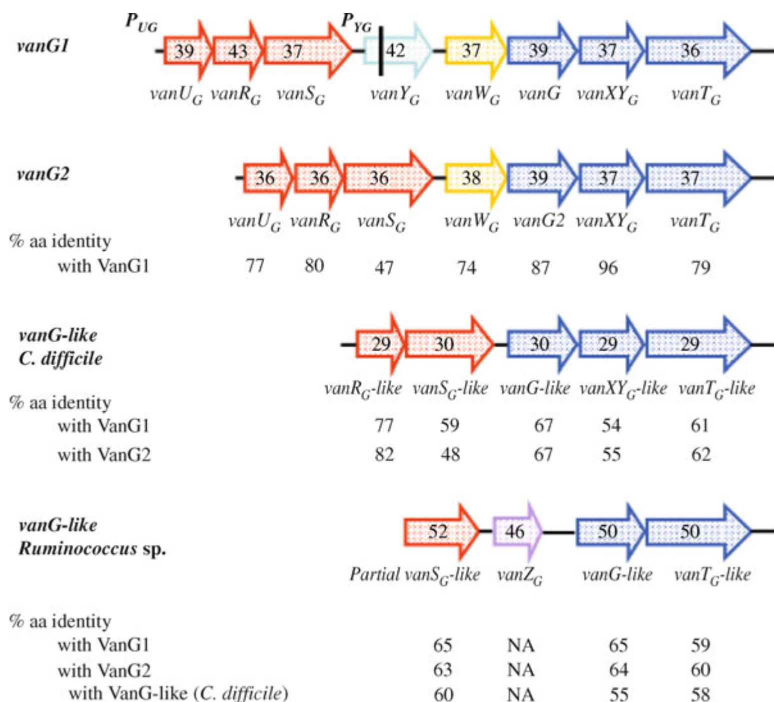

Fig. 15.6 Comparison of the *vanG1, vanG2, vanG-like* of *C. difficile,* and *VanG-like of Ruminococcus* sp. *Arrows* indicate coding sequences and direction of transcription. The percentage of amino acid (aa) identity between the deduced proteins is indicated under the *arrows*. The guanosine plus cytosine content (% GC) is indicated in the arrows. The vertical bar in $vanY_G$ indicates a frameshift mutation leading to a predicted truncated protein. *NA* not applicable

15.4.3 Intrinsic Resistance

Certain Gram-positive bacteria such as species of *Leuconostoc*, *Erysipelothrix*, *Pediococcus*, and many *Lactobacillus*, are intrinsically highly resistant to glycopeptides by synthesis of peptidoglycan precursors ending exclusively in D-Lac [22, 60]. Glycopeptide-producing actinomycetes are also resistant to glycopeptides by a mechanism, which will be discussed later.

The intrinsic VanC-type resistance is characterized by low-level resistance to vancomycin and is specific to *E. gallinarum* (VanC-1 type), *Enterococcus casseliflavus* (VanC-2 type), and *Enterococcus flavescens* (VanC-3 type). As in VanE, VanG, and VanL, resistance results from the replacement of the D-Ala C-terminal of the pentapeptide precursors by D-Ser. VanC resistance can be inducibly or constitutively expressed [105]. The chromosomally-located *vanC* gene cluster codes for three proteins responsible for resistance, VanC (D-Ala:D-Ser ligase), $VanXY_C$ (D,D-peptidase), and VanT (serine racemase) and two implicated in regulation of expression, $VanR_C$ (transcriptional regulator) and $VanS_C$ (sensor) (Fig. 15.5). The $vanXY_C$ protein possesses the bifunctional activity, D,D-dipeptidase and

D,D-carboxypeptidase, and is responsible for hydrolysis of precursors ending in D-Ala [6, 97]. $VanXY_C$ contains consensus sequences for binding zinc, stabilizing the binding of the substrate, and catalyzing hydrolysis that are present in both VanX- and VanY-type enzymes [6, 97]. However, amino acid sequence comparison indicated that $VanXY_C$ is more closely related to VanY than to VanX. The cytoplasmic domain of the membrane-bound serine racemase is able to convert L-Ser to D-Ser and also possess an alanine racemase activity [7, 8]. The transmembrane domain of VanT is probably also involved in the uptake of L-Ser from the external medium [8]. As opposed to *vanA*, *B*, *D*, *F*, and *G*, the $vanR_C/vanS_C$ genes are located downstream from the resistance genes. The constitutive phenotype observed in several strains is likely due to mutations in the $VanS_C$ sensor [85].

In addition to the host D-Ala:D-Ala and to the D-Ala:D-Ser ligase, the *E. gallinarum* VanC prototype strain also possesses a third ligase that has D-Ala:D-Ala activity. The gene encoding this third ligase (*ddl2*) is located downstream from the regulatory genes in an opposite direction [5]. This enzyme is active both *in vitro* and *in vivo*; however, its function in *E. gallinarum* remains unclear.

The organization of the *vanC-2* gene cluster of *E. casseliflavus* is identical to that of *vanC* and the deduced proteins display 65–91% of identity [42].

The *vanC-3* operon is highly similar to vanC-2 (from 97% to 100% of identity).

15.5 Glycopeptide Resistance in *Paenibacillus Popilliae*: The VanF-Type

The vancomycin resistance gene cluster *vanF* has been described in the vancomycin-resistant biopesticide *Paenibacillus popilliae* [48, 87, 102]. This cluster is composed of genes encoding $VanY_F$, $VanZ_F$, $VanH_F$, VanF, and $VanX_F$. Upstream from these genes, two other genes coding for $VanR_F/VanS_F$ have been found [48]. The identity of the proteins involved in resistance varies from 21% to 79% between the VanF- and VanA-type and that of the VanR/VanS system is 33% and 45%, respectively [48, 87]. However, for the two-component system, the greater similarity is found with a family of two-component regulators linked to VanY-like carboxypeptidases in several *Bacillus* species suggesting that *vanR*, *vanS* and *vanY* may be of different origin than the other genes of the *vanF* cluster [48]. Vancomycin resistance of the VanF-type is inducible in *P. popilliae*. This organism also possesses a D-Ala:D-Ala ligase [48].

15.6 Glycopeptide Resistance in *Streptomyces coelicolor*

A *van* gene cluster has been described in the non-pathogenic, non-glycopeptide-producing actinomycete *Streptomyces coelicolor*, which is highly resistant to vancomycin but susceptible to teicoplanin. This cluster is composed of seven genes that

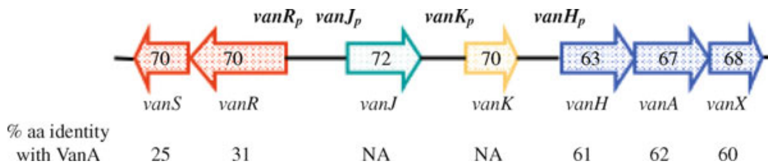

Fig. 15.7 The *van* gene cluster in *S. coelicolor*. *Arrows* indicate coding sequences and direction of transcription. The percentage of amino acid (aa) identity between the deduced proteins is indicated under the *arrows*. The guanosine plus cytosine content (% GC) is indicated in the *arrows*. Identified promoters ($vanR_p$, $vanJ_p$, $vanK_p$, and $vanH_p$) are indicated (From [63]). *NA* not applicable

are organized into four transcription units, the *vanR*/*vanS* genes being located upstream from the resistance genes, but in opposite orientation (Fig. 15.7) [66]. In the presence of vancomycin, the promoters of each unit are activated, leading to expression of resistance [66]. In this system, VanK, which is member of the Fem family of peptidyltransferases that add the cross-bridge amino acids to the stem pentapeptide of cell wall precursors, is essential for vancomycin resistance [66]. Interestingly, an orthologue of this gene was found in the *van*-like gene cluster of *Streptomyces toyocaensis* NRRL15009, producing the A47934 antibiotic [93].

15.7 Regulation of Expression of Glycopeptide Resistance

Expression of glycopeptide resistance is regulated by two genes, *vanR* and *vanS*, that encode a two-component regulatory system. To the best of our knowledge, the VanR/VanS system is the only one implicated in the control of the expression of genes mediating antibiotic resistance. VanS is a membrane-associated protein that contains an N-terminal sensor domain with two membrane spanning segments and a C-terminal cytoplasmic kinase domain. VanS has a bi-functional activity: kinase in presence of glycopeptides and phosphatase in the absence of inducer. In response to the presence of glycopeptides in the medium, a specific histidine residue of the cytoplasmic domain of VanS is autophosphorylated (Fig. 15.8). VanR acts as a transcriptional activator that can be phosphorylated on an aspartate residue present in the effector domain by acquisition of the phosphoryl group of activated VanS. Thus, VanS controls the level of phosphorylation of VanR. The resistance and regulatory genes in the *vanA*, *vanB*, and *vanD* operons are transcribed from two distinct promoters, P_{res} and P_{reg} respectively, that are coordinately regulated [10]. Phosphorylation of the VanR regulator enhances the affinity of this protein for the promoters and activates transcription of the two sets of genes, leading to an amplification loop (Fig. 15.8) [65]. VanS is not necessary for full activation of the promoters, since VanR can be phosphorylated independently by acetylphosphate or kinases encoded by the host chromosome [9].

As demonstrated with VanS, purified $VanS_B$ also act as both a histidine protein kinase and a phospho-$VanR_B$ phosphatase [36]. Enterococci that harbor the *vanB*

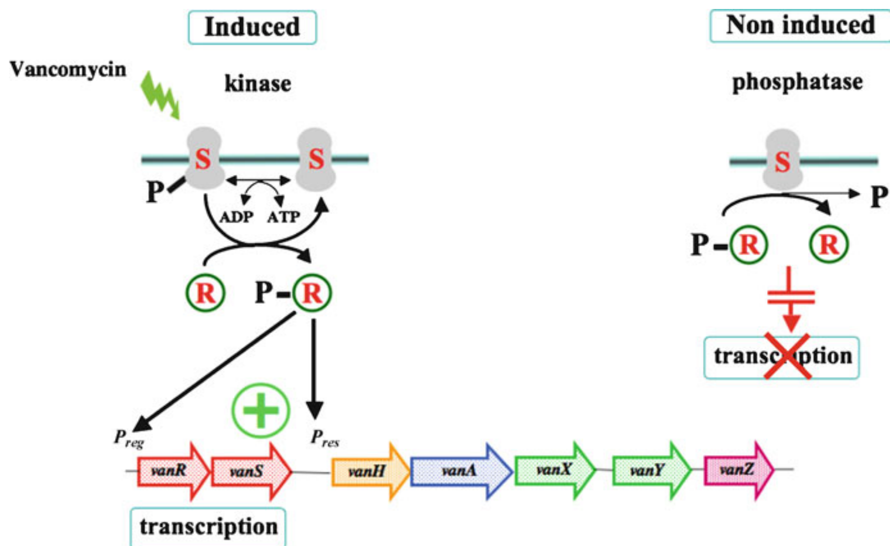

Fig. 15.8 Induction of VanA-type resistance by vancomycin. Schematic representation (1) of activation of the P_{reg} and P_{res} promoters of the *vanA* operon after phosphorylation of VanR by VanS (kinase activity) in response to the presence of vancomycin and (2) of lack of activation of the P_{reg} and P_{res} promoters due to dephosphorylation of VanR by VanS (phosphatase activity) in the absence of vancomycin

gene cluster remain susceptible to teicoplanin, since this antibiotic is not an inducer [13]. Mutations in the *vanS*$_B$ gene, obtained *in vitro* or *in vivo* in animal models, lead to teicoplanin resistance [17, 20]. Strains of VanB-type that are resistant to teicoplanin have also been isolated in patients [63, 68].

Recognition of the presence of glycopeptides in the environment by VanS and VanS$_B$ likely occurs by different mechanisms. VanA-type resistance is inducible by glycopeptides and moenomycin but not by drugs that inhibit the reactions preceding (e.g., ramoplanin) or following (e.g., bacitracin, penicillin G) transglycosylation [19, 58]. Accumulation of lipid II, resulting from inhibition of transglycosylation, may be the signal recognized by VanS, thus explaining the induction of the VanA-type resistance by antibiotics that inhibit the same step of peptidoglycan synthesis but have different structures and modes of action [19, 58].

The VanC-type resistance is constitutively expressed and two potential promoters have been located upstream from *vanC* and *vanR*$_C$ [6]. The five genes of the *vanE* operon in *E. faecalis* BM4405 are co-transcribed from a single promoter upstream from *vanE* [2].

As already described, the putative regulatory system of the *vanG* operon contains the following three genes: *VanR*$_G$, *vanS*$_G$, and *vanU*$_G$. Surprisingly, the regulatory genes are constitutively co-transcribed from a P_{UG} promoter whereas the five resistance genes are co-transcribed in an inducible manner from the P_{YG} promoter [35]. This is the first *van* operon to be regulated that way.

15.8 Glycopeptide Dependence

Some VanA- or VanB-type strains of enterococci require the presence of vancomycin in the culture medium to grow. This phenomenon, detected in *E. faecium* and *E. faecalis* [40, 49, 55, 115], but also in *Enterococcus avium* [103, 110], can occur *in vitro* [20], in animal models [17], and in patients, especially those treated for long periods of time with vancomycin [40, 49, 55, 115]. This particular phenotype is due to mutations in the *ddl* gene such as insertions, deletions, or single base-pair changes resulting in an amino acid substitution. It has been suggested that maintenance of the 3-D structure in the vicinity of the mutations in the active site is critical for D-Ala:D-Ala ligase activity [51]. If an alternative pathway is present in the bacteria, inactivation of the host Ddl is not lethal. Strains harboring a *van* gene cluster rely on the vancomycin-inducible activity of the acquired D-Ala:D-Lac ligase for growth. It is important to note that reversion to vancomycin independence has been observed following (1) a mutation in the two-component system leading to a constitutive expression of the resistance pathway or (2) a mutation in the D-Ala:D-Ala ligase that restores its activity leading to resistance inducible by vancomycin [20, 40, 115].

15.9 Origin of the Glycopeptide Resistance Genes

15.9.1 Acquired D-Ala:D-Lac Ligases

Leuconostoc mesenteroides, *Pediococcus pentosaceus*, and *Lactobacillus casei*, which are intrinsically highly resistant to glycopeptides by production of peptidoglycan precursors ending in D-Lac [22, 60], have been suspected to be the source of resistance ligases producing D-Ala-D-Lac. However, a phylogenetic tree based on the alignment of the deduced sequences of D-Ala:D-ala ligases and related enzymes revealed that VanA, VanB, and VanD exhibit only limited identity with D-Ala:D-Lac ligases of these naturally resistant species.

In the environment, the glycopeptide-producing organisms could represent a potential source of resistance for human pathogens. Indeed, they harbor resistance genes to protect themselves against suicide. Genes coding for homologues of VanH, VanA, and VanX have been found and with the same genetic organization in two glycopeptide-synthesizing organisms, *Amycolatopsis orientalis* C329.2, and *S. toyocaensis* NRRL15009, that produce vancomycin and the A47934 glycopeptide, respectively [74–77]. *vanHAX* homologues have also been detected in producers of chloroeremomycin (*A. oriental is*, 18098), ristocetin (*A. orientalis* subsp. Lurida), and teicoplanin-avoparcin (*Amycolatopsis coloradensis* subsp. Labeda) [75]. A two-component regulatory system has been detected in *S. toyocaensis* NRRL15009, but the *vanRst* and *vanSst* are located approximately at 20 kb from the *vanHAX* operon [93]. However, the base composition (G+C content) of the genes composing the *vanA*, *vanB*, and *vanD* clusters is significantly lower than that of the

vanRSHAX homologues in the producers, suggesting that acquisition of the genes is probably not a recent event. Furthermore, the clusters do not appear to be transferable under laboratory conditions [75, 86]. As already mentionned, a vancomycin resistance gene cluster, *vanF*, has been detected in the biopesticide *P. popilliae*. Orientation and alignment of the genes essential for resistance (*vanH*/*vanH*$_F$, *vanA*/*vanF*, and *vanX*/*vanX*$_F$) are identical in VanF and VanA and a putative Van$R_F S_F$-like system has been identified [48]. The base composition of the three resistance genes of *P. popilliae* is similar to that of the corresponding genes of *vanA* and *vanB*. Therefore, *P. popilliae* could represent an intermediate in the transfer from the producers to the clinical isolates. Such a transfer could have occured through a long chain of related organisms so that the first and the last member of this chain are only distantly related. Recently, glycopeptide resistance *vanA* operons were found in *Paenibacillus* isolated from soil [56, 57]. Their level of identity with the enterococcal operons is markedly higher than that of *vanF*. The close similarity of these operons with that of *Enterococcus* suggests that the gene clusters have evolved from a common ancestor or that the *vanA* operons from soil organisms were acquired by enterococci. The latter hypothesis seems to be more likely since (1) glycopeptide resistance was detected in *Paenibacillus apiarius* that were isolated in the early 1970s, when the use of glycopeptides was limited in clinical practice and before the detection in enterococci and (2) glycopeptide resistance is associated with mobile genetic elements in enterococci whereas resistance is chromosomal and seems to be intrinsic or at least acquired in very ancient times in *Paenibacillus* [56, 57]. The *vanA* gene clusters of *Paenibacillus thiaminolyticus* and *P. apiarius* can be heterologously and efficiently expressed in *E. faecalis* as opposed to a *van* gene cluster found in *Amycolatopsis coloradensis*, an avoparcin-producing soil bacterium [62].

It is important to note that the base composition differs between the essential and the non-essential genes for resistance within the *van* operons, suggesting that the genes could originate from different species. By collecting genes from various sources, the *van* gene clusters may thus have been composed.

Presence of the *vanB* operon on a Tn*1549*-like element in various anaerobes from the digestive tract has been demonstrated [18]. Transfer of the element from *Clostridium symbiosum* to *Enterococcus* spp. has been obtained *in vitro* and in the digestive tract of gnotobiotic mice [71]. The functionality of a Tn*1549*-like element and the transfer of the *vanB* operon between enterococci and human commensal anaerobes in the intestinal environment have been demonstrated. Anaerobic bacteria, which are also common in soil, could thus be an intermediate in the transfer of VanB-type vancomycin resistance from glycopeptide producers to enterococci.

15.9.2 Acquired D-Ala:D-Ser Ligases

No glycopeptide-producers were found to synthesize peptidoglycan precursors ending in D-Ala-D-Ser suggesting that the origin of the VanC, E, and G-type of resistance is different from that of VanA, B, and D.

The *vanC* and *vanE* gene clusters present a high degree of identity (41–60%) [2]. Thus, acquired resistance of the VanE-type could be due to acquisition of a chromosomal operon from another species of *Enterococcus* (*E. gallinarum*, *E. casseliflavus/flavescens*).

The *vanG* operon appears to be more heterogeneous. $VanR_G$ exhibits the highest identity (73%) with $VanR_D$; $VanY_G$ exhibits the highest identity with $VanY_B$ (56%), and $vanW_G$ has 49% identity with *vanW,* which is present only in the *vanB* operon. The 3′ part of the *vanG* cluster (*vanG*, $vanXY_G$, $vanT_G$) is more closely related to *vanC* and *vanE* than to the corresponding proteins of the other operons (Fig. 15.5) [35], apart from the VanG D-Ala:D-Ser resistance ligase, which is phylogenetically closer to the D-Ala:D-Lac ligases. Thus, the *vanG* operon is composed of genes probably recruited from various *van* operons.

15.10 Glycopeptide Resistance in *Staphylococcus aureus*

Staphylococcus aureus is one of the most common causes of hospital- and community-acquired infections. It was responsible for 20% of nosocomial bloodstream infections in US hospitals in 2002 and the proportion of methicillin-resistant *S. aureus* (MRSA) increased from 22% in 1995 to 57% in 2001 [123]. In Europe, the rates of MRSA vary, in 2008, from 1% to 52% (www.rivm.nl/earss/database/).

15.10.1 Vancomycin-Intermediate S. aureus (VISA Strains)

Due to emergence of methicillin resistance in *S. aureus*, vancomycin remained the last resort for MRSA treatment until recent years. However, the first *S. aureus* with decreased susceptibility to vancomycin (VISA, vancomycin-intermediate *S. aureus*) was reported in 1997 [64]. Since then, VISA strains have been isolated worldwide [118]. In these strains, decreased of susceptibility to vancomycin is probably due to the presence of a thickened cell wall with reduced levels of peptdoglycan cross-linking [118]. Mutations in several structural or regulatory genes as well as over- or under-expression of genes, including regulators, have been hypothesized to be involved in the resistance. It has also been proposed that anomalous diffusion of vancomycin through the VISA cell wall, caused by clogging of the cell wall with vancomycin itself, associated with cell wall thickening enables VISA to prevent vancomycin from reaching its true target in the cytoplasmic membrane [32].

15.10.2 Vancomycin-Resistant S. aureus (VRSA Strains)

In 2002, the first two clinical infections due to a MRSA exhibiting a high-level of resistance to glycopeptides were reported [79, 108]. These two VRSA (Vancomycin-Resistant *S. aureus*) strains harbor a plasmid-borne Tn*1546* element. Since then,

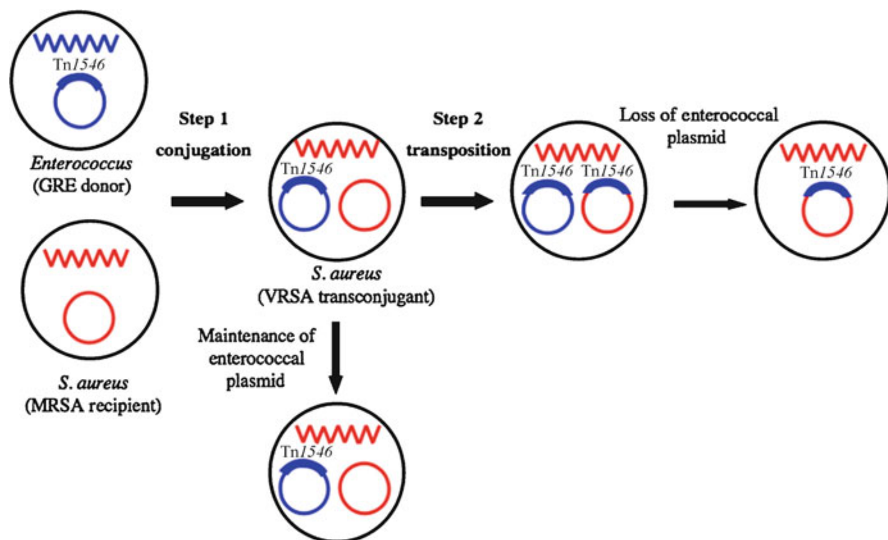

Fig. 15.9 Schematic representation of Tn*1546* transfer from *Enterococcus* spp. to *S. aureus*. *Blue* and *red* wavy lines represent chromosomal DNA of *Enterococcus* and *S. aureus*, respectively. *Blue circle*, enterococcal plasmid with a broad host range of transfer. *Red circle*, resident staphylococcal plasmid

seven other VRSA strains were isolated in the United-States [109], one in South Asia [104], and one in Iran [3]. However, a very few data are available for the Indian and Iranian strains. In all cases, the *vanA* gene cluster was located on a plasmid. Except for VRSA-8 a vancomycin-resistant enterococci (VRE) was co-isolated with the VRSA strains strongly suggesting that VRSA have acquired Tn*1546* from a VRE [90, 119, 126]. One or two genetic events are involved in the acquisition of the vancomycin resistance from a VRE donor to a MRSA recipient strain. The first step is acquisition by the MRSA of the enterococcal plasmid. The VRE plasmid bearing Tn*1546* could be maintained into the recipient MRSA or the Tn*1546* transposon could move in a second step from the enterococcal plasmid to a resident *S. aureus* replicon, the VRE plasmid thus behaving as a suicide delivery vector (Fig. 15.9).

The efficient heterologous expression as well as the inducibility of the glycopeptide resistance genes in the VRSA has been demonstrated [90, 91]. However, two VRSA strains differ from the others by their levels of resistance to glycopeptides [67, 79]. These strains are moderately resistant to vancomycin and to teicoplanin. As opposed to these two strains, all of the other VRSA exhibit high-level of resistance to both glycopeptides. In the low-level resistant strains, glycopeptide resistance is lost at a high frequency (ca. 50%) after overnight culture without pressure selection, whereas, in the same conditions, resistance is fully stable in the other VRSA [90, 91]. Of interest, the two low level resistant strains harbor an insertion sequence that is responsible for inactivation of the transposase of Tn*1546*. Thus, Tn*1546* is

frozen on the enterococcal plasmid that replicates inefficiently in *S. aureus*. Induction of resistance by vancomycin is greatly delayed (>8 h) in comparison with the highly resistant VRSA (ca. 3 h) [90, 91]. Delay before expression of resistance is due to the time necessary for synthesis of peptidoglycan precursors ending in D-Lac and for elimination of the susceptible pathway by the sequential action of VanX and VanY. Low-level resistance is not due to inefficient expression of the *van* operon, but is likely due to the combination of the long lag phase and instability of the genetic element carrying the *vanA* operon.

Interestingly, one of the low-level resistant strains was isolated from a patient who had not been hospitalized in the previous 5 years nor had received vancomycin during the 5 years period preceding the infection, suggesting that the organism was acquired in the community [120].

One VRSA strain has the peculiarity to be partially dependent of vancomycin to grow [80]. As observed in enterococci, vancomycin dependence in this strain is due to a mutation in the host D-Ala:D-Ala ligase.

To date, all the VRSA strains are resistant to glycopeptides and methicillin. Interestingly, association of a glycopeptide with oxacillin has a strong synergistic effect against VRSA strains both *in vitro* and in an animal model [47, 91]. This synergism is likely due to the fact that pentadepsipeptide precursors, which are mainly synthesized in the presence of glycopeptides, are likely not substrates for the PBP2′, which is the only transpeptidase that remains active in the presence of oxacillin [107]. Thus, it is conceivable that infections due to VanA-type MRSA strains, which are highly resistant to glycopeptides and oxacillin, could be treated by a combination of the two drug classes.

15.10.3 Biological Cost of the VanA-Type Resistance in VRSA

Biological cost is one of the factors that determines the stability and dissemination of antibiotic resistance. Worldwide dissemination of MRSA clones has been associated with their ability to compensate for the cost of harboring the staphylococcal chromosomal cassette *mec* (SCC*mec*) element that confers methicillin resistance [43]. Deletion of the *mecA* gene in certain VISA isolates suggests that simultaneous resistance to β-lactams and glycopeptides is too costly for *S. aureus* [82]. Quantification of the exponential growth rates revealed that (1) in the absence of vancomycin the growth rates of the VRSA were similar to that of susceptible MRSA, indicating that the fitness reduction of the resistant strains due to acquisition of Tn*1546* was minimal in the absence of induction, and (2) when resistance was induced by vancomycin, there was an important reduction of the growth rate of the VRSA relative to their non induced counterparts and to the susceptible MRSA [46]. The reduction in fitness following induction was evaluated as ca. 20–38%, indicating that VanA-type resistance is associated with an important biological cost for the host. Competition experiments between isogenic VRSA transconjugant and MRSA recipient revealed a competitive disadvantage of 0.4–3% per ten generations of the

transconjugant versus the recipient when the two strains were mixed in the same environment without inducer. This slight fitness burden can be attributed to the basal level of expression of the *van* operon combined with a gene dosage effect due to the presence of the cluster on a multicopy plasmid. The observation that, in competition experiments that mimic best natural conditions, the transconjugant is more rapidly eliminated than the MRSA recipient could explain, in part, the low level of dissemination of the VRSA clinical isolates. However, the minimal fitness cost in the absence of induction could lead to selection of compensatory mutations that will restore fitness of the host while retaining resistance.

15.10.4 Dissemination of VanA-Type Vancomycin Resistance among S. aureus

The spread of VRSA is, to date, limited. It has been hypothesized that the nature of the enterococcal plasmid bearing the vancomycin resistance gene cluster could play a role in the transfer of resistance [126]. Furthermore, it has been shown that a Sau1 type I restriction-modification system blocks transfer of mobile genetic elements from other species to *S. aureus* and limits the spread of resistance genes between isolates of different *S. aureus* lineages [117]. In addition, certain strains appear to be better recipients than others [111].

Associated to these molecular factors, several other factors such as population characteristics, prevalence of diabetes, of end-stage renal diseases, and of patients receiving dialysis (that is considered to be an important risk factor of MRSA infection and responsible for prolonged exposure to vancomycin), have been suggested to be implicated in a regional emergence of VRSA in Michigan [109].

Although it is of serious concern for patients infected with such bacteria, it seems that, due to several biological constraints, dissemination of VRSA has so far been limited.

15.11 Resistance to New Glycopeptides

The increase in multi-resistance of Gram-positive bacteria has resulted in the development of new antibiotics against these organisms. Among them, novel glycopeptides such as oritavancin, telavancin, and dalbavancin constitute an interesting advance.

15.11.1 Oritavancin

Binding affinity of oritavancin (previously known as LY333328), a derivative of the natural glycopeptide chloroeremomycin, which differs of vancomycin by having an

additional monosaccharide moiety (4-*epi*-vancosamine), to free D-Ala-D-Ala and D-Ala-D-Lac is similar to that of vancomycin and the general spectrum of activity is comparable between the two molecules (Table 15.2) [114]. Oritavancin inhibits late stages biosynthesis of peptidoglycan, as does vancomycin, but the addition of a p-chlorophenylbenzyl side chain to an epivancosamine carbohydrate seems to augment dimerization between oritavancin molecules and thereby facilitates hydrophobic interactions with the bacterial cytoplasmic membrane [4]. Oritavancin inhibits bacterial transglycosylase and transpeptidase. Although oritavancin is active against VanA- and VanB-type enterococci [12, 81] a reduced susceptibility (oritavancin MIC, 8–16 mg/L) could be obtained in these enterococci when the expression of the resistance genes was increased or after complete elimination of the peptidoglycan precursors ending in D-Ala-D-Ala due to a mutation in the *ddl* gene [12]. Cross-resistance to teicoplanin and oritavancin could occur in the VanB-type strains when the $vanS_B$ sensor suffered various mutations. A clinical vancomycin-dependent strain, which exhibits reduced susceptibility to oritavancin, was also isolated from a patient having received multiple courses of either vancomycin or teicoplanin [122]. Thus, it has been hypothesized that emergence of oritavancin resistance should be anticipated since mutations in *ddl* and $vanS_B$ could be selected under treatment and exist in natural population of VanA- and VanB-type enterococci [12].

15.11.2 Telavancin

Telavancin is another semi-synthetic derivative of vancomycin with a broad spectrum of activity against clinically important Gram-positive bacteria. This drug possesses multiple modes of action, including the depolarization and permeabilization of the bacterial membrane. Telavancin is active against vancomycin-resistant enterococci of the VanB-type (Table 15.2) [69, 70, 114]. To the best of our knowledge, no data concerning the resistance to this molecule are available.

15.11.3 Dalbavancin

The structure of dalbavancin, a semi-synthetic derivative of the natural glycopeptide A-40926, is related to that of teicoplanin. This new molecule is not more active than vancomycin or teicoplanin against VanA-type enterococci [29, 114] and is active against a low-level resistant VRSA [27]. As observed for teicoplanin, the VanB-type strains are susceptible to dalbavancin (Table 15.2). No stable mutant with decreased susceptibility to dalbavancin could be obtained in vitro with staphylococci [54].

Table 15.2 Pharmacological properties of glycopeptides

Properties	Vancomycin	Oritavancin	Telavancin	Teicoplanin	Dalbavancin
Semi-synrthetic derivative of	-	Chloroeremomycin	Vancomycin	–	A-40926
Binds to					
D-Ala-D-Ala	Yes	Yes	Yes	Yes	Yes
D-Ala-D-Lac	No	Yes	Yes	No	No
Pharmacodynamic profile					
Staphylococcus	Bactericidal	Bactericidal	Bactericidal	Bactericidal	Bactericidal
Enterococcus	Bacteriostatic	Bactericidal	Bactericidal	Bacteriostatic	Bactericidal
Activity against					
MSSA	S	S	S	s	S
MRSA	S	S	S	s	S
VISA	I	S	S	R	S
VanA-type VRSA	R	S	R	R	R
Enterococci	s	S	S	S	S
VanA-type VRE	R	S	R	R	R
VanB-type VRE	R	S	S	S	S
VanC-type VRE	R	S	S	S	S

MSSA methicillin-susceptible *S. aureus*, *MRSA* methicillin-resistant *S. aureus*, *s* active, *S* highly active, *R* resistant, *VISA* Vancomycin-intermediate *S. aureus*, *VRE* Vancomycin-resistant enterococci, *VRSA* Vancomycin-resistant *S. aureus*

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Chapter 16
Acquired Tetracycline Resistance Genes

Marilyn C. Roberts

16.1 Introduction

Tetracycline continues to be used as treatment for a variety of infections due to Gram-positive, Gram-negative, intracellular bacteria, some protozoan infections, and noninfectious conditions [12, 65]. Tetracycline is also an important antibiotic for prophylaxis or treatment either alone or in combination with other antibiotics and for agents of biological terrorism [*Bacillus anthracis*, *Francisella tularensis,* and/or *Yersinia pestis*] [64]. Hughes and Data [29] examined *Enterobacteriaceae* collected between 1917 and 1954 for tetracycline resistance [Tc^r] and found that 2% of the 433 isolates were Tc^r, with the first Tc^r bacteria found in the 1950s isolates. From this work, it was suggested that the discovery of Tc^r bacteria was a relatively recent event and an unintended consequence of tetracycline use in clinical, veterinary, and agricultural experiments since the 1950s.

The primary mechanism responsible for Tc^r bacteria was found due to the acquisition of new genes, which produce new proteins in the clinically relevant Tc^r bacteria. Their tetracycline resistance [*tet*] genes are often associated with conjugative and/or mobilizable plasmids and transposons. These elements allow transfer of the *tet* genes within and between species, genera, and ecosystems [63]. Mutations within the host chromosomal genes, which alter susceptibility to tetracyclines have recently been identified. Mutations that alter the 16S rRNA of *Brachyspira hyodysenteriae* and *Helicobacter pylori* are responsible for increased Tc^r, while mutations which alter expression of innate multidrug resistance genes [MDR] from *Acinetobacter baumannii, Enterobacter aerogenes, Neisseria gonorrhoeae, Pseudomonas aeruginosa, Serratia marcescens,* and *Stenotrophomonas* spp., also

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alter Tc^r [12, 35, 55, 60, 65, 79]. MDR genes are discussed in more detail elsewhere in the book, and this chapter will focus on Tc^r due to acquisition of new genes.

The first tetracycline resistant (Tc^r) and multidrug resistant, which included Tc^r, bacteria was Japanese *Shigella* and *E. coli,* were isolated in 1950s. The Tc^r and multidrug resistance phenotypes were transmissible to susceptible recipients under laboratory conditions and represent the first description of conjugative transmission of plasmids [100]. Later it was determined that these bacteria carried *tet* genes coding for efflux proteins on conjugative plasmids. In the Mendez et al. [48] paper, the authors describe heterogeneity among the Tc^r bacteria as well as the first indication that there were distinctive genes that could be identified in Gram-negative bacteria of *tet* genes. Different types of *tet* genes were defined as having $\leq$79% amino acid identity with all previous characterized *tet* genes. Thus two genes were considered part of the same type/class and given the same gene designation when they share $\geq$80% amino acid sequence identity over the entire length of the protein. In 1999, a nomenclature system was proposed, which is still followed today. For a *tet* gene to get a new designation, it has to be completely sequenced and its amino acid composition compared with all currently known classes of *tet* genes; then it must be submitted to the *tet* nomenclature clearing house, used for naming new *tet* resistance genes, and run by Dr. Stuart Levy and Ms. McMurry [85]. Before a potential gene can be given a new designation, it is necessary to conduct experiments to demonstrate that the gene confers Tc$^{r.}$.It is not adequate to show a gene sequence has characteristics in common with previously characterized *tet/otr* genes. Thus simple data mining of genomes looking for DNA and amino acid similarities to known *tet/otr* genes are not adequate. Obtaining the new *tet* gene name should be done prior to submitting the sequence to GenBank or on a manuscript for publication [41]. The new gene and its GenBank number will be forwarded to me where it will be added to the Tetracycline Nomenclature website (http://www.faculty.washington.edu/marilynr/).) More recently hybrid ribosomal protection *tet* genes have been described. These are *tet* genes that have regions corresponding to two or more different characterized *tet* genes. Various combinations of the different *tet* genes exist, such as combinations between the *tet*(O) and *tet*(W) genes, found in *Megasphaera elsdenii*, or the *tet*(O) and *tet*(32) genes found in a *Clostridium*-like bacteria [82, 84]. The only way mosaic *tet* genes can be identified is by sequencing the complete gene. These genes will be discussed further in a later in the chapter.

There are 43 different genes listed in Table 16.1, which confer resistance to tetracyclines. The *otr* genes were originally found in antibiotic producing *Streptomyces,* and their designation has not been changed even though the *otr* genes are now found in *Mycobacterium* spp. (Table 16.2). Twenty-seven genes code for energy-dependent efflux proteins, 11 to 12 genes, which code for ribosomal protection proteins, 3 genes that code for inactivating enzymes, and 1 gene has an unknown mechanism of resistance (Table 16.1). These genes are widely distributed and have been identified in 47 Gram-positive/cell-wall-free/*Mycobacterium/Nocardia/Streptomyces* and 78 Gram-negative genera (Table 16.2). In 2001, a website (http://faculty.washington.edu/marilynr/) was established that provides tables which list the mechanisms and

Table 16.1 Mechanism of resistance for characterized *tet* and *otr* genes

Efflux (27)	Ribosomal Protection (11)	Enzymatic[a] (3)	Unknown[b] (1)
tet(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E)	*tet*(M)[c], *tet*(O)[c], *tet*(S)[c], *tet*(W)[c]	*tet*(X)	*tet*(U)
tet(G), *tet*(H), *tet*(J), *tet*(V), *tet*(Y)	*tet*(32)[d] *tet*(Q)[c], *tet*(T), *tet*(36)	*tet*(37)	
tet(Z), *tet*(30), *tet*(31), *tet*(33)	*otr*(A), *tetB*(P)[e], *tet*(44)	*tet*(34)	
tet(39), *tet*(41)			
tet(K)[c], *tet*(L)[c], *tet*(38)			
tetA(P)[e], *tet*(40)			
otr(B), *otr*(C)			
tcr, *tet*(42)[f]			
tet(35)[g]			
tet(43)			

[a] *tet*(X) and *tet*(37) are unrelated but both are NADP-requiring oxidoreductases: *tet*(34) similar to the xanthine-guanine phosphoribosyl transferase genes of *V. cholerae*
[b] *tet*(U) has been sequenced but does not appear to be related to efflux, ribosomal protection proteins or enzymatic
[c] Found in both Gram-positive and Gram-negative bacteria
[d] The *tet*(32) gene has both ends of the gene which are 97–100% identical to the ends of *tet*(O) gene with the middle of the gene from bp 244 to 1,263 of unknown sequences which have been given the designation *tet*(32) thus the new name is *tet*(O/32/O) [84, 85]
[e] *tetB*(P) is not found alone and *tetA*(P) and *tetB*(P) are counted as one operon
[f] Found in subsurface Gram-positive and Gram-negative species
[g] Not related to other *tet* efflux genes. http://www.faculty.washington.edu/marilynr/

distribution of the *tet/otr* genes among different Gram-negative and Gram-positive genera and also representative GenBank numbers for each sequenced *tet/otr* gene. The information on the website and the Tables in this chapter are taken from the work of Dr. Roberts' laboratory as well as from published papers and abstracts presented at scientific meetings. From the later two sources, the data may not have been independently verified. The Gram-negative *tet* genes are found exclusively in Gram-negative bacteria, the Gram-positive *tet* genes may also be carried by Gram-negative and *Streptomyces* while the *otr* and Gram-positive *tet* genes are found in *Mycobacterium, Nocardia,* and *Streptomyces* (Table 16.2).

Carriage of multiple copies of the same ribosomal protection *tet* gene, carriage of two different ribosomal protection *tet* genes, and/or carriage of efflux and ribosomal protection *tet* genes have been identified in individual Gram-positive isolates [43, 90]. *Mycobacterium* and *Streptomyces* isolates have also been identified that carry multiple *tet/otr* genes [20]. However, multiple *tet* genes were found in < 10% of the Gram-negative isolates from early studies [49, 72]. This distinction has changed recently, as illustrated by a study of *E. coli* O157:H7 with four (33%) of the 12 human Tc^r isolates carrying two different *tet* genes [96]; another study [9] found that > 30% of the *E. coli* isolated from pigs, turkeys, and horses carried 2–3 different *tet* genes. In a 2004 study, on 52 Tc^r *Salmonella enterica* subsp., Enterica serovar Typhimurium isolated from animals, food, and humans in Italy, it was found that seven (71%) carried multiple different *tet* genes [58]. These studies suggest that Gram-negative bacteria from some host species now carry multiple *tet* genes and this trend is likely to continue.

Table 16.2 Distribution of *tet* and *otr* genes

Gene	G+C content (%)	Number genera	Genera
Efflux			
tet(A)	62–63	21	*Aeromonas, Actinobacillus, Acinetobacter, Bordetella, Citrobacter, Chryseobacterium, Edwardsiella, Enterobacter, Escherichia, Flavobacterium, Klebsiella, Laribacter, Plesiomonas, Proteus, Pseudomonas, Salmonella, Serratia, Shigella, Veillonella, Vibrio, Variovorax*
tet(B)	42–43	29	*Actinobacillus, Acinetobacter, Aeromonas, Aggregatibacter, Citrobacter, Brevundimonsa, Erwinia, Enterobacter, Escherichia, Gallibacterium, Haemophilus, Klebsiella, Mannheimia, Moraxella, Neisseria, Pantoea, Pasteurella, Photobacterium, Plesiomonas, Proteus, Providencia, Pseudomonas, Roseobacter, Treponema, Salmonella, Serratia, Shigella, Vibrio, Yersinia*
tet(C)	61–62	16	*Aeromonas, Bordetella, Citrobacter, Chlamydia, Enterobacter, Escherichia, Francisella, Halomonas, Klebsiella, Proteus, Pseudomonas, Roseobacter, Salmonella, Serratia, Shigella, Vibrio*
tet(D)	56–58	17	*Aeromonas, Alteromonas, Citrobacter, Edwardsiella, Enterobacter, Escherichia, Halomonas, Klebsiella, Morganella, Pasteurella, Photobacterium, Plesiomonas, Salmonella, Shewanella, Shigella, Yersinia, Vibrio*
tet(E)	47	10	*Aeromonas, Alcaligenes, Escherichia, Flavobacterium, Providencia, Proteus, Pseudomonas, Roseobacter, Serratia, Vibrio*
tet(G)	58	15	*Acinetobacter, Brevundimonsa, Enterobacter, Escherichia, Fusobacterium, Mannheimia, Ochrobacterum, Pasteurella, Providencia, Proteus, Pseudomonas, Roseobacter, Salmonella, Shewanella, Vibrio*
tet(H)	41	8	*Actinobacillus, Acinetobacter, Gallibacterium, Histophilus, Mannheimia, Moraxella, Pasteurella, Psychrobacter*
tet(J)	38	3	*Escherichia, Morganella, Proteus*
tet(K)	28	14	*Bacillus, Clostridium, Enterococcus, Eubacterium, Gallibacterium, Haemophilus, Lactobacillus, Listeria, Mycobacterium, Nocardia, Peptostreptococcus, Staphylococcus, Streptococcus, Streptomyces*
tet(L)	35–40	37	*Actinobacillus, Acinetobacter, Actinomyes, Bacillus, Bifidobacterium, Citrobacter, Clostridium, Enterococcus, Enterobacter, Escherichia Flavobacterium, Fusobacterium, Gallibacterium, Geobacillus, Kurthia, Listeria, Mannheimia, Morganella, Mycobacterium, Nocardia, Oceanobacillus, Ochrobactrum, Pasteurella, Paenibacillus, Pedicoccous, Peptostreptococcus, Proteus, Pseudomonas, Rahnella, Salmonella, Staphylococcus, Sporosarcins, Streptococcus, Streptomyces, Vagococcus, Variovorax, Veillonella*

tetA(P)	30	1	*Clostridium*
tet(V)	67	1	*Mycobacterium*
tet(Y)	48	2	*Aeromonas*, *Escherichia*
tet(Z)	63	2	*Corynebacterium*, *Lactobacillus*
tet(30)	59	1	*Agrobacterium*
tet(31)	41	1	*Aeromonas*
tet(33)	46	1	*Corynebacterium*
tet(35)	46	2	*Stentrophomonas*, *Vibrio*
tet(38)	32	1	*Staphylococcus*
tet(39)	40	9	*Acinetobacter, Alicaligenes, Bacillus, Brevundimonas, Cellulosimicrobium, Enterobacter, Lysinibacillus, Providencia, Stenotrophomonas*
tet(40)	55	1	*Clostridium*
tet(41)	67	1	*Serratia*
tet(42)	67	6	*Bacillus, Micrococcus, Microbacterium, Paenibacillus, Pseudomanas*, *Staphylococcus*
tet(43)		?	from metagenomic DNA
tcr	72	1	*Streptomyces*
otr(B)	71	2	*Mycobacterium, Streptomyces*
otr(C)	70	1	*Streptomyces*
Ribosomal protection			
tet(M)	35–36	67	*Abiotrophia, Acinetobacter, Actinomyces, Aerococcus*, *Aeromonas*, *Afipia*, *Anaerococcus, Arthrobacter*, *Bacteriodes, Bacillus, Bacterionema, Bifidobacterium, Brachybacterium, Citrobacter, Catenibacterium, Clostridium, Corynebacterium, Edwardsiella, Eikenella, Enterococcus, Enterobacter, Erysipelothrix, Escherichia,Eubacterium, Finegoldia, Flavobacterium, Fusobacterium, Gardnerella, Gemella, Granulicatella, Haemophilus, Hafnia, Kingella, Klebsiella, Kurthia, Lactobacillus, Lactococcus, Lawsonia, Listeria, Microbacterium, Mycoplasma, Mycobacterium, Neisseria, Pantoea, Paenibacillus, Pasteurella, Peptostreptococcus, Photobacterium, Prevotella, Proteus, Providencia, Pseudoalteromonas, Pseudomonas, Psychrobacter, Ralstonia, Rhanela, Selenomonas, Serratia, Shewanella, Shigella, Sporosarcina, Staphylococcus, Streptococcus, Streptomyces, Ureaplasma, Veillonella, Vibrio*

(continued)

Table 16.2 (continued)

Gene	G+C content (%)	Number genera	Genera
tet(O)	39–40	22	*Anaerovibrio, Aerococcus, Actinobacillus, Butyrivibrio, Bifidobacterium, Campylobacter, Citrobacter, Clostridium, Enterococcus, Eubacterium, Fusobacterium, Gemella, Granulicatella, Lactobacillus, Megasphaera, Mobiluncus, Neisseria, Pasteurella, Peptostreptococcus, Psychrobacter, Staphylococcus, Streptococcus*
tetB(P)	32	1	*Clostridium*
tet(Q)	39	19	*Anaerovibrio, Bacteriodes, Capnocytophaga, Clostridium, Eubacterium, Fusobacterium, Gardnerella, Lactobacillus, Mitsuokella, Mobiluncus, Neisseria, Peptostreptococcus, Prevotella, Porphyromonas, Ruminococcus, Selenomonas, Streptococcus, Subdoligranulum, Veillonella*
tet(S)	33–34	7	*Citrobacter, Enterococcus, Lactococcus, Lactobacillus, Listeria, Staphylococcus, Veillonella*
tet(T)	32	2	*Enterococcus, Streptococcus*
tet(W)	50–55	25	*Acidaminococcus, Actinomyces, Arcanobacterium, Bacillus, Bacteriodes, Bifidobacterium, Butyrivibrio, Citrobacter, Clostridium, Escherichia, Fusobacterium, Lactobacillus, Klebsiella, Mitsuokella, Megasphaera, Neisseria, Prevotella, Porphyromonas, Roseburia, Selenomonas, Staphylococcus, Streptococcus, Streptomyces, Subdoligranulum, Veillonella*
tet(32)	41	3	*Clostridium, Eubacterium, Streptococcus*
tet(36)	36	3	*Bacteroides, Clostridium, Lactobacillus*
tet		1	*Streptomycetes*
otr(A)	72	3	*Bacillus, Mycobacterium, Streptomycetes*
Enzymatic			
tet(X)	37	3	*Bacteroides, Pseudomonas, Spingobacterium*
tet(34)	43	4	*Aeromonas, Pseudomonas, Serratia, Vibrio*
tet(37)	38	?	from metagenomic DNA
Unknown mechanism			
tet(U)	38	2	*Enterococcus, Staphylococcus*

http://www.faculty.washington.edu/marilynr/ ([1, 30, 32–34, 47, 56]; Nonaka, Ikeno and Suzuki, 2007; [59, 63, 81])

16.2 Efflux Proteins

The efflux proteins are the best studied of the Tet protein and were first identified in the 1950s associated with mobile elements [100]. All 27 of these genes code for energy-dependent membrane-associated proteins, which export tetracycline and related compounds from the cell (Table 16.1). This action reduces intracellular concentrations and allows most of the ribosomes to continue to function. The efflux proteins exchange a proton for a tetracycline-cation complex against a concentration gradient and require intact cells to function. The efflux genes are the most commonly found *tet* genes in aerobic and facultative Gram-negative bacteria [65, 85]. Most of these Tet proteins confer resistance to tetracycline and doxycycline but not to minocycline or tigecycline, a newer glycylcycline. The exception is the Gram-negative *tet*(B) gene which confers resistance to tetracycline, doxycycline and minocycline but not tigecycline.

Upstream of the structural efflux gene is a divergently transcribed repressor gene that produces a protein that binds to the palindromic operator in the promoters for both the repressor and structural *tet* gene and blocks initiation of transcription. At ~1 nM tetracycline, a tetracycline-divalent cation complex interacts with the repressor protein, releasing it from the DNA and transcription of both genes occurs. A more detailed description of mechanism of tetracycline resistance due to the efflux proteins can be found in [62].

Over the last few years, the number of genera identified with known and new efflux *tet* genes has greatly increased, primarily due to the characterization of environmental isolates from soil and water sites (Table 16.2) [65]. Ten of the 27 efflux genes, *tetA*(P), *tet*(V), *tet*(30), *tet*(31), *tet*(33), *tet*(38), *tet*(40), *tet*(41)**,** *tcr*, and *otr*(C) have been found in a single genus each. However, most of these 10 genes have not been examined in surveillance studies, and thus no new genera have been identified. In other cases, the *tet* genes limited host range may be due to unique characteristics of the *tet* gene, lack of an associated mobile element, and/or the associated mobile element has a restricted host range. The G+C% of these 10 genes differ widely from the *tcr3* and *otr*(C) genes with high G+C of 69–72%, similar to the chromosome of *Streptomyces*, compared to the low G+C% of the *tetA*(P) [30%] and *tet*(38) genes [38%], which have similar G+C% in their bacterial host, *Clostridium* and *Staphylococcus* respectively.

The *tet*(J), *tet*(Y) and *tet*(35) genes are found in three, two, and two Gram-negative genera respectively. The *tet*(Z) gene is found in two Gram-positive genera, while the *tet*(K) [28%] and *tet*(L) genes [35–40%] are found in both Gram-positive and Gram-negative bacteria. The *tet*(42) [67%] has been identified in both Gram-positive and Gram-negative bacteria isolated from sediments extracted at 170–210 m below sea level [8]. The high G+C% of *tet*(42) gene was unexpected, since the other efflux genes, *tet*(K) and *tet*(L), found in both Gram-positive and Gram-negative genera have low G+C% and are thought to have originated in Gram-positive bacteria such as enterococci, streptococci, and staphylococci, which have chromosomal G+C%. The origin of the *tet*(42) gene is unclear. The *tet*(43) gene

has recently been isolated from metagenomic DNA, and little is known about this gene.

The *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), and *tet*(H) are found exclusively in Gram-negative bacteria, but only rarely in anaerobic Gram-negative genera. These seven *tet* genes differ in G+C% from *tet*(A) at 62–63% to *tet*(H) at 41%, and their distribution varies from two genera for the *tet*(35) gene to 29 genera for the *tet*(B) gene (Table 16.2).

The *tet*(A) gene has been identified in 21 different Gram-negative genera, and it is the only *tet* gene in *Chryseobacterium* and *Laribacter* spp. The *tet*(A) gene is usually associated with plasmids, but can be found in the 43 kb structure called *Salmonella* genomic island 1 [SGI1] in *Salmonella enterica* serotype Typhimurium [10, 73]. The *tet*(B) gene has the broadest host range, 67 genera, including enteric species and non-enteric species such as *Treponema denticola,* a bacteria related to *T. palladium* the causative agent of syphilis [70] (Table 16.2). The *tet*(B) gene is the only *tet* gene found in *Aggregatibacter*, *Erwinia*, and *Treponema*. In many cases, the *tet*(B) gene is associated with plasmids, although it can also be found in the chromosome in some *Clostridium perfringens*, *Haemophilus* spp., *Moraxella catarrhalis,* and *Treponema denticola* isolates [44, 45, 69, 70].

The *tet*(C) gene has been identified in 16 different genera, including an obligate intracellular Gram-negative bacteria, *Chlamydia suis* [22]. The *tet*(C) is usually associated with plasmids, though it is located in the chromosome in Tc^r *C. suis* that were originally isolated from the intestinal tract of pigs on Midwest farms, which had used tetracycline as growth promoters. The Tc^r *C. suis* isolates contained a13 kb of foreign DNA, including a truncated repressor gene, *tetR*(C), and a functional *tet*(C) gene. The 13 kb region has a high degree of identity with a pRAS3.2 plasmid from *Aeromonas salmonicida*, a microbe which does not grow in pigs [36]. More recently the same *tet*(C) gene was identified by PCR from Tc^r *C. suis* from pigs with conjunctival and/or reproductive disease in four different farms in the North and South of Italy, indicating that these *tet*(C) strains are not unique to North America [18]. This is the first report of a known acquired *tet* gene in an obligate intracellular bacteria and illustrates how the *tet* genes have spread through the bacterial populations and different ecosystems, despite the original hypotheses that intracellular bacteria such as *Chlamydia* spp., were unlikely to participate in conjugation with other genera because of the requirement for actively growing donor and recipient bacteria. This was assumed to be a major barrier, because most bacteria live outside eukaryotic cells, while *Chlamydia* spp., exist as physiologically inert particles outside eukaryotic cells and do not survive for long time periods [52]. Therefore, the only way an obligate intracellular *Chlamydia,* could have acquired the *tet*(C) gene is to assume that a second bacterium carrying the 13 kb region with the *tet*(C) gene co-infected the same eukaryotic cell as did the *C. suis*. *A. salmonicida* has an optimal growth temperature below 20°C, and since the *C. suis* and *A. salmonicida* have different optimal growth temperatures it is unlikely that the *A. salmonicida* was directly involved in the genetic transfer to the *C. suis*. Dugan et al. [22] suggested that the *tet*(C) gene and surrounding sequences may be on a moblizable element which was transferred from *A. salmonicida* into one or more other bacterial species,

which was able to exist within the pig intestinal tract and was the donor of the DNA into the *C. suis* within the pig. Sandoz et al. [71] have demonstrated that co-infection between Tc^r *C. suis* and susceptible *C. trachomatis* resulted in Tc^r *C. trachomatis,* which contain the 13 kb region from the pRAS3.2 plasmid inserted within the ribosomal cluster of their chromosome. These experiments provide support for the hypothesis that the 13 kb region is a mobile element. The presence of *tet*(C) positive Tc^r *C. suis* represent the first example of horizontal transfer of an antibiotic resistance gene by an obligate intracellular bacteria and now represents the first time a *tet* gene has been moved from one intracellular bacteria to another. It also suggests the potential for clinical *C. trachomatis* to acquire the *tet*(C), making them resistant to tetracycline therapy. *C. trachomatis* treatment failures need to be examined to determine if this resistance is occurring. The *tet*(C) gene is the only gene found in *Francisella*.

The *tet*(D) gene has been identified in 17 genera and is also normally associated with plasmids. The *tet*(D) gene is the only *tet* gene found in *Alteromonas*. The *tet*(E) gene is found in 10 genera and is more common among bacteria that live in water environments. The *tet*(E) gene has be associated with large nonconjugative plasmids, and it is not clear how it moves from one species to another [17]. The *tet*(E) gene is the only *tet* gene found in *Alcaligenes*. The *tet*(G) gene is found in 15 genera and may be found on plasmids or in the chromosome and in SGI1 and a new variant SGI1-O from *Proteus mirabilis* [6], while the *tet*(H) gene is found eight genera and is the only *tet* gene found in *Histophilus* (formerly part of the genus *Haemophilus*).

The *tet*(K) and *tet*(L) genes are found in both Gram-positive and Gram-negative genera as well as *Mycobacterium, Nocardia,* and *Streptomyces* spp. (Table 16.2). The *tet*(K) gene has been identified in 14 genera, including Gram-negative *Haemophilus*, *Gallibacterium, Mycobacterium, Nocardia,* and 10 classical Gram-positive genera. Ten of the fourteen of these genera carry both the *tet*(K) and *tet*(L) genes. The *tet*(K) gene is the only *tet* gene in *Nocardia* spp. [20]. The *tet*(L) gene is found in 18 Gram-positive and 19 Gram-negative genera (Table 16.2). The *tet*(L) gene is the only *tet* gene found Gram-negative *Rahnella*, *Sporosarcins,* and Gram-positive *Geobacillus,* O*ceanobacillus*, *Pediococcus*, *Vagococcus,* and *Virgibacillus*.

The *tet*(K) and *tet*(L) genes are associated with plasmids and are also found in the chromosomes of some bacteria such as *Bacillus subtilis* and *Staphylococcus aureus* [75, 88]. The *tet*(K) gene has been the most commonly found *tet* gene in *S. aureus* and methicillin resistant *S. aureus* [MRSA], though more recently environmental and carriage MRSA strains often carry the *tet*(M) gene (author's unpublished observations). The first well characterized *tet*(K) plasmid was pT181 from *S. aureus,* which was 4.4 kb. *S. aureus* strains isolated from Australian hospitals prior to 1970, commonly contained autonomous *tet*(K) positive pT181-like plasmids while *S. aureus* more recently typically carry the pT181-like plasmid integrated into the chromosome. Some MRSA strains have the *tet*(K) pT181-like plasmid inserted within the chromosomal *mec* region [75]. Plasmid integration within the *S. aureus* varies by country. The pT181 has also been found integrated into larger conjugative plasmids, which may code for mupirocin and/or other antibiotic resistances [53, 93].

The *tetA*(P) gene is the most commonly found *tet* gene in *Clostridium perfringens*; however, it is less common in other *Clostridium* spp. In our recent study characterizing USA environmental *C. perfringens*, we found 53% of the 160 isolates carried the *tetA*(P) gene [84]. The *tet*(39) gene is now found in nine genera, including three Gram-positive and six Gram-negative genera [1], while the *tet*(42) gene is found in five genera, two Gram-negative and four Gram-positive, isolated from deep terrestrial subsurface [8]. The *tet*(39) gene has been identified in 75% of the Tc^r *Acinetobacter* spp., isolated from water-sediment samples and manure in integrated fish farms in Thailand and on conjugative plasmids [2]. The *tet*(V) gene has been found in the chromosome of *Mycobacterium smegmatis* and *M. fortuitum* but not other *Mycobacterium* spp., [19], while the *tet*(35), *tet*(41), *tcr*, *otr*(B) and *otr*(C) genes are also located in the host chromosomes (Thompson et al. 2007; [93]). The *tet*(Z), *tet*(Y), and *tet*(33) genes are associated with plasmids [92]. It is not clear what sort of element the *tet*(42) gene is associated with, but since it is found in Gram-positive and Gram-negative genera, it is assumed that it is on a mobile element at least in some of the isolates characterized [8]. The new *tet*(43) gene has been isolated from metagenomic DNA and little is known about the gene (Table 16.2).

16.3 Ribosomal Protection Proteins

There have been 12 ribosomal protection genes characterized. These genes code for cytoplasmic proteins that protect the ribosomes from the action of tetracycline *in vitro* and *in vivo*. These genes confer resistance to tetracycline, doxycycline, and minocycline but not tigecycline [65]. The proteins have sequence similarity to the ribosomal elongation factors EF-G and EF-TU and are grouped in the translation factor super family of GTPases (Leipe et al. 2002). A model based on Tet(O)-mediated Tc^r biochemical and structural data for both Tet(M) and Tet(O) proteins has been proposed with the assumption that all of 11 proteins in this group have a similar mechanisms. In this model, the ribosomes without tetracycline function normally, and when tetracycline is added to the growth media, it binds to the ribosomes altering their conformational state, which interrupts the elongation cycle and protein synthesis stops. The ribosomal protection proteins are thought to interact with the base of h34 ribosomal protein, causing allosteric disruption of the primary tetracycline binding site(s), which releases the bound tetracycline. The ribosome returns to its normal conformational state and resumes protein synthesis. What is not clear is whether the *tet* ribosomal proteins actively prevent tetracycline from rebinding once it has been released or if the released tetracycline is able to rebind to the same or a different ribosome. More details can be found in previous publications [14, 15].

These *tet* genes predominate in Tc^r oral bacteria, anaerobic oral, and urogenital Gram-negative bacteria; however, they are less common among enteric Gram-negative bacteria. These genes have been found in most environments and ecosystems, and, as a group, have a host range varying from narrow (1 genus) to broad (67 genera). The *tet*(M) gene has been identified in clinical *Enterococcus* spp., isolated

between 1954 and 1955, which is approximately the same time as the first *tet* efflux genes were identified in Gram-negative Japanese isolates; however, the early *Enterococcus* spp., study was not published until 1997 [3, 91]. Thus both the *tet* efflux and *tet* ribosomal protection genes have been in the bacterial population for > 50 years. Forty-eight Gram-negative genera have been characterized which carry ≥ 1 ribosomal protection *tet* gene(s). Of these, 18 (38%) Gram-negative genera carry only ribosomal protection *tet* genes, while the remaining genera have isolates which carry efflux *tet* and/or ribosomal protection *tet* genes. Thirty-nine Gram-positive genera carry *tet* ribosomal protection genes, and 29 (74%) carry only ribosomal protection *tet* genes. The *tet*(M), *tet*(O), *tet*(Q), *tet*(S), *tet*(W) and *tet*(36) ribosomal protection genes are found in both Gram-positive and Gram-negative genera (Table 16.2).

Eight of the ribosomal protection genes have a G + C%, ranging from 32% to 40% and are thought to be of Gram-positive origin; however, the *tet*(W) gene has a 50–55% and its origin is unclear. The *Streptomycetes tet* and *otr*(A) genes have G + C% ranging from 68% to 78%, and their origin is thought to be *Streptomycetes*. Despite the difference in G + C%, the Tet(M) protein shares 68–72% amino acid identity with the Tet(O), Tet(S), Tet(W), and Tet(32) proteins. The Tet(Q) and Tet(36) proteins share 60% amino acid identity, and the Tet(T) shares 49% amino acid identity with both with the Tet(Q) and Tet(36) proteins. The ribosomal protection *tet* genes are the most common *tet* genes found in non-enteric Gram-negative, as well as anaerobic bacteria (Table 16.2).

The *tet*(32) gene is found in *Clostridium*, *Eubacterium*, *Streptococcus* and from the oral metagenome [38, 89]. The *tet*(36) has been identified in Gram-negative *Bacteroides* and Gram-positive *Clostridium* and *Lactobacillus* as well as from metagenomic DNA from manure pits [63, 95]. The *tet*(T) gene has been associated with *Enterococcus* and *Streptococcus*; the *tet* and *otr*(A) genes are found in *Streptomycetes* and *Mycobacterium*, and *Streptomycetes* respectively. The *tet*(W) gene has been identified in 15 Gram-negative and 10 Gram-positive genera and is the only *tet* gene identified in Gram-positive *Arcanobacterium* and *Roseburia* and Gram-negative *Acidaminococcus*. The *tet*(Q) gene is found in 12 Gram-negative and 10 Gram-positive genera. It is the only *tet* gene identified in Gram-negative *Capnocytophaga* and Gram-positive *Ruminococcus*. The *tet*(O) is the only *tet* gene currently found in Gram-negative *Campylobacter*. The *tet*(S) gene is found in four Gram-positive and three Gram-negative genera. The *tet*(M), *tet*(Q), and *tet*(W) are usually associated with conjugative transposons, while the *tet*(O) and *tet*(S) genes have commonly been associated with conjugative and nonconjugative plasmids (Table 16.2). These mobile elements will be discussed later in this chapter.

More papers have been written about the *tet*(M) gene than any other ribosomal protection *tet* gene. The *tet*(M) gene is commonly found in oral, urogenital, aerobic, and anaerobic Gram-positive and Gram-negative non-enteric bacteria; however, it is less common in enteric genera (Table 16.2). The *tet*(M) positive bacteria have been isolated from a variety of different species across the bacterial spectrum and from multiple, different ecosystems. Some variability at the base pair level is found and different *tet*(M) genes may have ≤11% of their base pairs which vary [85].

The *tet*(M) gene has been identified in 38 Gram-negative and 34 Gram-positive/cell-wall free genera; both aerobes and anaerobes and are commonly found in Tc^r oral and urogenital bacteria (Table 16.2). The *tet*(M) gene is the only gene found in 13 Gram-positive/cell-wall-free genera, including *Afipia*, *Abiotrophia*, *Anaerococcus*, *Arthrobacter, Bacterionema, Brachybacterium, Erysipelothrix, Granulicatella, Finegoldia*, *Catenibacterium*, *Mycoplasma*, *Sporosarcina,* and *Ureaplasma*. The *tet*(M) gene is found in a variety of conjugative transposons, which are often in the bacterial chromosome. In some *Clostridium perfringens* isolates, *tet*(M) gene is in the chromosome on an incomplete element and cannot move, while in other *C. perfringens* isolates the *tet*(M) gene is on complete transposons and can be conjugally transferred between isolates [84].

One exception of the chromosomal location for the *tet*(M) gene was found in the genus *Neisseria*. In *Neisseria gonorrhoeae,* the *tet*(M) gene was located on 25.2 Mda conjugative plasmids with the first *tet*(M) positive *N. gonorrhoeae* collected in 1983. These same *tet*(M) positive 25.2 Mda conjugative plasmids have since been found in Tc^r *N. meningitidis, Kingella denitrificans*, and *Eikenella corrodens* strains. All four species that carried these Tc^r plasmids could act as donors and conjugally transfer them to susceptible *N. gonorrhoeae, N. meningitidis*, commensal *Neisseria* spp., *Kingella denitrificans* recipients, *Eikenella corrodens,* and *Haemophilus influenzae* in the laboratory, but have not been normally identified in clinical commensal *Neisseria* spp., or *H. influenzae* [74, 75]. The *tet*(M) plasmids confer high levels of Tc^r (MIC $\geq$ 16 µg/ml) and are highly related to the 24.5 Mda indigenous *N. gonorrhoeae* conjugative plasmids [62]. It is thought that the *tet*(M) gene was transposed onto the *N. gonorrhoeae* 24.5 Mda conjugative plasmid creating the Tc^r plasmids. Two variance of the plasmids, with different amounts of the *tet*(M) conjugative element are widely distributed. Interestingly the Tc^r plasmids have a wider host range than the ancestral 24.5 Mda *N. gonorrhoeae* conjugative plasmid, but like the ancestral plasmid they were able to facilitate transfer of the small mobilizable gonococcal β-lactamase plasmids from one bacterium to another [62, 68]. Only part of the *tet*(M) transposon is found on the 25.2 Mda Tc^r *Neisseria* plasmids, while the complete *tet*(M) transposon was integrated into a Tc^r *Haemophilus ducreyi* conjugative plasmid [62]. The *tet*(M) gene is also found naturally in commensal *Neisseria* spp., but here the *tet*(M) genes are located in the chromosome. Today in some geographical locations, Asia, ~50% of the *N. gonorrhoeae* isolated carry these Tc^r plasmids.

The *tetB*(P) gene has been found only in the genus *Clostridium.* It is unique among the ribosomal protection genes because all isolates that carry this gene also carry a *tetA*(P) gene, which codes for an inducible efflux protein. The two genes are transcribed from a single promoter, which is located 529 bp upstream of the *tetA*(P) start codon and the *tetB*(P) gene overlaps the *tetA*(P) gene by 17 nucleotides [32]. The *tetA*(P) gene has been found alone where it does confer Tc^r to the bacterial host, while the *tetB*(P) gene has not. When the *tetB*(P) gene was cloned away from the *tetA*(P) gene and introduced into *Clostridium perfringens*, the natural host, and *E. coli* recipients, the resultant transformants had low-level Tc^r. Thus it is not clear if the *tetB*(P) gene contributes to the natural host's Tc^r phenotype. The *tetA*(P) and *tetB*(P) are often associated with conjugative and nonconjugative plasmids.

16.4 Mosaic Ribosomal Protection Proteins

Mosaic *tet* genes, consisting of regions from two known tet genes, have a descriptive designation such as *tet*(O/W) representing a hybrid between the *tet*(O) at one end and *tet*(W) at the other end of the gene while a *tet*(W/O/W) would represent a hybrid between the *tet*(O) and *tet*(W) genes with *tet*(O) sequence between the ends of the *tet*(W) gene. Mosaic genes can only be determined by sequencing the complete gene, and, at this time, the number of different genera with them is very limited. Three different hybrid genes have been sequenced from *Megasphaera elsdenii,* and the amino acids coded by the three genes share 95.8%, 89%, and 91.9% identity with the Tet W protein, with 13–43% of their sequences, at the ends of the gene, being related to *tet*(O) genes; although all three genes had 50–55% G+C similar to that of other *tet*(W) genes, and a hybrid designation was suggested for a new name for hybrids genes, which coded for proteins made of ≥50 amino acid residues in a single stretch which are from different genes [42].

The gene originally designated *tet*(32) from a *Clostridium*-like strain has been sequenced, and from bp 0 to 243, it had 100% identity with the same region in the *tet*(O) genes, and 158 bp non-coding region upstream of the structural gene showed 98% sequence homology with the upstream regions of the *tet*(O) genes from *S. mutans* and *Campylobacter jejuni*, GenBank # M20925, and M18896. The sequences at the end of the gene [1,262–1,782 bp] had a 98.8% sequence homology with the *tet*(O) gene. However, the sequences between bp 244–1,263 share < 70% similarity with any other known *tet* gene, and the overall DNA homology of the gene was < 80% to the *tet*(O) or any other *tet* gene and was given a new designation *tet*(O/32/O) [84]. In the original paper [46], they found by PCR that 6 of 9 rumen sheep samples and 8 of 11 pig fecal samples were positive for *tet*(32); however, in light of the more recent data on its hybrid nature, it is unclear if these positive samples were actually detecting the *tet*(O/32/O) sequence or different genes though nonmosaic *tet*(32) has been identified [63]). More recently, mosaic *tet* genes from *Bifidobacterium* and *Lactobacillus* have been identified, which combined sequences from *tet*(O), *tet*(W), and *tet*(32) genes [86].

16.5 Enzymatic Inactivation

Three genes coding for inactivating enzymes have been identified. The *tet*(X) gene encodes for a NADP-dependent monooxygenase, which requires oxygen to degrade tetracycline, including the recently introduced semi-synthetic drug tigecycline [51, 97]. The *tet*(X) gene was originally found in a strict anaerobe, *Bacteroides,* where it was linked to an rRNA methylase gene [(*erm*(F)], which confers resistance to macrolides, lincosamides, and streptogramin B, and is part of the conjugative transposon CTnDOT [94]. Because the TetX protein requires oxygen to degrade the tetracycline, the *tet*(X) gene does not confer Tc^r in the host *Bacteroides* spp. The *erm*(F) and *tet*(X) genes have a G+C% content of 36% and 37%, respectively, suggesting

that these gene did not originate in *Bacteroides* spp., and it has been suggested that the CTnDOT that was inserted within the *Bacteroides* spp., chromosome where the *tet*(X) gene has been maintained but not expressed while the *erm*(F) gene is functional [80]. Recently, a *tet*(X) positive aerobic Gram-negative Tc^r *Sphingobacterium* sp., isolated from agricultural soil, was identified, which degraded tetracycline, indicating that the *tet*(X) gene is functional in this aerobic host [25]. A12 kb region that included the *tet*(X) gene and up and downstream flanking regions were sequenced. The *Sphingobacterium* sp., *tet*(X) region shared organizational features and genes with the *Bacteroides tet*(X) gene and surrounding sequences found in the conjugative transposon CTnDOT [26, 74, 94]. However, unlike the *Bacteroides tet*(X) gene, the *Sphingobacterium tet*(X) gene was not linked to an *erm*(F) gene, though it did have features suggestive of those found in mobilizable transposons. However, we were unable to show conjugal transfer of the *tet*(X) gene under laboratory conditions [26]. In addition, the chromosome of *Sphingobacterium* sp., and the *tet*(X) gene had similar 37 G+C%, suggesting this or a related bacterium could be the ancestral source of the *tet*(X) gene now found in *Bacteroides* spp. This is the first report of the presence of the *tet*(X) gene in an aerobic bacterium. Whether the *tet*(X) gene is commonly carried by bacteria from agricultural soil or other environmental ecosystems is unknown.

The gene, *tet*(37), codes for a second NADP-dependent monooxygenase, which is unrelated to the *tet*(X) gene, but has a similar G+C% content of 37.9% [19]. Unlike all the other genes in Table 16.1, the *tet*(37) gene has only been cloned from the oral metagenome and no specific bacteria has been identified that carries this gene. The *tet*(34) gene was first described in *Vibrio cholerae* and codes for an enzyme that inactivates tetracycline but is similar to a xanthine-guanine phosphoribosyl transferase rather than a NADP-dependent monooxygenase [54]. More recently the *tet*(34) gene has been identified in *Aeromonas, Pseudomonas,* and *Serratia* (Table 16.2).

The enzymatic *tet* genes are found only in Gram-negative species. Six of the seven genera which carry one of these inactivating *tet* genes also may carry efflux and/or ribosomal protection *tet* genes, thus their contribution to bacterial Tc^r compared to the efflux and ribosomal protection *tet* genes is unclear. Perhaps, as more environmental bacteria are characterized, more genera carrying the enzymatic *tet* genes may be found and/or other inactivating *tet* genes will be characterized.

16.6 Unknown Gene, tet(U)

The *tet*(U) gene produces a small protein (105 amino acids), which confers low level tetracycline resistance (Chopra, [64]). The TetU protein has 21% similarity over its length to the TetM protein, but it does not include the consensus GTP-binding sequences, which are thought to be very important for tetracycline resistance in these proteins. The *tet*(U) gene has been identified in a vancomycin and tetracycline resistant *Staphylococcus aureus* that did not carry the *tet*(K), *tet*(L),

tet(M), or *tet*(O) genes. From the same patient, vancomycin resistant enterococci were cultured that carried both the *tet*(U) and *tet*(L) genes and a few isolates also carried the *tet*(K) and/or *tet*(M) genes [92]. The importance of the *tet*(U) gene is unclear, since both *Enterococcus* and *Staphylococcus* isolates are able to carry a variety of efflux and ribosomal protection *tet* genes.

16.7 Mobile Elements, Gene Transfer, and Linkages with Other Genes

Today, *tet* genes are found on conjugative, nonconjugative, and moblizable plasmids, transposons, conjugative transposons, and Salmonella Genomic Island 1. These mobile elements are primarily responsible for the lateral transfer of most antibiotic resistance genes and can lead to a rapid dissemination within and between bacterial communities from many different ecosystems. Mobile elements frequently carry other genes that confer resistance to antibiotics and/or heavy metals, that produce toxins, that have genes which allow for degradation of various compounds like toluene, and/or that code for other virulence factors. All the genes within a mobile element normally move as a unit from bacterium to bacterium [12]. It is currently assumed that mobility and type of element that a specific *tet* gene is associated with directly influences the genes host range and ability to spread to new genera and multiple ecosystems (Table 16.2) [12, 63].

Mobile elements include conjugative and moblizable plasmids, and transposons, which carry the genes needed to transfer between DNA sites within the cell, conjugative transposons, which carry the genes needed to transfer between bacterium, and integrons, which are elements that are able to collect gene cassettes containing different antibiotic resistance genes [61, 87]. However, plasmids often carry transposons and integrons, allowing for more mixing and rearranging of different antibiotic resistance genes to occur.

In general the *tet* genes coding for efflux proteins are associated with plasmids, and the very first Tc^r bacteria from Japan carried conjugative plasmids with multiple resistance genes [91]. Association with plasmids provides the flexibility of the *tet* genes to be linked with a large number of different antibiotic resistance genes. It also allows for the linked genes to be added or subtracted over time. In [50], Moller et al. found that patients treated for acne with low oral doses of tetracycline had an increase in the number of Tc^r and multidrug resistant *E. coli*. The level of multidrug resistant *E. coli* went from no patients before treatment to 50% of the patients by 4 week. The *tet* genes, coding for ribosomal protection are more often found on conjugative transposons in Gram-positive and Gram-negative bacteria in nature as well as transferred to Gram-positive and Gram-negative recipients in the laboratory (Table 16.2). In contrast, the Gram-negative *tet* genes are not found naturally in Gram-positive bacteria and usually do not confer Tc^r when cloned and placed into an *E. coli* recipient. This is also true for genes conferring antibiotic resistance to other classes of antibiotics such as macrolides [66].

Plasmids contain ≥ 1 entry exclusion gene, which reduces the host cell ability of acquiring other plasmids with the same origin of replication as the resident plasmid. Thus two plasmids with the same origin of replication are considered incompatible with each other whether they are conjugative or mobilizable [24]. Incompatibility of plasmids may limit the spread of some of plasmids and their associated *tet* genes between species and/or genera. Some *S. aureus* isolates have dealt with plasmid incompatibility by integrating their plasmids into their chromosome where the plasmid incompatibility does not function allowing the host to acquire multiple plasmids with the same origin of replication [27]. In contrast, plasmids with different origins of replication can co-exist within a single cell. Today, single plasmids may carry multiple different *tet* genes and/or an isolate may have different *tet* genes on different plasmids or some *tet* genes on plasmid(s) and other *tet* genes in the chromosome.

The *tet* plasmids come in a variety of sizes, 4.45 kb of *tet*(K) positive pT181 to large plasmids of ≥ 300 kb. Many of the large *tet* plasmids are conjugative or able to be mobilized; however, the *tet*(E) gene has been found on large plasmids ~170 kb, which are not conjugative or mobilizable under laboratory conditions [78]. Yet the *tet*(E) gene has been identified in 10 different genera, many of which are associated with water. How the *tet*(E) gene has spread between bacteria in nature is a mystery and indicates that we cannot yet reproduce all the possible mechanisms of gene exchange that occurs in nature under laboratory conditions [17, 78]. Gram-negative *tet* genes may be on transposons such as Tn*10,* which carries the *tet*(B) gene and is the best studied transposon [39]. Tn*10* is frequently associated with Gram-negative plasmids but can also be found in the chromosome of Gram-negative bacteria such as *H. influenzae* [45].

Transposons may link *tet* genes with other antibiotic resistance genes. For example, Tn*1545* a 15.3 kb conjugative transposon was first identified in the 1980s from *Streptococcus pneumoniae*. Tn*1545* carried the *tet*(M), *erm*(B), coding for a rRNA methylase, which confer resistance to macrolides, lincosamides, and streptogramin B, and an *aphA-3* gene, coding for kanamycin resistance. Multiple copies of this and other Tn*916*-Tn*1545* transposons were identified and integrated into a single bacterial chromosome [57]. Other types of elements have also been characterized such as the 43 kb chromosomal unit designated *Salmonella* genomic Island 1 (SGI1) found in the majority of epidemic multidrug resistant *Salmonella enterica* serovar Typhimurium phage type DT104 isolates. DT104 is pathogenic for both animals and man and has been isolated around the world. Within the SGI1 element, there is a 13 kb region that contains an antibiotic resistance gene cluster, which usually includes genes conferring resistance to tetracycline, ampicillin, chloramphenicol, spectinomycin, and sulfonamides [10]. The *tet*(A), or *tet*(B), or *tet*(G) have been found in this 13 kb region. The number and type of antibiotic resistance genes present varies over time and location, due to the gain, loss, and/or exchange of antibiotic resistance genes within the 13 kb region [87]. The SGI1 variants have been classified as SGI1, SGI1-A to SGI1-O [6, 10]. Normally the *tet* genes contain both the structural *tet* genes and the genes coding for the transcriptional repressors [*tetR* gene]. In some of the SGI1 variants, the *tet*(A) gene is downstream or upstream of

Tn*21* which contains seven genes of the mercury operon coding for organic and inorganic mercury resistance. In one isolate, the SGI1 element *tetR*(A) repressor gene was downstream of the *merR* gene, but the structural *tet*(A) gene was missing and thus this element did not confer Tc^r to its host bacteria [21]. Finding the *tetR*(A) gene without the *tet*(A) gene has not been commonly described in other bacteria. The SGI1 elements are able to conjugally transfer to non-SGI1 containing *S. enterica* and *E. coli* recipients under laboratory conditions. This may explain why the SGI1 is now found in a wide range of *S. enterica* serovars, and, more recently, why it has been identified in *Proteus mirabilis* strains [6, 21]. A new SGI2 element carrying a *tet*(G) gene has recently been described [40].

A 86 kb chromosomal antibiotic resistance island has been sequenced in an *Acinetobacter baumannii* strain AYE which carried two copies of the *tet*(A) gene along with a large number of other genes coding for antibiotic and heavy metal resistance [23]. It is not known if both the *tet*(A) genes in the *A. baumannii* strain AYE produce the structural TET protein. As more bacterial genomes are sequenced, it is likely that other chromosomal antibiotic resistance islands will be identified in other bacterial species. Interestingly, *tet* genes are not normally part of integrons.

The *tet*(M), *tet*(Q), and *tet*(W) genes are often found on conjugative transposons. Conjugative transposons carry all the genes needed to move from one bacterial cell to another by conjugation. The *tet* genes associated with conjugative transposons have fewer restrictions in moving between unrelated bacteria because they do not have incompatibility systems. As a result, multiple copies of the same or related conjugative transposons can be created in the laboratory and are found in natural isolates [57].

The Tn*916*-Tn*1545* transposons family is the most promiscuous of the conjugative transposons and one of the best characterized after Tn*10* transposons. The Tn*916*-Tn*1545* transposons family integrates site specifically in some species and relatively nonspecifically in other species [68]. The Tn*916* is 18 kb and has relatively few restriction sites. Why 70% of the unique restriction sites in Tn*916* are in a 2 kb region surrounding the *tet*(M) gene is not clear. Some investigators have suggested that the surrounding flanking regions represent ancient structures evolved for broad-host range transfer and the presence of the *tet*(M) gene in the Tn*916* is a relatively recent in the evolutionary progression of this element [68]. Low-dose exposure to tetracycline promotes the conjugal transfer of these transposons to neighboring bacterial cells. The Tn*916* can also mobilize co-resident plasmids.

The Tn*916*-Tn*1545* conjugative transposons are adaptable and able to form composite elements by integration of one transposon within another transposon. Both transposons encode for their own transfer, and the complete composite element may be transferred to another bacterium, or the embedded transposon can be transferred separately. Composite transposons may have multiple mobile elements, various types of insertion [IS] sequences as well as regions from plasmids and from different genera of bacteria. For example the 65 kb Tn*5385*, which carries resistance genes for penicillin, erythromycin, gentamicin, streptomycin, tetracycline, and mercury and has enterococci, streptococci, and staphylococci associated regions and sequences from three different transposons [68].

Conjugative transposons are able to exchange genes as demonstrated by a clinical isolate from 2004 with a Tn*916* element that had the *tet*(M) structural gene replaced by the highly related *tet*(S) gene. This modified element was conjugative under laboratory conditions and represents the first description of a *tet*(S) gene on a conjugative transposon [37]. Previously, the *tet*(S) gene was only found on conjugative and non-conjugative plasmids or in the chromosome where the *tet*(S) gene was unable to be conjugally transferred. Theoretically, the Tn*916*S element could have a host range similar to that of Tn*916* [56 genera] versus five genera with the *tet*(S) gene (Table 16.2). However, at this time no further identification of this Tn*916*S element has been reported.

New conjugative elements carrying *tet* genes continue to evolve as illustrated by the recent characterization of a ~ 60 kb conjugative transposon were the *tet*(O) gene was linked to an efflux *mef*(A)-*msr*(D) conjugative element. A 11,972 bp region was sequenced, and the *tet*(O) gene was linked to four new open reading frames downstream followed by three short sequences with homology for the mega element which includes *mef*(A)-*msr*(D)-*orf6*-*orf7*-*orf8* genes [7]. This conjugative element has been identified in the chromosome of *Streptococcus pyogenes* and has been transferred in the laboratory to *S. pyogenes* and unrelated *E. faecalis* recipients [28]. Whether the *tet*(O)-*mef* element was created in *S. pyogenes* or another bacteria is not clear.

In recent years, these transposons have acquired an increasing number of different antibiotic resistance genes and genes for heavy metal resistance (Table 16.3) [28, 37, 83]. For example, recently we described a novel non-composite conjugative transposon Tn*6009* containing a Tn*916* element linked to a *Staphylococcus aureus mer* operon carrying genes coding for inorganic mercury resistance [*merA*], an organic mercury resistance [*merB*], a regulatory protein [*merR*] and a mercury transporter [*merT*]. This transposon was identified in 66 isolates from two Gram-positive and three Gram-negative genera and is the first transposon in the Tn*916*-family to carry the Gram-positive *mer* genes directly linked to the *tet*(M) gene [83]. Other transposons related to the Tn*916*-Tn*1545* family include Tn*2009* and Tn*2010*, which have a *tet*(M) gene with an *erm*(B) gene inserted downstream of *tet*(M) gene in the same location as the *erm*(B) gene found in Tn*1545* which are linked to the macrolide resistance efflux genes, *mef*(A) and *msr*(D), upstream of the *tet*(M) gene [16]. Other assortments most certainly will be described in the future.

The *tet*(W) genes are also associated with one or more different types of conjugative transposons. Two different *tet*(W) transposons have been identified: one found in anaerobes and a second in the aerobic *Arcanobacterium pyogenes* [5]. In the last 4 years, 17 new genera have been identified carrying the *tet*(W) gene and many are associated with conjugative elements, but it is not clear if all of these *tet*(W) genes are associated with the same type of elements. The *tet*(W) gene is found among a number of different bifidobacteria species including a tetracycline susceptible isolate. In the susceptible isolate an IS30-like sequence of 736 bp interrupted the structural *tet*(W) gene [2].

Bacteroides spp., have conjugative transposons ranging in size [65-150 kb. One of the best studied is the CTnDOT 65 kb element which carries the tet(A) gene which is

Table 16.3 Tetracycline antibiotic resistance genes linked to other genes or elements

Gene	Linkage	Phenotype/element
Efflux		
tet(A)	bla_{TEM}	β-lactamase
	strA, strB	Streptomycin
	sul2	Sulfamethoxazole
	floR	Florfenicol/chloramphenicol
	SGI1	*Salmonella* genomic island 1
	mer operon	Mercury
	Tn*21*	Transposon
	Tn*1721*	Transposon
tet(B)	*tet*(M)	Tetracycline
	strA, strB	Streptomycin
	sul1, *sul2*	Sulfamethoxazole
	Tn*10*	Transposon
	bla_{TEM}	β-lactamase
	catA	Chloramphenicol
	int1	Class 1 integron
tet(G)	*floR*	Florfenicol/chloramphenicol
	sul1	Sulfamethoxazole
	cmlA9	Chloramphenicol
	SGI1	*Salmonella* genomic island 1
tet(H)	*sul2*	Sulfamethoxazole
	strA, *strB*	Streptomycin
tet(K)	*mec*	Methicillin
	dfrK	Trimethoprim
tet(L)	*dfrK*	Trimethoprim
	erm(T)	MLS_B[a]
tet(33)	*aadA9*	Aminoglycoside
	IS*6100*	Insertion sequence
tet(40)	*tet*(O/32/O)	Tetracycline (mosaic gene)
Ribosomal protection		
tet(M)	*erm*(B)	MLS_B
	mef(A)	Macrolide
	msr(D)	Macrolide
	aphA-3	Kanamycin
	tet(B)	Tetracycline
	mer operon	Mercury
	Tn*917*	Transposon
	Tn*916*-Tn*1545*	Transposon family
	Tn*5385*	Gm, SM, mer, Bla Tn*4001* Tn*552*
tet(O)	*mef*(A)	Macrolide
	msr(D)	Macrolide
tet(Q)	*erm*(B)	MLS_B
	erm(F)	MLS_B
	erm(G)	MLS_B
	mef(A)	Macrolide
	msr(D)	Macrolide
	rteABC	excision
	CTnDOT	*Bacteroides* conjugative transposon
tet(W)	Tn*B1230*	*Butyrivibrio* transposon
Enzymatic		
tet(X)	*erm*(F)	MLS_B

The Table illustrates examples of linkages found between *tet* genes and other antibiotic/heavy metal resistance genes, other important genes, and/or mobile elements. Many of the efflux *tet* genes are associated with large plasmids such as pK245 [98,265 kb] which carry multiple antibiotic resistance genes which are not all listed ([11, 21, 23, 31, 32]; Khachtryan et al. 2008;[66, 83, 84]

[a] MLS_B = resistance to macrolides, lincosamides and streptogramin B antibiotics

usually linked to the *erm*(F) gene, which codes for a methylase and confers resistance to macrolides, lincosamides and streptogramin B. In the presence of tetracycline, frequency of conjugal transfer increases and the CTnDOT was able to mobilize cor-resident nonconjugative transposons, mobilizable plasmids and unlinked integrated nonreplicating *Bacteroides* units [NBUs]. CTnDOT-like elements have been identified in a variety of different Gram-negative and Gram-positive genera [13].

Because the various *tet* genes are found in all of these different genetic elements they have been linked to a variety of other antibiotic resistance genes, genes coding for heavy metal resistance as well as specific mobile elements. A representative list of genes linked to *tet* gene are provided in Table 16.3. Only closely linked genes have been included in the table, though not every linkage described in the literature is represented. It is likely that new mobile elements will continue to be described over time allowing different *tet* genes to be linked with other resistance genes and the potential to expand their host range (Tables 16.2 and 16.3).

16.8 Future

The changes in the field over the last few years seem to be increasing more quickly than previously described (Tables 16.1 and 16.2). Whether this represents a true increase or whether the increase is due to screening of Tc^r bacteria from more diverse ecosystems, such as deep terrestrial sediments or from a larger number of geographic locations, including remote locations and/or a more diverse group of bacteria, is not clear. In addition, it is more common now to screen for the various ribosomal protection genes in Tc^r Gram-negative isolates which is illustrated in the diversity of genera now shown to carry the *tet*(M) or *tet*(W) genes (Table 16.2).

The distribution of different *tet* genes, even with the same mechanism of resistance, varies widely between different species of bacteria as well as different ecosystems (Table 16.2). Table 16.2 most likely underestimates the distribution of some of the *tet* gene. Updates to the distribution of *tet* gene can be found at the following URL: http://faculty.washington.edu/marilynr/, which is updated twice a year. Previously I had hypothesized that host range of specific *tet* genes could be influenced by their association with specific types of mobile elements and the *tet* genes associated with wide host range conjugative transposons are more likely to be found in a more diverse group of bacteria than *tet* genes associated with nonconjugative elements or plasmids with a narrow host range [63]. A good example is the *tet*(S) gene currently found in 11 genera. Now that the *tet*(S) has been integrated into a Tn*916*-like element, it may have the opportunity to spread more rapidly. Similarly, the recent discovery of the *tet*(O)-*mef*(A) element allowed easy transfer of the *tet*(O) gene to other unrelated recipients in the laboratory and the presence of this new mobile element may allow for a wider distribution of the *tet*(O) gene in nature as well [28]. Thus, future studies should include surveillance of the *tet*(O) gene.

Another question is the role, if any, of mosaic *tet* genes in the bacterial population. One possibility is that this may be a unique feature found in a small group of bacteria.

This theory is supported by finding the *tet*(O) or *tet*(W) genes in other bacteria from the same environments as the mosaic genes and finding a non-mosaic *tet*(O) gene in *M. elsdenii* [83]. Clearly more *tet*(W) and *tet*(O) genes need to be fully sequenced to answer this question. The ancestral source of the *tet*(32) gene, which also appears to have a mosaic structure is also of interest. These mosaic *tet* genes would not be detected if a PCR, which cover small regions of the gene and/or an individual probe, was used for genotyping *tet* genes.

The identification of two new *tet* genes coding for inactivating enzymes suggests that more work is needed to identify the ancestral origin(s) of these genes, their distribution in bacterial populations, and what role these enzymes might play in Tc^r in bacterial populations. It is also of interest in identifying the actual donor of the *tet*(C) gene to the *C. suis* and the steps leading to integration of this gene into the chromosome of an obligate intracellular bacteria. We also need to question whether the Tc^r *C. suis* is an indicator that other obligate intracellular bacteria may acquire *tet* or other commonly acquired antibiotic resistance genes in the future. The recent identification of the novel *tet*(42) efflux resistance gene, in bacteria from deep sediments, with the same distinctive characteristic as previously reported for other tetracycline efflux pumps suggests that tetracycline residue has blanketed the world and made its way into very remote and hostile corners of the world. One can only wonder what other unique *tet* genes may be isolated from bacteria taken from these unlikely places throughout the world as well as more common environmental sources.

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Part IV
Clinical Issues of Resistance: “Worst Offenders” List of Problematic Microbes Gram-positives

Chapter 17
Evolution of Molecular Techniques for the Characterization of MRSA Clones

Duarte C. Oliveira, Hermínia de Lencastre, and Alexander Tomasz

17.1 Introduction

The clinical impact of methicillin resistance in hospitals and in the community has seen numerous recent reviews. Various aspects of the mechanism of methicillin resistance [19], the putative origin of the heterologous mecA gene [15, 115, 119], and the spread of MRSA clones (in time and in geographic areas) have also been described in several recent reviews with appropriate narratives for general and/or expert readerships [10, 20, 21].

On the other hand, a detailed and critical discussion of the evolution of molecular typing techniques, which are increasingly used both in mechanistic and surveillance studies, both in research labs as well as clinical microbiology laboratories, and have produced unprecedented insights into the evolution of antibiotic resistant *Staphylococcus aureus* lineages, has seldom seen a detailed description. The purpose of this chapter is to fill this gap by a critical survey/update of molecular typing techniques in a historical context, from phage typing/antibiotyping to full genome sequencing.

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17.2 Emergence of MRSA

Methicillin-resistant *Staphylococcus aureus* (MRSA) was first described in 1961, soon after the first semi-synthetic beta-lactam antibiotic “celbenin” (methicillin) was introduced into clinical practice [50]. The novelty of these new beta-lactams was their resistance to degradation by penicillinase. By the late 1950s, the plasmid-born determinant of this enzyme was acquired by most clinical isolates of *S. aureus* virtually eliminating penicillin for therapeutic use.

Almost within a year of introduction of methicillin the first clinical failure, due to an MRSA strain was reported [26], and it was followed by the first MRSA outbreak in 1963 [99]. During the last five decades, MRSA have managed to spread in many hospitals worldwide to become one of the most important nosocomial pathogens [36]. Moreover, in the late 1990s, MRSA has also found its way into the community, and an increasing number of studies have reported MRSA infections in otherwise healthy ambulatory patients without identified risk factors [16, 22, 81]. This continuous changing epidemiology highlights the remarkable plasticity of *S. aureus* to adapt and survive in a wide range of environmental conditions and to cause an impressive spectrum of infections in the human host [4].

MRSA strains were shown to be resistant to virtually all beta-lactam antibiotics and eventually also acquired resistance traits to many mechanistically distinct antimicrobial agents as well [58, 61] often leaving physicians with few therapeutic options for the treatment of such multidrug resistant MRSA infections [102].

17.3 Two Mechanisms of Beta-Lactam Resistance in *S. Aureus*

The mechanism of beta-lactam resistance in MRSA strains shows at least two sharp contrasts to the mechanism of the historically first beta-lactam resistance, penicillin resistance, acquired by clinical isolates of *S. aureus*. In contrast to the plasmid-borne penicillin resistance gene which encodes for an enzyme capable of hydrolyzing the antibiotic, the genetic basis of methicillin resistance is a chromosomally located genetic determinant *mecA* [5, 62, 95], which encodes for a penicillin binding protein (PBP2A) with greatly reduced affinity for virtually all beta-lactam antibiotics [41, 83, 105]. Even today, some details of the mechanism of methicillin resistance are not fully understood. According to current thinking, PBP2A can continue to function as a “surrogate” transpeptidase catalyzing cell wall peptidoglycan biosynthesis in the presence of high concentrations of antibiotics which would inactivate the complement of native PBPs of *Staphylococcus aureus* and kill the bacterial cell [17].

In all MRSA strains, the methicillin resistance determinant *mecA* is part of a complex heterologous cassette, SCC*mec* (*S*taphylococcal *C*assette *C*hromosome *mec*), which incorporates into the chromosome of the recipient *S. aureus* strain at a specific site *orfX* close to the chromosomal replication origin [46]. While the DNA

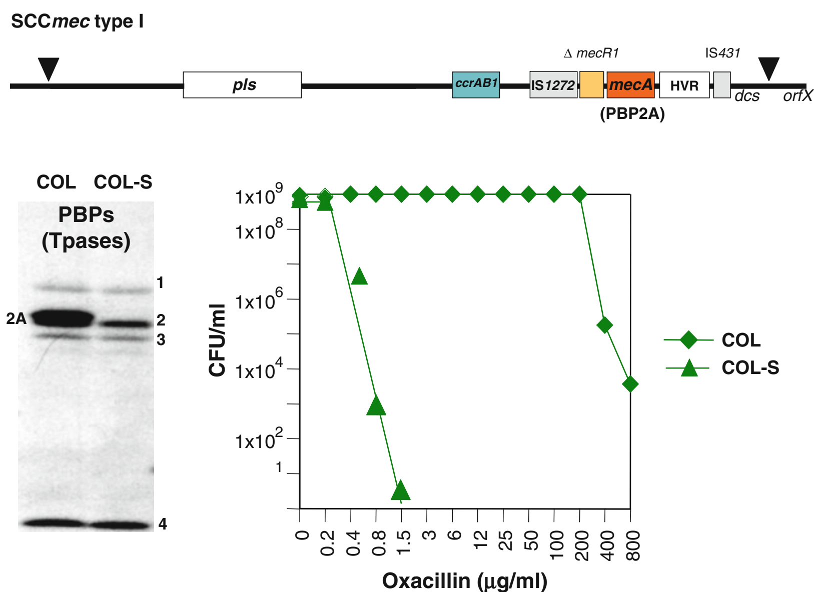


Fig. 17.1 Mechanism of methicillin resistance in *S. aureus*. The figure illustrates key components of the resistance mechanism in the MRSA strain COL, including the chromosomally located SCC*mec* type I structure; the protein product of *mecA,* PBP2A, identified by SDS-PAGE as a protein capable of covalently binding radioactive penicillin; and the high level and homogeneous resistance of strain COL to the beta-lactam antibiotic oxacillin, as demonstrated by the population analysis assay. Precise excision of the SCC*mec* cassette from strain COL generates the isogenic strain COL-S that lacks PBP2A and is fully susceptible to oxacillin

sequence of *mecA* appears to be conserved and virtually identical in all MRSA strains examined, the structure of the SCC*mec* cassette can vary widely from one MRSA clone to another. The nature of the SCC*mec* "donors" and the mechanism of their delivery into a recipient *S. aureus* are not known. Basic genetic and biochemical components of methicillin resistance and the resistant phenotype are illustrated for the MRSA strain COL in Fig. 17.1.

A second contrast between penicillin resistance and resistance to methicillin concerns their degree of spread. While the "plasmid epidemic" carrying the penicillinase gene (the mechanism of penicillin resistance) seemed to have swept through the entire species of *S. aureus,* the mechanism of methicillin resistance has remained linked to a limited number of unique *S. aureus* lineages or "clones." Much of what we know about the nature, the number, and the epidemiology of these clones, the composition and the genetic variance of SCC*mec* and estimates of the number of times these heterologous cassettes have entered *S. aureus* lineages to generate an MRSA clone (i.e., the entire evolutionary history of MRSA) is intimately linked to the rapid increasing resolving power of molecular typing techniques.

17.4 The Beginning of MRSA Typing: Phage Types and Antibiotypes

Phage typing is based on patterns of susceptibility in relation to a standard set of phages [7]. It relies on the outcome of a complex biological process, which may explain the difficulties experienced with the reproducibility of this technique. In addition, a large proportion of *S. aureus* strains are non-typable [6], which may be due to a "real" null phenotype (i.e., unable to support phage infection/replication) or to a phenotype for which reagents (i.e., phage lysates) are unavailable.

Regarding antibiotypes: identification of a new or unusual pattern of antibiotic resistance among isolates cultured from multiple patients may be the first indication of an outbreak. However, due to the extraordinary selective pressure associated with widespread antibiotic usage in contemporary hospitals, many nosocomial isolates representing different strains have the same phenotypic pattern of multiple antibiotic resistance. Moreover, resistance may happen as a result of a single point mutation or acquisition of plasmids and transposons, often carrying several resistance determinants. In the absence of specific selective pressure, such mobile elements may be lost. Different strains may develop similar resistance patterns and, conversely, sequential clinical isolates representing the same strain may differ for one or more antibiotics. All this can make antibiotyping an imperfect method for tracking MRSA strains.

Despite these limitations, the clonal nature of MRSA was first recognized through the application of phage typing to the newly emerged MRSA strains. The epidemic spread of MRSA in hospitals and intercontinental spread of a particular lineage was first demonstrated using this technique [87]. The unique antibiotic resistance pattern of MRSA strains in combination with phage typing was used to define the first MRSA clones [18, 29, 99].

17.5 Emergence of Molecular Typing Techniques

It was the introduction of molecular techniques that allowed the identification of the critical genetic determinant of beta-lactam resistance, *mecA,* as a component of a structurally complex heterologous cassette, the so-called SCC*mec* (for Staphylococcal Cassette Chromosome *mec*) [46]. The complexity of the SCC*mec* structure and its extensive clone-to-clone variation suggests that the assembly of this mobile form of the methicillin resistance mechanism involved a multi-stage evolutionary process. Identification of various structural features of SCC*mec* has become a challenge to molecular typing techniques, which developed gradually, in step with the recognition of the large number of structural variants of the SCC*mec* element.

A variety of molecular typing techniques, with increasing levels of sophistication, have been developed for the study of various aspects of MRSA epidemiology, from short term or local studies to more long term surveillance [3, 114]. Molecular typing strategies have become critical for infection control and surveillance programs,

enabling the prompt detection of outbreaks, reservoirs, and transmission routes in the hospital setting and also enabling the clinical management of infections, distinguishing relapse episodes from *de novo* infections and establishing the relationship between colonization and acute infection. Moreover, especially with the introduction of DNA sequence-based typing strategies applied to large international collections of clinical MRSA isolates, molecular typing efforts contributed significantly to the identification and to the tracing of the geographic dissemination of pandemic clones [80]; it also contributed to the study of the population structure and evolutionary pathways involved in MRSA origin and spread [28, 84].

17.6 Multiplicity of Molecular Typing Methods

The rapid advance of molecular biology techniques has provided an extensive list of DNA-based strain typing methods [107] such as restriction fragments length polymorphisms (RFLPs) analysis of plasmid DNA [64] or chromosomal DNA with probes for ribosomal operons (ribotyping) [101], *mecA* and transposon Tn*554* [56], insertion sequences IS*256* and IS*431* [70]. Advances on PCR technology and core reagents made it possible to develop a plethora of PCR-based methods [106], which can be divided in three conceptual categories: (1) RFLPs of PCR products of genes with a high level of allelic variability, such as the coagulase gene [34]; (2) amplification of repetitive chromosomal sequences (rep-PCR) such as short extragenic repetitive sequences present at many sites around the chromosome [109], the intergenic spacer region between the 16 S and 23 S genes in the rRNA operon characterized by extensive length and sequence variations [25], or the regions between the several copies of the insertion sequence IS*256* [24]; and (3) arbitrarily primed PCR (AP-PCR) or random amplified polymorphic DNA (RAPD) assay, using short primers not specific for any particular locus [112, 113].

Despite an abundance of such potential typing techniques, no single method has been adopted as an internationally recognized standard; although, in principle, each of these methods has the potential for typing *S. aureus* strains in short-term epidemiological studies [111]. Currently, there are four main MRSA typing strategies that have become widely implemented in hospitals and laboratories worldwide: PFGE, *spa*, MLST, and SCC*mec* typing. Even if some laboratories choose to use other molecular typing strategies, such as rep-PCR typing or multilocus VNTR analysis (MLVA) [23, 86, 89], those four typing methods are used either as benchmarking or for the definition of clonal lineages in an internationally recognized language.

17.7 Pulsed-Field Gel Electrophoresis (PFGE)

Pulsed-field gel electrophoresis (PFGE) is performed in a special gel electrophoresis apparatus in which the orientation of the electric field across the gel changes periodically enabling the effective resolution of larger DNA fragments (>25 kb)

when compared to conventional electrophoresis [90]. PFGE typing of bacterial isolates relies on the digestion of total DNA with a “rare-cutter” endonuclease, which produces a discrete set of DNA fragments and a reproducible “fingerprint” for the isolate. For *S. aureus* and many other species, SmaI endonuclease, which recognizes the rare sequence CCCGGG, is widely used producing patterns of 12–20 bands in the 48.5–582 kb range.

PFGE SmaI patterns may be regarded as chromosomal DNA fingerprints reflecting the distribution of the rare SmaI recognition sequences around the genome and/or the distances between them. Although a PFGE pattern may change by point mutation or insertion/deletions in the chromosome (e.g. pro-phage integration), patterns are relatively stable among genetic lineages enabling the definition and tracing of clones with a high degree of discrimination.

PFGE requires relatively expensive and specialized equipment. Moreover, due to a need for careful preparative procedures to minimize “nicking” the chromosomal DNA, PFGE is technically demanding and labor-intensive. However, the typability, discriminatory power, and the reproducibility of the macro-restriction patterns are excellent, and the method has been regarded as “the gold standard” of molecular typing for several bacteria species [103].

In 1995, Tenover and colleagues had proposed a set of guidelines for the visual interpretation of PFGE patterns [104], which assumed that some basic level of genetic change could be expected to occur in most nosocomial isolates as they move from patient to patient and, as such, allow variant patterns (≤6 band differences) to be included in outbreak assignments. Therefore, isolates differing by a single genetic event (e.g., point mutation, deletion, or insertion), reflected as a difference of two to three bands are regarded as closely related whereas those differing by four to six bands (representing two independent genetic events) are regarded as “possibly related” and those with seven or more band differences, are considered “unrelated.” PFGE patterns that are closely or possibly related are classified as subtypes of the same PFGE pattern or (type). These guidelines were of paramount importance in providing a useful framework for assessing relatedness of isolates in the context of local and short-term epidemiological investigations. Outside this context, (i.e., in more global and long-term studies), the application of these guidelines must be carefully validated.

Analysis of large numbers of isolates increases the number of patterns and subtypes from multiple gels that have to be compared. Development of sophisticated software programs (e.g. BioNumerics and GelCompar, Applied Maths) are now available to provide a full computer-assisted analysis of PFGE data, which may include the following: gel normalization for the correction of subtle electrophoresis-related variability; automatic band-assignments and clustering or related profiles according to a custom defined cut-off similarity coefficient that more or less mimics the Tenover et al. criteria [104] (Fig. 17.2). Moreover, these programs make it feasible to construct portable PFGE databases, which can integrate clinical data or data from other typing methods. Nevertheless, interlaboratory reproducibility and comparison of PFGE data requires a strict adherence to standardized and detailed protocols and

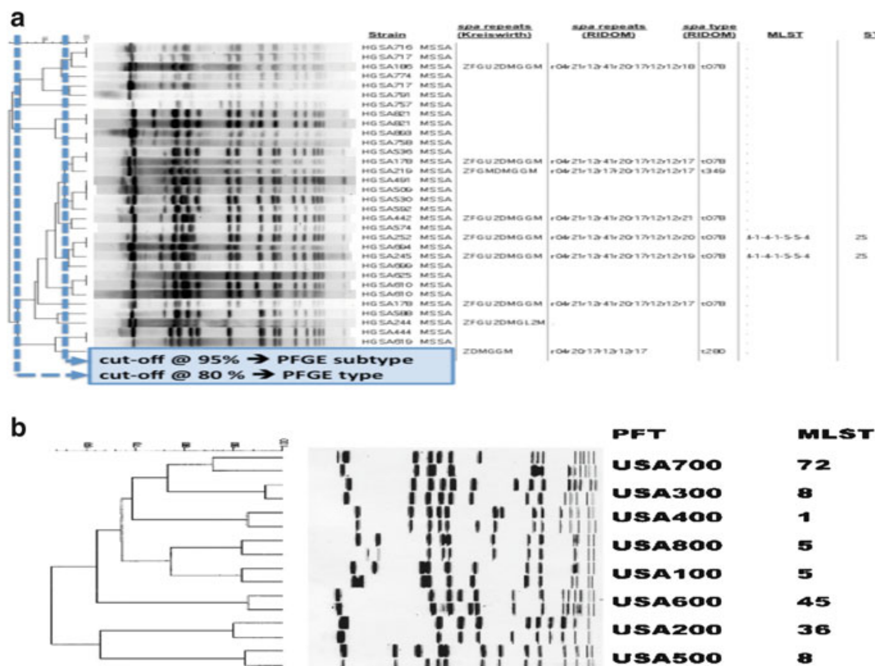


Fig. 17.2 Computer assisted PFGE analysis. Panel **A** – Custom defined cut-off values enable the automatic clustering of PFGE profiles in major types and subtypes in agreement with the Tenover et al. criteria [104]. The digital data-base of PFGE profiles also enables the integration of data from other typing strategies or clinical data [30]. Panel **B** – Standardization of protocols together with computer assisted analysis of PFGE profiles has enabled the creation of a database of MRSA clones circulating in the USA [63]

several successful national and international efforts have been made in order to improve the interlaboratory reproducibility of PFGE [13, 63, 71]. The chronologically first international molecular epidemiology study (RESIST) [88] used PFGE as the basic molecular typing method for the characterization of over 2000 MRSA isolates recovered in 120 hospitals from 20 different countries. A major conclusion of this study was the recognition for the first time that a few MRSA lineages (PFGE types) were responsible for over 80% of all MRSA hospital infections worldwide (Fig. 17.3).

17.8 *Spa* Typing

The first DNA sequence-based method developed specifically for the characterization of *S. aureus* isolates consists of the PCR amplification and sequencing of the polymorphic region of protein A (X region), which is specific for the species of *S.*

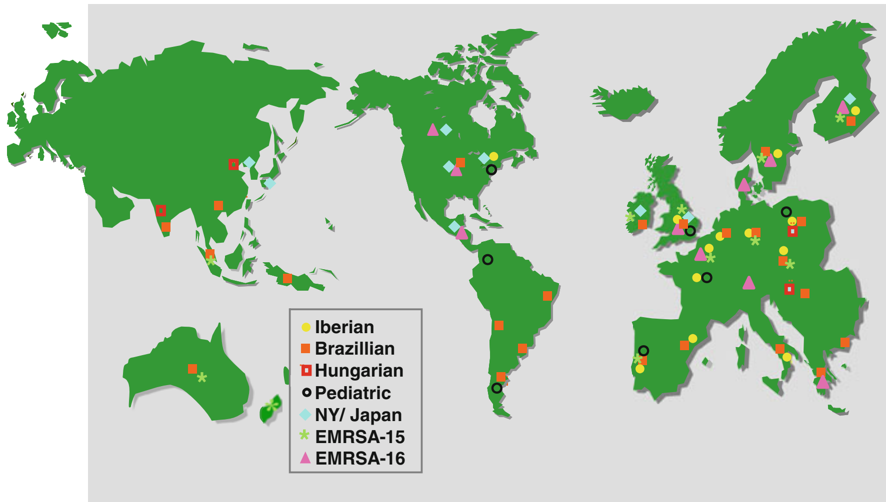


Fig. 17.3 Few epidemic MRSA clones cause most disease worldwide (Adapted from [1])

aureus. The X region (repeat region) of protein A consists of a variable number of tandem repeats (VNTRs) of 21–27 bp and polymorphic sequences. In *spa* typing, a numerical or letter code is assigned to each repeat and "*spa*" type is defined as the order of the specific alleles. Although by design (i.e., interrogation of a single polymorphic locus) *spa* typing is most suitable for local and short-term MRSA epidemiological studies [39, 92], *spa* type clusters seem to be stable and specifically associated with MRSA lineages, making this method also useful for global and long-term studies [14, 30, 38, 55, 100]. Recently, an algorithm, named "based upon repeat pattern" (BURP), has been described in order to explore *spa* typing data in long-term MRSA epidemiological studies [66, 67].

While two different nomenclature systems and databases have been developed for *spa* typing (Ridom and eGenomics) [39, 55], for comparison purposes it is always possible to interchange the *spa* type between the two nomenclature systems. Development of a dedicated software program and on-line database, the "Ridom Staph type," which enables the straightforward semi-automatic sequence analysis and type assignment via synchronization to a central server (www.SpaServer.ridom.de), has contributed to the wide use of *spa* typing and 100% reproducibility between different laboratories [2, 39]. This bioinformatics tool has also been used to establish a DNA-sequence based early warning system for MRSA outbreak investigations in hospitals [65]. In a major recent multinational epidemiology study, *spa* typing was used to identify the dominant clones of *S. aureus* recovered in 450 European hospitals located in 26 countries [35] (Fig. 17.4).

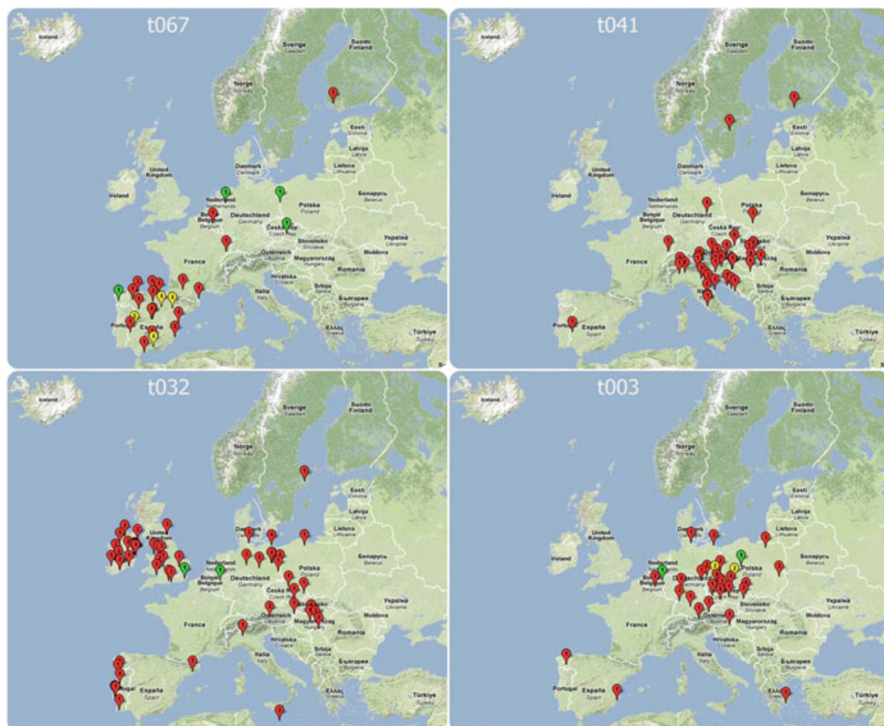


Fig. 17.4 Distribution of *S. aureus* clones in European hospitals in 2006 as determined by spa typing. Symbols, *red* – MRSA; *green* – MSSA; *yellow* – mixture of MSSA and MRSA (Reproduced from [35])

17.9 Multi-Locus Sequence Typing (MLST)

MLST is an adaptation of the multilocus enzyme electrophoresis (MLEE) principles. In contrast to MLEE, a phenotypic typing strategy in which isolates are tested for differences in the electrophoretic mobilities of a set of a metabolic enzymes [91], MLST consists of the assignment of alleles of seven house-keeping genes by nucleotide sequencing of internal fragments obtained by PCR amplification [60]. The different alleles are the products of neutral mutations in genes not subject to selective forces. Analysis of a single gene provides too little discrimination and the high discriminatory levels are only achieved by using several loci. The relative high discriminatory power, together with the fact that sequence variations accumulate slowly, make MLST a typing tool very suitable for global or long term epidemiology studies [96].

The MLST strategy has several intrinsic advantages. First, sequencing detects all variations, producing more alleles than MLEE where several mutations may be

needed in order to result in a shift of the electrophoretic mobility and different or very similar alleles may show the same mobility in the gel. Second, MLST data are unambiguous and amenable to the development and maintenance of a online database [96]. In fact, most MLST schemes are centralized in an online database (www.mlst.net) and use a universal language. Despite this advantage, MLST is still relatively laborious and expensive, since it involves 7 PCR reactions and 14 sequencing reactions per isolate, and as such it might not yet be appropriate as routine typing strategy of clinical isolates. However, the continuous and rapid improvement of automated DNA extraction, amplification and sequencing techniques, may make MLST, in the near future, a quite accessible option in terms of cost and simplicity.

Following the development of MLST schemes for various clinical important bacterial species, Feil and colleagues have developed the "based upon related sequence types" (BURST) algorithm for the interpretation of data and establishment of evolutionary relationships among isolates [32, 97]. MLST assigns to each isolate a sequence type (ST) (e.g. ST1), which corresponds to a numerical allelic profile for seven interrogated loci (e.g. 1-1-1-1-1-1-1). Isolates sharing the exact same ST are regarded as belonging to the same genetic lineage, whereas isolates differing in a single locus (SLV, single locus variants), or double locus (DLV, double locus variants) are regarded as belonging to a cluster of related lineages (CC, clonal cluster). The ancestor of each CC is the ST with the largest number of SLVs which should be present among the earliest isolates, is expected to be relatively prevalent in the population and have wide geographical dissemination, as compared to its descendents [31].

In 2000, Enright and colleagues developed and validated the MLST scheme for *S. aureus* [27]. Since then, MRSA lineages have been defined in terms of STs and for the first time there is an unambiguous universal language for MRSA clones. Moreover, when applied to the study of large collections, MLST has provided important insights into the structure of MRSA populations, confirming the previous empirical notions that were based mainly on PFGE data, namely that pandemic MRSA clones are restricted to a few genetic lineages and that the MRSA population has a very strong clonal structure [28, 84, 85] – (Fig. 17.5).

When compared to PFGE, MLST has a much lower discriminatory power. On the other hand, in the context of global epidemiology studies, the differences in macro-restriction patterns detected by PFGE may blur the evolutionary relationships between different clones [79]. In contrast to *spa* typing, which relies on the interrogation of a single polymorphic locus, MLST analysis of *S. aureus* isolates probes the allelic variation of seven neutral loci presumably with a much slower evolutionary clock speed. Data from *spa* typing have been shown to correlate well with MRSA lineages (i.e., with the sequence-types as defined by MLST). Considering the inherent simplicity of single locus analysis, *spa* typing is an attractive option as a first screening in global epidemiology studies [35].

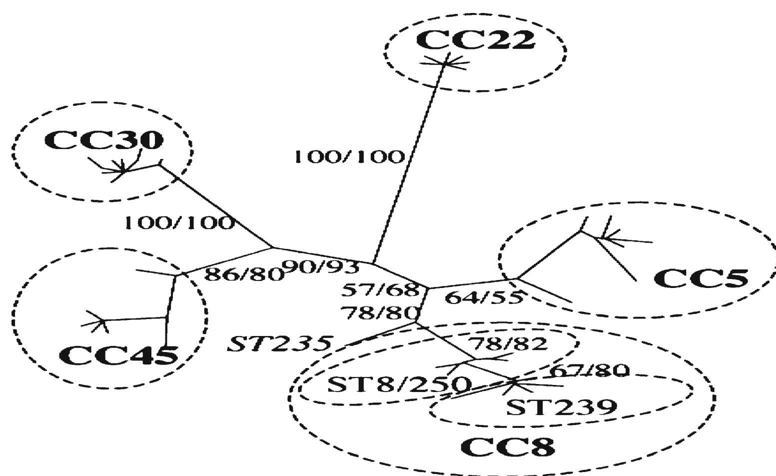


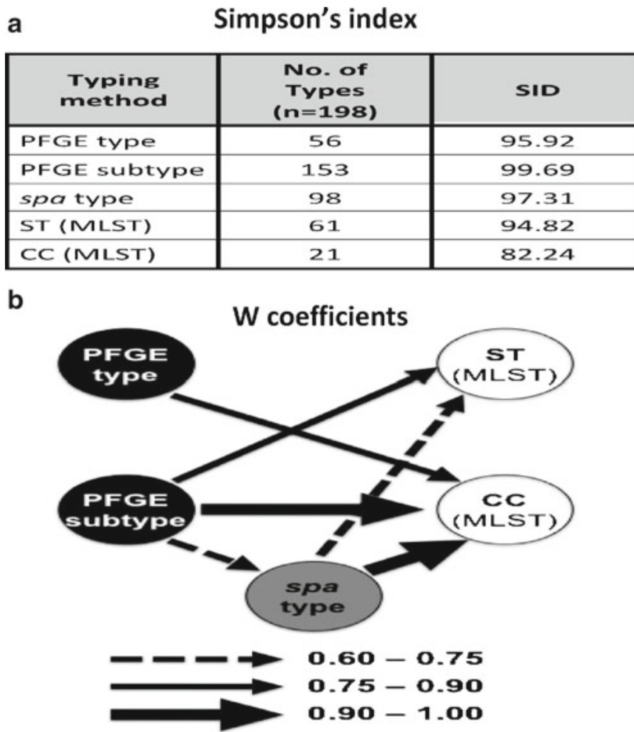
Fig. 17.5 MLST analysis of MRSA clonal evolution. Analysis of large international collections of MRSA isolates by MLST has enabled the definition of Clonal Clusters (CC) containing genetic related lineages and elucidation of the evolutionary relationships between lineages (Adapted from [84])

17.10 Congruence Analysis of Typing Methods

When choosing the most adequate typing strategy, it is important to take into account the study design and purpose, in order to decide critically what level of discrimination is most appropriate. Moreover, factors like associated costs, hands-on-time, required equipment etc., should also be considered. The Simpson's index of diversity (SID) [93] has been developed to serve as a measure of the discriminatory power of typing systems [44]. Basically, the SID index measures the probability that two isolates randomly sampled from a population belonged to two different types. In order to compare the discriminatory power of each typing method, Grundmann and colleagues have developed a method for determining confidence intervals for SID values [37]. The interpretation of SID value as a probability and the possibility of calculating a confidence interval makes it possible to use the SID in the evaluation of the molecular typing methods [9].

For the comparison of different typing methods, an objective measure of agreement is required and several methods have been developed for this purpose such as the Rand's index [82], Adjusted Rand's index [43], and the Wallace coefficient [110]. Recently, Carriço and colleagues have demonstrated the validity of these methods for a collection of *Streptococcus pyogenes* isolates characterized by several typing methods [9] and later this analysis was also applied to a large collection of MRSA and MSSA isolates characterized by PFGE, *spa*, and MLST typing [30].

When applied to a MRSA collection, this quantitative analysis ranks the typing methods in terms of discriminatory power (i.e., SID value); in addition, such a quantitative analysis also shows that both PFGE and *spa* typing data are good indicators


a Simpson's index

Typing method	No. of Types (n=198)	SID
PFGE type	56	95.92
PFGE subtype	153	99.69
spa type	98	97.31
ST (MLST)	61	94.82
CC (MLST)	21	82.24

Fig. 17.6 Congruence analysis of MRSA typing methods. Panel **A** – By determining the Simpson Index of Discrimination (SID) for each typing strategy, methods can be ranked according to the discriminatory power. For PFGE and MLST the analysis may be performed at different levels (type/subtype and sequence type/clonal cluster respectively). Panel **B** – The Wallace (W) coefficient permits to evaluate the probability for which a particular method predicts correctly the partitions defined by another method *(*Adapted from [30])

of the clonal complexes (CC) defined by MLST. In practical terms, this means that routine typing of MRSA strains should be performed by PFGE or spa typing and that MLST would only need to be performed for representative strains (Fig. 17.6).

17.11 SCC*mec* Typing

The staphylococcal cassette chromosome containing the *mecA* gene (SCC*mec* element) was first described by Ito and colleagues [46]. The genetic organization of the *mecA* vicinity defines the *mec* gene complex. In MRSA strains, three major classes have been described: class A containing the complete *mecA* regulon (*mecI*-*mecR1*-*mecA*), and classes B and C containing the *mecA* regulatory genes disrupted by insertion sequences, ΨIS*1272*-Δ*mecR1*-*mecA* (in class B) and IS*431*-Δ*mecR1*-*mecA* (in class C), respectively [52]. Class C is further classified into sub-classes C1 and C2, depending on the orientation of the IS*431* copy upstream to the *mecA* [42, 47].

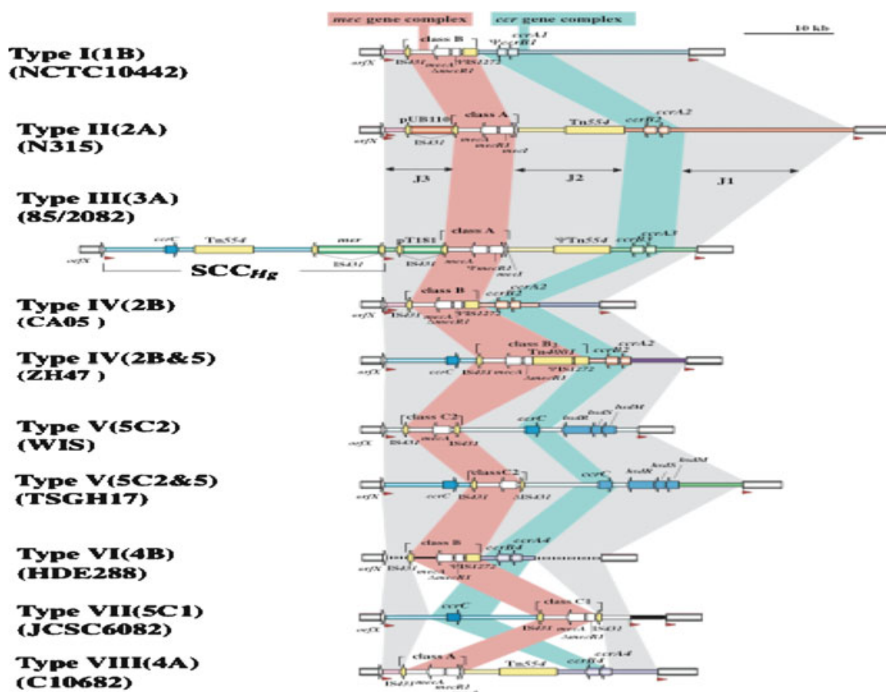


Fig. 17.7 Genetic organization of the major SCC*mec* types defined so far in MRSA. *Red* – *mec* gene complex; *green* – *ccr* gene complex; *gray* – J regions (Reproduced from [49])

The SCC*mec* element integrates into the *S. aureus* chromosome at a site-specific location (*attBscc*), located near the *S. aureus* origin of replication [57]. The mobility of SCC*mec* is in part due to the presence of the internal recombinases genes, which encode recombinases of the invertase/resolvase family which constitute the *ccr* gene complex of SCC*mec* [51]. If fully functional, *ccr* gene-products are able to catalyze the precise excision of the SCC*mec* element from the *S. aureus* chromosome. The *ccr* gene complex may have two genes, *ccrAB*, with four known allotypes [45, 79], or a single gene, *ccrC* not closely related to the *ccrAB* genes [47].

SCC*mec* types are defined by combining the class of the *mec* gene complex with the *ccr* allotype [42, 45–47, 49, 59, 77, 117]. Based on this definition, a new nomenclature has been proposed in addition to the historical system of Roman numerals [12]. In this new nomenclature SCC*mec* type definitions are as follows (proposed new names are in parenthesis): type I:*mec* class B and *ccrAB* allotype 1 (1B); type II – class A and *ccrAB2* (2A); type III: class A and *ccrAB3* (3A); type IV: class B and *ccrAB2* (2B); type V: class C2 and *ccrC* (5 C2); type VI: class B and *ccrAB4* (4B); type VII: class C1 and *ccrC* (5 C1); and type VIII: class A and *ccrAB4* (4A). Although, a few more SCC*mec* types have been described, only types I-VIII have been covered in the recently published guidelines for the classification of the SCC*mec* element [49] – (Fig. 17.7).

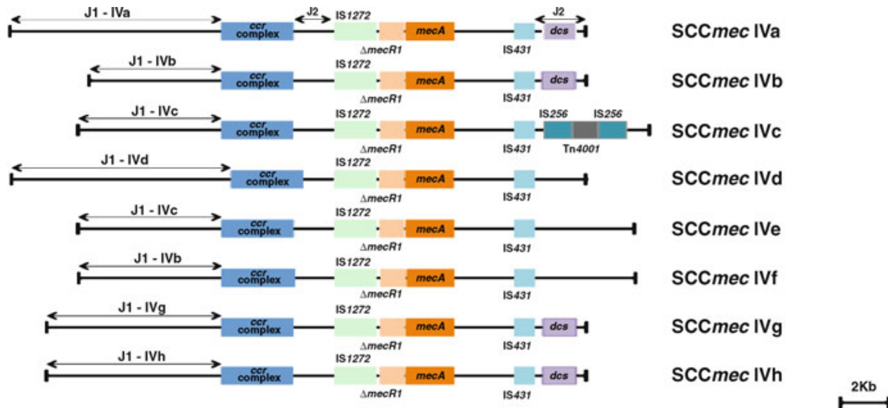


Fig. 17.8 Genetic organization of the sub-types of SCC*mec* type IV. SCC*mec* type IV is the most polymorphic structural variant of the SCC*mec* element and is dominant among CA-MRSA strains. Most of the variation is due to polymorphisms within the J1 region (Adapted from [68])

The remaining parts of SCC*mec* are called joining (J) regions (J1, J2 and J3), which in some cases carry additional antibiotic resistance determinants [48]. Starting clockwise from the origin of replication of *S. aureus* chromosome, the J3 is the region between the chromosomal left junction and the *mec* complex; J2 is the region between the *mec* complex and the *ccr* complex, and J1 is the region between the *ccr* complex and the chromosomal right junction. Therefore, SCC*mec* structural organization may be summarized as: J3-*mec*-J2-*ccr*-J1. Variations in the J regions (within the same *mec*-*ccr* combination) are used for defining SCC*mec* subtypes or variants – (Fig. 17.8).

The SCC*mec* element carries the *mecA* gene, the central element of the "broad-spectrum" beta-lactam resistance. Characterization of MRSA isolates in terms of SCC*mec* type has provided important insights into the origin(s) and transmission routes of the beta-lactam resistance. Enright and colleagues have proposed that MRSA clones should be named according to their MLST and SCC*mec* types (e.g., clone ST5-MRSA-II) [28]. A subcommittee of the International Union of Microbiology Societies in Tokyo accepted this in 2002. Under this rationale, rapid and easy assays for the detection of SCC*mec* types are critical tools for the proper characterization of MRSA clones.

In 2007, Stephens et al. have counted no less than 46 SCC*mec* variants [98], many of which differ in the presence of mobile elements such as insertion sequences, transposons, and/or linearized plasmids. Some of these SCCmec variants were found in sporadic or single isolates, making their epidemiological relevance questionable. Due to the ever-increasing number of SCC*mec* variants, the choice for the best SCC*mec* typing strategy (combining both routine feasibility and high discrimination index) is becoming extremely complex. Several PCR-based strategies have been developed for the characterization of SCC*mec* elements based on a variety of approaches that include conventional PCR detection of several type-specific loci [75]; multiplex PCR assays [8, 54, 68, 69, 76, 118]; RFLP analysis of PCR or

multiplex PCR products [108, 116]; allelic analysis by DNA sequencing of *ccrB* gene [78]; or multiplex real-time PCR assays [11, 33]. Currently, no single PCR assay is available to identify all SCC*mec* variants.

17.12 Evolutionary History of MRSA Clones as Traced by Molecular Typing Techniques

Several massive typing efforts of large MRSA collections have produced important insights concerning the origin, spread, and evolutionary pathways of contemporary MRSA clones. Initially, these typing efforts have used either PFGE or *spa* and MLST typing. The results of these studies suggest that before the acquisition of the SCC*mec* element populations of *S. aureus* were quite diverse but contained a few epidemic lineages. Under the selective pressure of beta-lactams, the SCC*mec* element was incorporated and maintained in some permissive lineages. Whenever, SCC*mec* acquisition occurred in an epidemic lineage of MSSA it originated an epidemic MRSA strain, which was able to spread in hospitals worldwide in the form of clonal expansion. This scenario would explain the relatively limited variation in MRSA population structure and the observation that the great majority of MRSA infections are caused by a few epidemic clones. This scenario also suggests that *de novo* SCC*mec* acquisition or horizontal transfer between *S. aureus* strains are relatively rare events and that resistance dissemination is due primarily to the clonal expansion of a few successful MRSA lineages.

The arrival of full genome sequencing has led to re-examination of these notions as well as attempts to identify genetic basis of such illusive properties of MRSA clones as virulence potential, epidemicity, and antibiotic resistance. Full genome sequencing was used in a recent attempt to identify the genetic basis of different degrees of virulence among some members of the CA-MRSA lineage USA300 [53]. Full genome sequencing was also used to identify stages in a "microevolution," (i.e., appearance of genetic determinants of vancomycin resistance in sequential blood isolates of an MRSA clone recovered from a patient undergoing antibiotic therapy [72]).

Nübel and colleagues [74] have concentrated their analysis on single-nucleotide polymorphisms (SNPs) in 108 loci of a global collection of 135 MRSA isolates that belonged to the MRSA clone ST5. They also performed SCC*mec* typing on the same isolates. The authors arrived at the surprising conclusion that in this particular clone's geographic spread over long distances may be a rare event when compared to the frequency of local acquisition of SCC*mec*.

In a landmark study, Harris and colleagues [40] used the power of full genome sequencing on 62 MRSA isolates belonging to the ST239-SCC*mec*III (originally named Brazilian clone). The availability of precise epidemiological data concerning dates and geographic sites of isolation has allowed the reconstruction of a detailed and multi-step evolutionary history for this clone, on a time scale of several decades, including identification of its putative geographic origin and its "migrations"

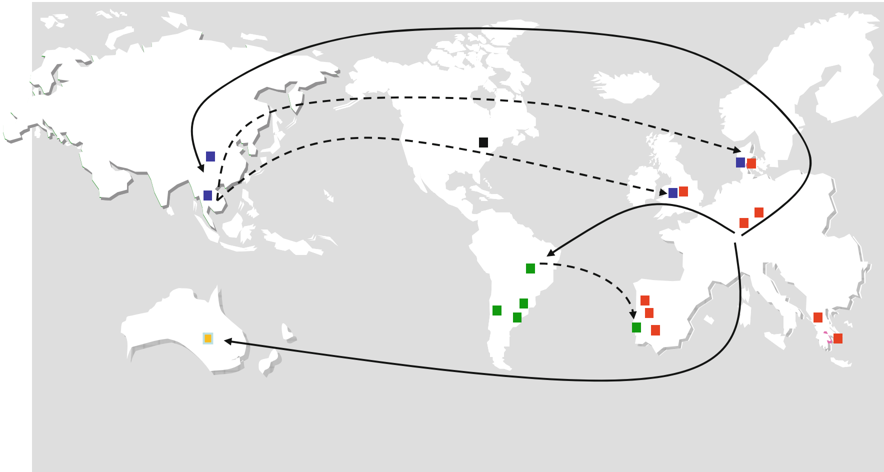


Fig. 17.9 "Birthplace" and migrations of the Brazilian MRSA clone ST239. Symbols: (*squares*), *red* – Europe; *green* – South America; *black* – North America; *blue* – Asia; *yellow* – Australia

between continents (Fig. 17.9). In the same study, sequencing of ST239 isolates recovered from a single hospital in Thailand, on a shorter time scale of a few weeks, has allowed precise tracing of the "movement" of individual invasive isolates among patients located in different wards.

Smyth et al [94] sequenced 32 loci in a global collection of the same ST239-III clone and arrived to very similar conclusions namely local genetic divergence of the clone during its geographic spread. SNP analysis of 269 loci, in isolates of yet another MRSA clone ST225 also showed evidence for geographic clustering [73].

17.13 Epidemiology and Molecular Biology in Alliance

It is important to realize that correct interpretation of data concerning evolutionary history will still depend ultimately on the availability of precise and detailed epidemiological information (place and time of isolation, microbiological as well as clinical data, etc.), even when the most modern molecular techniques are used. Retaining such an alliance of epidemiology and molecular biology will be critically important in future attempts to identify the molecular basis of complex properties of *S. aureus* clones such as epidemicity, virulence potential, or development of antibiotic resistance.

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Chapter 18
Mechanisms of Penicillin Resistance in *Streptococcus pneumoniae*: Targets, Gene Transfer and Mutations

Regine Hakenbeck, Dalia Denapaite, and Patrick Maurer

18.1 Evolution of Penicillin Resistance in *S. pneumoniae* – Epidemiological Aspects

S. pneumoniae belongs to the most penicillin-sensitive species. Unlike many other species, β-lactamase producing *S. pneumoniae* have not been discovered so far. Early attempts to isolate penicillin resistant mutants in the laboratory were extremely cumbersome. Nevertheless, after over 300 passages and during 24 months increasing selection pressure, variants were obtained with an over 1,000-fold increase in resistance to benzylpenicillin [46]. It therefore came as a surprise when the first reports of β-lactam resistant clinical isolates appeared. Initially, the increase in MIC values was not dramatic and the number of isolates was low (e.g., only 2 out of 200 strains had elevated MIC values in 1965 as reported by Kislak et al. (see Table 18.1)). However, the numbers increased to 12% in Papua, New Guinea in 1971, and it took only a few more years to document high level penicillin and multiple antibiotic resistant isolates in South Africa [64]. Meanwhile, penicillin-resistant *S. pneumoniae* (PRSP) are reported with increasing frequency worldwide ([32, 60, 96], and references within). The factor mainly responsible for this development is the use of antibiotics [42].

There are some features associated with this epidemiological scenario that are noteworthy (Table 18.1). The MIC ranges over a wide spectrum of antibiotic concentrations, indicating that the mode of resistance development is variable and/or complex. Second, in areas where the use of new generations cephalosporins was encouraged, high-level cephalosporin resistant strains appeared [95]. Last but not least in countries with a high number of resistant isolates, the frequency of resistant

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Table 18.1 Development of β-lactam resistant *Streptococcus* spp

		MIC PenG	MIC CTX	Reference
Streptococcus pneumoniae				
1940	Worldwide	0.01	0.02	[69]
1965	Boston	0.1–0.2		[73]
1967	Papua	0.6		[58, 59]
1977	South Africa	4–8	2	[64]
1990s	Hungary	>10		[90]
1990s	USA	0.01	32	[95]
Commensal Streptococcus spp				
1990s	Hungary/Spain	20	20	[112]
1994	Germany	>50	>60	[74]

CTX cefotaxime

strains of closely related commensal oral streptococci was also high, including those with MIC levels far above those reported for *S. pneumoniae* [24, 74].

In Spain, high level resistant *S. pneumoniae* in Europe were reported in the early 80s, with clones of the capsular type 9V, 6B, 14, 19F, 23F representing the majority of PRSP (penicillin-resistant *Streptococcus pneumoniae*), serotypes that are also common among healthy carriers. Clones have been named by an international network "The Pneumococcal Molecular Epidemiology Network" (PMEN) [97]. These prevalent clones have spread to varying degrees to other countries. The Spain^{23F}-1 clone represents the most widespread one, members of which having since been isolated in South Africa, the US, Europe, and Asia [96, 101]. Macrolide resistant variants of Spain^{23F}-1 contribute to the dissemination of this clone in Europe [114]. In contrast, clone Spain^{14}-5 apparently has spread less to other countries [87]. Type 19A clones with unusually high MIC values were described first in Hungary in the 1980s [90] and in the Czech Republic and Slovakia [34]. Other resistant 19A clones are increasing in many countries, probably since this serotype is not included in the 7-valent vaccine [18]. A remarkable epidemiological scenario in Iceland documents the increase of penicillin non-susceptible *S. pneumoniae* (PNSP) mainly due to the spread of a particular type 6B clone Spain^{6B}-2 in the late 1980s/early 1990s which carried resistance determinants against another five antibiotics [118]. High numbers of PSNP are now reported in Spain (30–50%) [108], Asian countries (up to over 90%) [81], and Italy with 69% [86].

No difference in disease potential was found between resistant and sensitive clones [128]. Although PNSP clones of a rare serotype 35B have only been found among patients with invasive disease in the US and only among carriers in Sweden, one cannot deduce a specific disease pattern since it is not known how common this clone is among carriers in the US [4, 61].

Serotype switching within clones has been noted, probably due to immunological pressure in the human population. Examples are 19F variants of the Spain^{23F}-1 clone, and type 14 variants of the Spain^{9V}-3 clone (for review, see [60, 96]). There might be an impact on the clonal structure of resistant *S. pneumoniae,* due to vaccination

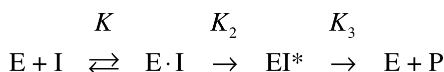
against the prevalent serotypes. In fact, an increase in the relative proportion of resistant *S. pneumoniae* causing invasive disease after the introduction of a 7-valent anti-pneumococcal vaccine has been reported [70].

18.2 Penicillin-Binding Proteins – Interaction with β-lactams

There are three main players involved in the evolution of resistance in *S. pneumoniae*: the penicillin target enzymes PBP2x, PBP2b, and PBP1a. Whereas PBPs of sensitive bacteria are inhibited at low concentrations by β-lactams, the altered PBPs of resistant isolates interact with and thus are functionally inhibited by the antibiotic at much higher concentrations. In clinical isolates these PBP genes have a mosaic structure (i.e., regions highly divergent from those of sensitive strains). The mosaicism of the PBP genes is the result of interspecies gene transfer fed by the common gene pool available to commensal and pathogenic streptococci, combined with the selection of point mutations. Apparently, mutations affecting the interaction with the antibiotics also have some impact on enzymatic activity, and thus compensatory mutations may occur. Mutations in non-PBP genes are also involved in the resistance process in clinical isolates and laboratory mutants as well.

PBPs are multidomain proteins, which are grouped into three main classes: the high molecular weight (hmw) PBPs of class A and B, and the low molecular weight (lmw) PBPs (for review see [40, 145]). Each species contains a set of PBPs, which are numbered in descending order according to their apparent molecular weight as revealed on SDS-polyacrylamide gels. The hmw PBPs are anchored into the membrane via a short N-terminal hydrophobic region, whereas lmw PBPs contain an amphiphilic helix at the C-terminus that functions as membrane attachment. In all cases, the functional domains are located in the periplasm outside the cytoplasmic membrane. All PBPs and the related β-lactamases contain a penicillin-binding domain which functions during late steps of murein (peptidoglycan) biosynthesis as transpeptidase/carboxypeptidase, representing the penicillin-sensitive steps. It contains three conserved motifs: SXXK with the active site serine which is directly involved in the transpeptidation reaction, and becomes acylated upon binding to β-lactam antibiotics, an (S/Y)XN and a (K/H)(S/T)G box. These sites are located in close proximity and represent crucial parts of the active site cavity in the three dimensional arrangement of the PBP. Mutations relevant for the resistance development resulting in a decreased affinity for β-lactams are located in this domain. The class A hmw PBPs contain an N-terminal transglycosylase domain, the target of the antibiotic moenomycin [93, 138, 141]; the function of the N-terminal domain of class B hmw PBPs is not known.

PBPs interact with β-lactams according to the following scheme [37]:



where E = active enzyme, I = β-lactam compound, E·I = non-covalent complex, EI* = covalent acyl-enzyme complex, and P = biologically inactive product. K is the dissociation constant, k_2 and k_3 are first-order rate constants for the acylation respectively the deacylation step, and the second order rate constant k_2/K represents the acylation efficiency.

Due to their ability to covalently bind β-lactams, PBP can easily be visualized after incubation with radioactive or fluorescent antibiotic and separation on SDS polyacrylamide gels. Since the hmw PBPs of *S. pneumoniae* are notoriously difficult to resolve on SDS gels, and PBP variants especially of clinical isolates may have a different electrophoretic mobility, specific antisera and monoclonal antibodies have been produced to label individual PBPs. The different ways to visualize PBPs have been reviewed recently [117]. *S. pneumoniae* contains six PBPs mysteriously named PBP1a, 1b, 2x, 2a, 2b, and 3. The nomenclature has evolved, due to improved resolution of 'first generation' SDS gels which resolved PBP1, 2, and 3 to PBP1a + 1b, and 2a + 2b in the late 1970s; PBP2x was discovered later during biochemical characterization of pneumococcal PBPs. PBP2x and PBP2b are class B hmw PBPs, and class A hmw PBPs are represented by PBP1a, 1b, and 2a.

18.3 PBP Function

Due to the lack of true substrates, thiolester compounds have been used to analyze the transpeptidation reaction of PBP2b and PBP2x, and the depsipeptide S2d has been used in many studies [1, 65, 94]. The thiolesters give rise to linear acyl-enzymes, which are easily hydrolysed thereby mimicking the transpeptidation reaction carried out by PBPs. Although PBPs may react differently with this compound compared to the natural muropeptide substrates, the kinetic parameters help to define the effect of mutations on peptide hydrolysis of PBP variants.

Isolated PBP2a derivatives contain transglycosylase activity in vitro [22]. The glycosyltransferase (GT) domain of class A PBP1b showed moenomycin sensitive binding to lipid II, an indirect evidence that it functions as transglycosylase as well [21]. The lmw PBP3 functions as a D,D-carboxypeptidase in vitro [52].

PBP2x and PBP2b are believed to be essential, since it is not possible to obtain deletion mutants in these two genes [72]. It is curious that in closely related bacteria, *S. thermophilus*, the PBP2b homologue could be deleted, resulting in altered morphology and defects in exopolysaccharide synthesis [137] and in *S. gordonii* as well, leading to aberrant septation and early lysis [47], indicating that special functions are associated with PBP2b of *S. pneumoniae* which are absent in the other species.

The class A hmw PBP1a, 1b and 2a are individually dispensable, suggesting that the putative transglycosylase and transpeptidase activities of these PBPs can complement each other. The *pbp2a* mutant showed a higher susceptibility to moenomycin, and PBP2a was therefore suggested to be the main transglycosylase in

S. pneumoniae [62, 107]. Double mutants have also been obtained except for the pair *pbp1a/pbp2a* [62, 107]. Nonetheless, the class A PBP double mutants were severely affected, being unable to synthesize regular division septa, and lysed earlier after reaching the stationary phase [107]. This indicates that PBP1b alone cannot complement for the activities of the other two hmw PBPs.

PBP3 deletion mutants can readily be obtained, but they grow poorly and have aberrant shapes: often multiple division septa are found irregularly distributed in the cells, they contain a thickened cell wall and shed wall material into the growth medium [120]. Biochemical alterations of the murein confirmed its D,D-carboxypeptidase activity [124]. Immunogold-labeling using anti-PBP3 antibodies revealed that PBP3 is evenly distributed over the entire surface [50]. It appears to be absent at the division septum in wild type cells, and the rings formed by hmw PBPs and that of FtsZ are no longer colocalized in PBP3 mutants [98].

18.4 Gene Transfer and the Evolution of Mosaic Genes in Clinical Isolates

The PBP genes in resistant isolates encoding low affinity PBPs are highly variable due to the presence of sequence blocks that differ approximately 20% on the DNA level resulting in up to 10% amino acid changes compared to corresponding sequences in sensitive strains. Mosaic genes have been described in all three key players of the resistance process: *pbp2b* [26], *pbp2x* [80], and *pbp1a* [89]. Despite extensive sequence variations, the number of amino acids is constant with a few exceptions in PBP2b and PBP1a as outlined below. The mosaic structure of PBPs might result in electrophoretic mobility shifts as has been detected already in the first reports describing PBP pattern in penicillin resistant clinical isolates [55, 109, 148], most prominent detectable in PBP1a variants [48, 80], although their calculated molecular weight is almost identical. Even among sensitive strains different PBP patterns can be distinguished [49]. Since these changes are generally clone specific, the PBP profiles on SDS PAGE in combination with antibody reactivity pattern have been used as clonal markers [48]. Restriction fragment length polymorphism (RFLP) of PBP genes has also been used as a DNA based screen to establish clonal relatedness [14, 101]. Although these methods are useful for screening a large number of isolates, small changes in the size of the mosaic blocks and point mutations in *pbp* genes that are important for the deduction of the evolutionary history might not affect the restriction sites or the epitopes and are thus missed in such analyses.

In general, the *S. pneumoniae* clones as identified by MLST analysis (multilocus sequence typing [85]) are either resistant or sensitive. However, there are a few cases where sensitive isolates were detected that belong to the same clone in agreement with the introduction of resistant genes into a sensitive population. Variation of PBPs has also been noted within resistant clones from Hungary and Poland,

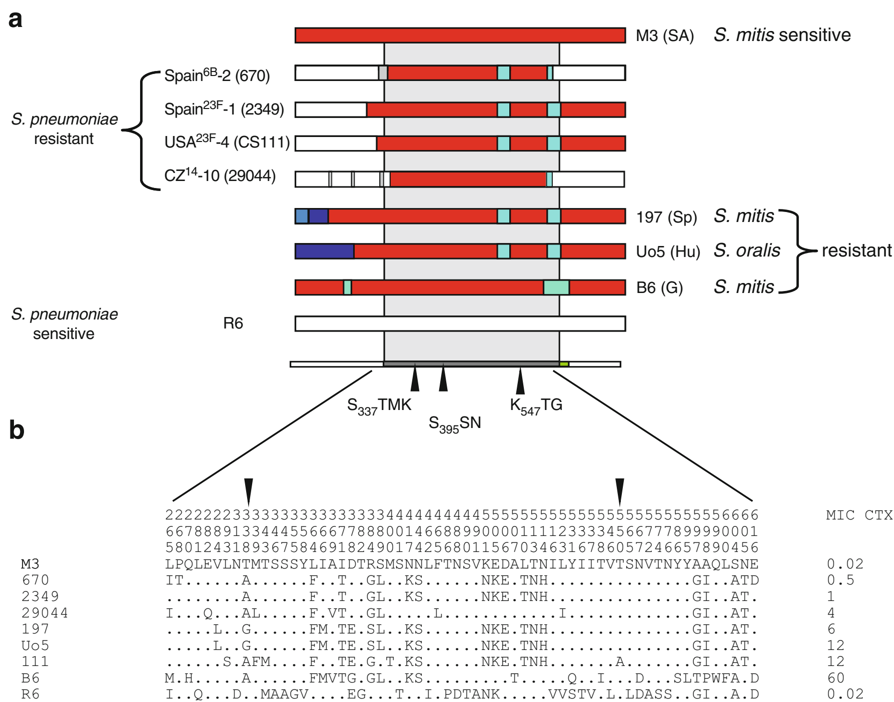


Fig. 18.1 Distribution of a family of mosaic PBP2x genes. (**a**) Mosaic structure. One group of mosaic PBP2x genes contain sequences highly related to the penicillin susceptible *S. mitis* M3 (*red*). Related mosaic *pbp2x* have been identified in *S. pneumoniae* and oral streptococci isolated in different geographic areas as indicated. *White*: homology to penicillin susceptible *S. pneumoniae*; *red*: homology to PBP2x of *S. mitis* M3. *Grey shading* indicates the transpeptidase domain. Arrows point to the active site motifs. (**b**) Amino acid variation of PBP2x of the strains shown in 1A with different MIC values for cefotaxime. Only changes within the transpeptidase domain are shown that are distinct from PBP2x of the sensitive *S. mitis* M3. It should be noted that the MIC values reflect alterations in other PBPs as well

indicating that PBP genes have been introduced into a clone on several occasions from different sources [63, 113].

Sequences highly related to mosaic regions of mosaic *pbp2x* [126] and *pbp2b* [25] of *S. pneumoniae* were detected in susceptible *S. mitis* strains, in agreement with the assumption that low affinity PBPs have evolved in commensal streptococci prior to interspecies gene transfer into the pneumococcal population. One major class of mosaic *pbp2x* can be recognized in different *S. pneumoniae* clones and in resistant *S. mitis* and *S. oralis* strains as well [11, 112] (Fig. 18.1). In addition, a surprisingly large number of distinct *pbp2x* variants exists with unique mosaic patterns all being approximately 20% divergent from each other [51, 99], indicating multiple intra- and interspecies gene transfer events. The high diversity of the

mosaic genes is surprising due to the fact that pbp2x is a highly variable gene in sensitive *S. mitis* and *S. oralis* ([126]; and own unpublished results).

Recombination events resulting in altered *pbp* genes can occur within the gene or in flanking regions. It has been observed in PBP2x from resistant isolates that the border of the mosaic blocks on the DNA level reflects the domain structure of the protein in many cases [89, 126]. This suggests a selective pressure on the function of the protein. The mosaic structure might extend into adjacent genes such as *ddl* upstream of *pbp2b* [31] and *ftsL* upstream of *pbp2x* (own unpublished results). Since the capsular gene locus is flanked by the *pbp1a* and *pbp2x* genes, intraspecies transformation of resistance can result in capsular switching as well [140] as has been shown to occur in natural populations [12, 15]. PBP genes that are located at a great distance on the chromosome such as *pbp2b* and *pbp2x*, or *pbp2a* and *pbp2x*, can be introduced in a single transformation step as has been shown with chromosomal DNA from resistant *S. mitis*, indicating that genes located elsewhere on the chromosome can be altered easily during interspecies transformation events [53, 112].

18.5 PBPs and β-Lactam Resistance: Physiology of Resistant Isolates

Only PBP2x and PBP2b are primary targets for β-lactams (i.e., alterations in PBP2x or PBP2b alone confer a resistance phenotype albeit to only low levels). PBP2x mutants can easily be selected with cefotaxime resulting in MIC values for cefotaxime between 0.03–0.3 μg/ml depending on the particular mutation (i.e. confer a 1.5–30-fold increase in resistance compared to sensitive strains (0.02 μg/ml)) [43, 77, 78, 127]. Single mutations in PBP2b result only in a 1.5–2-fold increase in piperacillin MICs [43, 54]. Since PBP2b does not interact with cefotaxime over a wide concentration range (or other third generation cephalosporins and aztreonam which has a similar side chain as well), it is not a target for this class of compounds [56]. Therefore, *pbp2x* together with *pbp1a* of resistant clinical isolates are sufficient for cefotaxime resistance [3, 27, 53, 102]. Indeed, high level cefotaxime resistant clones have been described in the USA and South Africa with altered PBP2x and 1a, but which did not contain alterations in PBP2b [13, 95, 129].

Whereas penicillin are highly lytic antibiotics for *S. pneumoniae*, cefotaxime leads to much slower lysis and cells are also killed at a much lower rate [56]. This suggests that inhibition of PBP2b is somehow coupled with cell lysis. In agreement with this notion, is the finding that high-level penicillin-resistant strains which usually contain a low affinity PBP2b appear to be tolerant [82]. *S. Pneumoniae* strains containing a low affinity PBP2b as the only altered PBP have also been shown to display a tolerant response for penicillin antibiotics [43, 112]. The fact that PBP2b mutants are less prone to lysis suggests that cells with a low affinity PBP2b are

better survivors; thus they might have an advantage over wild type cells even in the absence of antibiotic selection.

In this context, it is a curious observation that strains harboring either an altered PBP2b or PBP2x were significantly less virulent in a murine peritonitis model [115]. The PBP2x mutants remained stable in both resistance phenotype and virulence, and thus the authors suggested that PBP2x plays an essential role during growth, whereas virulent revertants of PBP2b mutants were obtained. The location of the compensatory mutations remains to be clarified.

18.6 Mutations in PBPs

18.6.1 PBP2x – Laboratory Mutants versus Clinical Isolates

Mutations in PBP2x have been extensively studied, due to the fact that they can be selected easily in the laboratory, and their effect can be tested directly via transformation of sensitive *S. pneumoniae* strains using cefotaxime for selection (Fig. 18.2a). Moreover, it was the first and for a considerable time the only PBP where soluble, active derivatives were available enabling biochemical studies in vitro. The diversity of mutations observed and physiological characterization of PBP2x mutants suggests a complex evolution of resistance by introduction of mutations that might affect its enzymatic function as well, coupled with complementing mutations in the protein or in other genes as well as outlined below. After all, PBP2x is an essential enzyme, and mutations that affect the interaction of the β-lactam (i.e., affect the overall active site topography) should not severely affect the interaction of the in vivo substrate.

Already after one selection step, different mutations in PBP2x occur [104, 127], and six independent laboratory mutants obtained after a multistep selection procedure resulted in six distinct PBP2x mutant proteins with up to four point mutations in the transpeptidase domain [77, 78]. It is remarkable that most of the mutations did not map close to the active site except for the two mutations: $T_{550}A$ and $Q_{552}E$ adjacent to the $K_{547}SG$ box, and $H_{394}Y$ next to the $S_{395}SN$ motif. The mutation T550A confers high level cefotaxime resistance and simultaneously hypersensitivity to penicillins in laboratory mutants [43, 78, 79]; occasionally, it occurs in mosaic PBP2x of high level cephalosporin resistant and penicillin sensitive clinical isolate [13, 119] and as a single PBP2x mutation in low level resistant strains as well [2]. A second substitution in the same codon 550 results in a $T_{550}G$ mutation which increases the cefotaxime resistance even further [43]. Curiously, the reverse substitution, $A_{235}T$, at the homologous site of TEM β-lactamase resulted in an enzyme with an extended substrate profile that could hydrolyze cefotaxime, an antibiotic which is not a substrate for the original protein [16]. It has therefore been speculated that the $T_{550}A$ substitution is directly related to cefotaxime selection [78]. The $T_{550}A$ mutation results in a 20-fold decrease acylation efficiency for cefotaxime [99],

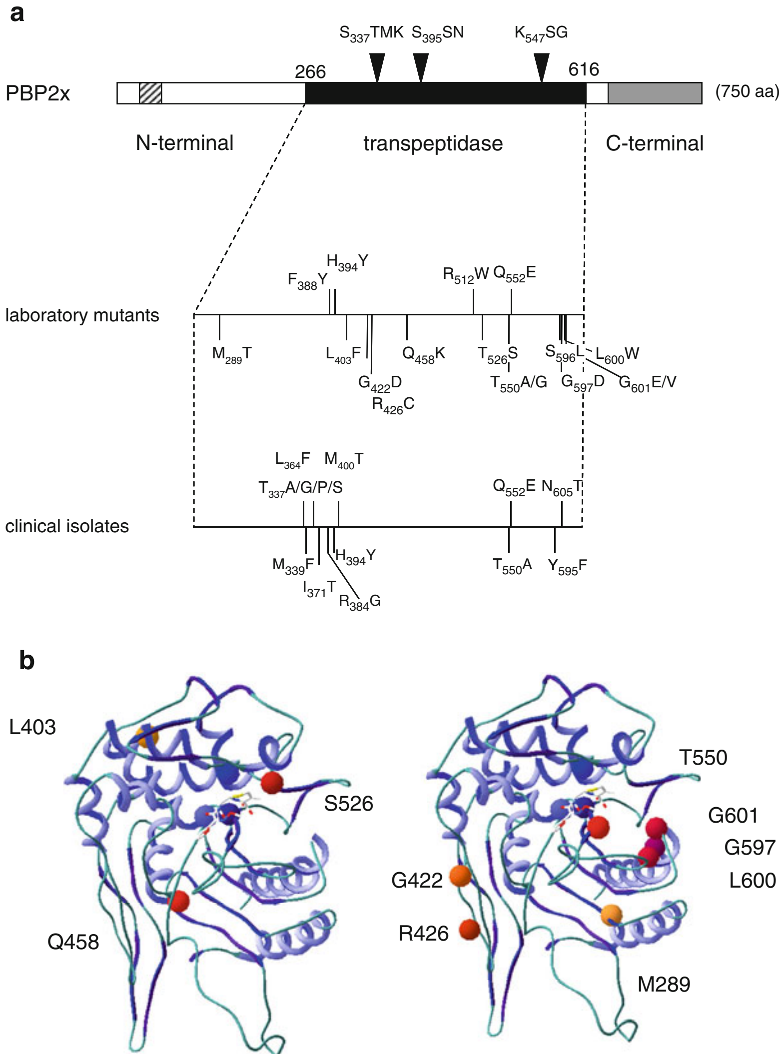


Fig. 18.2 Mutations in PBP2x. (**a**) Only mutations within the transpeptidase domain of PBP2x (*black*) have been described. The central *black* area indicates the transpeptidase domain, and the *hatched* area at the N-terminus indicates the hydrophobic membrane domain. The three active site boxes are indicated on *top*. Positions implicated in resistance identified in laboratory mutants and clinical isolates are indicated. (**b**) Three dimensional arrangement of mutations in two groups of laboratory mutants. *Left*: mutations occurring in group I; *right*: mutations in C505 (group II) which result in complete abolishment of β-lactam binding

probably due to the abolition of the hydrogen bond between T_{550} and the carboxylate moiety which is attached to the six-member ring of second and third-generation cephalosporins [41]. The $H_{394}Y$ change has also been identified in PBP2x of clinical

isolates [103]; the effect of $H_{394}L$ that occurs occasionally in clinical isolates has not been experimentally investigated.

At least two mutational patterns in PBP2x have been observed (Fig. 18.2b) [94]. In all PBP2x of group i: (1) mutations occur at the end of the transpeptidase domain (positions 596–601), (2) and mutations in other regions also are similar in some of the mutants. Noteworthy is the group ii PBP2x of one laboratory mutant C505 where no binding to any β-lactam could be detected, even if concentrations up to 10 μg/ml were applied and PBP visualized with anti-benzylpenicilloyl antibodies [79]. The combination of only three mutations in PBP2x-$C505_{T526S\text{-}L403F\text{-}Q458K}$ abolishes the interaction with cefotaxime as well as penicillin almost completely as measured in a purified soluble protein derivative, and $L_{403}F$ is crucial for this effect [94]. A possible impact of this mutational arrangement on the topology of the active site can be deduced from the three dimensional structure [94].

Mutations located at the end of the penicillin-binding domain in $C206_{G601V\text{-}G597D}$ only affect the acylation efficiency towards cefotaxime ($k_2/K = ^38{,}600$), possibly affecting indirectly the topology of the active site. Other combinations have an impact on both cefotaxime and penicillin binding [94]. The depsipeptide S2d has been used to determine the rate of hydrolysis for estimating the activity of PBP2x. In all cases studied so far, PBP2x mutants which showed a reduced acylation efficiency also reacted considerably poorer with the depsipeptide S2d [65, 94]. Curiously, the amount of PBP2x was also reduced in some mutants, but the molecular basis for this phenomenon is not clear [94].

Mutations in PBPs of resistant clinical isolates cannot easily be deduced from sequence analysis due to the multitude of amino acid alterations and the variability of the mosaic blocks, which is apparent even when comparing related mosaic PBPs (see Fig. 18.1b for examples). Comparison of a large number of diverse mosaic PBP2x revealed only two sites common to almost all highly divergent mosaic designs: T_{338} adjacent to the active site S_{337} is altered in one group of mosaic PBP2x (T_{338}(A/G/P/S)), whereas another group contains the mutation $Q_{552}E$ in most cases without the T_{338} mutation [51, 100]. All other mutations revealed so far occur only in subgroups of mosaic PBP2x or in single rare variants.

Kinetic parameters of isolated soluble PBP2x derivatives confirmed that the overall binding efficiency of a resistant PBP2x is much slower than that for sensitive PBP2x (k_2/K_d values of 100,000–200,000 M^{-1} s^{-1} for sensitive PBP2x compared to 11,000–85 M^{-1} s^{-1} and lower for resistant PBP2x containing multiple alterations) [6, 66, 84, 94]. The impact on resistance and β-lactam affinity has been demonstrated by a combination of mutagenesis and biochemical characterization of the protein for T_{338}(A/G/P), $M_{339}F$, and $Q_{552}E$ [9, 100, 110, 134, 146], which are close to active site residues. The T_{338}(A/G/P) mutations are special since they can be selected primarily with oxacillin [146], probably explaining why they have not been found in the cefotaxime-selected laboratory mutants. The side chain of T_{338} has been implicated in hydrogen bonding to a buried water molecule [100] and indeed this molecule is absent in a resistant PBP2x containing the $T_{338}A$ substitution [20]. The combination of $T_{338}A/M_{339}F$ reduced the acylation efficiency by penicillin over

1,000-fold, a result of slower acylation (300-fold lower k_2) and weaker pre-acylation binding (4-fold higher K_d) [9, 84]. The M_{339}F substitution also contributes to 40–70-fold faster deacylation kinetic [9, 23]. The structure of PBP2x$_{T338A/M339F}$ has been solved, revealing a distortion of the active site and a reorientation of the hydroxyl group of the catalytic Ser337 [9].

The structure of a mosaic resistant PBP2x carrying the Q_{552}E substitution reveals a distinct mechanism involved in resistance. The β3 strand with the K_{597}TG motif is displaced [110], resulting in narrowing of the active site. The negative charge of the glutamate residue also hinders binding of negatively charged β-lactams [110]. Whereas introduction of the single Q_{552}E mutation into the sensitive R6 PBP2x results in 3–4-fold reduction of the acylation efficiency, mosaic PBP2x with the Q_{552}E substitution have a 7-fold reduction [94] and 15-fold reduction was observed in a mosaic PBP2x which also contained the T_{338}A substitution [110].

Moreover, L_{364}F, I_{371}T, R_{384}G, M_{400}T, Y_{595}F, and N_{605}T [2, 6, 100, 134] are supposedly involved in resistance. The structure of a mosaic resistant PBP2x revealed that the substitutions I_{371}T and R_{384}G result in a slight displacement of the SXN motif, leading to a more accessible 'open' active site, and it has been suggested that thereby branched muropeptide substrates can be better accommodated [20].

Many alterations that have been suggested to contribute to resistance curiously occur also in PBPs of sensitive streptococci ([25, 126]; and own unpublished results). In PBP2x, they include Q_{447}M, S_{449}A and N_{514}H, which have been proposed to contribute to structural alterations of the active site in resistant strains [20, 110]. Also, the R_{384}G change, which has an impact on the susceptibility [6, 134] and altered the acylation efficiency of the protein [6], occurs in a sensitive *S. mitis* (own unpublished results). It is possible that different muropeptide substrates are used in sensitive *S. mitis*. Thus, the evolution of resistant PBPs in *S. pneumoniae* includes not only the reduction of β-lactam binding, but also the functional alterations in resistant PBPs as well. Some (but not all) resistant *S. pneumoniae* indeed contain a different cell wall with altered interpeptide bridges compared to sensitive isolates [38]. The acquisition of altered genes involved in the biosynthesis of such branched muropeptides might be a consequence of an altered PBP function affecting their substrate specificity (see below).

18.7 PBP2b

In PBP2b, G_{660}D at the C-terminal end of the protein, G_{617}A within the K_{615}TG motif [54], and T446A close to the S_{443}SN box [43] have been selected with piperacillin in the laboratory (see Fig. 18.3). A change within the KTG motif has also been observed in clinical isolates (T_{616}S; [136]). The T_{446}(A/S) change occurs in many resistant clinical isolates and E_{476}G as well [25, 33, 119, 133]; alterations at the C-terminal end of PBP2b have been implicated in the resistance process also of clinical isolates [10, 26]. The T446A substitution displays a 60% reduction in penicillin affinity in vitro, and in a PBP2b containing this, up to another 43 amino acids

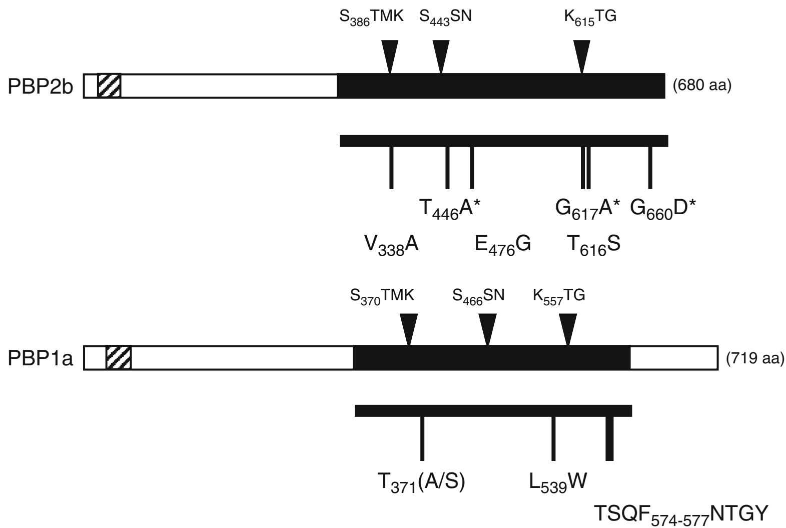


Fig. 18.3 Mutations in PBP2b and 1a. The central *black* area indicates the transpeptidase domain, and the *hatched* area at the N-terminus indicates the hydrophobic membrane domain. The three active site boxes are indicated on *top*. *: mutations in PBP2b whose impact on resistance has been demonstrated

change the affinity and is reduced up to 99% [106]. Only one case has been reported bearing a change within the SVVK motif ($V_{388}A$) [71]. Multiple changes in PBP2b between residues 590–641 have been observed in high amoxicillin resistant isolates and might contribute to this phenotype [28, 75]. Among resistant PBP2b are rare examples of the presence of additional amino acids in the protein: clinical isolates from Japan were found to contain a duplication of a region encoding three amino acid residues $S_{423}WY$ [143].

Recently, the structure of PBP2b from a wild-type and a high-level penicillin resistant strains has been resolved [17]. Similar to PBP2x and 1a variants, the main structural consequence of alterations concerned the active site, and it has been suggested that active site "breathing" could be a common mechanism employed by *S. pneumoniae* PBPs to interfere with β-lactam binding.

18.8 PBP1a

Resistance mediated by PBP1a can only be measured in combination with a low affinity PBP2x and/or PBP2b. In resistant PBP1a of clinical isolates, $T_{371}A$ or $T_{371}S$ close to the active site S_{370} occur frequently and contribute to resistance [2, 33, 89, 103, 105, 135]. $L_{539}W$ present in PBP1a present in a high level resistant Hungarian isolate [132], and the alteration of four consecutive residues $T_{574}SQF$ to NTGY have

been associated with resistance [68, 132, 135] as summarized in Fig. 18.3. The crystal structure of a resistant PBP1a derivative shows that the $T_{371}A$ mutation results in loss of a hydrogen bond, causing a shift of the active site S_{370} [67]. These changes in combination with the other alterations present the mosaic variant results in a narrower, discontinuous active site cavity. PBP1a mutants containing the $N_{574}TGY$ substitutions have a lower acylation efficiency in vitro [67]. Again, these positions are also altered in PBP1a genes of sensitive *S. mitis* (own unpublished results), and thus might also be related to functional properties in respect to the in vivo substrates. Generally, mosaic PBP1a derivatives have a greater effect on the interaction with penicillin compared to a cephalosporin with 8–164-fold decreased acylation rates towards penicillin G versus cefotaxime (2–25-fold) [67].

It has generally been accepted that the introduction of a low affinity PBP1a in a strain carrying a low affinity PBP2x results in elevated resistance. That the situation is much more complex has been shown recently, using low affinity PBP2x variants from laboratory mutants in comparison with mosaic PBP2x from resistant clinical isolates. Introduction of a mosaic *pbp1a* into the $PBP2x_{T338G}$ mutant, or into a PBP2x carrying three mutations of a laboratory mutant, did not lead to resistance increase [146]. It has been hypothesized that PBP2x and PBP1a interact with each other on some level and that alterations of both PBPs in resistant clinical isolates have evolved to ensure cooperation between both proteins. The data are in agreement with the observation that PBP1a variants can confer different levels of resistance although acylation efficiencies were very similar, and it has been postulated that dependent on the mosaic variant the physiological function of PBP1a varies [145].

18.9 PBP2a, 1b, and 3

Alterations in the other three PBPs associated with resistance have been described in rare cases. An altered PBP2a has first been observed in laboratory mutants, which contain a low affinity PBP2x [79]. Curiously, PBP2a in three such mutants could not be visualized using common labeling procedures, or even when high concentrations of penicillin and anti-penicilloyl antibodies were used for the detection of PBP- β-lactam complexes. In fact, PBP2a is absent in the mutants due to mutations in the genes that lead to premature termination of the transcript (M. van der Linden, J. Rutschmann, and R. Hakenbeck, unpublished results), but such mutations have not been found in clinical isolates.

Further evidence that PBP2a is involved in resistance development also of clinical isolates came from experiments where DNA from β-lactam resistant *S. mitis* or *S. oralis* was used to transfer the resistance into *S. pneumoniae*, resulting in transformants which contained a low affinity PBP2a [53, 112]. Some especially high level resistant clinical isolates of *S. pneumoniae* indeed contained a low affinity PBP2a, diverging from sensitive PBP2a only in up to 3% as changes ([7, 29, 53, 119, 130], and own unpublished results). Whereas in early studies an altered *pbp2a* of resistant *S. pneumoniae* could not be transformed using β-lactam selection [29];

this was possible with *pbp2a* of another isolate, confirming the potential role as resistance determinant for PBP2a [130]. It is remarkable that these resistant PBP2a mutations flanking the active site Ser_{410} ($T_{411}A$) occur frequently. PBP2a has a relatively low affinity especially to penicillins, and it has been suggested that therefore PBP1a mutations are selected before PBP2a becomes a player in the resistance development [147]; however, in transformation experiments using DNA of resistant commensal *Streptococcus* spp., a low affinity PBP2a is transferred into the recipient *S. pneumoniae* before a low affinity PBP1a is selected [53, 112]. Nevertheless, it is almost impossible to deduce the evolutionary history of high level resistant clinical isolates, and it is quite possible that different routes of gene acquisition occur in the natural environment.

In high level resistant *S. pneumoniae* strains, no changes in PBP1b could be detected [29, 53, 119]; however, PBP1b could not be labeled in particular resistant *S. pneumoniae* transformants obtained with DNA from a high level resistant *S. mitis* [53]. The PBP1b gene in the *S. mitis* strain contains a point mutation resulting in premature stop within the transpeptidase domain, probably resulting in absence of the entire protein (own unpublished results). This is the first case where a deletion mutation of a PBP has been identified in a resistant isolate. The fact that no growth defects are apparent in the *S. mitis* strain agrees with the assumption that the putative transglycosylation activities of the three class A hmw PBPs can complement each other. Whether the PBP1b mutation plays a role in resistance, whether it is associated with alterations in all other four hmw PBPs, or a rare coincidence unrelated to resistance remains to be clarified.

A PBP3 mutation $T_{242}I$ associated with resistance has only been described in one particular laboratory mutant C604, again in the immediate vicinity of the $K_{239}TG$ the mutation [76], and a reduced amount of PBP3, which occurs in some laboratory strains, due to mutations in the promoter region also seems to be related to cefotaxime resistance [122]. Particular clones of clinical isolates contain a PBP3 with altered electrophoretic mobility [76], but these variants do not affect the affinity towards β-lactams and are thus most likely unrelated to resistance.

18.10 Murein Chemistry and Penicillin Resistance

The peptidoglycan of Gram-positive bacteria contains interpeptide bridges which are L-Ser-L-Ala and L-Ala-L-Ala in *S. pneumoniae* [39]. These amino acids are added to the lipid II substrate by MurM and MurN, also named FibA and FibB [83, 111], encoded by the *murMN* (*fibAB*) operon. In the cell wall of some high level resistant clinical isolates, such 'branched' muropeptides are present in higher quantity compared to sensitive strains [38]. A mosaic structure of *murM* is associated with resistance increase in some clones [38, 131], but is not always involved in high level resistance [5, 125]. In vitro studies using lipid II substrates and recombinant MurM and MurN enzymes revealed that a much greater catalytic efficiency of MurM from resistant strains compared to the 'sensitive' MurM is mainly responsible

for the different murein structure [83] whereas MurN from both, resistant and sensitive strains, showed similar enzymatic function [19].

Curiously, disruption of *murM/fibA* results in an almost complete collapse of resistance to a level far below that mediated by the primary resistance determinants PBP2x and PBP2b, and such mutants contain an altered murein with a large reduction of crosslinked muropeptides [36, 142]. This is similar to *Staphylococcus aureus* where disruption of the *fem* genes ('factor essential for methicillin resistance') in MRSA resulted in a methicillin sensitive phenotype (for review, see [116]); moreover, *MurMN* mutants are hypersensitive to other cell wall antibiotics, whereas the overexpression of the MurMN genes reduces the lytic response to these compounds [35].

The reason for the resistance-breakdown in MurM mutants remains obscure. PBPs catalyze the crosslinking between two muropeptides, and thus must use the substrates which are the product of MurM/N function. PBP mediated resistance and altered muropeptide composition can be separated in transformation experiments [53, 123], and MurM mutants show no major growth defects [36, 142]. These experiments demonstrate that resistant PBPs can function with either linear or branched precursors in the absence of β-lactams [36, 142]. Thus, MurM (i.e., branched muropeptides) appears to be only crucial during MIC determination (i.e., in the presence of β-lactams), indicating that some of the low affinity PBPs responsible for resistance use branched muropeptides as substrates [53, 123]. It has been suggested that the branched muropeptide precursors are superior competitors against β-lactams for some resistant PBPs or that they might act as signals for some processes during cell wall biosynthesis [36]. Since muropeptides are also the substrate for the sortase enzyme attaching cell surface anchor proteins (LPXTG motif containing proteins) to the peptidoglycan layer, this reaction might also be affected by an altered murein chemistry interfering indirectly with the bacterial response to β-lactams.

However, clinical isolates containing identical MurM and PBP alleles differed significantly in their resistance level [8]. Pneumococcal transformants obtained with chromosomal DNA from high-level resistant oral streptococci also did not reach the resistance level of the donor strains by far, although transfer of PBP genes as well as MurM was achieved ([53, 123] and own unpublished results). These data strongly suggest that other still unknown factors are also involved in β-lactam resistance of clinical isolates.

18.11 Non-PBP Mutations in Laboratory Mutants

In laboratory mutants it was noted for some time that in addition to PBP changes, mutations in non-PBP genes also occur during the selection with β-lactams. Curiously, distinct mutational routes were detected when selection was done with the highly lytic β-lactam piperacillin compared to cefotaxime.

In piperacillin resistant mutants, mutations in a putative membrane associated glycosyltransferase CpoA were identified [44]. Its function as a lipid glycosyltrans-

ferase has recently been verified biochemically in vitro [30]. The *cpoA* mutants showed a pleiotropic phenotype, including a reduced susceptibility to piperacillin, less PBP1a, and a reduction in growth rate, genetic competence, and stationary phase lysis. CpoA has been verified as being responsible for the addition of the second sugar moiety to the major pneumococcal glycolipid GalGlcDAG, which suggests an indirect compensatory effect against the lytic action of piperacillin (C. Volz, B. Henrich, and R. Hakenbeck, unpublished results). GalGlcDAG represents the lipid anchor for LTA, confirming early suggestions that teichoic acid biosynthesis might be affected in CpoA mutants [44].

Some piperacillin and all cefotaxime-resistant mutants contained mutations in the histidine protein kinase CiaH, with every mutant containing a different *ciaH* allele [45, 144]. The CiaRH two component system apparently is required during cell wall stress: deletion mutants in *ciaR* are unusually lysis prone and hypersensitive to a wide variety of early and late cell wall inhibitors, whereas mutants with an activated CiaRH system were highly resistant to many different lysis inducing conditions [91]. Moreover, deletion of the response regulator in mutants containing a low affinity PBP2x showed severe growth defects and lysed rapidly [91]. This defect was especially marked with PBP2x from laboratory mutants containing the $T_{550}A$ change, whereas it was less pronounced in the presence of resistant PBP2x from clinical isolates. CiaR deletion mutants also revealed a complex interactive scenario concerning PBP2x and PBP1a, in that the presence of a mosaic PBP1a can compensate for growth defects apparent in *pbp2x/ciaR* double mutants [146]. This strongly suggests that PBP2x mutations are functionally not neutral, and that this defect can be balanced by a functional CiaRH system. Mutations in CiaH have not yet been observed in clinical isolates. Since CiaH mutations have a complex phenotype and affect the genetic competence as well [92], it might be required in the in vivo situation in agreement with the finding that CiaRH mutants are attenuated in mouse models [88, 121, 139]. The CiaRH regulon has been described on the basis of target sequences of the CiaR response regulator, present in 15 promoters including five regulatory RNAs [57], but the signal detected by the sensor kinase CiaH is still unknown.

These findings imply that inhibitors of LTA biosynthesis and histidine protein kinases are important targets for new antimicrobial agents. CiaR mutants containing a low affinity PBP2x could be screened for anti-histidine kinase antibiotics in that they are hypersensitive to such compounds, and gene products involved in LTA biosynthesis might represent useful proteins for in vitro screens.

In summary, the evolution of resistance in *S. pneumoniae* represents a highly complicated scenario, involving target proteins such as PBPs and non-PBP components as well. Laboratory experiments clearly documented that the kind of mutations and genes selected during resistance development varies enormously depending on the selective compound. Moreover, the complex mosaic structures found in resistant clinical isolates suggests that many different ways for the restructuring of PBPs exist, similar to what has been found in laboratory mutants.

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Chapter 19
Clinical Aspects of Multi-Drug Resistant Enterococci

German A. Contreras and Cesar A. Arias

Abbreviations

AAC	Aminoglycoside Phosphotransferase.
ANT	Aminoglycoside Nucleotidyltransferases.
APH	Aminoglycoside Phosphotransferase.
HCW	Health Care Worker.
HLR	High Level of Aminoglycoside Resistance.
MIC	Minimal Inhibitory Concentration.
MLS_B	Macrolides Lincosamides Streptogramine B.
MLST	Multi Locus Sequence Typing.
MRSA	Methicillin Resistant *Staphylococcus aureus.*
PBP	Penicillin Binding Protein.
VRE	Vancomycin Resistant Enterococci.
VREfm	Vancomycin Resistant *Enterococcus faecium.*
VREfs	Vancomycin Resistant *Enterococcus faecalis.*

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19.1 Introduction

Enterococci are Gram-positive microorganisms that form part of the normal flora of humans and vertebrae animals. They have the ability to grow and survive under extreme conditions and different environments, including soil, food, water, and medical devices [130]. The genus *Enterococcus* spp., contain several species, from which at least 12 have been described in human infections. Those species are the following: *E. faecalis, E. faecium, E. gallinarum, E. hirae, E. durans, E. mundtii, E. avium, E. raffinosus, E. casseliflavus, E. solitarius, E. malodoratus, and E. pseudoavium*. *E. faecalis* and *E. faecium* are the most common species isolated in the hospital environment [38]. According to the National Nosocomial Infections Surveillance System of the United States, enterococci are one of the most common causes of nosocomial infections in US hospitals, [11, 82, 147, 174, 197]. Among these two species, *E. faecalis* accounts approximately for 80–90% of the organisms isolated in the clinical laboratory; however, the number of cases of *E. faecium* (especially vancomycin-resistant) has increased during the last few years, and a recent report indicates that *E. faecium* are almost as frequent as *E. faecalis* in hospitals from the US (*E. faecalis/E.faecium* ratio of ca. 1.5:1) [82].

One the most important characteristics of enterococci is their ability to colonize the gastrointestinal tract of humans for long periods of time; inside this niche, enterococci are capable of recruiting and transferring antibiotic resistance genes, which confer resistance to several anti-enterococcal antibiotics, and as a result, the gastrointestinal tract serves as a reservoir for transmission of multidrug resistant organisms to other patients [144]. Classically, antimicrobial resistance in enterococci has been classified in two types: (1) intrinsic resistance, which refers to determinants (commonly chromosomally encoded) that are characteristic of the genus and are usually present in all enterococci (i.e., reduced susceptibility to aminoglycosides) and (2) acquired resistance, which occurs after acquisition of new DNA (via mobile elements such as plasmids and/or transposons) or by generation of DNA mutations resulting from antimicrobial pressure. The genetic plasticity of the enterococci is highlighted in the latter mechanisms, since these microorganisms have developed highly sophisticated methods to acquire and transfer genetic information between themselves and other bacterial species [39]. In this chapter, we will discuss the important clinical aspects related to the epidemiology and treatment of multi-drug resistant enterococcal infections.

19.2 Epidemiology of Enterococccal Infections

19.2.1 Colonization

As mentioned above, enterococci are natural colonizers of the human gastrointestinal (GI) tract and under nonselective circumstances are found in low numbers in the colon, which has a predominance of anaerobic species [130]. The administration of antibiotics, which are excreted in the bile and have low or no anti-enterococcal

activity promote the growth and expansion of enterococci in the GI tract, particularly vancomycin-resistant enterococci (VRE) [56, 156]. Once the enterococci have established themselves in the gut, two outcomes may be expected: (1) translocation of the microorganisms to the lymphatics and bloodstream producing disease and/or (2) persistent colonization, thus, becoming a silent reservoir for infection. Most patients colonized with VRE remain colonized for long periods of time; for instance, among 53 liver and kidney transplant recipients, 66% remained colonized for more than 3 weeks [145]. Similarly, in a cancer center, 44% of the individuals screened for the presence of enterococci had persistent colonization with the same strain for more than 3 weeks [160]. In a study carried out in a pediatric cancer unit, 43% (out of 73) of patients were persistent carriers and excreted VRE from an average of 19–331 days [80]. Moreover, enterococcal colonization has been shown to persist for more than a year with VRE isolates, which resemble genetically the initial colonizing strain [13, 80, 160].

The most relevant factors associated with the presence of VRE colonization include the physical proximity to patients infected or colonized with VRE, long periods of hospitalization, exposure to multiple and prolonged courses of antimicrobial therapy, hospitalization in long term facilities, surgical units and/or intensive care units, organ transplant recipients, presence of diabetes, renal failure, high APACHE score, and hemodialysis [33, 119, 163, 205]. As indicated above, the use of antimicrobials that lack activity against enterococci, which are excreted in substantial amounts in the gut, and have potent anti-anaerobic activity (i.e., clindamycin, metronidazole, second or third generation cephalosporins) have been strongly associated with promoting VRE colonization [182]. Therefore, colonized patients have the potential to become “efficient vectors” of environmental contamination, transmission and further infection.

19.2.2 Enterococcal Transmission

Multiple approaches have been implemented in order to understand the transmission dynamics in the hospital environment of pathogenic microorganisms such as enterococci in order to develop adequate infection control measures. The use of mathematical models for the study of the modes of transmission and epidemiology of resistant organisms have yielded important information that allow the following: (1) to track the natural history and duration of colonization and infection, (2) to identify transmission routes and the involvement of pathways of spread and, (3) to determine the host behavior and populations at risk. Based on these principles, a basic model of VRE transmission can be constructed based on two separated compartments that include non-colonized and colonized individuals where patients and health care workers (HCW) flow from one compartment to another (Fig. 19.1). This model resembles a vector borne disease where health-care workers (HCWs) play the mosquito role; thus, VRE transmission may result from contamination of the hands of HCWs followed by transmission to a patient; subsequently, those

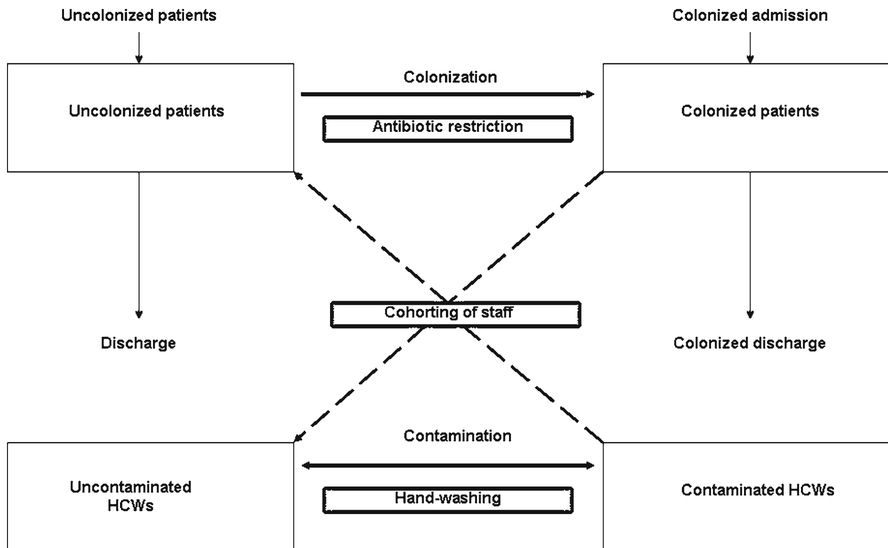

Fig. 19.1 Model of indirect vector mediated VRE transmission between patient-healthcare worker-patient. *HCW* Health care worker (Adapted from [74])

patients may become colonized and later develop a VRE infection. The dynamics of transmission can be summarized by the following formula:

$$\mathbf{R_o} = (\alpha \times \mathbf{N_{HCW}} \times \beta_1 \times \mathbf{D_P}) \times (\alpha \times \mathbf{D_{HCW}} \times \beta_2 \times \mathbf{N_P})$$

Where $\mathbf{R_o}$ represents the reproductive number or the number of VRE cases generated, α represents the number of contacts, $\mathbf{N_{HCW}}$ represent the number of HCWs, $\mathbf{N_P}$ represents the number of patients. The probability of transmission from patient to HCW is represented by β_1 and from HCW to patient is represented by β_2. $\mathbf{D_P}$ and $\mathbf{D_{HCW}}$ represent the duration of infectiousness for the patient and the HCW, respectively. This formula underscores the importance of the HCW role for VRE infection/colonization, which has been widely documented [11]. Thus, it becomes obvious that the pivotal (and cheapest) strategy to cut off one of the important components of this chain of transmission is to reinforce and to improve hand washing and isolation techniques as well as reducing the number of contacts (either colonized individuals and/or environmental contamination). The important role that environmental contamination plays in VRE transmission is supported by the fact that isolates from patients during outbreaks are genetically identical to those that are present on environmental surfaces [51]. Thus, the Healthcare Infection Control Practices Advisory Committee of the Centers for Disease Control and Prevention developed specific guidelines for infection control which make emphasis on disinfection of inanimate surfaces in patient care areas (e.g., bed rails, bedside tables, telephones, stethoscopes, and computer keyboards) [50, 183]. In addition to the HCW and

environment, it is quite important to identify populations at risk of VRE infection and/or colonization. The hospital areas most likely to have VRE colonized patients include intensive care units (medical and surgical), organ transplant services, oncology, dialysis, and AIDS units [10, 46, 56, 93, 111]. These wards tend to keep a constant flow of VRE in and out the hospital setting. Indeed, newly admitted patients may become colonized with VRE "*de novo*" and later discharged from the units to the floor or the community after the patient's discharge [183]. With this rationale, targeted screening of patients at high risk of harboring VRE at hospital admission (e.g., patients with a history of previous isolation of methicillin-resistant *S. aureus* (MRSA), chronic hemodialysis, admission from long term care facilities or another hospital, antibiotic exposure within 30 days, hospitalization within 1 year, and age >60 years) appears to be the best strategy to curtail the chain of transmission [183].

19.2.3 Enterococcal Disease

The most common infections produced by enterococci include urinary tract infection, bloodstream infections, endocarditis, and surgical wound infections (see below). Furthermore, enterococci are well known to produce neonatal sepsis and meningitis. Infections by species different from *E. faecalis* or *E. faecium* such as *E. gallinarum, E. hirae, E. durans,* and *E. casseliflavus* are rarely reported in the literature and most of them are found among critically and chronically ill individuals such as patients with chronic renal failure, diabetes, and malignancies [34, 48, 152]. Strains of both *E. faecalis* and *E. faecium* can cause nosocomial outbreaks and, recently, *E. gallinarum* has been documented to be capable of hospital spread (albeit, uncommonly) [42, 44].

Once a patient is colonized with VRE, the risk of infection is substantially increased among colonized individuals compared to those patients who are free of VRE colonization; however, the risk of infection is dependent on the specific characteristics of the patient population (i.e., patients with hematological malignancies, organ transplant recipients, and allogenic blood and marrow transplants have the highest rates of bloodstream VRE infections, where the urinary, GI, genitourinary tracts, wounds and intravascular catheters are the principal portals of VRE entry) [144]. Furthermore, among patients with leukemia, concurrent *Clostridium difficile* infection was associated with increased risk of developing a VRE bloodstream infection [159].

The fact that infections with VRE produce an increase in mortality has been the object of many years of debate, mainly because most of the infected patients have serious underlying conditions which confound the results obtained in clinical studies [100,179]. Two meta-analyses have showed that the presence of any VRE infection increased the risk of mortality, independently of the clinical status when compared with individuals infected with a susceptible strain of enterococci [52, 165]. This finding may be explained by the lack of administration of appropriate therapy

against VRE and/or delay in receiving such therapy; therefore, early and adequate antimicrobial therapy as well as appropriate control of each one of comorbidities would have a significant impact in the reduction of mortality.

19.2.4 Bacteremia and Endocarditis

Bacteremia and endocarditis are the most common enterococcal infections reported worldwide. In the United States, enterococci is the third cause of mono-microbial bacteremia among the internal medicine, hematology/oncology, cardiothoracic, neurosurgery and obstetrics services (60% and 2% of *E. faecium* and *E. faecalis* were resistant to vancomycin, respectively), where intravascular or urinary catheters, intra-abdominal, and pelvic infections are the principal sources of infection [201]. In Europe, enterococci is recognized as a common cause of bloodstream infections, but the prevalence of VRE is diverse and ranges from <1% to >40%; for instance, the prevalence of VRE among Swedish enterococcal bloodstream isolates was below 0.5% in 2006 whereas the proportion of enterococcal bacteremia attributable to VRE in the UK was 8.5–12.5% for all enterococci species in 2007 (20–25% for *E. faecium* and 1.6–2.5% for *E. faecalis*) [140, 188, 195]. A common feature found among the majority of the European hospitals is that vancomycin-resistant *E. faecium* (VREfm) has also started to emerge as an important cause of nosocomial infections (as is the case in the USA) [24, 195, 197].

In the case of endocarditis, enterococci are reported to be the third most common cause of endocarditis in most of the series published [62]. Enterococcal endocarditis tend be more common among individuals with pre-existing cardiac diseases (i.e., heart valve disease, congenital cardiac diseases, previous history of endocarditis), presence of prosthetic valves, elderly populations with serious underlying conditions, and patients who have undergone diagnostic or therapeutic instrumentation of GI and/or genitourinary tract [62]. The majority of enterococcal endocarditis cases reported in the literature are secondary to *E. faecalis* and *E. faecium,* and just a small percentage of the series documented other species such as *E. durans*, *E. hirae,* and *E. avium* [62, 123, 139, 151]. Furthermore, the risk of developing endocarditis in individuals with *E. faecalis* bacteremia has been postulated to be ca. 6%, but appears to increase substantially in the presence of a cardiac condition such as valvulopathy [62].

19.2.5 Urinary Tract Infections (UTIs)

Enterococci are one of the most common etiologic agents of UTI, and according to the National Healthcare Safety program, enterococci was the third most common pathogen associated with catheter urinary tract infections between 2006 and 2007

(9,377 cases); *E. faecium* (vancomycin- and ampicillin- resistant) and *E. faecalis* were the most common enterococcal species isolated [82]. In addition to UTIs, enterococci are common causes of prostatitis, pyelonephritis, and periphrenic abscesses that can lead to bacteremic episodes [130]. The most relevant risk factors for enterococcal nosocomial UTIs include the presence of an indwelling urinary catheter, long duration of catheterization, high severity of illness at admission, prior and current use of broad spectrum antibiotics, diabetes mellitus, immunosuppression, and structural urologic or anatomic abnormalities [14].

19.2.6 Intra-abdominal Infections

Enterococci are commonly recovered from nosocomial surgical site infections, which mostly include abdominal and pelvic infections; *E. faecalis* is the principal enterococcal species isolated, followed by *E. faecium* [82]. The clinical significance of enterococcal abdominal infections has been argued for many years; for instance, surgery and the use of antibiotics without any enterococcal activity are sufficient for the management of complicated (defined as extending beyond the hollow viscus of origin into the peritoneal space and causing abscess formation or peritonitis) community acquired intra-abdominal infections, despite the presence of positive enterococcal cultures. Due to this fact, the Infectious Diseases Society of America does not recommend anti-enterococcal therapy in the treatment of these intra-abdominal infections [76, 176]. Nevertheless, when the clinical scenario involves high risk populations (i.e., immuno-comprimised patients, high APACHE scores, patients with valvulopathy or patients with persistent intra-abdominal infections or any hematological or solid malignancy) the probability of therapeutic failure increases significantly; in fact, the lack of adequate anti-enterococcal therapy in the above "at risk" populations increased the risk of mortality in several studies [71, 76]. Thus, the use of antibiotics with activity against enterococci may be recommended for high-risk individuals in specific clinical settings.

19.2.7 Meningitis and Neonatal Infections

Meningitis is a rare clinical presentation of enterococcal disease, and most cases reported are associated with individuals who have a previous history of central nervous pathology, surgery, or trauma. *E. faecalis* is the most common species isolated followed by *E. faecium*, *E. gallinarum*, *E. avium,* and *E. casseliflavus* [55]. Complications of enterococcal meningitis included hydrocephalus, stroke, and brain abscesses; accompanying bacteremia can be found in more than half of the cases [55]. Enterococci are also an important cause of neonatal sepsis and they may produce ca. 6% of the cases of late onset sepsis, 5% of pneumonias, 9% of surgical site

infections, 10% of bloodstream infections and 17% of UTIs in neonatal services [31, 72]. These infections are found among newborns who have been hospitalized for more than 1 month, with low birth weight, and have undergone selective invasive procedures [72, 122].

19.3 Therapeutic Options

19.3.1 β-Lactams and Synergism with Aminoglycosides

The bacterial cell wall, which is a polymer of polysaccharides and pentapeptides, is one of the most vital bacterial structures with the function to protect against the high osmotic pressures originated from the bacterial metabolism. The β-lactam antibiotics are bactericidal inhibitors of the cell wall synthesis, since they interact with membrane-bound proteins responsible for the polymerization of the bacterial peptidoglycan (designated PBPs, for penicillin binding proteins), resulting in the inhibition of the transpeptidation reactions that are necessary for the synthesis of the cell wall. An important characteristic of enterococci is their tolerance to the activity of penicillin and other β-lactams, a phenomenon that was initially described during the late 1980s and was defined as a decrease in the rate of killing of enterococci during exposure to bactericidal antibiotics when compared with other bacteria (i.e., streptococci) [95]. Thus, between the 1960s and 1970s, multiple clinical reports highlighted the persistent therapeutic failure of β-lactam monotheraphy for the management of endovascular infections; for instance, in an analysis of 38 patients with enterococcal endocarditis by Mandel et al. [118], 11 individuals who received cephalotin, penicillin, or ampicillin alone showed a bacteriologic relapse after 1 month of therapy; similarly Geraci et al. [67], described six individuals with enterococcal endocarditis treated with penicillin; two of these patients continued to have positive blood cultures during therapy and four had a bacteriologic relapse when penicillin was discontinued [67, 118]. Later on, this clinical observation of penicillin tolerance was described in an endocarditis model; animals infected with a tolerant strain of enterococci showed a significant higher number of bacterial counts in vegetations and significant lower rates of sterilization after 10 days of therapy with penicillin G when compared to a non-tolerant isolate ([95]). In addition to β-lactam tolerance, enterococci (*E. faecalis*) have also been found to produce β-lactamase (Murray et al. 1992; Murray and Mederski-Samaroj, 1983; Patterson et al. 1991); fortunately, *E. faecalis* isolates producing β-lactamase continue to be extremely rare, with the exception of outbreak isolates from the USA and Argentina (Lopardo et al. 1990; Murray et al. 1992). It is important to emphasize that the presence of this enzyme is not easy to detect by routine susceptibility testing, and, therefore, testing specifically for β-lactamase in endocarditis or serious enterococcal infections should be always considered. Although rare, non-β-lactamase mediated resistance to ampicillin and imipenem has also been reported in *E. faecalis* and appears to be associated with mutations of

the *pbp4* gene [141]. Conversely, most *E. faecium* strains have developed a different mechanism of β-lactam resistance to the one described in *E. faecalis* and involves mutations or over production of the PBP5, yielding MICs concentration of > 256 mg/L in some strains [131]. Of note, more than 90% of *E. faecium* isolated from US hospitals are now resistant to ampicillin, often with high level of resistance (MIC > 256 mg/L) [102, 132]. A novel mechanism of β-lactam resistance was described in a laboratory mutant of *E. faecium* involving an alternate pathway of peptidoglycan cross-linking, although no clinical isolates have so far been found to exhibit this mechanism [117].

Amongst the penicillins, ampicillin has the most potent activity against enterococci, followed by the carbapenems (imipenem) whereas the cephalosporins lack any activity against enterococci (except the new generation cephalosporins, see below) [5]. As mentioned above, previous *in vitro* and *in vivo* studies have shown that β-lactam monotherapy is associated with a poor outcome in patients with endovascular enterococcal infections; thus, synergistic therapy that involves the combination of β-lactams and aminoglycosides has become the standard of care for severe enterococcal infections [5, 124]. Synergism is defined as a 2-$\log_{10}$ or greater increase in killing by 24 h, compared with the cell wall agent alone and a 99.9% decrease from the starting inoculum, resulting from the combination of a cell wall agent and an aminoglycoside and when the concentration of the latter has no effect on the growth curve [127]. The development of β-lactam resistance precludes the use of these compounds for the treatment of severe enterococcal infections with the following caveats: (1) infections caused by β-lactamase- producing *E. faecalis* may respond to a combination of β-lactam/β-lactamase inhibitor (in combination with aminoglycoside), (Rice et al. 1991; Wells et al. 1992) and, (2) strains of *E. faecium* with MICs to ampicillin of ≤ 64 mg/L may respond to high dose ampicillin therapy (18–30 g per day associated with one of the recommended aminoglycosides), since high plasma concentrations (superior to 150 mg/L, well above the MICs) can be achieved at the high dose regimen (Tables 19.1 and 19.2) [131].

Gentamicin and streptomycin are the recommended aminoglycosides for synergistic therapy in combination with cell wall agents. This class of antibiotics bind to the 30 S bacterial ribosomal subunit, inhibiting the translocation of the peptidyl-tRNA from the A-site to the P-site, also causing misreading of the mRNA [99]. As indicated above, enterococci exhibit reduced susceptibility to aminoglycosides, a phenomenon that is thought to be mediated by a decrease in the uptake of the drugs associated with specific proteins localized within the cell wall. However, the addition of an agent that blocks cell wall synthesis (ampicillin or vancomycin) markedly increases the uptake of these antibiotics [36, 127, 207]. In clinical practice, one of the most challenging situations related to the treatment of endovascular enterococcal infections is the acquisition of high levels of resistance (HLR) to both streptomycin and gentamicin, defined as the growth of enterococci at concentrations of 2,000 and 500 mg/L of streptomycin and gentamicin, respectively (on brain heart infusion agar) or 1,000 mg/L of streptomycin when using BHI broth; thereby eliminating the synergistic effect previously described ([36]; Mederski-Samoraj and Murray, 1983). It took 12 years for enterococci to develop resistance to the combi-

Table 19.1 Suggested regimens for the management of vancomycin-resistant *Enterococcus faecalis* infections

Clinical syndrome	β-lactams	HLR to aminoglycosides	Therapeutic option
Endocarditis			
	Susceptible[a]	No	Penicillin or ampicillin *plus* an aminoglycoside[b]
	Susceptible[a]	Yes	Ampicillin *plus* ceftriaxone (or cefotaxime)
			HD daptomycin[c] ± another active agent[d]
			Ampicillin *plus* imipenem (± another active agent)[d]
Non-endovascular infections			
	Susceptible	No	Ampicillin or penicillin[e]
	Susceptible	Yes	Penicillin or ampicillin[f]
			HD daptomycin[g]
Urinary tract infections	NA	NA	Nitrofurantoin, fosfomycin, or amoxicillin[h]

NA, not applicable, *HD*, High dose

[a] In rare cases of β-lactamase-producing isolates, the use of ampicillin-sulbactam (12–24 g/day) is suggested. Continuous infusion is recommended by some experts

[b] Gentamicin or streptomycin

[c] Consider doses of 8–10 mg/kg day

[d] An active agent may include a fluoroquinolone (if susceptible) or tigecycline

[e] The addition of an aminoglycoside may be considered in severe infections

[f] The addition of ceftriaxone (or cefotaxime) may be considered in severe infections

[g] Daptomycin, 8–10 mg/kg/day

[h] Only for urinary tract infections

nation of penicillin and streptomycin, which had been recommended since 1947 [30, 85]. A dramatic report in 1959 describes an individual with left side endocarditis (aortic valve) whose *E. faecalis* isolate was tolerant to penicillin and resistant to streptomycin; the patient was successfully treated with penicillin and neomycin, but the therapy left the patient deaf [77]. Later in the early 1970s, several reports emerged describing isolates of *E. faecalis* exhibiting HLR to aminoglycosides [58] and since then, the prevalence has increased worldwide. The mechanisms of resistance mainly involve the following: (1) ribosomal mutations (streptomycin) and (2) enzymatic modification (both streptomycin and gentamicin) [36]. There are three types of common aminoglycoside enzymes in enterococci with acetyltransferase (AAC), nucleotidyltransferase (ANT) and phosphotransferase (APH) activities [87, 175, 202]. The most frequent includes the bifunctional enzyme AAC(6')-Ie-APH(2')-Ia, which confers resistance to all available aminoglycosides, except streptomycin. Other enzymes include the following: (1) the APH(3')-IIIa that confers resistance to amikacin and kanamycin, (2) the ANT(6')-Ia that confers resistance to streptomycin, (3) the APH(2')-Ic that confers resistance to gentamicin and, (4) the

Table 19.2 Suggested regimens for the management of vancomycin-resistant *Enterococcus faecium* infections

Clinical syndrome	β- lactams	HLR to aminoglycosides	Therapeutic options
Endocarditis			
	MIC ≤ 64 μg/ml	No	HD ampicillin[a] *plus* an aminoglycoside[b]
	MIC > 64 μg/ml	No	HD daptomycin[c] *plus* an aminoglycoside[b] ± another active agent[d] Q/D[e] ± another active agent[d] Linezolid[e] ± another active agent[d]
	MIC ≤ 64 μg/ml	Yes	HD ampicillin[a] *plus* HD daptomycin[c] Q/D[e] *plus* HD ampicillin or doxycycline with rifampin HD ampicillin *plus* imipenem[f]
	MIC > 64 μg/ml	Yes	HD daptomycin[c] *plus* another active agent[d] Q/D[e] *plus* doxycycline with rifampin Linezolid[e] ± another active agent[4]
Non-endovascular infections			
	MIC ≤ 64 μg/ml	No	HD ampicillin[a,g]
	MIC > 64 μg/ml	No	Q/D[e] HD daptomycin[c,g] Linezolid[e]
	MIC ≤ 64 μg/ml	Yes	Q/D[e] ± HD ampicillin HD daptomycin[b] ± HD ampicillin Linezolid[e]
	MIC > 64 μg/ml	Yes	Q/D[e] ± another active agent[d] HD daptomycin[c] ± another active agent[d] Linezolid[e] ± another active agent[d]
Urinary tract infections			
	NA	-	Nitrofurantoin, fosfomycin or amoxicillin

[a]Doses to up 30 g/day may be considered
[b]Gentamicin or streptomycin.
[c]Daptomycin, 8–10 mg/kg/day
[d]Agents with potential activity include tigecycline [Jenkins, 2007; Schutt & Bohm, 2009] and doxycycline with rifampin (if susceptible to each agent).
[e]Quinupristin-dalfopristin or linezolid are listed in the American Heart Association recommendations for the treatment of vancomycin and ampicillin resistant *E. faecium.*
[f]If imipenem MIC < 32 μg/ml.
[g]Consider adding an aminoglycoside in severe infections
HLR, high levels of resistance; HD, high dose; NA, not applicable

(6')-AAC found in *E. faecium* and confers resistance to tobramycin, kanamycin, netilmicin and sisomicin.[36, 37, 57, 97, 203]. In Japan, the aminoglycoside arbekacin has been shown to be more stable to the action of the AAC(6')-Ie-APH(2')-Ia enzyme, exhibiting synergism *in vitro* and *in vivo* when combined with β-lactams in the presence of this enzyme, although human studies are lacking [94].

As mentioned above, the presence of HLR to aminoglycosides poses an immense therapeutic challenge for the treatment of endovascular enterococcal infections since the outcomes of β-lactam monotherapy are poor. Therefore, other alternatives have been studied; the most promising involves the combination of ampicillin and ceftriaxone (or cefotaxime). In an observational, open label, non-randomized trial that involved 13 hospitals in Spain, the efficacy of the combination of ceftriaxone (2 g every 12 h) and ampicillin (2 g every 4 h) for 6 weeks for the management of *E. faecalis* endocarditis, which was caused by isolates with HLR to aminoglycosides, was evaluated. The study showed a clinical cure rate of 67.4% and a microbiology cure of 100% at 3 months in those individuals who completed the protocol. The rate of clinical cure for *E. faecalis* isolates with HLR and no-HLR to aminoglycosides was 71.4% and 72.7% at 6 weeks, respectively, and 71.4% and 63.6% at 3 months, respectively [66]. The efficacy of this synergism relies on the observation that ceftriaxone may be able to saturate PBP proteins (e.g., PBP 2 and 3) that are involved in cell wall synthesis in the presence of other β-lactam such as ampicillin (which mainly interacts with PBP4); thus, most of the PBPs that participate in cell wall synthesis are inhibited resulting in a synergistic effect [116]; Of note, the efficacy of this combination is not observed in clinical isolates of *E. faecium*. In addition to the previous combination, the association of ampicillin, imipenem, and vancomycin for the management of *E. faecalis* with HLR to aminoglycosides has been successfully reported and in *E. faecium* with low level of resistance to ampicillin (16 mg/L); the association of ampicillin and imipenem produced a substantial decrease in bacterial counts from vegetations (5-$\log_{10}$) compared with the most active single agent in an animal model of endocarditis (Antony et al. 1997; Brandt et al. 1996).

19.3.2 Glycopeptides

Vancomycin and teicoplanin are the only two glycopeptides currently used in the treatment of gram-positive infections. Vancomycin was the first glycopeptide antibiotic developed for clinical use, and in 1958, it was introduced into clinical practice as an agent active against penicillin-resistant *Staphylococcus aureus.* Initially, the use of vancomycin was eventually limited as a result of toxicity issues originating from the impurities contained in the initial preparations of the antibiotic, and the drug was eventually replaced by the development of cephalosporins and other β-lactams. However, the emergence of MRSA and β-lactam resistance in enterococci, by the end of 1979 and during the 1980s, resulted in a renewed interest for vancomycin as a therapeutic option for Gram-positives [150]. Certainly, vancomycin was in clinical use for over four decades before the first resistant clinical bacterial strains (*E. faecium*) were isolated in France and England in 1986 from patients with leukemia and renal failure, respectively; both strains were resistant to vancomycin and teicoplanin (later classified as VanA type of resistance) (Leclercq et al. 1988; Uttley

et al. 1988). One year later (1987), two VRE isolates were recovered from blood cultures of a patient hospitalized in an intensive care unit in Missouri; 3 days after the isolation of the second strain, a third strain was recovered in an urine specimen from a patient who was in the same intensive care unit as the first patient. Later on, the three isolates were identified as *E. faecalis* and were resistant to vancomycin and susceptible to teicoplanin (subsequently classified as VanB type) [162]. By 1989, almost all enterococcal isolates were susceptible to vancomycin in the United States, but in the following years, the proportion of resistant strains associated with nosocomial infections increased to 12.8%, 25.9% and 28.5 in 1995, 2000, and 2004, respectively (mostly *E. faecium*) [135]. According to the most recent National Healthcare Safety Network at the Centers for Disease Control and Prevention surveillance study, of 983 *E. faecium* isolates associated with nosocomial infection, 80% and 90.4% were resistant to vancomycin and ampicillin, respectively. Conversely, just 6.9% and 3.8% of *E. faecalis* isolates were resistant to vancomycin and ampicillin, respectively (n = 1,542). [82].

In Europe, the prevalence of VRE has increased gradually but not as much as in the United States. Initially, the emergence of VRE in Europe was correlated with the use of avorpacin (glycopeptide antibiotic), which was used as a growth promoter in the agriculture industry and was clearly associated with the presence of a high number of VRE in animal feces and meat samples with the subsequent colonization of healthy humans via the food chain, either by eating or direct contact with contaminated products [29]. The presence of an animal reservoir clearly influenced the rates of VRE transmission and dissemination in the hospital environment in Europe. Thus, the European Union banned the use of avorpacin in 1997, leading to a markedly decrease in the rates of VRE colonization and infection [192, 193]. Most of the cases reported to date are secondary to VREfm, whereas vancomycin-resistant *E. faecalis* (VREfs) are still rare [195]. Similar to the United States, Europe is now facing an epidemic of VREfm as the principal enterococcal agent of nosocomial infection [28, 49, 109, 187, 191]. The situation is rather different in other parts of the world. In Latin-Amercia, for example, a recent study that involved 32 hospitals of four Latin-American countries (Colombia, Venezuela, Peru and Ecuador) showed that *E. faecalis* (77.4%) was by far the most common microorganism associated with hospital infections while *E. faecium* accounted for only 15.4% of all isolates (743 enterococcal isolates recovered). The overall frequency of VRE was 6.2%, although *E. faecium* also accounted for most of the vancomycin resistant isolates [143]; therefore, it appears that several factors influence the local behavior and epidemiology of VRE.

Molecular typing data have revealed interesting patters of evolution, transmission, and population genetics of a large number of bacterial pathogens, including VRE. In the case of *E. faecium*, multi-locus sequence typing (MLST) has led to the identification of a distinct enterococcal clonal complex, designated clonal complex 17 (CC-17), which appears to have acquired resistance and pathogenic determinants that enable them to have an enhanced ability for dissemination and disease potential in the hospital environment [23, 69, 96, 98, 103, 115, 184, 200]. The CC17 is mainly

characterized by high levels of ampicillin and quinolone resistance [103, 199, 200] and isolates from this clonal cluster are enriched with several genes that include the presence of a functional *acm* (a gene encoding for a collagen adhesin) [134], enterococcal surface protein gene (esp_{Efm}), which is contained on a putative pathogenicity island (PAI) and has an important role in biofilm formation, [200] and the hyl_{Efm} gene, which encodes a putative glycosyl-hydrolase and has been associated with *E. faecium* isolates of nosocomial origin (as opposed to those from healthy volunteers and animals) and appears to be important for colonization and possibly virulence [156, 157]. In the case of *E. faecalis*, CC21, CC9, CC2, CC8, and CC40, represent the most common genetic lineages, where CC2 and CC9 are the predominant enterococcal clonal complexes associated with hospitals outbreaks and life threatening infections [121, 133]. The most relevant characteristics of *E. faecalis* isolates, belonging to these clonal complexes, include the presence of vancomycin resistance, high-level of resistance to gentamicin (due to the presence of the bifunctional enzyme AAC(6')-Ie-APH(2')-Ia,) and in some sporadic isolates, resistance to ampicillin due to the production of β-lactamase. Furthermore, all members of this clonal complex contain a previously described PAI, which harbors a gene that encodes for the Esp protein that contributes to colonization of epithelial cells and biofilm formation [168, 186].

19.3.3 Lipopeptides: Daptomycin

Daptomycin is a 13 member lipopeptide antibiotic containing a hydrophilic core with a lipophilic tail. It is derived from the fermentation of *Streptomyces roseosporus* and has rapid bactericidal activity against most of the Gram-positive organisms [60, 166]. Daptomycin has been approved for the treatment of complicated skin and soft tissue infections caused by susceptible strains of Gram positive organisms, including vancomycin susceptible *E. faecalis* and for bacteremia caused by *S. aureus*; however, it is not FDA-approved for the treatment of *E. faecium* or for VRE. The mechanism of action is not completely understood and most of the studies suggest that interacts with the cytoplasmic membrane via the insertion of its lipid tail in a calcium dependent manner [78]. This binding is followed by oligomerization of daptomycin molecules, which leads to an increase in the efflux of K^+, causing a change of membrane potential that subsequently leads to bacterial cell death [170]. Currently, there are several case reports documenting daptomycin resistance in enterococci; these are usually found among patients with serious underlying conditions such as end stage renal diseases, hematology malignancies, congestive heart failure, and diabetes mellitus and also in individuals with preexposure to either vancomycin or daptomycin or with an undrained focus of infection [8, 63, 73, 83, 92, 108, 110, 114, 128]. Daptomycin has a concentration dependent bactericidal activity against enterococci in *in vitro* models and, the area under the curve/MIC ratio in 24 h (AUC/MIC) appears to be the best pharmacokinetic parameter that predicts its efficacy *in vivo* [1, 45, 47]. Furthermore, it has

a prolonged post-antibiotic effect that together with its pharmacokinetic profile support a once daily dosing regimen [75]. Emergence of daptomycin-resistant strains with treatment failures have been documented with standard dose daptomycin monotherapy [8] and it has been postulated that the combination of high-dose daptomycin with other agents such as gentamicin, rifampicin or β-lactams may offer certain clinical advantages in the setting of enterococcal endocarditis. As examples, the use of the combination of high-dose daptomycin (8 mg/kg/day), high-dose ampicillin and gentamicin or, daptomycin, gentamicin, and rifampin, successfully treated two cases of *E. faecium* endocarditis, which had failed to other therapies, respectively [8, 178]. More recently, the combination of daptomycin with tigecycline was able to eradicate a multi-drug resistant *E. faecium* isolate causing endocarditis [89, 167]. In conclusion, high-dose daptomycin should be considered as an alternative for the treatment of severe enterococcal infections and the association with another active agent may add some clinical benefit and should be carefully considered in the setting of endovascular infections (Tables 19.1 and 19.2).

19.3.4 The Oxazolidinones: Linezolid

The oxazolidinones represent a unique class of synthetic antimicrobials agents discovered by DuPont Pharmaceuticals in the late 1970's [126, 173]. Linezolid has broad activity against the most common gram positive pathogens, anaerobes, and non-tuberculous mycobacteria [125]. Linezolid is currently approved for the treatment of methicillin-susceptible and methicillin-resistant *Staphylococcus aureus* strains and VRE infections. Linezolid is an inhibitor of bacterial ribosomal protein synthesis, and, according to current studies, it binds to the A site of bacterial ribosomes interfering to protein synthesis. Linezolid resistance is mediated by two mechanisms (1) specific mutations in the central loop of the domain V of the 23 S rRNA which alter the binding site of the antibiotic (which is the most common mechanism in enterococci) and, (2) methylation of the 23 S rRNA at position A2503, catalyzed by the presence of a methyltransferase (encoded by the *cfr* gene and only described in *S. aureus* with a possible enterococcal donor) [9, 185]. Linezolid resistance has been reported in both *E. faecalis* and *E. faecium* and risk factors for acquisition of linezolid-resistant enterococci are similar to those described for vancomycin (i.e., presence of invasive medical devices, immuno-supression, broad spectrum antibiotics, amongst others), including prolonged therapy with the antibiotic [53, 70, 81, 149].

Skin and soft tissue infections and pneumonia are the principal indications for linezolid use; however, controversial data exist for the use of linezolid in the treatment of endovascular infections caused by VRE, due in part, to the lack of randomized clinical trials. In 2003, an open-label, non-comparative, and non-randomized study that evaluated the efficacy of linezolid against gram-positive infections found that the clinical cure and microbiology eradication rate for VREfm bacteremia were 78% and 85%, respectively (for endocarditis cases only, the values were 76% and

63%, respectively); however, the study included a small number of individuals and a significant number of the patients were lost to follow up; therefore, the actual efficacy of linezolid may have been overestimated [22]. On the other hand, a recent meta-analysis evaluated the available evidence related with the efficacy of linezolid for the management of endocarditis; of eight cases of enterococcal endocarditis treated with linezolid (two vancomycin-resistant *E. faecalis,* four VREfm and two vancomycin-susceptible *E. faecalis*), seven were cured or achieved resolution of the infectious episode after a median duration of linezolid therapy of 48 days; two of the seven patients had a surgical procedure in addition to linezolid therapy and just one case presented treatment failure (vancomycin-susceptible *E. faecalis*) after treatment for 98 days. Two out of eight patients presented thrombocytopenia whereas in the remainder of the individuals the information of adverse effects was not available. The use of a failed regimen and/or allergy were the principal reasons for linezolid therapy [61]. In contrast, numerous case reports have documented the failure of linezolid monotherapy in the treatment of either bacteremia or endocarditis [17, 190, 206]; thus, until further data are available (randomized controlled trials), linezolid should be used with caution in the treatment of VRE endocarditis and only when resistance, side effects or allergy prevent the use of combinations of β-lactams and aminoglycosides, high-dose daptomycin or daptomycin plus aminoglycosides (Tables 19.1 and 19.2).

19.3.5 Pristinamycins: Quinupristin-Dalfopristin

Quinupristin-dalfopristin (Synercid) is a combination of two naturally occurring compounds produced by *Streptomyces pristinaspiralis* [32]. This compound consists of a 30:70 mixture of quinupristin (type B streptogramin) and dalfopristin (type A streptogramin), which independently are bacteriostatic, but in combination, they exhibit *in vitro* bactericidal activity against MSSA, MRSA, *Streptococcus pyogenes* and *E. faecium* [16]. The combination is ineffective against *E. faecalis,* due to the presence of a gene (designated *lsa*), which likely encodes a component of an ABC (ATP-binding cassette) transporter [2]. The main mechanism of action of this compound is inhibition of the bacterial protein synthesis by a serial of interactions within the 50 S ribosomal subunit of the 70 S unit during the elongation phase [26, 40]. The efficacy and safety of quinupristin-dalfopristin for the treatment of VREfm infections were assessed in a prospective, multicenter non-comparative study. The investigators enrolled severely ill patients who had signs and symptoms of active VREfm infection and with no appropriate alternative antibiotic therapy. The overall clinical response was 65.5%, and, according with the type of infection, the highest rates of success were found in urinary tract infection (80.0%) and bacteremia of unknown origin (71.9%); arthralgias and myalgias were the most frequently reported adverse events, which also led to the discontinuation of therapy [112]. Quinupristin-dalfopristin has also been used as part of a combination regimen in enterococcal endocarditis; *Matsumura et al.,* showed that blood cultures remained positive after

2 weeks of quinupristin/dalfopristin monotherapy and sterilization of the blood was only obtained when doxycycline and rifampin were added to the initial regimen. A similar finding was reported when high doses of ampicillin (32 g/day) were added to quinupristin/dalfopristin for the treatment of VREfm endocarditis in a cancer patient [19, 120]. Furthermore, in a rabbit model of endocarditis, the combination of quinupristin-dalfopristin with imipenem or levofloxacin showed a better response in comparison with quinupristin-dalfopristin alone [147]. We may conclude that quinupristin-dalfopristin may be an option for the management of VREfm infections when used in combination with other active agents (i.e., doxycycline, rifampin, ampicillin, imipenem, or levofloxacin), although clinical data are lacking and the compound has an unfavorable side effect profile (Tables 19.1 and 19.2).

The mechanism of quinupristin-dalfopristin resistance in enterococci is due to the methylation within a single adenine residue of the 23 S RNA. This residue is located within a region of domain V of 23 S RNA and takes part in the binding of the MLS_B antibiotics (macrolides, lincosamides, and streptogramins B); this change impairs the binding and produces cross-resistance between all MLS_B antibiotics, due to the overlapping binding sites in 23 S rRNA. This biochemical change is mediated by the erythromycin ribosomal methylases (Erm), which are encoded by a variety of genes [194]. Resistance to quinupristin-dalfopristin is also associated with the presence of the *vat*(D) and *vat*(E) genes, which encode acetyltransferases that inactivate streptogramin A and by the expression of possible efflux pumps (e.g., Lsa) [171, 172].

19.3.6 The Glycylcyclines: Tigecycline

Tigecycline is a novel semisynthetic antibiotic from the glycylcycline group of antimicrobials and the first new tetracycline analogue since minocycline was brought to the market 30 years ago [113]. Tigecycline has a primary backbone of minocycline with an *N*-alkyl-glycylamido group substituted in position 9, which enhances the spectrum of activity and also offers protection against the most common mechanisms of tetracycline resistance such as production of active efflux pumps, ribosomal protection, enzymatic degradation and 16 S rRNA mutations [35, 41, 68, 161, 174, 177, 189]. This antibiotic binds to the bacterial 30 S ribosomal subunit and blocks the entry of amino-acyl tRNA molecules into the A site of the ribosome; therefore, amino acid residues are prevented from becoming incorporated into elongating peptide chains [18, 138]. Animals and humans studies have demonstrated that tigecycline distributes extensively into various tissues and body fluids such as lungs, skin, gallbladder, bone, synovial fluid, bile, and cerebro-spinal fluid [105, 154]. The antibiotic is active against a wide variety of microorganisms and in a multicenter study across the United states, the MIC_{90} for tigecycline in *E. faecalis* and *E. faecium* was 0.12 mg/L [54], which were similar to isolates recovered in Europe and Latin-America [64, 158]. In experimental endocarditis, tigecycline has achieved 2–4 log_{10} decrease in bacterial colony forming units counts in cardiac

vegetations for both vancomycin-susceptible and resistant enterococci at 48 h compared with untreated controls [105, 129]. However, clinical data to support the use of tigecycline in enterococcal endocarditis or any other endovascular infection are still lacking. Furthermore, the maximum serum concentration of tigecycline at the recommended dose is ca. 1 mg/L, which is a concern in the treatment of endovascular infections [146, 153]. Nonetheless, two recent case reports indicate that tigecycline (100 mg intravenously followed by 50 mg every 12 h) may have a role in the treatment of multi-drug resistant *E. faecium* endocarditis when combined with high-dose daptomycin (6 mg/kg or 8 mg/kg) [89, 167] (Table 19.2). In the management of soft tissue infections due to vancomycin-susceptible *E. faecalis*, tigecycline showed a microbiology eradication rate of 87.5%; however, it was not superior to the combination of vancomycin – aztreonam (91.7%) [59]. In a trial evaluating the treatment of complicated abdominal infections, tigecycline and imipenem-cilastatin exhibited similar rates of microbiological eradication for vancomycin-susceptible *E. faecalis* (78.8% and 74.5%, respectively) [12]. Thus, tigecycline appears to be an alternative for the treatment of certain enterococcal infections but its use in severe VRE bloodstream and endocarditis infections is still questionable.

Tigecycline resistance has been reported in *E. faecalis* and it was described in a 65-year-old patient with history of an intra-abdominal surgery, who presented several post-operative complications such us colon perforation, peritonitis, nosocomial pneumonia, and renal failure. She was treated with several courses of antibiotics including tigecycline for more than 2 weeks. Subsequently, an *E. faecalis* with a MIC of 2 mg/L by Etest for tigecycline (resistant strains are defined as having an MIC > 0.5 mg/L) was isolated from the urine; however, the exact mechanism of resistance has not been elucidated [196].

19.4 Future Therapeutic Options

19.4.1 Lipoglycopeptides

Oritavancin was obtained from the natural glycopeptide cloroeremomycin and has a similar spectrum of activity to vancomycin with the major difference that it remains active against VRE and vancomycin-resistant staphylococci [43]. The activity against VRE appears to be related to an increase in the affinity for both pentapeptide (ending in D-Ala-D-Ala) and pentadepsipeptide (ending in D-Ala-D-Lac) of peptidoglycan precursors. This enhanced affinity appears to be related to the ability of the molecule to dimerize in solution, anchor to the cytosolic membrane of the bacteria, and alter the membrane potential (Mckay et al. 2008). Typical MICs of oritavancin against VRE are between 1 and 2 mg/L [15, 136]; however, the inclusion of polysorbate 80 0.002% to the standard Mueller-Hinton broth media reduced the MICs of oritavancin 16–32 fold against susceptible organisms, suggesting that

the potency of this compound against enterococci might have been underestimated using standard susceptibility testing [4]. Oritavancin is characterized to have a long half-life [mean range was 195.4 h (135.8–273.8)] at [20]. Furthermore, it shows a concentration-dependent bactericidal activity and post antibiotic effect against VRE. In a rabbit model of endocarditis caused by strains of *E. faecalis* exhibiting the VanA or VanB phenotype, an intramuscular regimen of 20 mg/kg produced a significant reduction in the bacterial counts of vegetations as compared with controls; however, oritavancin selected mutants with increased oritavancin MICs in VanA strains at a rate of 10^{-7}, generating approximately ten mutants per animal, although the addition of gentamicin proved to be synergistic and able to prevent emergence of resistant mutants [104, 164]. In skin and soft tissue infections, oritavancin was comparable to vancomycin for both clinical and bacteriological cure (about 78%) [106].

Telavancin (TD-6424) is a derivate of vancomycin that has a hydrophobic side chain on the vancomycin sugar and an aminomethyl substituent on the cyclic peptide core [91]. Telavancin produces inhibition of the late stages of peptidoglycan synthesis by binding to the D-Ala-D-Ala terminus; in addition, it produces disruption of bacterial membrane potential, which led to an increased cell permeability [107]. It has activity against a wide number of gram-positive microorganisms; however, it appears to have limited activity against glycopeptide-resistant enterococci with MIC_{90} for vancomycin resistant *E. faecalis* and *E. faecium* ranging between 4 and 16 mg/L and 2 and 16 mg/L, respectively; although these MICs are several fold lower than those for vancomycin [5]. Telavancin has a long half life (7–9 h at doses above 5 mg/kg), long post-antibiotic effect, and time dependent killing [79, 169]. Telavancin has showed a similar clinical success (85%) in comparison to vancomycin in the management of skin and soft tissue infections; in clinical trials, *E. faecalis* (vancomycin susceptibility was not mentioned) represented 5% (32 isolates) and 6% (43 isolates) of the total of isolates in the telavancin (n = 680) and vancomycin (n = 703) arms [181], respectively. Thus, telavancin may be useful for the management of skin infections secondary to vancomycin susceptible enterococci.

Dalbavancin is derived from a teicoplanin-like glycopeptide agent by modification of the functional groups and sugar moieties, while preserving the D-Ala-D-Ala affinity [21]. This antibiotic interferes with the cross-linking and polymerization of the peptidoglycan molecule. In addition, it attaches to the bacterial cell membrane, but unlike oritavancin, the ability of dalbavancin to dimerize appears to be poor, making it less active against the depsipeptide peptidoglycan [3]. Dalbavancin is active against VRE expressing the *vanB* or *vanC* gene cluster, but is inactive against strains that harbor the *vanA* gene cluster (which is the most clinically relevant) [27, 65, 180]. The typical dalbavancin MIC_{90} values for vancomycin susceptible *E. faecalis* and *E. faecium* are 0.06 and 0.12 mg/L, respectively [5]. In phase III trial, dalbavancin (two doses, 1 week apart) was non-inferior to linezolid (600 mg twice daily for 14 days) in the treatment of skin and soft tissue infections with 89% of clinical success in the dalbavancin arm versus 91.2% in the linezolid arm; the microbiology success was similar in both arms (85%) [88].

19.4.2 New Cephalosporins with Anti-Enterococcal Activity

Ceftobiprole is a new broad-spectrum cephalosporin characterized by its stability against the most common β-lactamases and by high affinity for the PBPs of gram-positive microorganisms, including PBP2a of *S. aureus* and PBP2x of *S. pneumoniae*. It has a potent bactericidal activity against *E. faecalis* (including VRE) but not *E. faecium* [90]. The MIC_{90} for 62 *E. faecalis* isolates was 4 mg/L, but it was >32 mg/L for 52 isolates of *E. faecium* ([90]). In experimental peritonitis, ceftobiprole showed a comparable activity to ampicillin against vancomycin-resistant and ampicillin- susceptible *E. faecalis*, but was superior to ampicillin against a β-lactamase producing isolate of *E. faecalis* [6]. Furthermore, synergistic activity was observed for the combination of ceftobiprole (0.5 μg/mL) plus gentamicin (10 μg/mL) or streptomycin (25 μg/mL) against β-lactamase producing *E. faecalis* [7], indicating that this cephalosporin may be a good alternative for the management of *E. faecalis* infections, although is not likely be useful against *E. faecium*. The other cephalosporin with similar profile to that of ceftobiprole is ceftaroline. The typical $MICs_{90}$ for ceftaroline against *E. faecalis* are 4 μg/mL (regardless of vancomycin susceptibility), lacking activity against *E. faecium* (MICs between 16 μg/mL and >32 μg/mL, respectively) [25]. In an animal model of *E. faecalis* endocarditis, ceftaroline (10 mg/kg/12 h) showed a significant reduction in the bacterial counts in cardiac vegetations in comparison to vancomycin and linezolid (10 mg/kg/12 h) after 4 days of treatment [86]. Of note, spontaneous mutants (1.25×10^{-7}) were obtained after serial passages [84]. Clinical studies are therefore needed to determine the role of these compounds in the treatment of enterococcal infections.

19.5 Alternative Therapeutic Options

Nitrofurantoin is a useful alternative for the management of UTIs due to enterococcal species including VRE since most strains are still susceptible. The *in vitro* activity of nitrofurantoin was evaluated in ca. 300 enterococcal isolates from urinary tract infections, including *E. faecium* (carrying the *vanA* and *vanB* gene clusters), *E. faecalis*, and *E. gallinarum*. None of the isolates tested had MICs ≥ 128 mg/L [204], indicating that nitrofurantoin was a potentially active compound in UTIs caused by these isolates. Furthermore, successful treatment of urinary infection caused by vancomycin and ampicillin resistant *E. faecium* has been documented in a nosocomial outbreak setting [142]. Similarly, fosfomycin tromethamine has activity against many urinary tract pathogens, including enterococci and is currently FDA approved for the treatment of urinary tract infections caused by *E. faecalis*. A study from the United States showed that 75 VRE isolates recovered from UTIs (23 *E. faecalis* and 52 *E. faecium*) had MICs between 8–256 μg/mL and 16–64 μg/mL for *E. faecium* and *E. faecalis*, respectively [148]. Although the current breakpoints for

fosfomycin are only available for *E. faecalis* (R ≥ 256 μg/mL), this antibiotic appears to be useful for the treatment of *E. faecium* UTI.

The quinolones have been sporadically used in the treatment of enterococcal infections, mainly as part of a combination regimen. However, the increased rates of resistance observed in different countries, the high frequency of selection of resistant mutants during therapy, and the lack of any effect in some animal models make the quinolones a less attractive alternative for enterococcal infections. A potential role may be as long-term suppressive therapy against fluoroquinolone-susceptible enterococci in endovascular infections in combination with amoxicillin although clinical trials have not been performed [198].

Chloramphenicol is another antibiotic that has been used in the treatment of enterococcal infections, since rates of resistance amongst clinical isolates appear to be low even among VRE isolates. In a retrospective study of 14 patients who received chloramphenicol for the treatment of serious VRE infections, eight (57%) showed clinical improvement after treatment and 73% (8 out 11 available for microbiological evaluation) had cleared the bacteria from the blood after treatment and no major adverse side effects of the drug were noted in the study [137]. In a series of 51 patients with VRE bloodstream infections treated with this antibiotic, 61% demonstrated clinical response and 79% exhibited microbiological eradication; similar results have been observed for the treatment of prosthetic valve endocarditis and meningitis [101, 155]. Therefore, chloramphenicol may be useful in certain circumstances (when available) and in cases when no other choices are appropriate due to allergy, resistance, or toxicity; the risk of bone marrow suppression and emergence of resistance during therapy are still major concerns with this compound.

19.6 Conclusion

Enterococci represent one of the most versatile gram-positive microorganisms and their ability to survive under extreme conditions, colonize, disseminate across different environments and produce substantial disease make them a cause of increased concern and a top clinical challenge. The emergence of multi-resistant organisms for which no effective therapy is available has become more common and physicians are becoming increasingly “desperate” when faced with severe enterococcal infections. The enormous genetic plasticity and adaptation that these organisms have developed during recent years put them on the cusp of evolution. Although, in recent years, new therapeutic options have emerged for the treatment of infections caused by enterococci, the organisms have responded quickly and swiftly to the biological challenge, developing sophisticated mechanisms of resistance. A comprehensive approach that involves a deep understanding of the biological characteristics of the organisms, mechanisms of resistance and means of dissemination in the hospital is the only way to deal with these pathogens in the near future.

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Part V
Gram-negatives

Chapter 20
Clinical Issues of Resistance: Problematic Microbes: Enterobacteriaceae

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Abbreviations

CAP	Community-acquired pneumonia
CFU	Colony-forming unit
CLSI	Clinical and Laboratory Standards Institute
DAEC	Diffusely adherent *E. coli*
EAEC	Enteroaggregative *E. coli*
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ESBL	Extended spectrum β-lactamase
ETEC	Enterotoxigenic *E. coli*
GNB	Gram-negative bacilli
HUS	Hemolytic-uremic syndrome
ICU	Intensive care unit
IM	Inner membrane
LPS	Lipopolysaccharides
LTCF	Long term care facility
MBL	Metallo-carbapenemase
MIC	Minimum inhibitory concentration
NNIS	National nosocomial infections surveillance
OM	Outer membrane

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OMP	Outer membrane proteins
PBP	Penicillin binding protein
SSTI	Skin and soft tissue infection
STEC	Shiga toxin-producing *E. coli*
Stx	Shiga-like toxin
TMP/SMX	Trimethoprim/Sulfamethoxazole
UTI	Urinary tract infection

20.1 Classification and Structure

The family *Enterobacteriaceae* falls within the domain Bacteria, phylum Proteobacteria, class Gammaproteobacteria, and order Enterobacteriales. They are non–spore-forming gram-negative organisms that ferment glucose and other sugars, reduce nitrates to nitrites, and produce catalase but, in general, do not produce oxidase. They are rod-shaped, and measure 1–3 μm in length and 0.5 μm in diameter; several structures are found among some members of the family and include a capsule, a cell wall with an inner and an outer membrane, pili and flagellae (exceptions include *Shigella* spp. and *Klebsiella* spp. which are non-motile).

20.1.1 Capsule

The capsule corresponds to a layer of polysaccharides with antigenic properties called the glycocalyx. Classically, the K antigen has been described as the main component of the capsule. It functions as an anti-phagocytosis, anti-complement, protective structure for bacteria; these properties allow *Enterobacteriaceae* not only to avoid host defenses but to act as enhancers of virulence. In *E. coli* for example, more than 80 K antigens have been described some involved in virulence and infection like the K1 antigen associated with meningitis and urinary tract infections in humans [62, 90, 92], and the M antigen, also called colonic acid, implicated in protection against desiccation of bacteria and as a component of biofilms.

20.1.2 Outer Membrane (OM)

The OM consists of an asymmetrical bilayer of phospholipids in the inner aspect and lipopolysaccharides (LPS) in contact with the extracellular medium. LPS acts as an important antigenic molecule and it has been implicated as a mediator in Gram-negative sepsis. The OM serves as a protective barrier against harmful environmental substances and possesses specialized protein channels through which molecules can pass called the outer membrane proteins (OMP) or porins. Antibiotics often are porin-specific and can pass only through OMP [7]. Passage of compounds

is almost always driven by passive diffusion, although energy-dependant systems have been described in *E. coli* [15].

20.1.3 Inner Membrane (IM)

The IM is a phospholipid bilayer with proteins attached to this cellular component; they typically span the membrane as α-helical structures and are involved in important cellular processes such as cell division, signal transduction, molecular transport and energy generation [50]. Embedded in the IM are the penicillin binding proteins (PBP's), which are responsible for cell wall synthesis in the key step of transpeptidation.

20.2 Mechanisms of Resistance

Resistance is a major issue when considering therapy. There are four mechanisms which may confer resistance in Gram-negative bacteria including *Enterobacteriaceae*, (a) Enzymatic modification of the antibiotic like ß-lactamases and aminoglycoside modifying enzymes, (b) pump-mediated efflux of antibiotics through the outer membrane, (c) loss of permeability due to reduced synthesis of porins, and (d) target modifications as seen in PBP's for ß -lactams or DNA gyrase and topoisomerase IV, which confer resistance to quinolones.

20.2.1 β-Lactamases

ß -lactamases, which are located in the periplasmic space, are the main mechanism of resistance in Gram-negative bacteria against β-lactam antibiotics. These can be either chromosomally encoded or plasmid mediated. The latter confer transferability among bacteria, due to their capacity for spreading and posing a challenge for controlling the dissemination of resistance. ß-Lactamases are most commonly classified according to two general classifications: the Ambler *molecular* classification system and the Bush-Jacoby-Medeiros *functional* classification system. The former use sequence-based classification recognizing only four classes designated A to D. Classes A, C, and D compromise evolutionary distinct groups of serine enzymes, and class B contains the zinc types. The second classifies β-lactamases by their substrate preference among penicillin, oxacillin, carbenicillin, cephaloride, expanded-spectrum cephalosporins, and imipenem and also by their susceptibility to inhibition by clavulanate. This phenotypic classification faces the problem that point mutations can greatly alter substrate specificity and inhibitor susceptibility, changing the group to which an enzyme is assigned [65]. We will review the most frequent and clinically relevant β-lactamases.

20.2.1.1 AmpC-Type β-Lactamases

AmpC β-lactamases have been isolated in multiple members of the family *Enterobacteriaceae.* Typically, AmpC β-lactamases provide resistance to cephamycins as well as to oxyimino-β-lactams, and monobactams and are also resistant to inhibition by β-lactamase inhibitors [83].

Organisms harboring AmpC-type β-lactamases are a major clinical problem, due to their proclivity for selecting resistance during therapy due to overexpression of the enzyme which occurs either by derepression of the *ampC* chromosomal gene or by acquisition of a transferable *ampC* gene by a plasmid. The molecular details are beyond the scope of this chapter, but can be found in other chapters.

Among the *Enterobacteriaceae* that carry the AmpC gene and can de-repress during treatment when using a third generation cephalosporin are the following: *Aeromonas* spp., *Morganella morganii, Providencia stuartii*, *Providencia rettgeri, Proteus* spp. (indole-positive), *Citrobacter freundii, Enterobacter* spp., and *Serratia* spp. [34, 69, 71]. The acronym AMPCES may help to remember the members of this group. Once derepression occurs, the hyperproduction of AmpC is stable. Many clinical isolates fit this pattern. For example, in the United Kingdom 30–40% of *E. cloacae* isolates from inpatients are AmpC hyperproducers [48] as are 15–25% of North American isolates [37].

Since bla_{AMPC} genes occur on transmissible plasmids, the clinical microbiologist needs to consider this resistance mechanism whenever the identification of these organisms occurs. Plasmid-mediated AmpC enzymes were first reported in nosocomial isolates of *Klebsiella pneumoniae* and *E. coli* in the late 1980s. Minor differences in amino acid sequence have given rise to families. Forty-three CMY variants are currently known (http://www.lahey.org/Studies/) and in GenBank, sequence data can be found (some of it unpublished) for seven varieties of FOX; four varieties of ACC, LAT, and MIR; three varieties of ACT and MOX; and two varieties of DHA [33].

Fortunately, in the United States and the rest of the world plasmid mediated AmpC β-lactamases are still uncommon, with rates in most pathogens of less than 10% [72]; however, vigilance is still warranted [64].

20.2.1.2 Extended-Spectrum β-Lactamases (ESBLs)

ESBLs are derivatives, predominantly, of Amber class A: TEM and SHV type, and class D: OXA type β-lactamases. Though over the past several years other classes have been reported, including BES, GES, PER, TLA, VEB, and CTX-M. They have been found in a wide range of Gram-negative organisms, particularly members of the *Enterobacteriaceae*, most commonly in *E. coli, K. pneumoniae,* and Proteus [8]. The first reported ESBL dates back to 1983, shortly after the introduction of third generation cephalosporins into clinical practice.

ESBLs arose due to point mutations in TEM-1 and SHV-1 β-lactamases in *E. coli* and *K. pneumoniae.* TEM was named after the Greek patient from whom the first

sample was obtained in 1965 from an *E. coli* isolate, while SHV denotes a variable response to sulfhydryl inhibitors [65].

ESBLs confer resistance to penicillins, narrow-spectrum cephalosporins, oxymino-cephalosporins (third-generation cephalosporins) and aztreonam. Usually they do not hydrolyze cephamycins (cefoxitin and cefotetan), carbapenems, and β-lactamase inhibitors. The latter characteristic differentiates these enzymes from the AmpC-type β-lactamases, which are resistant to β-lactamase inhibitors.

ESBLs can be carried on chromosomes, but typically are plasmid encoded and often found in association with integrons while conferring the capacity to spread within both the same and different species.

Plasmids encoding ESBLs may harbor genes that give co-resistance to aminoglycosides, tetracycline's, and trimethoprim/sulfamethoxazole (TMP/SMX); many ESBL producers are also resistant to quinolones, and in some of these resistant strains, a plasmid-mediated quinolone resistance determinant defined as *qnr*, has been found [35, 64].

Among the risk factors for acquisition of a nosocomial ESBL producers are the following: prolonged duration of hospital stay, residence in intensive care units (ICUs), invasive medical devices, prior antibiotic use, renal failure, burns, and receipt of total parenteral nutrition [66].

Historically ESBLs have been a nosocomial problem, but recent reports show spread to *E. coli* in ambulatory patients with CTX-M enzymes as the predominant enzyme. According to Ben-Ami et al. risk factors for fecal carriage of ESBL-producing *Enterobacteriaceae* found in 26 out of 246 patients (10.8%) who were colonized or infected at admission to a hospital were the following: dependent functional state (OR, 4.2; $P = .004$), current use of antibiotics (OR, 3.4; $P = .015$), chronic renal insufficiency (OR, 2.8; $P = .03$), liver disease (OR, 11.1; $P = .02$), and use of a histamine receptor antagonist (OR, 2.8; $P = .03$) [6].

A recent report by Lewis et al. confirmed CTX-M as the predominant ESBL isolated in a U.S. Health Care System [44]. Also CTX-M isolates can be hypervirulent [10]; CTX-M-15 for example is not only the dominant ESBL in Europe but has been found in multi-resistant hypervirulent clones of *E. coli*, associated with extra-intestinal infections [39, 40, 49, 73].

Recognition of ESBLs is critical, since multiple studies have shown that inadequate antibiotic therapy of infections due to organisms of this type is associated with a high mortality rate; the carbapenems appear to be the agents of choice but selection of OprD deficient imipenem-resistant *P. aeruginosa* has been shown to occur after wide use [76]. Carbapenem resistance can also occur in *Enterobacteriaceae* harboring CTX-M when associated with the lack of expression of the OMPK36 porin [55].

Finally, OXA type ESBLs are comparatively rare and have been found mainly in *P. aeruginosa* in specimens from Turkey and France [34]. Most OXA-type ESBLs are relatively resistant to inhibition by clavulanic acid and can hydrolyze carbapenems, so are also classified as carbapenemases, as will be discussed below.

20.2.1.3 Carbapenemases

Carbapenemases are a diverse group of enzymes capable of hydrolyzing almost all β-lactams, including the carbapenems. They may be chromosomally encoded or carried on mobile genetic elements. Endemic rates of carbapenemase production are highly regional, being more common in eastern and southern Europe and in some developing countries. They are mostly found in *Acinetobacter* spp., and *P. aeruginosa,* and more recently in *Enterobacteriaceae*. The most important types include the following:

1. Class D enzymes (OXA-23, -40, -51, -58 and related) that are present in several successful *Acinetobacter* clones.
2. The metallo-carbapenemases (MBLs) such as IMP, VIM, SPM, and GIM, which belong to serine carbapenemases, are derived from Ambler molecular Class B. These are strongly active against carbapenems in vitro and also against all other β-lactams except aztreonam. They mostly occur in *P. aeruginosa* and *Acinetobacter* spp., with producers sometimes causing major outbreaks. A common feature as with ESBLs and AmpC enzymes, these MBLs only confer resistance to carbapenems in *Enterobacteriaceae,* if their activity is augmented by the impermeability caused by porin loss.
3. The plasmid-mediated class A enzymes of the KPC family have become the dominant carbapenemase in enterics worldwide, so we will describe this family in more detail.

KPC-1 (for *Klebsiella pneumoniae* carbapenemase), was first discovered in North Carolina, USA, in a *K. pneumoniae* clinical isolate in 1996. This was followed by detection of KPC-2 in *K. pneumoniae* and afterwards in other species such as *Salmonella enterica*, *K. oxytoca*, *Enterobacter* spp., and *E. coli*. Recently KPC 1 and 2 have been shown to be the same enzyme.

Dissemination to other countries soon occurred. In South America, Villegas et al. reported in 2006 the first KPC-2 in clinical isolates of *K. pneumoniae* from the continent [86] followed in 2007 by the first identification of a KPC-harboring *P. aeruginosa* [87]. As variants of KPC usually differ from one another by one amino acid substitution, new KPCs have been described in rapid sequence. The most recent reports were KPC-5 in a *P. aeruginosa* as well as KPC-6 in a *K. pneumoniae* isolate from Puerto Rico [75, 77, 93].

KPC-producing strains have generally been shown to exhibit multidrug resistance to piperacillin/tazobactam, third- and fourth-generation cephalosporins, fluoroquinolones, and aminoglycosides, as well as carbapenems. Moreover, there is a report of a unique KPC *K. pneumoniae* strain producing at least eight different β-lactamases [23].

Detection of KPC enzymes in the clinical lab is difficult. Levels of resistance may vary according to the type and amount of enzyme, the species in which it is found, and the confounding presence of additional resistance mechanisms like the concomitant loss of an outer membrane protein, which is required for high-level resistance to carbapenems [64].

Recently, Endimiani et al. showed the limitations of the current CLSI breakpoints to detect KPC. In the case of carbapenems, two thirds of imipenem and meropenem MICs for *K. pneumoniae* strains with KPC were in the susceptible range, while 95% of ertapenem MICs were in the resistant range. Hence, the authors recommend that for *K. pneumoniae* isolates showing imipenem, meropenem, or doripenem MICs ≥1 mg/L or ertapenem MICs ≥2 mg/L, screening for KPC should be done using PCR or a phenotypic method (i.e., modified Hodge test) [23].

Few studies have measured the clinical impact of these enzymes, so the outcomes are still highly variable and uncertain. One of the studies which tried to approach it was a city wide surveillance in New York; from 602 *K. pneumoniae* studied, 43.5% had ESBLs and 1.5% of those had also KPC, and these KPC producing strains represented a major clonal outbreak at several New York hospitals with up to 47% mortality rate at 14 days [9, 94, 96].

It is unclear if a common genetic element is responsible for the transmission of KPC genes around the world. The gene arrangements associated with KPC have been studied in *Klebsiella* and *P. aeruginosa* isolates from Greece, France, and Colombia. In each case, a unique transposon, Tn4401, was detected [56]. These studies should be conducted on additional strains from other countries.

20.2.2 Target Modifications

Cellular structure modification is an important mechanism of resistance in *Enterobacteriaceae*.

PBP's are crucial for cell wall transpeptidation; thus, altering cell wall architecture leads to disruption of cell homeostasis causing cell death. Gram-positive organisms commonly employ PBP mutations to acquire resistance to β-lactam antibiotics. In contrast, this is rare among Gram negatives.

In *Enterobacteriaceae* the alteration or disruption of the enzymes gyrase and topoisomerase IV to confer quinolone resistance is quite common. In humans, increasing rates of resistance to this class of antibiotics has been associated to a greater consumption of the drug [46]. In animals, the use of quinolones in poultry by veterinarians and the food industry might be associated with a parallel increase in quinolone resistant-rates in humans [24]. Both gyrase and topoisomerase IV are enzymatic complexes composed by two pairs of subunits; GyrA encoded by the *gyrA* gene, and GyrB, encoded by the *gyrB* gene. The subunits in topoisomerase IV are ParC and ParE. Quinolones bind to the enzymes, creating a drug-DNA-enzyme complex altering cellular processes and releasing DNA breaks that are lethal to bacteria [32]. Amino acid substitutions in the Quinolone-Resistance-Determining Region (QRDR) in GyrA or ParC can lead to resistance. In *E. coli,* most mutations are encountered at amino acid positions 83 and 87 [28].

Recently, a novel mechanism of resistance was described in *K. pneumoniae* [54] consisting of a plasmid-mediated quinolone resistance conferred by a group of proteins termed Qnr and encoded by the *qnrA, qnrB, qnrD, and qnrS* genes [14].

These pentapeptides bind to gyrase and topoisomerase IV and protect DNA from the inhibition by quinolones: *qnr* genes are now prevalent in different countries and different species of *Enterobacteriaceae* including *E. coli, K. pneumoniae, E. cloacae, C. freundii, and Salmonella* spp.*,* in Asia, Europe, South America and the United states [13, 63, 68, 81]. Very often, genes for ESBLs are found in the same plasmid where *qnr* genes are explaining in part the co-resistance to multiple antibiotic families commonly found in *Enterobacteriaceae.*

Methylation of ribosomal RNA (rRNA) by 16 S methylases is another mechanism of target modification. Its importance has been well characterized in resistance against aminoglycosides.

So far, six different enzymes have been classified: RmtA, RmtB, RmtC, RmtD, ArmA, and NpmA. The NpmA enzyme is the only one that methylates residue A1408, whereas the others methylate residue G1405 within the aminoacyl site (A site) of the ribosomal subunit [21].

20.2.3 Efflux Pumps

Efflux pumps have been recognized for many years and are present in every living cell. There has been growing evidence, indicating their crucial role in resistance to antimicrobials. While efflux as a mechanism of resistance to antibiotics has been known for some time, multidrug efflux systems have only recently been identified and appreciated as significant determinants of resistance. Essentially, these protein components span the outer membrane and actively extrude from the bacteria a vast array of molecules including metabolites, detergents, organic solvents, as well as antibiotics, using either ATP hydrolysis or an ion-antiport mechanism as its source of energy. The main goal of this mechanism is to limit the accumulation of toxic components inside the cell. Efflux pumps can be either drug-specific, which tends to be plasmid-encoded and are thus transmissible, or nonspecific, which are normally expressed on the chromosome. If a pump is overproduced in the latter case, then it may confer extended cross-resistance to multiple drug class using this single mechanism.

Typically, efflux alone leads to small increases in MIC. However, when multiple mechanisms come into play simultaneously, susceptibility may change in a dramatic fashion. Recently, a new plasmid-mediated fluoroquinolone efflux pump called QepA was found in *E. coli,* showing significant increased levels of resistance to norfloxacin, ciprofloxacin and enrofloxacin [95].

20.2.4 Porin Loss

Porins are outer membrane channels that work as a filter in a permeability barrier. Mutational loss retards the access of antibiotics into the bacterial cell. OMPs can be either specific or nonspecific, allowing the passage of multiple molecules in the latter case.

Many studies on the structure and regulation of porins in *E. coli* K-12 are available, but there is little information concerning clinical isolates of this species. In *K. pneumoniae*, two major porins, OmpK35 and OmpK36, are produced, but many ESBL-producing *K. pneumoniae* isolates do not express OmpK35. Loss of both OmpK35 and OmpK36 in ESBL-producing *K. pneumoniae* causes resistance to cefoxitin, increased resistance to expanded-spectrum cephalosporins, and decreased susceptibility to carbapenems, particularly ertapenem. Porin loss also decreases the susceptibility to other non-β-lactam compounds, such as fluoroquinolones [53].

20.3 *Escherichia coli*

20.3.1 Microbiology and Laboratory Diagnosis

E. coli grows readily on simple culture media or synthetic media with minimal nutritional requirements such as glucose or glycerol. *E. coli* strains are typed according to their antigens: lipopolysaccharide (O), capsule (K) and flagellum (H). Hundreds of such antigens have been described. However, certain O serogroups may predominate for various groups of intestinal *E. coli* infections as well as some K serogroups predominate for certain extraintestinal infections [61]. Specific pathogenic strains will be discussed later.

20.3.2 Epidemiology

Data reported to the National Nosocomial Infections Surveillance (NNIS) System during 2003 showed that *E. coli* is the most important Gram-negative causing nosocomial infections, being the most prevalent agent responsible for urinary tract infections (26% of the total reported) and it is one of the top five causes of nosocomial pneumonia, surgical site infection, and bacteremia [30].

E. coli is ubiquitous and present in almost all animal intestinal tracts. In humans, it is the major aerobic organism residing in the intestine, typically with around 10^6–10^9 CFU per gram of stool [22]. Commensal *E. coli* variants constitute most of the normal facultative intestinal flora in healthy humans. They confer benefits to the host, including resistance to colonization with pathogenic organisms. These strains usually lack the specialized virulence traits that enable pathogenic *E. coli* strains to cause disease either outside or within the gastrointestinal tract. However, even commensal *E. coli* strains can be involved in extraintestinal infections in the presence of a predisposing factor, such as a urinary catheter, urinary or biliary tract obstruction, systemic immunocompromise, or an inoculum that is large enough or contains a mixture of bacterial species (e.g., peritonitis).

The clinically important strains of *E. coli* are not morphologically or biochemically distinct from the commensal ones; however, as discussed later, certain serogroups may predominate in given types of *E. coli* infections and are known to have specific virulence factors that make them responsible for several clinical infections.

20.3.3 Clinical Syndromes

20.3.3.1 Extra-Intestinal Pathogenic Strains

The majority of *E. coli* isolates from symptomatic infections of the urinary tract, bloodstream, cerebrospinal fluid, respiratory tract, and peritonitis can be differentiated from commensal and diarrheagenic strains of *E. coli* by virtue of their distinctive virulence factor profiles and phylogenetic background. Such strains express adhesive organelles like type 1 and P pili that allow them to bind and invade host cells and tissues like the urinary tract [57].

Expression of iron-chelating factors (siderophores) enables extraintestinal *E. coli* to steal host iron stores. Deployment of an array of toxins, including hemolysin and cytotoxic necrotizing factor 1, allows extensive tissue damage facilitating bacterial dissemination as well as releasing host nutrients and disabling immune cells. These toxins also have the capacity to modulate, in more subtle ways, host signaling pathways affecting myriad processes, including inflammatory responses, host cell survival, and cytoskeletal dynamics [91].

Like commensal *E. coli* (but in contrast to intestinal pathogenic *E. coli*), extraintestinal pathogenic strains are often found in the normal intestinal flora and do not cause gastroenteritis in humans.

Urinary Tract Infection (UTI)

The urinary tract is the site most frequently infected by extraintestinal pathogenic strains. An exceedingly common infection among ambulatory patients, UTI accounts for 1% of ambulatory care visits in the United States and is second only to lower respiratory tract infection among infections responsible for hospitalization. UTIs are divided into a variety of clinical syndromes including uncomplicated cystitis, pyelonephritis, and catheter-associated UTIs. *E. coli* is the most common pathogen for all UTIs, which are the precipitating cause for seven million patient visits per year with total costs exceeding 1 billion dollars in the United States alone [3].

Uncomplicated cystitis, the most common acute UTI syndrome, presents as dysuria, frequency, and suprapubic pain. In contrast, the appearance of fever or back pain suggests progression to pyelonephritis. Persistently elevated or increasing fever and neutrophil counts should prompt evaluation for intrarenal or perinephric abscess or obstruction. Renal parenchymal damage and loss of renal function during pyelonephritis occur primarily with urinary obstruction. Pregnant women are at unusually high risk for developing pyelonephritis, which can adversely affect the outcome of pregnancy. Therefore, prenatal screening for and treatment of asymptomatic bacteriuria are

standard. Prostatic infection is a potential complication of UTI in men. The diagnosis and treatment of UTI should be tailored to the individual host, the nature and site of infection, and local patterns of antimicrobial susceptibility.

Abdominal and Pelvic Infection

The abdomen and pelvis are the second most common sites of extraintestinal infection due to *E. coli*. A wide variety of clinical syndromes occur in this location, including acute peritonitis secondary to fecal contamination, spontaneous bacterial peritonitis, dialysis-associated peritonitis, diverticulitis, appendicitis, intraperitoneal, or visceral abscesses (including hepatic, pancreatic, splenic), infected pancreatic pseudocysts, and septic cholangitis and/or cholecystitis. In intraabdominal infections, *E. coli* can be isolated either alone or along with other facultative and/or anaerobic members of the intestinal flora.

Bacteremia

E. coli bacteremia arises mainly from primary infection at any extraintestinal site. In addition, primary *E. coli* bacteremia can arise from percutaneous intravascular devices or transrectal prostate biopsy or can result from the increased intestinal mucosal permeability seen in neonates and in the settings of neutropenia and chemotherapy-induced mucositis, trauma, and burns. Roughly equal proportions of *E. coli* bacteremia cases originate in the community and in the hospital. In most studies, *E. coli* and *Staphylococcus aureus* are the two most common blood isolates of clinical significance. *E. coli*, which is isolated in 17–37% of cases, is the gram-negative bacillus most often isolated from the blood in the ambulatory setting and in most long term care facilities (LTCFs) and hospital settings [5].

Isolation of *E. coli* from the blood is almost always clinically significant and typically is accompanied by the sepsis syndrome, severe sepsis (sepsis-induced dysfunction of at least one organ or system), or septic shock. Calculations based on a conservative estimate for the proportional contribution of *E. coli* to severe sepsis (i.e., 17% of all cases) translate into an estimated 40,000 deaths among the affected patients in the United States in 2001.

The urinary tract is the most common source of *E. coli* bacteremia, accounting for one half to two-thirds of episodes. Bacteremia from a urinary tract source is particularly common in patients with pyelonephritis, urinary tract obstruction, or instrumentation in the presence of infected urine. The abdomen is the second most common source, accounting for 25% of episodes. Although biliary obstruction (stones, tumor) and overt bowel disruption are responsible for many of these cases, some abdominal sources (e.g., abscesses) are remarkably silent clinically and require identification via imaging studies (e.g., CT). Therefore, the physician should be cautious in designating the urinary tract as the source of *E. coli* bacteremia in the absence of characteristic signs and symptoms of UTI. Soft tissue, bone, pulmonary, and intravascular catheter infections are other sources of *E. coli* bacteremia.

Pneumonia

E. coli is not usually considered a cause of pneumonia. *Enterobacteriaceae* account for only 2–5% of cases of community-acquired pneumonia (CAP), in part because these organisms only transiently colonize the oropharynx of a minority of healthy individuals. However, rates of oral colonization with *E. coli* and other Gram-negative bacilli (GNB) increase with the severity of illness and with antibiotic use. Thus, GNB are a common cause of pneumonia among residents of LTCFs and are an important cause of hospital-acquired pneumonia, particularly among postoperative and critically ill patients. Pulmonary infection is usually acquired by aspiration, but it also occurs via hematogenous spread in which case multifocal nodular infiltrates can be seen. Tissue necrosis, probably due to cytotoxins produced by GNB, is common. Despite significant institutional variation, *E. coli* is generally one of the most commonly isolated GNB in hospital-acquired pneumonia, accounting for 5% of episodes in U.S.-based studies [30]. Regardless of the host, pneumonia due to enteric GNB is a serious disease, with high crude and attributable mortality.

Meningitis

E. coli is one of the two leading causes of neonatal meningitis (the other being group B *Streptococcus*) and the most common cause among developing countries. Most of the responsible strains possess the K1 capsular antigen [43]. After the first month of life, *E. coli* meningitis is rare. *E. coli* meningitis after this age usually implies disruption of the meninges from neurosurgery or after trauma or in the presence of cirrhosis. In patients with cirrhosis, the meninges are thought to be seeded with *E. coli* because of the lack of hepatic clearance of portal vein bacteremia.

Skin and Soft Tissue Infections (SSTI)

E. coli contributes frequently to infection of decubitus ulcers and occasionally to infection of ulcers and wounds of the lower extremity in diabetic patients and other hosts with neurovascular compromise. In addition, occasionally it causes cellulitis or infections of burn sites or surgical wounds, especially when the infection originates close to the perineum. Myositis or fasciitis due to *E. coli* in the thigh should prompt an evaluation for an abdominal source with contiguous spread.

Endovascular Infection

Despite being one of the most common causes of bacteremia, *E. coli* rarely seeds native or prosthetic heart valves. It lacks many of the virulence and adherence factors that allow bacteria to stick to the endothelium and produce disease. When the organism does seed native valves, it usually does so in the setting of prior valve disease. *E. coli* infections of aneurysms and vascular grafts are quite uncommon.

20.3.3.2 Intestinal (Diarrheagenic) Pathogenic Strains

Certain strains of *E. coli* are capable of causing diarrheal disease. At least in the industrialized world, these strains of *E. coli* are rarely found in the fecal flora of healthy persons and instead appear to be always involved in disease. These strains have evolved a special ability to cause gastroenteric syndromes when ingested in sufficient quantities by a naive host. There are at least five distinct types of diarrheagenic *E. coli*: (1) Shiga toxin–producing *E. coli*/enterohemorrhagic *E. coli* (EHEC), (2) enterotoxigenic *E. coli* (ETEC), (3) enteropathogenic *E. coli* (EPEC), (4) enteroinvasive *E. coli* (EIEC), and (5) enteroaggregative *E. coli* (EAEC). Diffusely adherent *E. coli* (DAEC) and cytodetaching *E. coli* have also been described. Transmission occurs predominantly via contaminated food and water for ETEC, EHEC, EIEC, and probably EAEC and by person-to-person spread for EPEC (and occasionally EHEC). Gastric acidity confers some protection against infection; therefore, persons with decreased stomach acid levels are especially susceptible. Humans are the major reservoir (except for EHEC); host range appears to be dictated by species-specific attachment factors. Although there is some overlap, each type possesses a unique combination of virulence traits that results in a distinctive intestinal pathogenic mechanism. These strains are largely incapable of causing disease outside the intestinal tract. Except in the case of EHEC and perhaps EAEC, disease due to this group of pathogens occurs primarily in developing countries.

20.3.4 Antibiotic Considerations

In the past, most *E. coli* isolates were highly susceptible to a broad range of antibiotic agents; this situation has changed. In general, the frequency of ampicillin resistance precludes its empirical use, even in community-acquired infections. The prevalence of resistance to first-generation cephalosporins and TMP/SMX is increasing among community-acquired strains [97]. Until recently, TMP/SMX was the drug of choice for the treatment of uncomplicated cystitis, but resistance in the United States increased from 7% in 1990 to 18% in 1998 [78]. Nowadays, this trend has reached a resistance rate ≥20% (range, 13–45%) [82]. Thus, treatment guidelines switched to alternative agents including fluoroquinolones. Resistance to fluoroquinolones has increased steadily over the last decade all over the world. In Europe, the trend between 2001 and 2007 is shown in Figs. 20.1 and 20.2. The prevalence of resistance is higher in settings where fluoroquinolone prophylaxis is used extensively (e.g., in patients with leukemia, transplant recipients, and patients with cirrhosis) and among isolates from LTCFs and hospitals [88]. Among quinolone-resistant strains, a significant prevalence of multi-drug resistance has been well described; in a collaborative study, only 10.8% of fluoroquinolone resistant strains were found to be resistant only to this class [41].

The prevalence of resistance to third and fourth generation cephalosporins, monobactams, piperacillin-tazobactam, and the non-amikacin aminoglycosides is

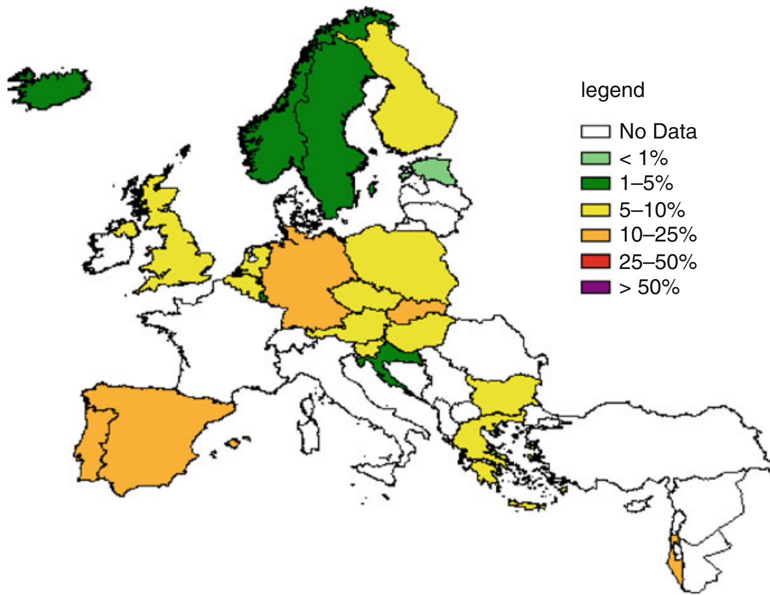

Fig. 20.1 EARRS, resistance to fluoroquinolones in *E. coli* (2001)

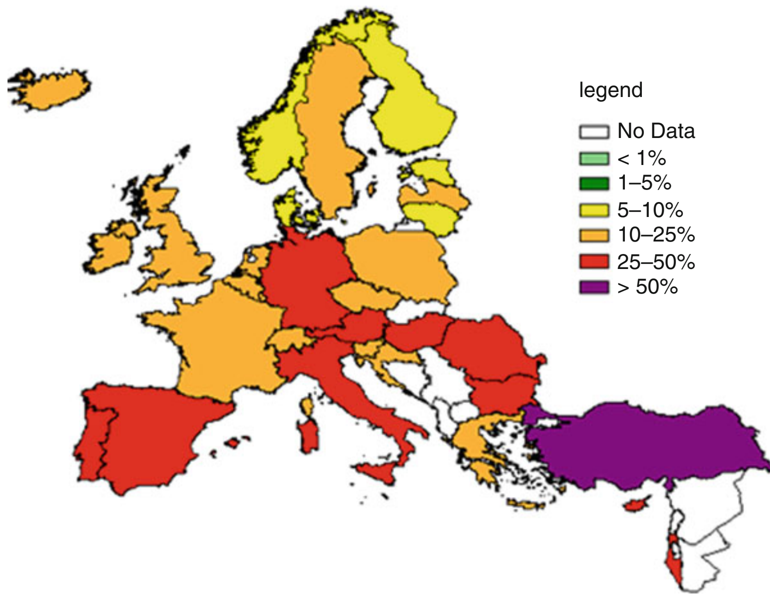

Fig. 20.2 EARRS, resistance to fluoroquinolones in *E. coli* (2007)

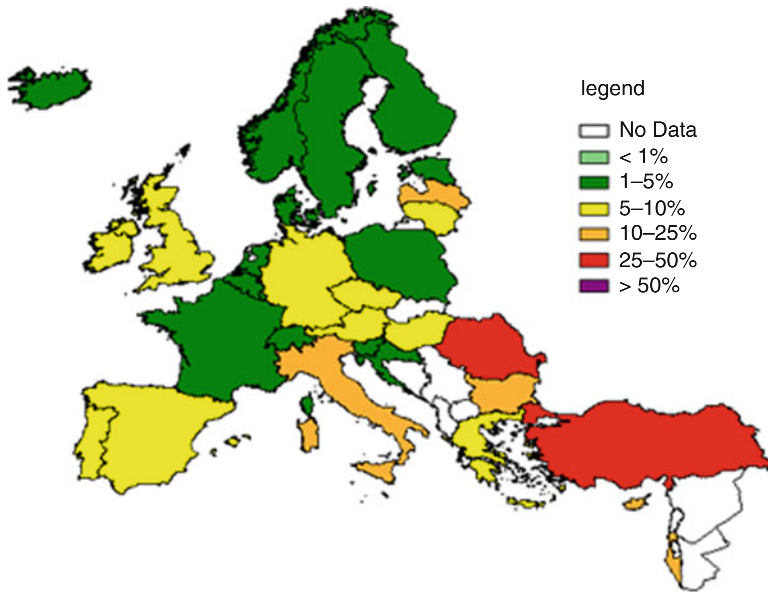

Fig. 20.3 EARRS, proportion of third gen. cephalosporins resistant *E. coli* in 2007

increasing but is still generally <10% [41]. In Europe, the resistance to third generation cephalosporins is shown in Fig. 20.3.

Carbapenems and amikacin are the most predictably active agents. Among the carbapenem class, some prefer to use ertapenem for enterics in order to avoid unnecessary coverage of nonfermenters. Although relevant clinical experience is limited, tigecycline and polymyxin B or E are highly active in vitro. However, blood and urine concentrations of tigecycline are lower than those seen in gallbladder and skin [47, 70], therefore use of this agent should be restricted to its FDA approved indications in skin and intra-abdominal infection. Several reports have described treatment failure in bacteremia and this agent should not be used in that setting [1].

The mainstay of treatment for all diarrheal syndromes is replacement of fluids and electrolytes. The use of prophylactic antibiotics to prevent traveler's diarrhea generally should be discouraged, especially in light of high rates of antimicrobial resistance. However, in selected patients including those in whom an infection would be lethal, the use of rifaximin, which is non-absorbable and well tolerated, is reasonable. When stools are free of mucus and blood, early patient-initiated treatment of traveler's diarrhea with a quinolone or azithromycin decreases the duration of illness, and the use of loperamide may halt symptoms within a few hours. Although dysentery caused by EIEC is self-limited, treatment hastens the resolution of symptoms, particularly in severe cases. Antimicrobial therapy for Shiga Toxin-Producing *Escherichia coli* (STEC)/EHEC infection (the presence of which is suggested by bloody diarrhea without fever) should be avoided, since antibiotics may increase the incidence of Hemolytic-uremic syndrome (HUS) possibly via increased production/

release of shiga-like toxin (Stx). Current in vitro data suggest that antibiotic exposure increases the risk of HUS in children infected with STEC by inducing expression of Stx through replication of phages that carry *stx* genes [38, 67].

20.4 *Klebsiella pneumoniae*

20.4.1 Microbiology

The genus *Klebsiella* consists of non-motile, aerobic and facultative anaerobic, gram-negative rods ranging from 0.3 to 1.0 μm in width to 0.6–6.0 μm in length. The genus is heterogeneous, and can be arranged into three clusters proposed by Orskov [74]: first, the subspecies of *K. pneumoniae*: *pneumoniae*, *ozaenae,* and *rhinoscleromatis*; second, *K. oxytoca;* and third, the other species with common features (growth at 10°C and uses L-sorbose as a carbon source). The vast majority of *Klebsiella* spp. have prominent capsules composed of complex acidic polysaccharides which are essential virulence factors. Most strains grow on standard media. *K. pneumoniae* can be readily distinguished by tests for lysine decarboxylase, malonate utilization, the Voges-Proskauer reaction and gas from lactose at 44.5°C. The latter is the single test that can distinguish the subspecies *pneumoniae*.

20.4.2 Epidemiology

Klebsiella spp., is ubiquitous in nature. They have two common habitats, one being the environment and the other being the mucosal surfaces of mammals including humans, which they colonize. In this respect, the genus *Klebsiella* is like *Enterobacter* and *Citrobacter*. In humans, *K. pneumoniae* is present as a saprophyte in the nasopharynx and in the intestinal tract. Reported carriage rates in hospitalized patients are 77% in stool, 19% in the pharynx and 44% on the hands. They are amongst the most common causes of a variety of community-acquired and hospital-acquired infections. *K. pneumoniae* subsp., *pneumoniae* is the leading cause of disease followed by *K. oxytoca*. In the United States, it accounts for 3–8% of all nosocomial bacterial infections, which is similar to the European statistics. The NNIS system report (2003) states that among patients in intensive care units in the United States, 20.6% of all *K pneumoniae* isolates were non-susceptible to third generation cephalosporins. This represented a 47% increase compared with resistance rates from 1998 to 2002 [64]. In Europe, the resistance to third generation cephalosporins is shown in Fig. 20.4.

By the end of the 1990s, carbapenem-resistant *K. pneumoniae* was isolated harboring the KPC enzymes. Currently, within the *Enterobacteriaceae* family, *K. pneumoniae* has the highest rates of carbapenem resistance. These are highly focal causing outbreaks worldwide and occasionally becoming endemic in some medical centers.

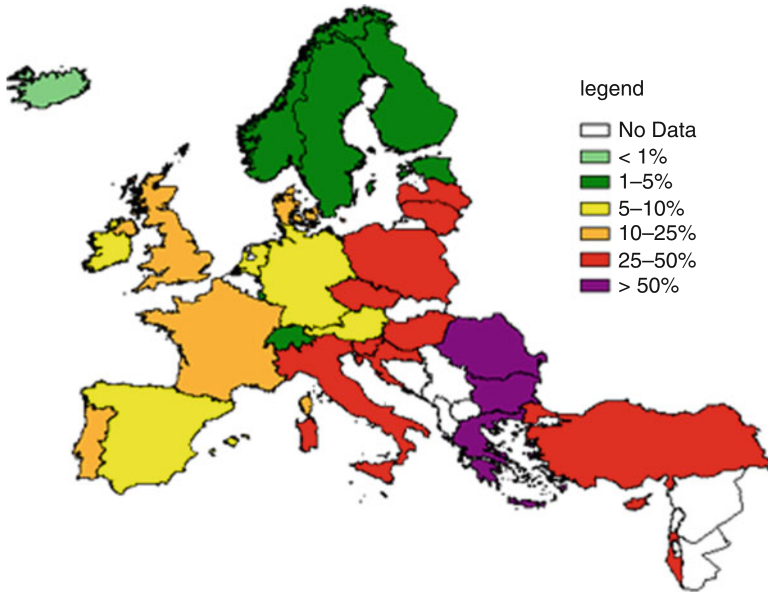

Fig. 20.4 EARRS, proportion third gen. cephalosporins resistant *K. pneumoniae* in 2007

20.4.3 Clinical Syndromes

K. pneumoniae is a pathogen capable of causing urinary tract infections (UTIs) and pneumonia in otherwise healthy people; however, most infections are nosocomial in origin. In addition to UTIs and pneumonia, it can cause a wide spectrum of diseases: wound infections, infections of intravascular and other invasive devices, biliary tract infections, peritonitis, meningitis, endophthalmitis and rarely, endocarditis. It is second in incidence after *E. coli* as a cause of Gram-negative bacteremia.

Pneumonia caused by *K. pneumoniae* cannot be distinguished on clinical grounds from that caused by other organisms; however, some have described particular distinguishing features, conferring it the eponym "Friedländer's disease." Among these features are severe pneumonitis (especially in alcoholics, in whom mortality rates exceeding 50% has been reported), propensity to affect the upper lobes, the production of "currant jelly" sputum resulting from hemoptysis, the "bulging fissure sign" on roentgenography caused by edematous lobar consolidation, and its tendency for abscess formation.

K. pneumoniae meningitis is a relatively common cause of neonatal meningitis in developing countries in contrast to developed countries where it is less common. In adults, it can occur extremely rarely as a community acquired infection, but more commonly as a nosocomial disease complicating neurosurgery. These infections have traditionally had high mortality. As with all infections associated with devices, the therapeutic approach should be combined antibiotic use and removal of any devices.

20.4.4 Antibiotic Considerations

Selection of antimicrobial therapy for *K. pneumoniae* infections should be based on local susceptibility patterns. A critical consideration is if the isolate is an ESBL producer or not. Critically ill patients with nosocomial *Klebsiella* infections should be treated with antibiotics covering ESBL producers, such as carbapenems.

The vast majority of *K. pneumoniae* strains produce the chromosomally encoded β-lactamase SHV-1 conferring resistance to the following: penicillin, ampicillin, amoxicillin, oxacillin, carbenicillin, and ticarcillin. As previously discussed, 20% of ICU strains in teaching centers in the US produce ESBLs conferring resistance to third generation cephalosporins as well as aztreonam. Usually the cephamycins, carbapenems and β-lactamase inhibitors are not hydrolyzed by these.

K. pneumoniae can also express plasmid mediated AmpC β-lactamases, increasing the resistance profile to include aminopenicillins, carboxy and ureidopenicillins, third generation cephalosporins, cephamycins and β-lactamase inhibitors. It should be noted that the same organism can harbor multiple β-lactamases.

ESBL producing *K. pneumoniae* display broad resistance to antimicrobial agents. Carbapenems are the most active agents in vitro. A study of *K. pneumoniae* bacteremia by Paterson et al. which included 455 isolates (85 were ESBL-producers) showed lower mortality rates after a 14 day follow up when a carbapenem was used in the first 5 days of treatment versus other antibiotics (mortality rates of 4.8% vs 27.6%, $P = .012$) [66].

However, as already highlighted, *Klebsiella* species can also be resistant to carbapenems. KPC enzymes have been discussed, and MBLs occur sporadically. Vatopoulos described an increasing trend through 5 years of imipenem-resistant, VIM-1-producing *K. pneumoniae* in a Greek hospital. The author shows an increase from less than 1% in 2001, to 20% in isolates from hospital wards and to 50% in isolates from intensive care units (ICUs) in 2006 [84]. Cagnacci et al. described the first report on the emergence of a multidrug resistant (MDR) *K. pneumoniae* producing the VIM-1 MBL, causing an outbreak of bloodstream infections in an Italian hospital [12].

Of note, is that plasmid containing ESBLs frequently carry aminoglycoside-modifying enzymes, specially AAC(3)-V, APH(3″) and APH(3′)-I. Furthermore, at least 20% of *K. pneumoniae* harboring ESBLs may also be resistant to quinolones.

20.5 Proteus Species

20.5.1 Microbiology

The genus *Proteus* consists of motile, aerobic and facultative anaerobic Gram-negative rods. *Proteus* is a member of the tribe *Proteeae*, which includes *Morganella* and *Providencia*. *Proteus* spp. are identified by the following biochemical characteristics:

positive methyl-red reaction, negative Voges-Proskauer reaction, phenylalanine deaminase production, growth on KCN (potassium cyanide), and lipase, urease and hydrogen sulphide production. The genus *Proteus* currently consists of five named species: *P. mirabilis*, *P. vulgaris*, *P. penneri*, *P. myxofaciens,* and *P. hauseri*, and three unnamed genomospecies: *Proteus* genomospecies 4, 5 and 6. The ability to ferment glucose, sucrose, and maltose serves to further subdivide the strains into two groups: *P. vulgaris* ferments glucose, sucrose, and maltose readily, while *P. mirabilis* ferments glucose readily and sucrose slowly and does not ferment maltose. These two strains account for the vast majority of clinical isolates in this genus. Both produce urease, and the latter is indole negative.

The term *Proteus* means "changeability of form", as illustrated in the Homeric poems where "Proteus … has the gift of endless transformation." This attribute of changeability reminded early microbiologists of the morphologic variability of the *Protei* on subculture, including their ability to swarm.

Swarming capacity and Gelatin liquefaction (22°C) are key microbiological features. Swarming appears macroscopically as concentric rings of growth emanating from a single colony or inoculums. On a cellular level, swarming results from bacterial transformation by expressing fimbriae and flagellae into highly elongated rods with thousands of flagellae that translocate rapidly across the surface of blood agar plates.

20.5.2 Epidemiology

Members of the *Proteus* genus are widespread in the environment and are part of the human gastrointestinal tract commensal flora. Among the genus *Proteus*, *P. mirabilis* is by far the most common species identified in clinical specimens. It accounts for approximately 3% of nosocomial infections in the United States. The most common infections caused by *Proteus* spp., are UTIs. It causes about 7% of community-acquired UTIs. Additionally, in hospital-acquired UTI, it ranks as a leading organism after *E. coli* [60].

20.5.3 Clinical Syndromes

As mentioned above, UTI is the most common clinical manifestation of *Proteus* infections. It frequently affects persons with indwelling catheters or anatomic or functional abnormalities of the urinary tract. Infections tend to be more severe than those caused by *E. coli*, with a higher proportion representing pyelonephritis.

The ability of *P. mirabilis* to produce a potent urease makes it an important virulent pathogen. It hydrolyzes urea to form CO_2 and ammonia, alkalinizes the urine, which leads to the precipitation of struvite (ammonium magnesium phosphate), formation of calculi, and obstruction of urinary catheters. These kidney stones serve as

foreign bodies in which the bacteria are embedded and from which they emerge to cause recurrent infections.

P. mirabilis has also been implicated in bacteremia, neonatal meningoencephalitis, meningitis, empyema, osteomyelitis and endocarditis. An unusual case of transfusion-transmitted *P. mirabilis* bacteremia was described in a patient transfused with a contaminated unit of platelet concentrate [60].

20.5.4 Antibiotic Considerations

P. mirabilis has intrinsic resistance to nitrofurantoin and tetracycline. Tigecycline has minimal activity against it [79]. It long has been regarded as an organism susceptible to a wide range of antibiotics. However, in the last decade there have been numerous reports of ESBL production. Among *P. mirabilis*, ESBLs prevalence appears highest in South America and Europe, 22.4% and 11.1%, respectively, in contrast to the much lower values seen in the US, 4.9%, and Canada, 3.1% [60].

UTI in most case can probably be treated with amoxicillin/clavulanate or a quinolone. The latter option is problematic for ESBL-producing strains since quinolone resistance in these is substantial (61.2% in Europe and 34.5% in the US). Moreover, amoxicillin/clavulanate would not be the best option if the strain harbors plasmid-mediated AmpC β-lactamases.

Luzarro et al. described the first report of a rapid spread of AmpC β-lactamase-positive *P. mirabilis* strains. They showed that the prevalence of AmpC β-lactamase-positive isolates increased from 0.3% in 2004 to 4.6% in 2006, whereas that of ESBL-positive isolates remained constant. AmpC β-lactamase-positive isolates were multidrug-resistant and carried the CMY-16 determinant [51].

Aragon et al. have described the diversity of β-lactamases found in this species. They found CTX-M and CMY-type as well as VEB-4. Also, inhibitor-resistant TEM-type β-lactamases were found [2]. Therefore, therapeutics targeting *P. mirabilis* may be complicated. As always, empiric therapy should be guided by local resistance rates. As is true for other enterics, carbapenems are the treatment of choice for ESBL producing isolates causing bacteremia.

20.6 Enterobacter Species

20.6.1 Microbiology

The major species of the genera are *E. cloacae, E. aerogenes* and *E. agglomerans*. *Enterobacter* can be readily isolated and separated in the laboratory from other members of the family. They grow in ordinary agar, ferment glucose, and possess peritrichous flagella. Some strains are endowed with a capsule. *E. cloacae* accounts for more than half of all infections and has been representative of the genera in its mechanism of resistance, that is, overexpression of AmpC enzymes.

20.6.2 Epidemiology

Enterobacter is well adapted to the nosocomial environment and infections have been reported especially in the ICU. It has been found over the skin, dry surfaces, and contaminated fluids. Its clinical importance was recognized in 1976 after an outbreak of septicemia in 378 patients at 25 hospitals from a contaminated intravenous solution [52]. Other outbreaks have been described in glucose-containing parenteral fluids, enteral feeding, humidifiers and respiratory therapy equipment [80, 85, 89]. According to the NNIS, which included data collected from 1998 to 2004, mean resistance in the ICU to third-generation cephalosporins and carbapenems was 27.7% and 0.7%, respectively; in non ICU, hospitalized patients, resistance to third-generation cephaolosporins was 21% while resistance to carbapenems was 1% (2004). Although resistance in outpatient areas was lower than that reported in hospitals, about 10% of the isolates were resistant to third generation cephalosporins [58]. These findings are correlated with the institutional use of antibiotics.

As mentioned previously, AmpC-derepressed mutants can be selected during therapy with third-generation cephalosporins. Earlier studies placed special emphasis on horizontal transmission in hospitalized patients colonized with endogenous gut flora and prophylaxis with cephalosporins. In 1987, a study by Flynn and colleagues showed that 58 of 87 patients who received cefazolin prophylaxis for cardiac surgery became colonized, 28 by the time of admission to Coronary ICU. Colonization increased after prophylaxis by 45%; statistical significance was found when compared with patients who received no prophylaxis. *E. cloacae* was the predominant species in this study [26].

Enterobacter may also be transmitted from patient to patient. A PCR molecular typing study that included 185 clinical isolates of *E. aerogenes* from two ICUs over a year found that two-thirds of the isolates belonged to the same clone [19]. This study emphasizes the importance of cross transmission and the impact hand washing can have when appropriately done, especially in the ICUs.

20.6.3 Clinical Syndromes

Similar to other *Enterobacteriaceae*, *Enterobacter* species are capable of causing a wide variety of nosocomial disease including pneumonia and infections of the urinary tract, the abdominal cavity and those associated to intravascular devices. Risk factors for pneumonia include severity of illness, mechanical ventilation, and prior use of antibiotics. *E. sakazakii* has been implicated in neonatal sepsis and meningitis, especially in premature and low-weight infants [4, 59]. Although *E. sakazakii* has been isolated from infant formula, confirmation of the strains as the source of the disease has not always been found. Meningitis by other members of the genera is uncommon and includes *E. cloacae, E. aerogenes,* and *E. agglomerans* [27]. Neurosurgery and neurotrauma are the main risk factors.

20.6.4 Antibiotic Considerations

The propensity to select resistant strains during therapy is an important consideration when treating *Enterobacter*, since earlier studies have shown how inappropriate treatment can have a negative impact in development of resistance and patient outcome. Chow and colleagues studied 129 patients with *Enterobacter* bacteremia in three tertiary-care university hospitals. Multi-resistance was statistically greater in *Enterobacter* when a third-generation cephalosporin had been administered previously, when compared with other antibiotics (69% and 20%, respectively; $p<0.001$). Furthermore, emergence of resistance during treatment of initially susceptible strains was higher for third-generation cephalosporins as compared to aminoglycosides (19% vs 1%; $p<0.001$). Multi-resistant *Enterobacter* was associated with a higher mortality when compared with infection due to a sensitive isolate (32% vs 15%; $p<0.003$). The authors concluded that judicious use of third- generation cephalosporins must be applied and they advised against their use in bacteremia as monotherapy, regardless of the susceptibility profile [17].

A recent study by Choi and colleagues showed that among AmpC-producing *Enterobacteriaceae, Enterobacter* was the main organism capable of selecting resistance to broad-spectrum cephalosporins (8.3%) but overall risk of mortality was low [16]. Kaye et al. reported in 2001 that 19% of patients with *Enterobacter*-positive isolates will develop resistance when treated with broad-spectrum cephalosporins. The authors report the resistance rate as higher if the *Enterobacter* is isolated from blood than from tissue, wounds or urine. Resistance occurred more frequently among *E. aerogenes* than *E. cloacae* (17% vs 9%; $p=0.03$); mortality rate due to multi-resistant *Enterobacter* was 26% [18, 42].

Paterson et al. reported that approximately 33% of *E. cloacae* bacterial bloodstream isolates had co-existence of ESBLs and AmpC β-lactamases [64].

Resistance to carbapenems is rare, presumably because two mutations, namely AmpC derepression and porin loss, are needed in combination to cause full resistance [45]. In the class, ertapenem can be used when *P. aeruginosa* or *A. baumannii* infection have been ruled out.

A report from the SENTRY surveillance system in the USA between 1997 and 2000 showed excellent susceptibility to cefepime and carbapenems (>99%) [36]. Although no statistical differences in clinical improvement, bacteriological eradication and 30-day mortality were found in a study comparing cefepime and carbapenems for the treatment of infections caused by *E. aerogenes*, treatment failure was documented in those isolates with higher cefepime MIC [31]. Despite the high susceptibility in many studies, cefepime resistance have been described recently due to the production of CTX-M enzymes, AmpC β-lactamases hyperproduction and porin loss [25]. Other antibiotics like aminoglycosides retain good activity but usually are combined with other agents.

20.7 *Serratia marcescens*

20.7.1 Microbiology

S. marcescens is a motile, aerobic rod that is also easy to differentiate in the microbiology laboratory. Strains of *Serratia* usually produce extracellular deoxyribonuclease (DNase), lipase and gelatinase. Strains are usually indole and lactose negative. Some strains are red pigmented. Other members of the genera *Serratia* include *S. liquefaciens, S. marinorubra,* and *S. odorifera* but *S. marcescens* accounts for more than 90% of infections.

20.7.2 Epidemiology

S. marcescens is an opportunistic pathogen which, unlike other *Enterobacteriaceae* like *E. coli*, has a very low rate of intestinal carriage in the healthy, inmunocompetent hosts and community acquired infections are uncommon. In hospitalized patients, *Serratia* carriage can be as high as 21% and gut colonization can be persistent over time [11]. Transmission of the organism is predominantly carried on the hands of healthcare personnel as has been described in several studies. Surveillance studies in the U.S. has shown *S. marcescens* as the cause of nosocomial respiratory infections in 4.6% of the cases; regarding other infections, *S. marcescens* is the etiological agent in 2% of bacteremias, 1.9% of urinary tract and 2.4% skin, soft tissue infections [29]. Regarding resistance, in the 2000–2004 SENTRY report more than 90% of *Serratia* spp. isolates were susceptible to most tested antibiotics, including carbapenems, gentamicin, ciprofloxacin, penicillins and cephalosporins with the highest susceptibility reported for carbapenems [20].

20.7.3 Clinical Syndromes

Like other members of the family *Enterobacteriaceae*, *S. marcescens* has been implicated in multiple clinical syndromes including pneumonia, urinary tract infections, skin and soft tissue infections, meningitis and, less commonly in endocarditis. Invasive procedures like intravenous catheters, endotracheal tubes and urinary catheters are risk factors for the acquisition of these infections.

20.7.4 Antibiotic Considerations

S. marcescens is another AmpC hyper-producer. As in the case of serious *Enterobacter* infection, third generation cephalosporins should be avoided. Aminoglycosides have been used but induction of resistance and nephro/ototoxicity are issues to consider. Carbapenems remain active against almost all strains.

20.8 Recommendations and Perspective

There is an emerging problem with resistance in enterics, with similar trends observed throughout the world. In general, antibiotic choices should be based on local epidemiology as well as an understanding of specific mechanisms of resistance. The fluoroquinolones in particular have seen rapid erosion of their utility in many regions.

Carbapenems should be considered first line empiric therapy in serious infections due to ampC hyperproducers and ESBLs; however, the spread of carbapenemases worldwide, especially KPC, is a major threat.

There is an urgent need for new agents as older classes fall victim to emerging resistance. Unfortunately the pharmaceutical pipeline for gram negatives is bare. In the meanwhile, antibiotic stewardship and infection control strategies must be rigorously applied to preserve existing therapies.

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Chapter 21
Pseudomonas aeruginosa: A Persistent Pathogen in Cystic Fibrosis and Hospital-Associated Infections

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21.1 Introduction

Pseudomonas aeruginosa is a motile, non-fermenting, Gram-negative organism belonging to the family Pseudomonadaceae. Its history as a recognized human pathogen dates back to the 1850s when Sédillot observed a blue-green discharge was frequently present and associated with infection in surgical wound dressings. The infectious organism, a rod shaped, blue-green pigmented bacterium, was isolated in 1882 and initially named *Bacillus pyocyaneus*. Five years later in France, the pathogenicity of the organism was demonstrated in animal models by Charrin who described it as a pyocyanic illness in his 1898 publication "La maladie pyocyanique." The later naming of the organism as *P. aeruginosa* was also a reflection of the bacterium's typical blue-green pigmentation on solid media. In 1925, Osler distinguished the organism as an opportunistic pathogen capable of infecting and invading only damaged tissue sites. As modern medicine advanced in the first half of the twentieth century, extending the life expectancy of highly susceptible immunocompromised groups, *P. aeruginosa* established a more prominent role in hospital infections. In the 1960s, *P. aeruginosa* emerged as a major human pathogen, due to improved treatment of burn and wound victims as well as surgical, neutropenic, and cystic fibrosis (CF) patients [48]. Despite anti-pseudomonas activity being one of the holy grails of pharmaceutical drug discovery for several decades, it still remains one of the most recalcitrant and difficult to treat organisms, and as therapeutic options run out, *P. aeruginosa* has achieved Superbug status.

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Many of the difficulties with infection control of *P. aeruginosa* result from its superb adaptability. Its large genome encodes approximately 5,500 predicted genes [203], lending to the organism's adaptability to varying environments. *P. aeruginosa* is ubiquitous in nature, with many inert surfaces and many moist environments serving as reservoirs including soil, water, plants and vegetables. *P. aeruginosa* has a broad range of growth substrates, minimal nutrient requirements and is able to survive even in distilled water [162]. The organism is tolerant of temperatures as high as 50°C and is capable of growing under both aerobic conditions as well as anaerobic conditions using nitrate or arginine as a terminal electron acceptor [214]. Despite possessing a large number of virulence factors, compromised host defenses are required for initiation of infection with *P. aeruginosa. P. aeruginosa* is truly a challenging pathogen in the hospital setting, as it is intrinsically resistant to many antibiotics and is capable of forming hardy biofilms, both within the body and on the surfaces of medical instruments.

This chapter will begin by discussing the nature of *P. aeruginosa* as an opportunistic pathogen, highlighting the importance of its large arsenal of virulence factors that play specific and distinct roles in virulence and pathogenicity in various infections. We have limited discussions to several of the most prominent infections caused by *P. aeruginosa* citing *in vitro*, *in vivo,* and epidemiological data that contribute to the repertoire of knowledge of the pathogen. The focus of each section will be on difficulties in infection prevention, treatment, and eradication and will describe some of the highlights and limitations of studies to date.

21.2 Pathogenesis and Major Virulence Factors

P. aeruginosa stands out as a unique and threatening organism, as it is both capable of causing severe invasive disease and of evading immune defenses causing persistent infections that are nearly impossible to eradicate. Furthermore, *P. aeruginosa* has been found to either colonize or cause infection at nearly every site of the body [162]. A period of prior colonization at a local site is strongly associated with a later state of disease. Predisposing factors that present an opportunity for the colonizing bacterium to initiate infection include the breach of primary defense barriers either by use of invasive mechanical devices, wound trauma, broad-spectrum antibiotic use, chemotherapy, or other host immunodeficiencies [40, 54, 173]. Colonization and initial growth may lead to a larger local focus of infection, as is seen with CF lung infection, but in nosocomial infections can frequently precede dissemination either systemically or to another tissue site. The subsequent tissue damage, invasion, and dissemination of *P. aeruginosa* can be directly attributed to the many virulence factors it produces. These virulence factors include pili and flagella, which play an initial role in motility and adhesion to the epithelium, as well as the endotoxin lipopolysaccharide (LPS) and a number of secreted toxins including exotoxin A, exoenzyme S (ExoS), exoenzyme U (ExoU), exoenzyme T (ExoT), exoenzyme Y (ExoY), elastase, and alkaline protease. These factors are thought to be critical for

maximum virulence of *P. aeruginosa*; however, based on observations in diverse animal models, the relative contribution of any given factor may vary with the type of infection. Several of these virulence factors have also been studied for their roles as potential vaccine candidates, although there is currently no generally accepted vaccine. The following section briefly outlines the various virulence factors produced by *P. aeruginosa* and their roles in contributing to disease.

21.2.1 Lipopolysaccharide

The LPS is a predominant component in the outer membrane of *P. aeruginosa.* LPS plays a prominent role in activating the host's innate and acquired immune responses and dysregulated inflammatory responses that contribute to morbidity and mortality [92]. The molecule produced by *P. aeruginosa* is a typical Gram-negative bacterial LPS, with a basic Lipid A structure inserted into the outer leaflet of the outer membrane, a relatively conserved core polysaccharide and a more variable O-antigen capping. The *P. aeruginosa* LPS tends to be less endotoxic to host cells compared to *Escherichia coli* LPS. This is thought to arise from the lower acylation of the *P. aeruginosa* Lipid A component which comprises an N- and O-acylated diglucosamine bisphosphate backbone [5, 206]. The inner core is composed of two D-*manno*-2-keto- octulosonic acid residues and two L-*glycero*-D-*manno*-heptose residues while the outer core contains an N-alanylated galactosamine residue, three D-glucose residues and one L-rhamnose as well as variable substitution with amino acids, phosphate, pyrophosphate and ethanolamine [118, 160].

Significant variability in LPS composition can be observed in the Lipid A moiety and in the outer core. The Lipid A component can be either penta-, hexa-, or hepta-acylated, and the phosphate residues variably capped with aminoarabinose. The variations in the acylation patterns result in differing immune activation potencies, due to varied binding to Toll-like receptor 4 [58] and together with the aminoarabinose capping influence susceptibility to host defense (antimicrobial) peptides as well as aminoglycosides and polymyxins. The outer core can either be uncapped (85% of molecules, also lack the terminal core rhamnose molecule) or capped by a variable number of repeated saccharide (O-antigen) units of 3–5 sugars with defined linkage. The composition of this repeating unit determines the serotype of the *P. aeruginosa* isolate, and there are 20 serotypes based on serological reactivity of the O-antigen [132]. Despite the observation that only one in six molecules of LPS is capped by O-antigen in most isolates, these polysaccharide chains form a capsule-like layer on the surface of cells [124]. In addition to this type of LPS (often called B-band LPS), there is a common antigen LPS (A-band LPS) that is capped by poly-D-rhamnose. Isolates lacking the O-antigen have a distinct rough colony morphology compared to the smooth isolates that predominantly produce an LPS containing the O-antigen [117]. The O-antigen has been shown to be a major target for protective immunity in numerous studies. The relative amounts of O-antigen expressing and rough LPS that are produced can vary in a given strain dependant on the growth conditions, and

can influence the pathogenicity of a given strain [57], with rough strains being less able to cause a systemic infection in experimental animals possibly due to their susceptibility to complement killing [86]. Intriguingly, while blood stream isolates are normally smooth, O-antigen expressing, cystic fibrosis chronic respiratory isolates commonly express rough LPS, an observation that may reflect the organism's attempt to evade the adaptive immune system and maintain chronic infections in the lung [86, 161].

A number of LPS vaccines have been investigated for use in CF patients in phase II and III clinical trials; however, these have not been successful [51, 87, 126, 157, 159]. The LPS based vaccines provided little immunity and did not appear to protect the patients from infection with *P. aeruginosa.* In several trials with different LPS based vaccines, the vaccinated groups actually demonstrated overall worse clinical status than the control groups.

21.2.2 Flagellum

The single unsheathed polar flagellum of *P. aeruginosa* is responsible for the swimming motility of this organism and on semi-solid media flagella plays a role in swarming motility [121]. Nonetheless, its role in virulence goes beyond simple motility. Flagellar proteins have been shown to play critical roles in attachment, invasion, biofilm formation and in the mediation of inflammatory responses. Flagellar protein synthesis, assembly and regulation involves over 40 genes and is intricately controlled through transcriptional and post-translational events by the four main regulators RpoN, FleQ, FleR and FliA [41].

Although adhesion of *P. aeruginosa* is primarily mediated via type IV pili, flagella have been shown to mediate adhesion to corneal cells and have been implicated in the adhesion to cell bound mucins [166]; in particular, FliD, the flagella cap protein, specifically binds to the oligosaccharides of respiratory mucins [4].

Non-flagellated mutants are often isolated from chronic infections in CF patients due to the repressor activity of AlgT, which acts on the FleQ regulator [207]. The loss of flagella in these isolates is believed to be an event involved in the evasion of the host immune system, as flagellin mediates the inflammatory response via the innate immune system through its specific interaction with a leucine-rich repeat region of host Toll-like receptor 5 (TLR5) [216]. Intriguingly epithelial cells with the CFTR protein mutated are hyper-inflammatory, especially when stimulated through flagellin-TLR5 interactions [17]. Again CF isolates are often found to be lacking flagella, possibly as a strategy for evading innate immunity.

Flagellar vaccines have been investigated in pre-clinical studies in mouse models and have reached phase III clinical trials for CF patients; however, limited protection was observed with a monovalent vaccine and future development of a bivalent vaccine has been terminated [51].

21.2.3 Type IV Pili

The type IV pili of *P. aeruginosa* have a role in adhesion to many cell types and this is likely important in such phenomena as tissue tropism (attachment to particular tissues), initiation of biofilm formation and non-opsonic phagocytosis [11, 165]. These pili also mediate twitching motility, a factor found to be important in the formation of *in vitro* biofilms [116] as well as in the initiation of dissemination from an initial point of colonization [81, 115]. This surface associated motility occurs by the coordinated extension and retraction of these polar pili [24]. Although more than 50 genes have been identified to play either a direct or indirect role in the synthesis, functioning and control of the type IV pili of *P. aeruginosa,* the pili are composed of a single type IVa pilin protein encoded by *pilA* [88]. Five phylogenetically distinct alleles of *pilA* have been identified, with group I pili being the most prevalent in CF and environmental isolates [123].

21.2.4 Type III Secretion

P. aeruginosa has a variety of secretion systems of which at least four likely play a role in virulence (Type I, II, III, and VI). One of the most intriguing is Type III secretion that involves a flagellum-basal-body related system for delivering proteins directly from the cytoplasm of *Pseudomonas* into the cytosol of host cells. A functional Type III secretion system contributes to the successful evasion of phagocytosis by *P. aeruginosa* as well as damage of host tissues, promotion of immune avoidance, and bacterial dissemination. The Type III secretion system of *P. aeruginosa* delivers up to four cytotoxins ExoS, ExoT, ExoU, and ExoY directly to host cells. The gene clusters *psc, pcr, exs,* and *pop* encode proteins of the *Pseudomonas* Type-III secretion apparatus and proteins involved in regulation of this apparatus [64, 76, 220].

ExoS and ExoT are bifunctional cytotoxins that possess both Rho GTPase-activating protein and ADP ribosyltransferase activities. These molecules can inhibit phagocytosis by disrupting actin cytoskeletal rearrangement, focal adhesins and signal transduction [9]. ExoU is a phospholipase that contributes directly to acute cytotoxicity towards epithelial cells and macrophages. ExoY is an adenylate cyclase that affects intracellular cAMP levels and cytoskeleton reorganization [182, 221].

Recent evidence has implicated a role for the Type III secretion system in virulence in humans. The presence, in *P. aeruginosa* cultures from intubated patients, of large amounts of Type III secretion products, particularly ExoU, was linked to increased mortality regardless of whether these patients had symptoms or confirmation of ventilator-associated pneumonia [222].

21.2.5 Exotoxin A

There are several key virulence factors that are secreted through Type II secretion mechanisms (which use a pilus-like apparatus to secrete proteins into the extracellular environment), including exotoxin A, lipases, phospholipases, alkaline phosphatase, and proteases; animal experiments have indicated the important role of these factors in model infections. Exotoxin A has been demonstrated to be involved in local tissue damage and invasion. This cytotoxin is encoded by the gene *toxA* and has been found to be present in most clinical isolates of *P. aeruginosa,* although its role in virulence is poorly understood. Exotoxin A is also an ADP-ribosylating factor although it is distinct from the ADP ribosylating activity of ExoS and ExoT in that it inhibits protein biosynthesis in host cells by catalyzing the inactivation of elongation factor 2 [155].

21.2.6 Proteases

P. aeruginosa produces several secreted proteases including the zinc metalloprotease/elastase LasB, the metallo-endopeptidase LasA, and alkaline protease. These proteases work in a concerted fashion to destroy host tissue, thus playing an important role in both acute lung infections and in burn wound infections. LasB elastase is capable of degrading a number of connective tissue proteins, including elastin, fibrin and collagen. Destruction of elastin by these enzymes starts with the activity of LasB, which nicks the elastin providing a substrate for degradation by other proteases including *Pseudomonas* LasA, elastin and alkaline protease, and host neutrophil elastase [70]. A clear role for these destructive proteases in acute infections has been established; however, LasA and LasB elastases have also been found in the sputum of CF patients suffering from pulmonary exacerbations of infection [103, 110]. Thus while their role in chronic infection is not well understood, they may play a role in interference with host defense mechanisms.

21.2.7 Alginate

P. aeruginosa can produce a mucoid exopolysaccharide capsule, comprised of alginate, an acetylated random co-polymer of β1-4 linked D-mannuronic acid and L-guluronic acid [69]. All *P. aeruginosa* strains investigated possess the *alg* genes for alginate production; however, most environmental and many clinical isolates, with the prominent exception of CF isolates, do not typically produce a mucoid phenotype. Overexpression of the alginate-producing genes is required for the mucoid phenotype. Conversion to mucoidy has been observed in the laboratory under nutrient limiting conditions, in the presence of the major lung surfactant, lecithin, and in

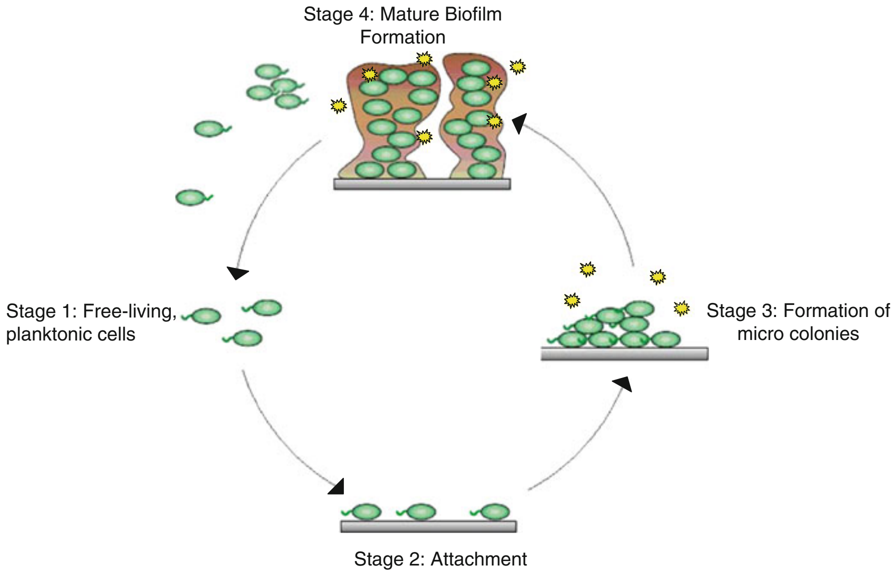

Fig. 21.1 *Development of a P. aeruginosa biofilm.* Stage 1: Cells are free-swimming (planktonic), with predictable antimicrobial susceptibilities. Stage 2: Planktonic cells initiate attachment to a surface via their type IV pili and flagellum. Stage 3: Small aggregative communities begin to form and quorum-sensing signals begin to accumulate. Stage 4: A critical threshold of quorum sensing signals is reached. Microcolonies become encased in an extracellular matrix. Cells enter a sessile phase of growth and become highly resistant to antimicrobials. Individual cells and small micro-colonies slough from the mature biofilm initiating further biofilm development [68]

response to sub-lethal concentrations of antibiotics. Stable conversion to mucoidy in the clinic is typically due to mutations in the regulatory *muc* genes, which act to negatively regulate the alternative sigma factor AlgU that is responsible for transcriptional activation of the *alg* genes [22, 23, 43]. The overproduction of alginate plays a role in cell adherence within the CF lung and is also thought to be involved in resistance to host defenses by reducing susceptibility to phagocytosis [163].

21.2.8 Biofilm Formation

P. aeruginosa is capable of forming complex organised structures called biofilms. These highly structured biofilms are characterized by an extracellular matrix, enclosing an aggregated population of slow growing cells and adopting under most *in vitro* circumstances a heterogeneous, mushroom shape. Biofilm development is a complex process (Fig. 21.1) and is in part controlled by quorum sensing signals. Type IV pili and flagella play a role in the initial attachment of cells to a surface [116]. As the microcolony develops, the extracellular matrix forms and encloses the biofilm (Fig. 21.1). This matrix is composed of polysaccharides (particularly Pel

and Psl), proteins and nucleic acids. During biofilm formation, cell differentiation occurs, and oxygen and water filled channels are formed to provide nutrients to the deep rooted cells of the mature biofilm [42, 66, 114, 176].

P. aeruginosa has been demonstrated to form biofilms on a variety of indwelling medical devices [35, 113]. It is particularly problematic for patients requiring mechanical ventilation and catheterization, as the surfaces of medical devices can readily develop *P. aeruginosa* biofilms that are difficult to remove. Furthermore, *P. aeruginosa* has been demonstrated to grow as a biofilm within the body, particularly at the site of burn wounds. It has been proposed that *P. aeruginosa* exists as a biofilm in the CF lung [38, 191] and this has been observed in a mouse model of CF lung infection [99]. Support for this belief has also come from reports detecting the presence of quorum sensing molecules in sputum from CF patients. The concept that *P. aeruginosa* exhibits a biofilm or biofilm-like mode of growth within the CF lung helps to further explain the persistence of these infections, as biofilms have demonstrated the ability to evade the host immune system and resist antibiotic treatment [106].

In addition to evasion of the host immune system, the highly antibiotic resistant nature of biofilms to killing by bactericidal antibiotics contributes to bacterial persistence in chronic infections [137]. It is in part the slow growth and poor accessibility of biofilms that contributes to their high antibiotic resistance [28, 196], although adaptive mechanisms involved in biofilm differentiation may also be influential. It has been demonstrated that cells growing in a biofilm can be up to 1,000 fold more resistant to antibiotics than free-swimming, planktonic cells [105]. Biofilms present not only a diffusion barrier to antibiotics, but the cells in a biofilm have been demonstrated to have significantly different expression patterns compared to their planktonic counterparts; with the observed differentially expressed genes being dependent on the region of the biofilm where the cells are located [183].

21.2.9 Quorum Sensing

Quorum-sensing is a mechanism of cell to cell communication in which a critical number of bacteria (the quorum) are required to produce a sufficient amount of a secreted signal molecule (termed an autoinducer) to trigger expression of a large regulon. In *P. aeruginosa*, quorum-sensing systems regulate biofilm formation, swarming motility and a broad array of virulence factors via two separate secreted homoserine lactone (HSL) autoinducer molecules, N-(3-oxododecanoyl) homoserine lactone (Las system) and N-butyryl homoserine lactone (Rhl system), and three quinolines, 2-heptyl-4-hydroxyquinoline N-oxide (HQNO), 4-hydroxy-2-heptylquinoline (HHQ), and Pseudomonas quinolone signal (3,4-dihydroxy-2-heptylquinoline; PQS) [46, 94, 193]. These quorum-sensing signal molecules have been found to act in a cell density dependent manner. The HSL molecules are secreted from the cell, and as population density increases, as does the concentration of signalling molecules. Once a critical threshold concentration has been

attained, the bacterial cell can sense the signals and undergoes broad changes in gene expression. The two HSL quorum-sensing regulatory systems, present in *P. aeruginosa*, LasIR and RhlIR [27], act through LasR and RhlR at the transcriptional level, which interact with a variety of sigma factors and transcriptional regulators. Regulation of these systems is also intertwined with the expression of the two-component regulatory system GacAS and the small regulatory RNAs encoded by the *rsm* system, thus forming a complex network of regulation in *P. aeruginosa* [172]. In addition to biofilm formation these systems collectively regulate the production of hydrogen cyanide, rhamnolipid, pyocyanin, elastase, alkaline protease, superoxide dismutase, LasA, and swarming motility [219].

Recent advances in the understanding of quorum-sensing in *P. aeruginosa* have generated interest in using quorum sensing as a target for therapeutics. The macrolide antibiotic, azithromycin, has been a promising candidate in this regard, as it has been demonstrated to be capable of both penetrating biofilms and interfering with quorum sensing [100]. Screening of synthetic compound libraries has led to the identification of a number potential quorum sensing inhibitors; in addition, several natural compounds including extracts of garlic, yellow pepper, carrot, chamomile and bean sprout have demonstrated an inhibitory effect on quorum sensing [168].

21.3 Antimicrobial Resistance

P. aeruginosa can be a particularly challenging organism to treat once infection has been established, as it is intrinsically resistant to many of the available antibiotics. In addition, it readily acquires plasmids that can harbour multiple antibiotic resistance cassettes, and is capable of adapting and mutating leading to alterations in gene expression that can cause resistance. This section will provide an overview of the main mechanisms of resistance present in clinical isolates of *P. aeruginosa.*

21.3.1 Intrinsic Resistance

P. aeruginosa exhibits intrinsic resistance to almost all of the available antibiotics, indicating that the wild type strain possesses a number of genetic mechanisms that contribute to reduced susceptibility of the organism. One of the major factors contributing to this intrinsic resistance is the low permeability of its outer membrane. The outer membrane is important for passively determining the rate of uptake of antibiotics and small molecules, and the outer membrane permeability of *P. aeruginosa* is 12–100 fold lower than that of *E. coli* [83]. However, by itself this is insufficient to mediate resistance as antibiotics will still quite rapidly equilibrate across the outer membrane. Intrinsic resistance arises from the combination of slow uptake and secondary mechanisms that benefit from this slow uptake such as degradative enzymes like periplasmic β-lactamase, and particularly multidrug efflux [83].

The *P. aeruginosa* outer membrane is an asymmetric membrane composed of an inner leaflet of phosopholipid, predominantly phosphatidyl ethanolamine, and an outer layer of polyanionic LPS. The latter presents a negatively charged surface, which, together with the divalent cations bridging the individual LPS molecules, forms a matrix around the cell that is relatively impermeable to polar compounds except polycations [82]. Several classes of polycationic antimicrobials, including the aminoglycosides, polymyxins and cationic antimicrobial peptides employ the mechanism of self-promoted uptake wherein they competitively displace the divalent cations that bridge adjacent LPS molecules and are important for maintaining membrane integrity [85, 145]. This causes a perturbation of membrane structure allowing the perturbing cationic antimicrobials to gain entry into the periplasm, located between the outer and cytoplasmic membranes. Conversely, many hydrophilic antibiotics, particularly the β-lactams, rely on the presence of the water filled channels of proteins termed porins, to gain entry to the periplasm. These porins are often an abundant (major) protein component of the outer membrane and are involved in controlling its selectivity as a barrier to extracellular molecules. Porins are divided into several classes including general porins, specific porins, and gated porins [147]. OprF is an extensively studied general or non-specific porin found in *P. aeruginosa* and is responsible for the large exclusion limit (MW ~ 3,000) of the organism although it is very inefficient as a channel, leading to the overall low outer membrane permeability of *Pseudomonas*. Reduced expression of this porin alone appears to have little effect on the susceptibility of the organism to β-lactams and fluoroquinolones [15]. Another well characterized porin is the basic amino acid/peptide-specific porin, OprD, which is the major porin mediating the uptake of the carbapenem β-lactam imipenem [107, 108]. Gated porins include the iron regulated outer membrane proteins that mediate the uptake of specific siderophore-iron complexes and are thus essential for virulence, as well as certain catechol containing antibiotics.

As mentioned above, in mediating resistance to many classes of antibiotics, low outer membrane permeability works in collaboration with multidrug efflux whereby the slow uptake of antibiotics is countered by rapid efflux. The *P. aeruginosa* genome contains a large number of drug efflux systems [203], predominantly of the Resistance-Nodulation-Division (RND) and major facilitator superfamily (MFS) types. RND efflux pumps comprise an outer membrane channel-tunnel of distinct architecture (which form a family of 17 proteins) as well as cytoplasmic membrane pump and linker proteins. The exact number of RND systems contributing to antibiotic resistance is uncertain, but four major RND efflux pump systems have been well characterized in *P. aeruginosa*, MexAB-OprM, MexXY-OprM/OpmG, MexCD-OprJ, and MexEF-OprN with regard to their contributions to antimicrobial resistance. Channel-tunnel OprM together with pump MexB and linker protein MexA operates constitutively to mediate general efflux of antibiotics, including β-lactams, fluoroquinolones, tetracyclines, and macrolides [77, 120, 139, 140]; together with OprF this system is primarily responsible for the high intrinsic resistance to these antibiotics in *P. aeruginosa*. The MexXY efflux proteins together with channel-tunnel protein OprM and/or OpmG mediates aminoglyscoside intrinsic

resistance [112, 134]. The other two efflux systems are normally very weakly expressed in *P. aeruginosa*.

Low outer membrane permeability also works in concert with β-lactamases to degrade many β-lactam antibiotics. B-lactamases are hydrolyzing enzymes that cleave the lactam ring of penicillins, carbapenems, cephalosporins and monobactams, thus leading to inactivation of the antibiotic [174, 181, 204]. In *P. aeruginosa,* this activity is due to a chromosomally encoded AmpC β-lactamase. The AmpC β-lactamase of *P. aeruginosa* can degrade and contribute to intrinsic resistance to ticarcillin, piperacillin and the third generation cephalosporins. It is strongly induced by carbapenems, particularly imipenem, although these inducing carbapenems are stable against its hydrolytic activity.

It is important to reiterate, that the high intrinsic resistance exhibited by *P. aeruginosa* involves the concerted activity of all of the above mentioned mechanisms which together can act synergistically to reduce active intracellular levels of many antibiotics [84, 146]. Recent evidence supports the notion that *P. aeruginosa* has a much larger resistome (intrinsic and mutational) than was previously believed, with >100 genes have been identified that when mutated lead to altered susceptibility of the wild type to ciprofloxacin and tobramycin [26, 186]. These findings highlight the vast number of mutations that could sequentially arise, potentiating gradually increasing levels of resistance.

21.3.2 Acquired Resistance

Hyperproduction of β-lactamases in *P. aeruginosa* can be the result of plasmid acquisition, but more usually results from mutations that derepress AmpC β-lactamase regulatory genes [125]. In the latter situation, regulatory mutations lead to the overexpression of AmpC β-lactamase, increasing resistance to penicillins, cephalosporins and to a lesser extent meropenem. In *P. aeruginosa* clinical isolates, overexpression of AmpC β-lactamase has been predominantly associated with mutations in the *ampC* repressor gene *ampD* [125]. Specific point mutations in the transcriptional regulator, *ampR*, have also been found to result in overexpression of *ampC,* but these mutations are relatively rare [135]. Regarding plasmid encoded enzymes, a broad variety of β-lactamases, including *Pseudomonas* specific enzymes (PSE), OXA-type β-lactamases, ESBLs and metallo-carbapenemases have been detected in *P. aeruginosa* clinical isolates, and can each lead to high level resistance to specific β-lactams [178].

Although mutations leading to the overproduction of AmpC have little effect on the carbapenems, which are relatively unaffected by its hydrolytic activity, mutations leading to the loss of the specific porin, OprD, can significantly affect this class of antibiotic [153]. Mutations leading to the loss of OprD can be either in OprD itself or in one of the regulatory proteins (e.g., MexT (NfxC)), controlling its expression. Interestingly, *mexT* regulatory mutations leading to the loss of this porin can be selected for by fluoroquinolones, but not by the carbapenems themselves,

since this regulatory gene controls a major efflux pump involved in fluoroquinolone resistance, MexEF-OprN, in addition to the OprD porin [150]. Thus, mutations at the *mexT*/*nfxC* locus contribute not only to imipenem resistance via loss of a porin but also to multidrug resistance via overexpression of an efflux pump [67].

Similarly, overexpression of the MexAB-OprM and MexCD-OprJ operons, due largely to *nalB* and *nfxB* regulatory mutations respectively, lead to multidrug resistance [130, 138, 164], while overexpression of MexXY-OprM/OpmG, due to *amrR*/*mexZ* regulatory mutations and is thought to be a prominent cause of broad spectrum, low level (so-called impermeability-type) aminoglycoside resistance [134, 164, 217, 223].

In addition to selection of efflux regulatory mutations by fluoroquinolones, these antibiotics more frequently select for mutations in the genes encoding DNA gyrase and topoisomerase IV. As *P. aeruginosa* tends to exhibit reduced susceptibility to fluoroquinolones compared to the Enterobacteriaceae, mutations in DNA gyrase and topoisomerase IV occur at a higher rate and an individual mutation in DNA gyrase can lead to clinically relevant levels of resistance. Higher levels of resistance are typically associated with mutations in both DNA gyrase and topoisomerase IV [111].

Other than the impermeability type resistance, which accounts for up to 50% of clinical resistance to aminoglycosides, aminoglycoside resistance in *P. aeruginosa* is often caused by the presence of aminoglycoside modifying enzymes found on mobile genetic elements. Three major classes of modifying enzymes exist, including the aminoglycoside acetyltransferases (AAC), the nucleotidyltransferases (ANT), and the phosphoryltransferases (APH) [188]. The individual enzymes in each class are highly specific in their activity, affecting only specific subsets of aminoglycosides and no individual enzyme acts on all aminoglycosides. Nonetheless, the occurrence of strains possessing multiple enzymes is not uncommon and so the combined effect of the enzymes can inactivate a broader range of aminoglycosides.

Another type of impermeability type resistance is mediated through the two component regulators PhoPQ and PmrAB with mutations in these genes leading to an altered phenotype [144]. This phenotype, which is particularly found in CF isolates, results from LPS modifications (e.g., addition of aminoarabinose to Lipid A phosphate blocks self promoted uptake across the outer membrane). A similar mechanism of resistance can be observed for the polymyxins including colistin which also depend on LPS binding for entry into the cell. More recently, high-level resistance to all aminoglycosides has been attributable to 16S ribosomal RNA methylases [50]. These resistance determinants are typically located on transposons and are carried on transmissible plasmids.

21.3.3 Adaptive Resistance

Discrepancies in *in vitro* susceptibility determinations for *P. aeruginosa* isolates and treatment outcomes in CF patients have been observed and can be attributed to

the phenomenon of adaptive resistance. Adaptive resistance occurs when cell populations are exposed to non-lethal concentrations of antibiotic and undergo specific changes in gene expression that result in reduced susceptibility. It is a form of inducible resistance that does not require the presence of mutations and has been demonstrated both *in vitro* using CF isolates, and in mouse models, when isolates are pre-incubated with subinhibitory concentrations of antibiotics [10, 25, 75]. The best described mechanisms of adaptive resistance in *P. aeruginosa* overlap significantly with the above-described acquired mechanisms of resistance, and may in fact precede the development of mutational resistance. For example, β-lactamase production from the chromosomally encoded *ampC* β-lactamase is inducible by many β-lactams, particularly imipenem, and although this does not cause imipenem resistance, it can lead to resistance to other β-lactams including the third generation cephalosporins. Interestingly however, this strong induction may not result in large differences in susceptibility during MIC testing as in the time required for induction significant killing may still occur [133]. Nonetheless the concern arises that this induction may allow small populations to survive and acquire stably resistant mutations. Similarly, adaptive resistance to polymyxins and antimicrobial peptides has been shown to occur through altered expression of the PhoPQ and PmrAB systems in response to these agents and leads to modulation of lipid A fatty acid composition [144]. Finally, inducible efflux of fluoroquinolones has been demonstated to be mediated through MexCD-OprJ. As mentioned above recent evidence indicates that for some antibiotics, *P. aeruginosa* has a very large resistome (e.g., >100 genes when mutated alter susceptibility to ciprofloxacin and tobramycin). We have proposed that these mutants may contribute to adaptive and/or stepwise mutational resistance [26, 186].

21.3.4 *P. aeruginosa* as a Superbug

The accumulation of multiple resistance mechanisms in clinical isolates of *P. aeruginosa* has resulted in strains that are resistant to all available antibiotics. This pandrug resistance, together with high attributable mortality, has thrust *P. aeruginosa* into the spotlight as an emerging superbug. According to previous reports by the National Nosocomial Infections Surveillance (NNIS) System, which focused on nosocomial infections in intensive care units (ICU), not only were resistance rates increasing, but the incidence of occurrence in most infection types was also increasing [72]. In 2003, the NNIS reported a 9% increase in resistance to the third generation cephalosporins, a 15% increase in ciprofloxacin resistance, and most alarming, a 47% increase in imipenem resistance over a 5 year period [149]. According to the European Antimicrobial Resistance Surveillance System 18% of *P. aeruginosa* isolates were multidrug resistant with 6% of all isolates being resistant to piperacillin, ceftazidime, fluoroquinolones, aminoglycosides and carbapenems [195]. The drug of last resort for infections with multidrug resistant *P. aeruginosa* is colistin, and

while resistance rates remain low (approximately 1% in most countries), mortality of 80% has been observed for infections caused by colistin resistant Gram-negative bacilli [16].

21.4 Hospital-Associated *P. aeruginosa* Infections

Hospital-acquired (nosocomial) infections are those not present or incubating (i.e., non-obvious) at the time of hospital admission and usually developing more than 48 h post admission. *P. aeruginosa* infections are typically of the late onset category, often presenting more than 5 days after admission. The 2006–2007 report by the National Healthcare Safety Network (NHSN) at the Centers for Disease Control and Prevention ranked *P. aeruginosa* as the 6th most common healthcare associated pathogen causing infection, and it is typically found at even higher rates in studies focused on the ICU [149]. The NHSN reports that in the US in 2006–2007, 8% of all hospital associated infections were due to *P. aeruginosa,* with *P. aeruginosa* causing 3% of central line associated bloodstream infections, 6% of surgical site infections, 10% of catheter associated urinary tract infections and 16% of ventilator associated pneumonia infections [95]. It is worth noting, however, that difficulties in treatment of such infections, and the associated morbidity and mortality, have made *Pseudomonas* one of the most feared hospital pathogens.

Of major concern with nosocomial infections are the high rates of antibiotic resistance. Recent reports from the NHSN annual update suggest that the highest resistance rates amongst pathogenic nosocomial isolates of *P. aeruginosa* were towards fluoroquinolones, with over 30% being considered resistant. Resistance rates for other therapeutics were as follows: carbapenems 25%, piperacillin-tazobactam 18%, and cefepime 11%, while the agent with the lowest rate was the aminoglycoside amikacin at 6% [95]. However it is worth noting that in other countries rates vary dramatically and for some selected multidrug resistant isolates there are virtually no therapeutic options [195].

Mechanical ventilation, antibiotic therapy, surgery and chemotherapy are the major predisposing factors contributing to the acquisition of a *P. aeruginosa* infection in the hospital. The frequency with which patients come into contact with *P. aeruginosa* is high and this likely also plays a significant role in contributing to infection. Many inanimate surfaces which patients are exposed to, including countertops, sinks, toilets, flower vases and cleaning supplies, harbour *P. aeruginosa*. Furthermore patients themselves tend to have high colonization rates. In contrast to the minority of healthy individuals that carry *P. aeruginosa* in the gut, nasal mucosa, throat, or on the skin, in the hospital *P. aeruginosa* frequently colonizes patients in the armpit, ear and perineum. Studies have shown that while only 7% of healthy individuals are colonized in the oropharynx and recovery rates of *P. aeruginosa* from stool approach only 24%, up to 50% of hospitalized patients may be colonized with *P. aeruginosa* [143]. A period of prior colonization with *P. aeruginosa* is strongly associated with approximately 50% of all cases of invasive infection with *P. aeruginosa* [19].

21.4.1 Burn Wound Infections

A breach of the skin barrier is the hallmark of thermal injury allowing for the rapid colonization and infection of the dermal tissues by *P. aeruginosa*. The gastrointestinal tract and the patient's surrounding environment are the most common sources of infection of these burn wound infections. Although *P. aeruginosa* is usually introduced locally through the skin, and *P. aeruginosa* is the leading cause of invasive infections in burn patients. The threat of invasive disease posed by *P. aeruginosa* in burn patients has been demonstrated in a mouse burn model where inoculation of the burned skin with as few as 10 organisms led to systemic dissemination and death [200]. Thus, the breadth of infection sites observed in burn patients goes beyond local infection at the wound site. Most notable is the fact that 75% of all deaths in patients with severe burns are related to sepsis from invasive burn wound infection [6, 8, 14]. In addition to wounded skin injury, inhalation injury is common in burn patients. This results in edema and sloughing of the respiratory tract mucosa and impairment of the normal mucociliary clearance mechanism, thus making these patients more susceptible to upper respiratory tract infections as well as *P. aeruginosa* pneumonia [36].

Although Gram-positive organisms such as *Staphylococcus aureus* and *Streptococcus pyogenes* are typically the first microorganisms to colonize the site of infection, after an average of 5–7 days other microbes including *P. aeruginosa* begin to colonize these wounds [1, 136]. Due to available effective antimicrobial therapies for the early colonizing Gram-positives and the innate antimicrobial resistance of *P. aeruginosa, P. aeruginosa* is a common bacterial cause of serious burn wound infections in many centres. Recent successes with early wound excision practices were shown to contribute to the prevention of invasive infections disseminating from the wound site [13]. As well, topical antibacterials with anti-pseudomonal activity, such as silver nitrate, silver sulfadiazine, and mafenide acetate, have proven useful for controlling the colonization and growth on the surface of wound.

Prior to the inception of the practice of excision of necrotic wound tissue, burn wound sepsis was predominantly caused by invasive wound infections with *Pseudomonas* originating from the burn wound site. In addition, older treatment strategies such as immersion hydrotherapy contributed to increased exposure to *P. aeruginosa* that was frequently found growing in the water baths [30]. During these pre-excision times, morbidity and mortality were extremely high as adequate penetration of the necrotic tissue at the wound site with either topical or systemic antimicrobials was difficult to achieve. Animal studies of partial-thickness cutaneous burns showed that mature biofilms could develop in 48–72 h, indicating a major potential source of further difficulties in antimicrobial therapy at these sites [210].

In addition to the *P. aeruginosa* virulence factors described in Sect. 2, which undoubtedly contribute to the success of *P. aeruginosa* as a pathogen in the burn patients, the impairment of host immunity, beyond simple loss of the skin's physical barrier, plays a role in enhancing susceptibility to infection. Recent studies have demonstrated that thermal injury causes impaired production of the host defense peptides β-defensins in the tissues surrounding the wound. These immunomodulatory peptides have been proposed to play an important role in primary defense against

P. aeruginosa and synthesized β-defensin was recently shown to be protective against *P. aeruginosa* infection in a burned mouse model [119].

While the rates of sepsis caused by dissemination from the wound site have now been significantly reduced due to early wound excision practices, *P. aeruginosa* sepsis still poses a major risk to burn patients, as many of these patients require mechanical ventilation or catheterization [36].

21.4.2 Bacteremia

P. aeruginosa is among the five leading causes of nosocomial bacteremia and frequently leads to sepsis. In the 1960s and early 1970s, aminoglycosides and polymyxins were the only options for treatment of *P. aeruginosa* bacteremia and were found to be fairly ineffective for these infections. Mortality of greater than 50% was reported when crude mortality was used as the end point [63, 218], and was as high as 70% in febrile neutropenic patients [21]. Despite the introduction of effective anti-pseudomonal β-lactams and the associated reduction in mortality rates, *P. aeruginosa* bacteremia is still one of the most feared nosocomial infections. These infections are generally still associated with higher mortality than with other infecting pathogens, and persistence, particularly associated with device-related bacteremia, continues to plague patients.

The clinical presentation of *P. aeruginosa* bacteremia is difficult to distinguish from sepsis in general. Patients are typically febrile, although more severely ill patients may present signs of shock and hypothermia. The main distinguishing feature of *P. aeruginosa* sepsis is the presence of ecthyma gangrenosum and these infarcted skin lesions occur only in markedly neutropenic patients [162]. As *P. aeruginosa* bacteremia rarely has notable distinguishing features, antimicrobial treatment tends to not be specific for the organism and standard empirical therapy for bacteremia and sepsis are usually administered until laboratory results are available; this may add to later complications as the antibiotics that can be successfully utilized to treat *P. aeruginosa* infections are often distinct and limited.

Historically, burn victims and neutropenic patients were the most commonly afflicted by *P. aeruginosa* bacteremia, with the skin and the gastrointestinal tract being the most common sources of *P. aeruginosa* [71]. As mentioned in Sect. 4.1, dissemination from burn wounds has now been significantly reduced due to appropriate preventative measures, and the incidence of *P. aeruginosa* infections has been reduced [36]. Although *P. aeruginosa* bacteremia still occurs in both patient populations, the origins of *P. aeruginosa* in hospitals have changed. The use of foreign devices such as catheters and mechanical ventilators now plays a significant role, initiating most cases of *P. aeruginosa* bacteremia, and therefore dissemination is mainly found to be from the respiratory and urinary tracts. Removal of an infected catheter is often necessary to control device-related bacteremia, since these devices frequently contain biofilms that can continuously shed *P. aeruginosa* [18, 35].

When *P. aeruginosa* disseminates from a site of local infection, it gains access to the bloodstream by breaking down the epithelial and endothelial tissue barriers [122]. To evade the bactericidal activity of the serum complement *P. aeruginosa* must produce a smooth LPS [86, 161]. Animal models have demonstrated that once in the blood stream, sepsis is related to the release of pro-inflammatory mediators like tumor necrosis factor-α (TNF-α). In a rabbit lung model of sepsis, provision of antibodies to TNF-α or the anti-inflammatory cytokine IL-10 improved both the signs of septic shock and the levels of bacteremia [79, 185].

Initial treatments for bacteremia are typically empirical and should include those compounds with the lowest resistance rates for that institution, until antimicrobial susceptibility results are returned. Standard treatment regimens for *P. aeruginosa* include the use of the anti-pseudomonal β-lactams ceftazidime, cefepime, meropenem, imipenem and piperacillin. Aztreonam may be used for patients with β-lactam allergy [18, 162]. The addition of an aminoglycoside is frequently used but is generally at the discretion of the physician for non-neutropenic patients. At the time when aminoglycosides and polymyxins were the only drugs available for treatment of *P. aeruginosa* infections, the prognosis for bacteremic patients was dismal. With the introduction of carbenicillin, the first antipseudomonal β-lactam, patient outcomes significantly improved [20, 184]. However it is worth noting that β-lactam and multi-drug resistance threatens to reverse these gains and, for example, carbenicillin is now rarely used therapeutically for such infections.

Analyses of treatment of *P. aeruginosa* bacteremia have included retrospective studies performed mostly for febrile neutropenic patients. These have included retrospective analyses of β-lactam monotherapy, β-lactam combination therapy, and the synergistic effects of combination therapy [21, 31, 128, 129, 156, 215]. *In vitro* and animal experiments performed around this time suggested a synergistic effect between the anti-pseudomonal β-lactams and aminoglycosides against *P. aeruginosa,* and so this, together with occasional reports of treatment failure with β-lactam monotherapy, was used to promote the practice of combination therapy [3, 104, 187]. While a prospective trial of combination therapy with anti-pseudomonal β-lactams and aminoglycosides demonstrated superiority to monotherapy with aminoglycosides [96], there has not been strong evidence that combination therapy is superior to antipseudomonal β-lactam monotherapy.

Pharmacokinetic and pharmacodynamic studies of anti-pseudomonal therapeutics have offered insights as to effective dosing regimens, particularly for aminoglycosides [39]. It is now understood that aminoglycosides kill in a concentration dependent manner, and so it is predicted that a single daily high dose of aminoglycosides would result in more effective killing, without increased toxicity, than would multiple daily does or continuous infusion [55, 89]. Although these pharmacokinetic and pharmacodynamic studies offer the hope of better outcomes than were observed in prior studies with aminoglycoside monotherapy, no prospective trials of single daily dosing with aminoglycosides have been conducted for *P. aeruginosa* bacteremia.

In contrast to the concentration-dependent killing observed with aminoglycosides, β–lactams act in a time-dependent manner. Efficacy is correlated to the percentage

of time for which tissue concentrations exceed the MIC, and for *P. aeruginosa* the ideal dosing would have a target attainment of 60–70% of the dosing interval [2].

Support for the use of combination therapy can also be found in the fight against the development of antimicrobial resistance, which is thought to be delayed when using two antibiotics with distinct mechanisms of action. Development of resistance is a major concern in treating *P. aeruginosa* infections, as the organism is intrinsically resistant to many antibiotics and further development of resistance leaves very few effective options for treatment. However, it is not really known if combination therapy increases the rate of development of multi-drug resistance (e.g., through efflux pump upregulation), and progressive development of resistance to each of the two agents in a combination remains a risk.

21.4.3 Hospital-Associated Pneumonia

The human respiratory tract presents a favourable environment to which *P. aeruginosa* has become particularly well adapted. *P. aeruginosa* has the formidable ability to cause both chronic infections in the lungs of CF patients and acute nosocomial pneumonia. Acute *P. aeruginosa* infections are rarely early onset and typical *P. aeruginosa* pneumonia is characterized by slow onset pneumonia, occurring after 5 days of hospitalization, and is associated with prior use of broad spectrum antimicrobial therapy, structural lung disease, or mechanical ventilation [18].

Animal model studies of *P. aeruginosa* pneumonia have demonstrated the involvement in virulence of proteases, flagella, pili, and LPS O side chains as well as the delivery of the extracellular toxins ExoS, ExoT, and ExoU via a type III secretion system. For example, administration of anti-*pcrV* antibodies blocking the type III secretion system has been shown to offer protection against acute *P. aeruginosa* pneumonia when tested in animal studies [61, 190].

Adequate clinical trials focusing on appropriate antimicrobial therapy for acute nosocomial *P. aeruginosa* pneumonia have not been performed and issues involving differentiation between colonization and infection as well as defining Pseudomonas-attributable mortality complicate available studies. Most recent studies have focused on the treatment of *P. aeruginosa* VAP and will be discussed in Sect. 4.3.1. The standard treatment for *P. aeruginosa* pneumonia is similar to treatment for *P. aeruginosa* bacteremia, although the inclusion of IV administration of an aminoglycoside in combination therapy for fully susceptible organisms is arguable given that aminoglycosides are not optimally active in the lungs at the concentrations normally used with IV administration [154, 158, 212]. Nonetheless the rates of antibiotic resistance and multidrug resistance in nosocomial isolates of *P. aeruginosa* are on the rise and so an adjunct to β-lactam monotherapy should be considered. Aerosolized antibiotics, particularly aerosolized aminoglycosides, have been developed to circumvent the issues relating to the poor activity of aminoglycosides in the lungs and to deliver higher concentrations of drug directly to the respiratory tract [127].

The inhaled aminoglycoside tobramycin is commonly used for treatment of respiratory tract infections in patients with CF, however, trials for non-CF patients with *P. aeruginosa* bronchiectasis were not as successful [12, 53, 152]. Its efficacy in the treatment of acute pneumonia has not been demonstrated to date in controlled prospective trials with the exception of a few preliminary studies related to ventilator-associated pneumonia (VAP).

21.4.3.1 Ventilator-Associated Pneumonia

P. aeruginosa appears to have a distinct advantage as a pathogen in device-related infections and is commonly found to be the first or second major pathogen causing VAP [95]. It is the most common multidrug resistant pathogen involved in this disease and recovery rate of *P. aeruginosa* is increased with increased duration of mechanical ventilation. *P. aeruginosa* VAP infections are characterized by their late onset, and early inappropriate antimicrobial therapy may provide an added selective advantage predisposing to *Pseudomonas* infections. In addition to being amongst the most common pathogens causing VAP, *P. aeruginosa* is also amongst the most lethal pathogens, since reports suggest up to 70–80% mortality when the organism remains confined to the lungs [33], with directly attributable mortality rates reaching 38% [59].

Antimicrobial treatment of VAP has been the subject of recent studies investigating the optimal length of treatment. A comparison of 8 versus 15-day antibiotic therapy for VAP found that patients treated for only 8 days did not have significantly higher mortality and in general did not have significantly higher rates of recurring infection. However, in patients suffering from VAP caused by non-fermenting Gram-negative bacteria, including *P. aeruginosa,* the shorter treatment length was associated with a higher rate of recurring infections [32], highlighting one of the major issues specific to *P. aeruginosa*, namely persistence. Despite appropriate treatment of these infections, *P. aeruginosa* has been isolated from the alveolar space 8 days after the onset of VAP [56] and relapsing infections by susceptible isolates has been seen at a rate of 18% [90]. This persistence is in fact one of the main challenges of treatment of VAP and has recently been shown to be associated with the expression of the Type III secretion systems [45, 56], although it is likely that growth in a biofilm mode plays a role in persistence in the face of seemingly appropriate treatment.

Recent pilot studies have begun to assess the utility of aerosolized tobramycin for the treatment of VAP and have focused on the use of aerosolized antimicrobials as a preventative measure, as an adjunct to systemic therapy and due to its efficacy in treating multidrug resistant organisms. Earlier studies of preventative use of inhaled antibiotics used aerosolized polymyxin B administered every 4 h. These investigations revealed that although overall rates of pneumonia were reduced, there was an increase in pneumonia caused by multidrug resistant organisms and that these were associated with higher mortality rates [62]. More recent investigations of

the preventative use of aerosolized antimicrobials for VAP have centered around the use of inhaled tobramycin, ceftazidime, and colistin, and have demonstrated that these agents could reduce the occurrence of VAP without promoting the emergence of drug resistant organisms; however there was no reduction in mortality in patients receiving these measures [60] and thus standard therapy for VAP involves treatment with broad-spectrum systemic antimicrobials upon establishment of VAP.

Adjunct aerosolized therapy for VAP in general is arguable; however, in studies focusing on *Pseudomonas* species and *Enterobacteriaceae* species, reports of 96% clinical cure rates have been cited when combination systemic and aerosolized antimicrobial therapy were used in conjunction with selective topical gastrointestinal decontamination [202]. Unfortunately, these investigations were limited by the lack of a control group and other supportive data is limited to isolated case reports reporting successful treatment with combinations of systemic and aerosolized antibiotics [141, 194, 201]. A number of devices have been evaluated for their ability to efficaciously deliver aerosolized antimicrobials to the lungs, and, although there is evidence that inhaled aminoglycosides can achieve drug levels in the tracheobronchial tree capable of reducing the density of *P. aeruginosa* and diminishing inflammatory markers, strong evidence has yet to be presented to indicate that the inhaled antibiotics can penetrate further into the distal lung parenchyma for effective treatment of acute pneumonia [44].

21.5 *Pseudomonas aeruginosa* Infections in Cystic Fibrosis

CF is an autosomal, recessive, multi-organ disorder affecting 1:2,500 in the Caucasian population [169]. Mortality in this afflicted population is mainly attributed to chronic respiratory infections and the associated gradual deterioration of lung function. There are several pathogens known to play a role in CF lung infection, with *S. aureus* and *Haemophilius influenza* being the predominant pathogens colonizing younger patients, and *Stenotrophomonas maltophilia*, *Burkholderia cepacia* and *Pseudomonas aeruginosa* in adults. However, *P. aeruginosa* is often isolated from patients less than 2 years of age and is the most predominant concern in adults [73, 109]. The nature of this disease is important in understanding why *P. aeruginosa* dominates as the primary pathogen in CF patients and so host pathology is addressed below.

The defective gene involved in CF encodes for the CF transmembrane conductance regulator (CFTR) resulting in pathological changes in organs that express CFTR, including secretory cells, the liver, the pancreas and the lungs. In a normal airway epithelial cell, the gene encoding for CFTR regulates the transport of chloride, sodium and water. Abnormalities of the CFTR gene product lead to a thick and dehydrated mucus secretion that impairs mucociliary clearance of bacterial pathogens [37]. In the normal lung, the mucus layers function in binding and clearance of inhaled pathogens, and although the bacterial load can be quite high in the upper airways, the lower airways remain free of bacteria [7]. Due to the characteristic

thickened mucus associated with CF lung disease, and an inability of ciliary beating to remove this mucus (due to the thickened mucus and inhibition by *Pseudomonas* factors), the invading pathogens become trapped in the mucus layer and consequently damage and then adhere to the epithelial surfaces more readily than in healthy individuals.

P. aeruginosa airway colonization frequently occurs after initial airway colonization with *S. aureus* and subsequent antimicrobial therapy [102]. *P. aeruginosa* thereby replaces the other bacterium becoming the predominant bacterium in the CF lung. Undoubtedly the adaptive behaviour of *Pseudomonas* plays a critical role in establishment of a chronic infection that may last more than a decade; some of these adaptations include quorum sensing, the development of anti-immunity measures such as the mucoid phenotype, development of a rough LPS lacking O-antigen, and loss of flagella and/or pili, biofilm and/or microcolony development, adaptation and eventual mutation to antibiotic resistance, and often development of a mutator phenotype, as discussed in more detail below. Furthermore, *P. aeruginosa*'s ability to grow in microaerophilic and anaerobic environments offer this pathogen an added survival advantage over strict aerobic pathogens as steep hypoxic gradients have been observed in the thickened mucus in the lung. *P. aeruginosa* infections in the CF lung differ from the classical definition of infection wherein symptoms arise as a result of an invading pathogen. Instead, CF infection is more accurately described as a state of chronic colonization characterized by high bacterial load that ultimately triggers a prolonged inflammatory response. It is this prolonged and pronounced inflammatory response that causes the variety of symptoms accompanying these chronic infections (cough, sputum production, damage to the epithelial layer, loss of lung function, and breathing problems), and leads to respiratory distress and eventual failure and death. Acute infections due to *P. aeruginosa* are not typically the cause of mortality in CF patients, although with increasing bacterial loads, patients frequently suffer from acute exacerbations of respiratory symptoms.

21.5.1 Adaptations Occuring During Chronic Infection

CF patients frequently become colonized in the upper airways by environmental isolates of *P. aeruginosa*, although epidemic isolates (e.g., the Liverpool epidemic strain) are also known [180]. During the process of infection, this bacterium undergoes a number of adaptations lending to the characteristic persistence and antibiotic resistance of this organism during chronic infection. Amongst the most common adaptations found in CF isolates of *P. aeruginosa*, is the conversion to mucoid phenotype due to overexpression of alginate [78]. While the environmental isolates involved in initial colonization and attachment usually present a non-mucoid phenotype, as the organism penetrates the thickened mucus lining of the airways, travelling down the oxygen gradient, increased expression of alginate and a switch to a mucoid phenotype occur [80, 197]. This phenotype often occurs coincidently with the establishment of chronic infection, and becomes stabilized by regulatory mutations as described above.

The mucoid form of *P. aeruginosa* is associated with 90% of *P. aeruginosa* CF infections compared to only 2% of *P. aeruginosa* non-CF infections [47, 49].

Other easily identified morphological adaptations include the switch from smooth to rough colony morphology and the development of small colony variants. The rough colony morphology is representative of strains that have lost the LPS O-antigen [86]. As the O-antigen is the immunodominant portion of the LPS, this adaptation leads to a less virulent phenotype, which is perhaps more adept at evading host immune defenses thus causing persistent infections. It also makes rough isolates more susceptible to complement killing and perhaps explains in part why these organisms virtually never cause invasive infections. The small colony phenotype is less well understood, but is of considerable interest as these isolates exhibit increased antibiotic resistance. This phenotype is observed as smaller colonies after 2 days of incubation at 37°C, and has been associated with prior aminoglycoside therapy. Isolates exhibiting this phenotype have been found to be hyperpiliated with increased abilities in twitching and biofilm formation, and with decreased ability for swimming [91].

Another phenotype of relevance to antimicrobial therapy and resistance is the hypermutator phenotype, which is frequently observed in CF isolates, but less commonly in other nosocomial isolates of *P. aeruginosa* [151]. This phenotype, characterized by an up to 1,000-fold increased mutation frequency, has been attributed to mutations in genes encoding DNA replication and repair mechanisms, such as *mutS, mutL* and *mutY.* The higher mutation frequency gives an added advantage for survival in harsh environments such as the CF lung since it facilitates the aforementioned adaptations, by stabilizing these changes through mutations. Most importantly, these hypermutator isolates have the ability to develop mutational resistance more readily during a course of antimicrobial therapy than do non-mutator isolates (e.g., at frequencies of 10^{-6} instead of 10^{-9}). The hypermutator phenotype can give rise to a variety of mixed morphologies, including those described above, within the lung [101]. These mixed populations can colonize or infect different compartments within the lung and often have differing antimicrobial susceptibilities and virulence properties.

Comparison of *P. aeruginosa* isolates from the CF lung to strains from non-CF patients showed clearly that CF isolates tend to demonstrate an overproduction of β-lactamase, loss of OprD and an overproduction of MexXY. This efflux pump overproduction leads to high-level aminoglycoside resistance and the overproduction of this and other efflux systems also lead to quinolone resistance, amongst which MexCD-OprJ was the most frequent [93].

21.5.2 Antimicrobial Therapy for Treatment of P. aeruginosa

Chronic colonization and infection with *P. aeruginosa* is an inevitable reality for the majority of adults with CF, as over 80% of adults over the age of 18 years return

positive cultures for *P. aeruginosa* [97]. It is the state of chronic lung infection and the resulting inflammatory response that lead to their gradual deterioration of lung function. Although these chronic infections are generally deemed impossible to eradicate, antimicrobial therapy has been used to effectively delay the onset of chronic infection and to decrease bacterial load, thereby reducing the deleterious effects associated with increased inflammation in established infections [205, 213].

Treatment strategies have been developed for prevention of colonization, for eradication of early stage colonization and for clearance of established infections [73, 211]. Common practices for treatment of infections during these stages are outlined in Table 21.1. Although prevention of colonization seems an appealing strategy, prophylactic treatment for prevention has gained only modest support as a therapeutic option. In fact, prophylactic antimicrobial use was initially developed for prevention of *S. aureus* infection, but was found to be associated with increased acquisition of *P. aeruginosa* [171].

The strategies used for patients with early stage infections may differ from those adopted for individuals with established chronic infections, as the organism changes and adapts to the lung environment. Nonetheless, the drugs of choice for treatment of *P. aeruginosa* colonization and infection are all bactericidal agents including β-lactams, aminoglycosides, fluoroquinolones and colistin [65, 131, 199, 205, 213].

P. aeruginosa isolates from CF patients frequently develop multi-drug resistance. Combination therapy can be used to avoid resistance development and to exploit the synergistic effects of the bactericidal antibiotics. In a study of 1,240 *P. aeruginosa* isolates from CF patients, susceptibility to tobramycin, amikacin, gentamicin, aztreonam, ceftazidime, ticarcillin and ciprofloxacin was examined [189]. Cross-resistance was found amongst the aminoglycosides, however 70% of isolates resistant to β-lactams were susceptible to aminoglycosides and up to 60% of tobramycin resistant isolates were susceptible to β-lactams; thus, supporting the use of combination therapy with a β-lactam and an aminoglycoside antibiotic.

The use of aerosols allows for drugs to be delivered directly to the site of infection and a number of antibiotics including gentamicin, tobramycin, colistin, ceftazidime, carbenicillin, aztreonam, and amikacin have been administered as aerosols to CF patients, although approved formulations and adequate controlled studies have not been performed for most of these [98, 198]. In contrast, aminoglycosides have several advantages for use as aerosols. They are highly polar, chemically stable, and less toxic than when administered intravenously. The most promising aminoglycoside currently used for inhalation is the tobramycin formulation TOBI (Chiron, CA, USA) which is capable of delivering a high dose of tobramycin to the lower respiratory tract of CF patients [34, 167].

Gilead Science has recently developed an aerosolized formulation of the monobactam aztreonam and lysine (AZLI) [142]. Clinical trials have demonstrated the efficacy of AZLI as an adjunct therapy to TOBI. AZLI appeared to improve lung function and was well tolerated by the patients. Regardless, AZLI was not approved after its phase III clinical trial (Sept 2008) and additional studies over a longer time period are required before FDA approval.

Table 21.1 Empiric therapy for the treatment of *P. aeruginosa* infections in patients with cystic fibrosis [29, 73]

	Antibiotic	Pediatric dose (> 6 years)	Adult dose	Daily dosing interval	Route of administration	Duration of treatment
First isolation of *P. aeruginosa* (without clinical symptoms)	Ciprofloxacin	15 mg/kg	15 mg/kg	12 h	Oral	3–4 weeks
	+					
	Tobramycin	300 mg	300 mg	12 h	Inhalation	
	or Colistin	150 mg	150 mg	12 h	Inhalation	
Pulmonary exacerbations	Ceftazidime	50 mg/kg	2 g	8 h	IV	2–3 weeks or longer if no signs of improvement
	or Piperacillin	100 mg/kg	3 g	6 h	IV	
	or Imipenem	15–25 mg/kg	0.5–1 g	6 h	IV	
	or Meropenem	40 mg/kg	2 g	8 h	IV	
	or Aztreonam	50 mg/kg	2 g	8 h	IV	
	+					
	Tobramycin	3 mg/kg	3 mg/kg	8 h	IV	
	or Amikacin	5–7.5 mg/kg	2 g	8 h	IV	
Mild exacerbations	Ciprofloxacin	15 mg/kg	2 g	8 h	Oral	2–3 weeks
Maintenance therapy	Tobramycin	300 mg	300 mg	12 h	Inhalation	28 day on/off cycle
	or Colistin	150 mg	150 mg	12 h	Inhalation	
	+/–					
	Ciprofloxacin	10–15 mg/kg	0.5–0.75 g	12 h	Oral	2–4 weeks cycled every 3–4 month

21.5.2.1 Antimicrobial Therapy for Colonization and Initial Infection with *P. aeruginosa*

Eradication of *P. aeruginosa* from the CF lung is possible only in the early stages of colonization. At this stage, the bacterial load tends to be low, and the organism is non-mucoid and has not begun to undergo significant morphological changes. Aggressive antimicrobial treatment upon first isolation of *P. aeruginosa* has been demonstrated in most cases to delay or occasionally prevent the onset of chronic infections resulting in a better quality of life and a greater life expectancy [148, 175]. Successful eradication is judged by the observation of at least three consecutive negative cultures at intervals of at least 1 month. After 1 year of negative cultures following the onset of antimicrobial therapy, any isolation of *P. aeruginosa* is considered to represent a new isolate [73]. Aggressive antimicrobial use at the early stage has proven in certain cases to be successful, with a number of patients having remained culture negative for *P. aeruginosa* for several years after treatment [65, 170].

A number of differing antimicrobial regimens have been used for treatment of early colonization of *P. aeruginosa.* While earlier regimens included the use of intravenous ciprofloxacin, or nebulized tobramycin or colistin [131, 199], more current recommended therapies typically use a combination of oral and aerosolized antibiotics [65, 213]. These latter two studies used a combination of oral ciprofloxacin and aerosolized colistin administered twice daily over a 3-week period. During the 27 months observed, 80% of the treated group did not go on to develop chronic infections with *P. aeruginosa.* Unfortunately, most investigations to date have been limited by small sample size and lack of proper control groups. Furthermore, microbiological samples are generally taken from the upper respiratory tract, which gives a poor prediction of the microbiology of the lower airways. The only controlled study using lower respiratory tract samples investigated the effect of inhaled tobramycin (300 mg) over a 28-day period, but was terminated early due to adverse affects [74].

21.5.2.2 Antimicrobial Therapy for Chronic Infections

Once chronic infection has been established by *P. aeruginosa,* antimicrobial therapy becomes complicated due to the high bacterial load present in the lung as well as the phenotypic changes occurring in the pathogen. The high bacterial load and thickened mucus act as barriers to the attainment of sufficient exposure of the entire bacterial population and to the bactericidal concentrations of antibiotics. Administration of insufficient concentrations of antibiotics adds increased selective pressure for resistant phenotypes, thereby enhancing the diversity of the population, lending further difficulties to effective treatment. As such, a variety of morphological phenotypes, termed colony variants and partially described above, may be present within the lung and demonstrate varying susceptibilities. Susceptibility testing should be performed for each morphological variant identified in a sample.

Despite the improved means of antibiotic delivery using aerosolized antibiotics, antimicrobial therapy at this stage has been shown to be insufficient for eradication

of *P. aeruginosa.* The goal of therapy at this point is largely to reduce bacterial load. Antimicrobial therapy is used during chronic infections in CF for two main purposes: maintenance therapy and treatment of acute exacerbations of infection [52].

Maintenance Therapy

Maintenance therapy is recommended for CF patients with chronic *P. aeruginosa* infections in order to reduce bacterial load and maintain overall lung function. Unfortunately, a number of side effects are associated with long term antimicrobial use, including loss of hearing, increased cough, alterations of the voice, and the appearance of antibiotic resistant strains. The use of on/off cycles of intermittent drug administration led to the reduced occurrence of these side effects [167].

Multiple studies have investigated the effect of intermittent administration of TOBI on lung function in CF patients [34, 167]. In the case of Cheer et al. [34], patients were treated with 300 mg of TOBI twice daily in a cycle of 28-days on/28-days off. This treatment regimen led to a significant improvement in lung function and a reduction of *P. aeruginosa* in the sputum. Similar observations were made by Ramsey et al. [167] who showed that patients receiving inhaled tobramycin for 4 weeks, followed by 4 weeks without tobramycin, over a period of 24 weeks, had better lung function compared to the placebo group. Patients receiving tobramycin in this study were also less likely to be hospitalized (26%) than the placebo group.

Oral ciprofloxacin has also been studied for its use in maintenance therapy and has shown promising results regarding improved lung function. The risk of developing fluoroquinolone resistance in both *S. aureus* and *P. aeruginosa* favours its intermittent use, and as with tobramycin no longer than 4 consecutive weeks on ciprofloxacin is recommended [29].

Intermittent delivery of aerosolized colistin has also been used, particularly in Europe, although randomized placebo controlled studies are lacking. Colistin is generally regarded as a last resort option for isolates that are resistant to the common antibiotics. Colistin exhibits high activity against multi-drug resistant strains and resistance of *P. aeruginosa* to colistin rarely occurs [177].

Recent interest in macrolide antibiotics for the treatment of CF infections has been sparked by the findings that these agents are capable of acting in a direct antimicrobial manner, penetrating biofilms and interfering with quorum sensing signals in *P. aeruginosa* [208, 209] as well as acting in an anti-inflammatory manner. Phase III trials using thrice weekly azithromycin have demonstrated promising results with regards to decreased occurrence of pulmonary exacerbations, with only mild side effects including nausea, diarrhea and wheezing [179].

Treatment of Acute Pulmonary Exacerbations

Given the high bacterial load that can accumulate within the CF lung, patients suffering from chronic infections with *P. aeruginosa* have frequent acute exacerbations that are characterized by strong inflammatory responses. Antimicrobial treatment of these exacerbations can effectively reduce bacterial load and reduce the inflammatory

response. Limited data is available for evaluation of effective treatments of pulmonary exacerbations and the results of antibiotic susceptibility profiling play an important role in guiding treatment. The use of aerosolized antimicrobials in the treatment of acute exacerbations requires further investigation ([29, 74].

Mild exacerbations during chronic lung infections can typically be treated in an outpatient setting using oral ciprofloxacin [29]. Moderate and severe exacerbations, however, require intravenous administration of two antimicrobial agents, generally, an antipseudomonal β-lactam and either tobramycin or amikacin, which are recommended for use in combination for 2–3 weeks [192]. While severe symptoms require the patient to be hospitalized to permit monitoring of renal function and for adjunct respiratory therapy, it is becoming more common to treat moderate exacerbations at home [29].

21.6 Concluding Remarks

P. aeruginosa is truly a unique pathogen and is generally regarded as one of the most lethal organisms causing nosocomial and CF infections. There is no doubt that its high intrinsic resistance to antibiotics, together with its impressive ability to adapt has led to its prominence as a pathogen, and it remains one of the most challenging organisms to treat. As we gradually accumulate knowledge regarding this elusive pathogen and its mechanisms of pathogenesis, it seems as though behind each discovery is another complex regulatory network waiting to be revealed. While significant advances have been made in the anti-infective treatment of other bacterial species, these advances have effectively paved the way for the emergence of *P. aeruginosa* as an important pathogen. Due to its high level of intrinsic resistance, the use of broad-spectrum antimicrobials without anti-pseudomonal efficacy eliminates competing organisms, thus removing competitors and permitting *P. aeruginosa* to initiate colonization. Its high propensity for development of high-level resistance to all of the available anti-pseudomonal antibiotics only exacerbates the dire situation we face in terms of the scarcity of effective anti-pseudomonals. Indeed there is gathering evidence that *Pseudomonas* has become the most recent prominent entry into the category of Superbugs, for which therapeutic options are extremely limited and becoming more so. Not only is there a need for additional anti-pseudomonal drugs, but it is also evident that there is a need for additional studies to determine the most effective therapeutic dosing regimens for the currently-available antibiotics, so as to extend the life of available tools. Without effective dosing regimens and adequate surveillance, we will likely re-enter a time when polymyxins are the only therapeutic option for treatment of *P. aeruginosa* infections, and, of great concern, resistance is on the rise for this class also.

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Part VI
Mycobacteria

Chapter 22
Drug Resistant and Persistent Tuberculosis: Mechanisms and Drug Development

Ying Zhang

22.1 Introduction

Mycobacterium tuberculosis is a particularly successful pathogen that latently infects about one third of world population (about two billion people). Despite the availability of chemotherapy and BCG vaccine, tuberculosis (TB) remains a leading cause of infectious morbidity and mortality, causing about nine million new cases and nearly 1.5–2 million deaths annually [142]. The increasing emergence of drug-resistant TB, especially multidrug-resistant TB (MDR-TB) (resistant to at least isoniazid [INH] and rifampin [RIF]) and more recently extensively drug-resistant TB (XDR-TB), has caused a great deal of concern. XDR-TB is defined as MDR-TB plus additional resistance to two major second-line drugs fluoroquinolones and one of the three injectables (capreomycin, kanamycin, and amikacin) [141]. The recent outbreak of XDR-TB in South Africa where 52 of 53 HIV-positive individuals infected with XDR-TB died in an average of 16 days is particularly alarming [44]. There are about 500,000 new cases of MDR-TB and 40,000 XDR-TB cases a year [141]. The TB situation is further complicated by the spread of HIV pandemic worldwide, which weakens the host immune system and allows latent TB to reactivate or makes the person more susceptible to reinfection with drug susceptible and even drug-resistant strains [123]. It is estimated that of about 40 million people infected with HIV, one third are also co-infected with the tubercle bacillus. These individuals are 20–30 times more likely to develop TB and have five times higher mortality than those without HIV [95]. The current TB control regimen is seriously undermined by MDR/XDR-TB and HIV infection, which is a lethal combination and presents significant challenges for effective TB control.

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The MDR/XDR-TB problem is at least partly attributable to the suboptimal 6–8 month chemotherapy. The lengthy therapy not only has significant side effects, but also creates poor patient compliance, which frequently selects drug-resistant TB bacteria. Due to the increasing drug-resistant TB problem and the increasing realization of the problem with lengthy TB therapy, there is currently significant interest in developing new TB drugs that are not only active against drug-resistant TB but more importantly also can shorten the TB therapy [40,46,149]. New progress is being made in this area, and there is hope that more effective TB therapy may be in sight in the near future. However, there are various challenges in TB drug development. This chapter will cover a brief history of TB treatment and the problem of current therapy, followed by discussions of mechanisms of drug resistance and persistence, and finally the current status of new TB drug development.

22.2 The Current TB Chemotherapy

22.2.1 A Brief History

The TB chemotherapy began in 1944 when Schatz and Waksman discovered streptomycin (SM), the first effective TB drug that marked the beginning of modern effective TB treatment [116]. Two years after the discovery of SM, Lehmann discovered para-aminosalicylic acid (PAS) as an effective TB drug in 1946 [74]. Isoniazid (INH) was discovered in 1952 based on the nicotinamide activity against tubercle bacilli in the animal model observed by Chorine in 1945 [26] and the reshuffling of chemical groups in thiosemicarbazone [8,42,97]. The nicotinamide lead also led to the discovery of pyrazinamide (PZA) in 1952 [81,124] and ethionamide (ETH)/Prothionamide (PTH) in 1956 [77]. Ethambutol (EMB) was discovered in 1961 at Lederle [131]. Further screening for antibiotics from soil microbes led to the discovery of cycloserine [72], kanamycin [134] and its derivative amikacin, viomycin [7], capreomycin [55], and rifamycins [119] and its derivative rifampin [120]. The broad-spectrum quinolones developed in 1980s have high activity against mycobacteria and are used as second-line drugs for the treatment of MDR-TB [132,147].

Evaluation of TB drugs and treatment regimens started in 1946 when the British Medical Research Council performed the first ever randomized clinical trial (RCT) with any medicine, proving SM's efficacy [43], but resistance to SM was soon observed. It was noted that addition of PAS to SM prevented SM resistance, thus establishing the important principle of drug combination for the treatment of TB. The same principle of drug combination was subsequently used to treat various infectious diseases such as AIDS and *Helicobacter pylori* and also cancer. Addition of INH to SM and PAS in the 1950s finally led to a consistent cure in 18–24 months. The use of RIF in the 1970s in combination with INH further shortened treatment to 9 months [43]. In developing countries where cost is a factor, RIF in the continuation phase can be replaced by thioacetazone or ethambutol (EMB) but the treatment

with INH and thioacetazone or INH and EMB has to be extended to 6 months instead of 4 months of INH and RIF in the continuation phase to achieve comparable results. In the 1970s and 1980s, reevaluation of PZA in combination with INH and RIF led to the current 6-month TB chemotherapy. Since 1995, WHO recommends the 6-month therapy (part of the Directly Observed Therapy – short course (DOTS) strategy) as the preferred standard TB chemotherapy for treating all TB patients. DOTS is now widely used by most countries to treat drug-susceptible TB [142].

22.2.2 Current TB Chemotherapy and Its Problems

The current recommended standard TB chemotherapy takes at least 6–8 months to treat drug-susceptible TB. WHO recommends a 6-month therapy consisting of an initial phase of treatment with four first-line drugs, isoniazid (INH), rifampin (RIF), pyrazinamide (PZA), and ethambutol (EMB) for 2 months, followed by a continuation phase of treatment with INH and RIF daily or three times a week for another 4 months [12]. The International Union Against Tuberculosis and Lung Diseases (IUATLD) recommends an 8-month therapy consisting of a 2-month initial phase of INH, RIF, PZA, and EMB given by DOT, followed by a 6-month continuation phase of daily INH and thioacetazone, self-administered [12]. DOTS is currently the best TB treatment strategy with a cure rate of 78–95% [142]. However, DOTS therapy is lengthy and toxic, and the development of drug resistance is mainly due to poor patient compliance. In addition, DOTS may not work in areas where there is high incidence of MDR-TB, with a cure rate as low as 50%. In such situations, DOTS-Plus, which is DOTS plus second-line TB drugs for the treatment of MDR-TB and TB, is recommended [142]. However, treatment of MDR-TB requires the guidance of drug susceptibility testing and can take 18–24 months, which is not only costly but also has significant toxicity.

TB therapy is exceptionally long compared to treatment of other bacterial infections, most of which are cured with usually no longer than 1 or 2 weeks antibiotic treatment. Several factors underlie lengthy TB therapy. First, the nature of the disease pathology can influence the efficacy and duration of chemotherapy. For example, open cavities in the lung containing large numbers of bacilli present a particular problem for eradication of the bacilli by chemotherapy and can facilitate development of drug resistance [21]. The intracellular location of the bacilli could render some drugs such as streptomycin inactive against these intracellular bacilli. However, while most drugs seem to penetrate the necrotic tissues [21] the drugs cannot effectively kill non-replicating bacilli in the lesions. Second, the phenotypic resistance in non-replicating persisters presents a major problem for the current TB therapy. It is well known that antibiotics are active against growing bacteria but are ineffective against non-growing bacteria [84]. The remaining persister population accounts for posttreatment relapses, especially when the duration of therapy is inadequate. Third, the host immune system may not effectively eliminate tubercle bacilli in the lesions with single drug or short-term treatment. In many bacterial infections, small numbers

of residual bacteria after antibiotic therapy can be effectively controlled by the immune system. However, it appears that the host immune system is not very effective in controlling the residual TB bacteria not killed by TB chemotherapy. Indeed, *M. tuberculosis* can manipulate the immune system by stimulating immune suppressive cytokines TGF–β and IL-10 to avoid clearance by the immune system [45].

To understand why the current TB therapy is this long, it is necessary to examine the conceptual framework of the current chemotherapy. Tubercle bacilli during the disease process reside in different microenvironments, with varying oxygen contents from high oxygen in a lung cavity to low oxygen in host macrophages and virtually no oxygen in closed lesions, and acid pH during active inflammation, all of which affect the metabolic status of tubercle bacilli and are the basis for producing different bacterial populations that have varying susceptibilities to different TB drugs. Tubercle bacilli in lesions are divided into four different subpopulations according to Mitchison [87]: (a) those that are actively growing such as those in cavities exposed to sufficient oxygen are killed primarily by INH (but in case of INH resistance, the bacilli are killed by RIF or SM or inhibited by EMB); (b) those that have spurts of metabolism are killed by RIF, (c) those that have low metabolic activity in acid pH environment are killed by PZA; (d) those that are "dormant" or persisters are not killed by current TB drugs. The use of different drugs, INH, RIF, and PZA, in combination is to kill different populations of bacilli in different types of lesions, to improve the efficacy of the therapy, and to prevent drug resistance. RIF and PZA have the greatest sterilizing activity and significantly reduce the persister population in the lesions and play an important role in shortening the therapy from 12–18 months to 6 months. The bactericidal activity of INH is only short-lived, and kills only growing bacilli. The sterilizing activity of RIF persists throughout the therapy, but the sterilizing activity of PZA is confined to the first 2 months of the initial phase of treatment [43]. Importantly, in RIF-containing regimens, PZA provides additional sterilizing activity by killing a special population of bacilli not killed by other TB drugs [87]. However, there are still other persister populations that are not killed by RIF or PZA [149]. Despite the fact that TB chemotherapy renders a patient noninfectious a few weeks after the initiation of the therapy, the whole 6-month therapy is necessary to kill a population of slowly metabolizing persister bacilli and to allow the host to develop sufficient immunity to prevent relapse. It is worth noting that the current chemotherapy, while achieving a clinical cure, does not achieve a bacteriological cure for certain, i.e., the therapy cannot completely eradicate all bacilli in the lesion and relies on host immune mechanisms to mop up the remaining small number of persisters left after chemotherapy [21,84]. In cases of immune deficiency and high bacterial load, treatment failure and relapse can occur after treatment. However, if the number of persisters or dormant bacilli is sufficiently small, and the immune response is adequate, the residual persisters may "die off" or be cleared by the immune system and a stable cure can be achieved without relapse.

Although the above Mitchison model of TB treatment is useful in explaining the effects of different drugs on different bacterial populations, it does not explain the

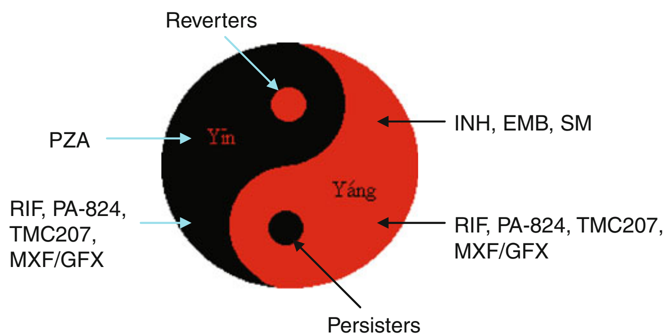

Fig. 22.1 Yin-Yang model of TB life cycle: effects of different drugs

practice of the current two-phase chemotherapy and the use of INH for prophylaxis of latent TB infections (LTBI). In the case of the current TB therapy; why, after 2 month intensive treatment with INH, RIF, PZA, and EMB, do we still use INH and RIF which mainly kill growing bacilli and some portion of bacilli with low metabolism in the continuation phase of treatment? The first 2 months' treatment should kill all the growing and part of the nongrowing bacilli, and thus INH and RIF should not work in the continuation phase. But why are INH and RIF still used and actually work in the continuation phase? Furthermore, why do we treat LTBI with INH which is only active against growing bacilli? I proposed a new Yin-Yang model which seems to better explain the current TB therapy and treatment of LTBI [150] (Fig. 22.1). This model postulates that a large bacterial population consists of varying dynamic subpopulations in a continuum with different metabolic status and that these populations can interconvert, i.e., growing bacteria can become persisters and persisters can revert back to growing forms. In growing bacterial population (Yang) there is a small population of nongrowing (Yin) or slowly growing persisters. As the bacteria enter stationary phase more persisters form but the number of growing bacteria is now the minority. INH, SM, or EMB only kill or inhibit growing bacteria, whereas RIF, moxifloxacin, gatifloxacin, and the new drug candidate TMC207 kill both growing bacterial population and a portion of nongrowing persisters, while PZA only kills nongrowing persisters at acid pH. While some persisters are killed by some antibiotics like RIF, others are killed by PZA, and still others are not killed by RIF or PZA, and can revert to growing form when antibiotics are removed. The reverted growing form (reverter) now becomes susceptible to INH and RIF. This model explains why after a 2-month intensive phase of treatment with the four first-line drugs (INH, RIF, PZA and EMB) some bacilli with low metabolism that are left (persisters) can revert to the growing forms (reverters) that can still be killed by INH and RIF in the subsequent 4-month continuation phase of treatment. Similarly, during LTBI, persister bacteria revert to growing forms which are then susceptible to INH used in prophylaxis. This Yin-Yang model could have general applicability to other organisms and treatment including cancer.

22.3 Mechanisms of Drug Resistance in *M. tuberculosis*

Antibiotic resistance in bacteria can occur through five major mechanisms [149, 152]: (i) decreased uptake or impermeability, (ii) increased efflux, (iii) enzymatic inactivation, (iv) modification or overexpression of drug target, and (v) inactivation of drug activating enzymes or defective prodrug activation. The last is a new mechanism of drug resistance that was first convincingly demonstrated with INH resistance mediated by mutations in KatG which activates INH [154].

M. tuberculosis has several means to achieve intrinsic resistance. For example, *M. tuberculosis* has a very hydrophobic cell envelope, which provides a permeability barrier for some antibiotics [14]. *M. tuberculosis* also possesses some enzymes such as beta-lactamase necessary to inactivate penicillin [118]. In addition, *M. tuberculosis erm37* encodes a 23 S rRNA methyltransferase, which adds a single methyl group to its primary target at A2058 and also attaches an additional methyl groups to the neighboring nucleotides A2057 and A2059 of the 23 S rRNA [80]. The gene *erm37* is present in the *M. tuberculosis* complex but absent in many non-tuberculous mycobacteria, and is responsible for the natural macrolide resistance in *M. tuberculosis* but susceptibility to macrolide in non-tuberculous mycobacteria [16]. *whiB7,* a transcriptional activator present only in Actinomycetes, was found to be responsible for intrinsic resistance to various antibiotics of different structures including chloramphenicol, clarithromycin, erythromycin, lincomycin, spectinomycin, streptomycin, and tetracycline in Streptomyces and *M. tuberculosis* [89]. Inactivation of the *whiB7* in *M. tuberculosis* caused increased susceptibility to macrolides, a lincosamide, and streptomycin [89]. Antibiotics (erythromycin, tetracycline, streptomycin) and fatty acids (palmitic acid being most active), and other stimuli could induce the expression of WhiB7, causing an inducible antibiotic resistance [89]. *whiB7* was the initially induced regulator that may be involved in subsequent induction of a regulon of eight genes including the known efflux gene *tap,* encoding an efflux pump that confers low-level resistance to aminoglycosides and tetracycline, and *erm37 (Rv1988)*, encoding a ribosomal methyltransferase which confers macrolide, lincosamide, and streptogramin resistance by modification of 23 S rRNA in response to antibiotics [89]. There are seven *whiB* genes, *whiB1-7,* in *M. tuberculosis,* which are induced by different stress conditions and antibiotics [48]. It was found that the cell wall active agents like INH, EMB, and cycloserine induced *whiB2,* whereas aminoglycosides induced *whiB7* primarily and also other *whiB* genes such as *whiB2, 3, and 6* to a lesser extent [48]. However, it remains to be determined if the *whiB* genes are involved in clinically relevant TB drug resistance.

Acquired drug resistance in *M. tuberculosis* is mediated by spontaneous mutations in chromosomal genes [151] (Table 22.1). The MDR-TB phenotype is caused by accumulation of mutations in different genes involved in individual resistance [56]. Unlike many other bacterial species, mobile genetic elements like plasmids or transposons play no role in drug resistance in *M. tuberculosis* [151]. The frequency of mutations that confer resistance is about 10^{-6} for INH and 10^{-8} for RIF using in vitro planktonic cultures [145]; thus, the frequency of concurrent mutations to resistance

Table 22.1 Mechanisms of drug action and resistance in *M. tuberculosis*

Drugs (year of discovery)	MIC (μg/ml)	Gene(s) involved in Resistance	Gene function	Role	Mechanism of action
Isoniazid (1952)	0.02–0.2	*katG*	Catalase-peroxidase	Prodrug conversion	Inhibition of mycolic acid biosynthesis and other multiple effects on DNA, lipids, carbohydrates, and NAD metabolism
		inhA	Enoyl ACP reductase	Drug target	
Rifampin (1966)	0.05–1	*rpoB*	β-subunit of RNA polymerase	Drug target	Inhibition of RNA synthesis
Pyrazinamide (1952)	16–50 (pH5.5)	*pncA*	Nicotinamidase/pyrazinamidase	Prodrug conversion	Depletion of membrane energy
Ethambutol (1961)	1–5	*embB*	Arabinosyl transferase	Drug target	Inhibition of arabinogalactan synthesis
Streptomycin (1944)	2–8	*rpsL*	S12 ribosomal protein	Drug target	Inhibition of protein synthesis
		rrs	16 S rRNA	Drug target	
		gidB	rRNA Methyltransferase (G527 in 530 loop)	Drug target	
Amikacin/ Kanamycin (1957) Capreomycin (1960)	2–4	*rrs*	16 S rRNA	Drug target	Inhibition of protein synthesis
		tlyA	16 S rRNA2'-O-methy ltransferase		
Quinolones(1963)	0.5–2.5	*gyrA*	DNA gyrase subunit A	Drug target	Inhibition of DNA gyrase
		gyrB	DNA gyrase subunit B		
Ethionamide (1956)	2.5–10	*etaA/ethA*	Flavin monooxygenase	Prodrug conversion	Inhibition of mycolic acid synthesis
		inhA		Drug target	
PAS (1946)	1–8	*thyA*	Thymidylate synthase	Drug activation?	Inhibition of folic acid and iron metabolism?

[142a]

to both INH and RIF would be 10^{-14}, which is a highly unlikely event. Therefore, at least in theory, combination of INH and RIF can prevent development of resistance to INH and RIF. However, in reality, because of poor patient compliance to therapy or because bacteria are in biofilm-like or L-form structures, bacteria are exposed to suboptimal concentration of drugs or show phenotypic resistance in vivo, which may provide an advantage for subsequent selection of genetic drug-resistant mutants. Inappropriate adherence to the current lengthy 6-month therapy has led to frequent emergence of drug-resistance including MDR-TB and XDR-TB throughout the world, which poses a major challenge to effective treatment and control.

The increasing drug-resistant TB problem has highlighted the importance of understanding the mechanisms of resistance in *M. tuberculosis*. Mechanisms of resistance to all first-line TB drugs (INH, RIF, PZA, EMB) and also most second-line drugs have been identified (see Table 22.1). It is worth mentioning that inactivation of prodrug-activating enzymes KatG, PncA, and EthA/EtaA, responsible for INH, PZA, and ethionamide resistance, respectively, is a fairly common mechanism of resistance in *M. tuberculosi*s. In addition, target alterations as in mutations in *inhA, rpoB, rpsL* or *rrs, embB*, and *gyrA*, responsible for INH, RIF, streptomycin, ethambutol, and quinolone resistances, respectively, are also frequent mechanisms of drug resistance in *M. tuberculosis* [152]. Due to space limitation and to focus on the topic of drug discovery, detailed mechanisms of drug resistance will not be covered here. For a recent review on this topic, please refer to Zhang and Jacobs [152] and Zhang and Yew [153].

22.4 The Problem of Persisters

Mycobacterial persistence has been demonstrated in the mouse model (Cornell model) [83] and in patients as underlying the lengthy therapy and relapse after treatment. Despite the importance of mycobacterial persistence and the recent interest in this topic [53,148], the mechanisms involved in TB persistence and in fact in overall bacterial persistence are still poorly understood. Since persistence is a general property of virtually all organisms (with varying ability to persist) including tubercle bacilli, it is useful to start with a brief overview of the persistence phenomenon and our current understanding of this important topic.

The phenomenon of bacterial persisters was first described by Gladys Hobby in 1942 when it was observed that penicillin kills only 99% of the bacterial population while 1% (still a large number!) are not killed [57]. In 1944, Joseph Bigger, who is more commonly credited with the discovery of persisters, confirmed Hobby's observation and coined the term "persisters" to describe the 1% of bacteria not killed by penicillin [10]. The small numbers of persister bacteria not killed by the antibiotic are still susceptible to the same antibiotic when they start growing again in fresh medium. The insusceptibility to antibiotics in persisters is phenotypic and distinct from stable genetic resistance. The persister bacteria are due to preexisting metabolically quiescent bacteria that are not susceptible to antibiotics [5]. The persister

phenomenon is presumably a protective strategy bacteria employ to survive under adverse conditions such as starvation, stress, and antibiotic exposure. The persister bacteria present in biofilms [75,126], and also L-forms during natural infection in the host with or without antibiotic treatment [84], pose a formidable challenge for effective control of a diverse range of bacterial infections such as tuberculosis and biofilm infections including catheter infections, heart valve infections, pseudomonas infections in cystic fibrosis, etc. [75,84,149].

Despite the discovery of the persister phenomenon over 60 years ago [10], the mechanisms by which bacteria form persisters have been elusive as the persisters represent a small fraction of bacterial population and constantly changing. The first molecular study of bacterial persistence was carried out by Moyed and colleagues in 1983 when a gene in *Escherichia coli* called *hipA* was identified whose mutation caused about 100–1000-fold increase in penicillin-tolerant persister bacteria [90]. *hipA* forms an operon with *hipB* as a toxin-antitoxin (TA) module where HipA as a toxin is tightly regulated by repressor HipB, which forms a complex with HipA [11]. Based on the microarray analysis of *E. coli* persisters not killed by ampicillin [66], Lewis and colleagues extended the TA module–based persister model where persister formation is dependent on various TA modules such as HipBA and RelBE, which could inhibit peptidoglycan, RNA and DNA synthesis, and protein synthesis [11,100], leading to persistence and multidrug tolerance [66]. Overexpression of toxins such as HipA [41,68,136], RelE [66], and MazF [68,136] could increase persister formation. Yet, deletion of *hipA* has no effect on persister formation [76]. More recently, HipA has been shown to have serine kinase activity [29] and interact with the translation elongation factor EF-Tu (which is probably the most abundant protein) in *E. coli,* which was suggested to inhibit protein synthesis and thus cause persister formation [117]. However, this proposition remains to be confirmed. Given that HipA is involved in persister formation, how the environmental cues are transmitted to HipA which then causes persister formation is unclear. Given the significance of HipAB in bacterial persistence in Gram-negative bacteria that have HipA homologs [41,69], it may not explain the universal persister phenomenon in Gram-positive bacteria that do not have HipA homologs or in other bacteria that do not have TA modules. Overexpression of unrelated toxic proteins such as heat shock protein DnaJ and protein PmrC also caused higher persister formation [136]. These findings raise the question whether TA modules serve as a specific and universal mechanism for persister formation and suggest other mechanisms of persistence may exist. Although there are multiple TA modules in *M. tuberculosis* [98], their role in mycobacterial persistence is unclear.

DNA repair may play a role in persister formation or survival [32]. An *E. coli* LexA mutant is more susceptible to different antibiotics [32]. It has been suggested that persisters do not grow because of ongoing DNA repair in such cells. Using an overexpression approach, Lewis and colleagues identified *glpD* (glycerol-3-phosphate dehydrogenase) and *plsB* (glycerol-3-phosphate acetyltransferase) [125]. However, the effect of overexpression of *glpD* or *plsB* on persister formation is not very high, i.e., 100–1000-fold [125]. Yet, how expression of *glpD* or *plsB* causes increased persister formation is not clear. Overexpression of RelA (ppGpp synthetase),

responsible for stringent response involved in stationary phase survival, also caused increased persisters in *E. coli* [69]. In *M. tuberculosis,* inactivation of RelA led to a defect in persistence in the mouse model [31,102]. Isocitrate lyase (ICL) required for fatty acid catabolism and virulence in *M. tuberculosis* was found to be involved in mycobacterial persistence [85].

Using a transposon mutagenesis approach to identify mutants that show defects in persister formation, we recently identified a new persister gene *phoU* in *E. coli* whose inactivation leads to a generalized higher susceptibility to a diverse range of antibiotics and stresses, especially in the stationary phase, over that seen in the parent strain [76]. The PhoU mutant phenotype could be complemented by a functional *phoU* gene. Mutation in PhoU leads to a metabolically hyperactive status of the cell, as shown by increased expression of energy production genes, flagella and chemotaxis genes and a defect in persister formation. These findings suggest that PhoU is a global negative regulator beyond its role in phosphate metabolism and facilitates persister formation by suppression of many important cellular metabolic processes. PhoU is not expressed in log phase cultures but is expressed in stationary phase [76] and in biofilms [38], conditions that are associated with persister formation. Conditional PhoU expression can cause increased persister formation in *E. coli* (W. Shi and Y. Zhang, unpublished observation). These results suggest that PhoU expression is correlated with persister formation. A new model of persister formation was proposed based on PhoU as a persister switch that senses external changes to modulate cellular metabolism and facilitate persister formation [76]. In *M. tuberculosis*, which is notorious for its persistence, there are two PhoU homologs, PhoY1 and PhoY2. We have shown that PhoY2 is the equivalent of *E. coli* PhoU as its mutation caused increased susceptibility to TB drugs, a defect in persister formation, and defective persistence in mice [122a]. We have also found that PhoU has kinase and phosphatase activity modulated by environmental cues such as iron and phosphate which then alter persister numbers [122a]. Work is ongoing to identify inhibitors of PhoY2. Since PhoU is a ubiquitous protein that is present in both Gram-negative and Gram-positive bacteria, PhoU could be an attractive persister drug target for development of broad-spectrum antibiotics for persisters.

Unlike planktonic persisters in liquid culture, L-form bacteria, like biofilms, represent another form of persisters that live in a community of organisms. L-forms have been shown to occur among many species of bacteria and are suspected to be involved in chronic and persistent infections. Since their discovery in 1935 [67], numerous studies characterizing their morphology, growth, and pathogenic potential have been conducted [35,37]. However, the molecular mechanisms underlying the formation and survival of L-forms remain unknown. Using an unstable L-form *E. coli* as a model, we performed microarray analysis and screened a deletion mutant library to identify the molecular mechanisms involved in the formation and survival of *E. coli* L-forms. Microarray analysis revealed many up-regulated genes of unknown function as well as multiple overexpressed stress pathways in L-forms that are common in persister cells and biofilms. Mutant screens identified mutants with varying degrees of defect in L-form colony formation. The identified mutations map to pathways involved in cell envelope stress, DNA repair, iron homeostasis,

membrane biogenesis, and drug efflux/ABC transporters. Complementation of selected mutants allowed restoration of L-form growth to the mutants [144a]. These results provide new insights into the molecular mechanisms underlying L-form formation and survival, help to improve our understanding of bacterial persistence, and have implications for developing new drugs that target this form of persistence.

The relevance of the L-form research for TB is that in clinical situation, L-forms exist and are suspected to be underlying persistent infections and relapse after therapy [84]. In addition, the emergence of genetically drug-resistant TB bacteria in vivo may occur not only through selection of preexisting mutants but also through initially phenotypic resistance in L-form bacteria which subsequently acquire more stable genetic drug resistance. It is disquieting to note that some strains of *M. tuberculosis* can develop both genetic and phenotypic drug resistance and that some TB drugs like RIF can stimulate or enhance the growth of tubercle bacilli in a peculiar form of RIF-dependent/enhanced MDR-TB. We have recently shown that such RIF-dependent/enhanced bacteria occur in the clinical setting and treatment regimens containing RIF or other rifamycin like rifapentine can paradoxically aggravate the disease as a result of RIF-dependent/enhanced tubercle bacilli [86]. Future studies are needed to determine how such RIF-dependent/enhanced MDR-TB strains occur and how to develop new drugs that target the L-form bacteria for improved treatment.

22.5 New Drug Development

22.5.1 Challenges of New TB Drug Development

The goals of new TB drug development are new drugs should treat MDR-TB, shorten the therapy, shorten treatment for LTBI, and be given safely with antiretroviral therapy [46]. Development of new TB drugs has unique and additional challenges besides the usual problems and risks of any new drug development. There is currently significant interest in the TB field toward developing new drugs that target persister bacilli in the hope of shortening the lengthy TB therapy. However, a major obstacle facing the development of such drugs is that there is currently no reliable in vitro model that predicts in vivo sterilizing activity [149]. This makes the development of new drugs that shorten the TB therapy quite difficult and unpredictable. Moreover, it can take a long time to evaluate TB drug candidates due to the slow growth of the organisms, lengthy animal testing, and the time-consuming clinical trials needed to assess the drugs that shorten therapy. Furthermore, the activity of a new drug candidate cannot be evaluated as a single agent for the treatment of TB in patients but instead can mainly be evaluated in a relatively short 1–2 week early bactericidal activity (EBA) study in patients, which only measures bactericidal activity for killing growing bacilli but not sterilizing activity against persisters. The sterilizing activity of a new agent is usually measured in combination with other TB drugs. Identifying the best drug combinations that shorten therapy also presents a challenge, as there may be limitations to just adding the new agent to the

current standard regimen. One may have to try novel drug combinations not restricted to current regimens to achieve a more efficacious therapy. Adequate funding for TB drug development is critical. In the USA, the NIH sponsored TB drug discovery program [52], Global Alliance for TB Drug Development [50], and the Bill and Melinda Gates Foundation, Lilly-NIAID-Infectious Disease Research Institute partnership are currently providing the much needed support for TB drug development.

22.5.2 New Drug Targets and Novel Approaches to New TB Drug Discovery

22.5.2.1 Novel Drug Targets

Desirable targets should be absent from the human host but involved in vital aspects of bacterial growth, metabolism, and viability, and their inactivation should lead to death of the bacteria or its inability to persist. Currently used TB drugs inhibit particular targets in DNA and RNA synthesis, cell wall synthesis, and energy metabolism pathways (Table 22.1). Clearly, enzymes in the above pathways that are not inhibited by the current TB drugs could also be good targets. The following is a list of novel drug targets for which new drugs may be developed.

Essential Genes

Transposon mutagenesis and signature-tagged mutagenesis have been used to identify essential genes for *M. tuberculosis* important for growth in vitro [114] and survival in vivo [113]. The genes that are essential for survival under in vitro and in vivo conditions are grouped into the following categories[113,114]: lipid metabolism, carbohydrate and amino acid transport and metabolism, inorganic ion transport and metabolism, nucleotide transport and metabolism, energy production and conversion, secretion, cell envelope biogenesis, cell division, DNA replication, recombination and repair, transcription and translation, posttranslational modification, chaperones, coenzyme metabolism, and signal transduction. The functions of a significant number of essential genes are unknown. The mycobacterial essential genes should be good targets for TB drug development. However, a limitation of the essential gene approach is that the "essentiality" is only relative and is highly dependent on the conditions of selection. For example, in vitro essential NAD biosynthetic genes are not essential in vivo [13]. It is likely the in vivo essential genes may be more relevant targets for drug development. In addition, the current genetic approach examines only a single gene a time and identifies only dominant genes involved in viability and the combination effect of multiple genes is missed.

Persister Targets

Since the presence of persister bacteria is considered to be the major reason for lengthy TB therapy, a great deal of research activity is focused on understanding the biology of persistence in the tubercle bacillus and also developing new drugs that target the persister bacteria [149]. Gene products involved in mycobacterial persistence such as isocitrate lyase (ICL) [85], RelA (ppGpp synthase) [102], and DosR (controlling a 48-gene regulon involved in mycobacterial survival under hypoxic conditions) [139], as well as other genes identified by microarray [9] and confirmed by mutant analysis in persister models, the PhoU homolog PhoY2 [122a] and those involved in L-form formation or survival, could be good targets for development of drugs that target persister bacilli.

Energy Production Pathways

Bacteria require energy to remain viable. While the energy production pathways in *M. tuberculosis* are not well characterized, their importance as drug targets was first demonstrated in 2003 when PZA, a frontline TB drug that is more active against nongrowing persisters than growing bacilli and shortens the TB therapy, was found to act by disrupting membrane potential (part of the proton motive force) and that PZA activity is enhanced greatly by energy inhibitor DCCD, an F1F0 ATPase inhibitor [155]. This study implies the energy production or maintenance is important for the viability of persister tubercle bacilli. The subsequent discovery of the highly effective TB drug diarylquinoline, which acts on the same target F1F0 ATPase as DCCD, enhances PZA activity just like DCCD, and shortens TB therapy in mice [3], further confirms the importance of energy production pathways for persistent mycobacteria. In addition, clofazimine, an agent used to treat leprosy and other non-tuberculous mycobacterial infections, has recently been shown to inhibit Ndh-2, an NADH dehydrogenase in electron transport chain and energy production [140]. The critical role of proton motive force for viability of non-replicating persister bacilli has also been demonstrated [104]. Thus, the energy production pathways could represent good targets for TB drug development.

NAD Metabolism

NAD biosynthesis is an attractive drug target for growing and also nongrowing *M. tuberculosis* [13]. Boshoff et al. showed inhibition of both NAD biosynthetic and salvage pathways caused cell death in *M. tuberculosis* [13]. Of particular interest are NAD synthetase inhibitors that showed good activity for non-replicating persisters in the Wayne hypoxic model [13], indicating that NAD metabolic pathways can be good targets for design of new TB drugs.

Virulence Factors

A recent study identified rhodanines as inhibitors of *M. tuberculosis* dihydrolipoamide acyltransferase (DlaT), an enzyme required to cause TB in guinea pigs and used by the bacterium to resist host-derived reactive nitrogen intermediates, and as having high bactericidal activity for non-replicating mycobacteria in synergy with host nitric oxide and hypoxia [15]. PhoP, a transcription factor that controls the expression of toxic cell wall lipids, is a well-known virulence factor in *M. tuberculosis* [101] and could be a good drug target for design of new TB drugs. In addition, mycobacterial two-component systems, sigma factors, and other virulence factors have also been proposed as targets for TB drug development [149] and will not be detailed here.

Toxin-Antitoxin (TA) Modules

Studies primarily in *E. coli* have identified toxin and antitoxin modules (TA modules) that are involved in cell death and persistence [49]. Inappropriate or uncontrolled expression of the toxin component or a decrease in the expression of antitoxin can cause bacterial cell death. In *E. coli*, antibiotics such as chloramphenicol, spectinomycin, rifampin, and sulfa drugs that inhibit transcription, translation, and cause thymine starvation, respectively, kill bacteria by inducing the toxin MazF [115], which in turn inhibits translation leading to cell growth arrest and programmed cell death by cleaving mRNA [146]. *M. tuberculosis* genome contains at least 40 TA modules including 2 *parDE*, 3 *relBE*, 9 *mazEF*, 33 *vapBC* loci [49]. Recently, it was shown that overexpression of the 3 *M. tuberculosis rel* toxin genes *relE, relG*, and *relK* induced growth arrest in *M. smegmatis* [70]. Upon further confirmation, the TA modules might represent attractive targets for drug development in *M. tuberculosis*.

22.5.2.2 Novel Approaches in Drug Discovery

Current antituberculosis drugs primarily target cellular processes involved in bacterial growth and are either bacteriostatic or bactericidal. These include cell wall synthesis inhibitors, nucleic acid synthesis inhibitors, protein synthesis inhibitors, and energy inhibitors [149]. With the exception of RIF and PZA, these drugs are mainly active against replicating bacilli but are considerably less potent against persister bacteria [149]. Persistent bacterial populations must be taken into consideration when developing new TB drugs. It is increasingly appreciated in the field of TB drug development that new drugs should not only be active against drug-resistant TB but also kill persisters and shorten the lengthy TB treatment, which underlies the problem of drug resistance due to poor compliance to the lengthy therapy.

Activity against nongrowing persister bacilli is correlated with good sterilizing activity and ability to shorten therapy in vivo as exemplified with PZA and RIF. Novel drug screens that mimic the in vivo conditions in lesions such as acidic pH,

hypoxia, and against old stationary phase nongrowing bacilli may be important to identify drugs that kill persisters and shorten the treatment. Some models utilizing these conditions have recently been developed and are being used for drug screens to identify persister active agents, such as 100-day-old RIF-tolerant bacilli model [58], the low oxygen recovery assay (LORA) [25], etc. The dithiocarbamates DETC and PDTC were found to have activity against nongrowing tubercle bacilli in a persister model under acid pH and hypoxic conditions [20]. It is worth noting that there are different persister populations, and different persister models only look at certain population of persisters and thus have limitations. Besides the persisters in planktonic cultures, there are also persisters in the form of biofilms and L-form bacteria that need to be taken into account in developing new persister active drugs.

A recent study has shown that inhibitors of the repressor EthR for the drug-activating enzyme EthA could enhance the activity of ethionamide by tenfold in vitro and in the mouse model [143]. This study represents a new approach to drug discovery based on identification of inhibitors of a repressor of drug-activating enzymes to increase the activity of existing prodrugs. It will be of interest to determine if this approach can be applied to other prodrugs like INH and PZA, which are activated by KatG and PncA, respectively.

There is recent interest in the systems biology approach for drug discovery [128]. Instead of the conventional reductionist approach of finding a single drug that hits a single target, the systems biology approach proposes to inhibit multiple targets in different pathways of the cell to achieve better killing. Combinations of compounds may produce unexpected results that cannot be achieved by compounds used singly. Thus, drug combination screens may be performed to identify drugs that have a synergistic effect. Systems biology approaches can be used for identifying novel drugs or drug combinations for improved TB treatment.

22.5.3 Promising Development with Existing Drugs

The more active rifamycin rifapentine (RPT) offers some promise to shorten the TB treatment. Once-weekly RPT and moxifloxacin was far better than the twice-weekly INH+RIF regimen, but there was no difference between once weekly INH+RPT compared with twice weekly INH+RIF [106]. Twice weekly RPT plus INH or RPT plus moxifloxacin was even more effective and could shorten the therapy to 4 months in the mouse model [107]. In a recent mouse study, it was found that replacing RIF with rifapentine and INH with moxifloxacin in conjunction with PZA produced a better killing activity than the standard INH+RIF+PZA regimen [108]. After 2 months of treatment, mice receiving rifapentine+moxifloxacin+PZA had negative lung cultures, while those given the standard INH+RIF+PZA regimen still had 3.17 log10 colony-forming units in the lungs. Importantly, no relapse was observed after 3 months of treatment with daily and thrice-weekly rifapentine+moxifloxacin+PZA, whereas the standard INH+RIF+PZA daily regimen required 6 months

to prevent relapse in all mice [108]. In a subsequent study to assess the relative importance of INH and moxifloxacin in combination with rifapentine + PZA in the mouse model, it was found that moxifloxacin + rifapentine + PZA was superior to INH + rifapentine + PZA in the 4-week and 8-week treatment experiments but required a 10-week treatment to achieve a stable cure for the moxifloxacin + rifapentine + PZA regimen [105]. Further clinical studies are needed to determine whether the rifapentine + moxifloxacin + PZA regimen can shorten the TB treatment in humans.

PZA is an important sterilizing drug that shortens TB therapy. It is interesting to note that the clinically used weak acids aspirin and ibuprofen could enhance PZA activity in the mouse model [18]. It will be of interest to further evaluate if aspirin or ibuprofen could improve PZA activity in humans and shorten the therapy even further in combination with other TB drugs.

β-Lactam antibiotics are generally not active against *M. tuberculosis*, due to β-lactamase enzyme present in the bacilli. However, when β-lactam antibiotics are used together with the beta-lactamase inhibitor clavulanate, they show good antituberculosis activity in vitro [24], and in mice and humans [23]. More recently, the carbapenem antibiotic meropenem, when combined with clavulanate, had potent anti-TB activity with MIC < 1 μg/ml [60]. In addition, meropenem plus clavulanate also had good activity against persister bacilli under anaerobic conditions and also was active for XDR-TB [60]. Further clinical studies are needed to evaluate active β-lactam antibiotics plus clavulanate for treatment of MDR/XDR-TB in combination with other drugs.

22.5.4 Promising New Drug Candidates

The increasing emergence of drug-resistant TB has highlighted the need for new and more effective TB drugs. There is renewed interest in developing new TB drugs that are not only active against drug-resistant TB but more importantly also shorten TB therapy ([40]; 2001; [96,149]). Various drug candidates and active compounds have been identified. For a more detailed review, please refer to ([40]; 2001; [96,149]). Here only the most promising drug candidates that are in clinical trials are briefly discussed below (see Table 22.2). The structures of these TB drug candidates are shown in Fig. 22.2.

New fluoroquinolones. Moxifloxacin (MXF) and gatifloxacin (GFX) are more active against *M. tuberculosis* than ofloxacin and ciprofloxacin [1,63]. MXF was active against *M. tuberculosis* comparable to INH in a mouse model [88]. Early bactericidal activity (EBA) of the new quinolones in TB patients demonstrated that while day 0–2 EBA of levofloxacin, moxifloxacin, and GFX was not as high as INH, day 2–7 EBA for the quinolones was higher than INH [65]. MXF was well tolerated and combination therapy with MXF seems to be as effective as current standard drug combination [135]. To determine if MXF decreases the infectious period and shortens therapy, a preliminary study was conducted and showed that

Table 22.2 New drug candidates in clinical trials

Drugs	MIC (μg/ml)	Mechanism of action	Development stage	Developer
Rifapentine	0.03–0.06	Inhibition of RNA synthesis	Phase 4	Sanofi-Aventis
Moxifloxacin	0.03–0.5	Inhibition of DNA synthesis	Phase 2, 3	Bayer, CDC, TB Alliance, UCL, Johns Hopkins
Gatifloxacin	0.03–0.5	Inhibition of DNA synthesis	Phase 3	Bristol-Myers Squibb, WHO, European Commission, OFLOTUB Consortium
Oxazolidinone PNU100480	0.125–1	Inhibition of protein synthesis	Phase 1	Pfizer
Diarylquinoline (TMC207)	0.03	Inhibition of ATP synthesis	Phase 2	Tibotec (Johnson & Johnson)
Nitroimidazolepyran (PA-824)	0.015–0.25	Inhibition of cell wall synthesis	Phase 1, 2	TB Alliance
Nitro-dihydro-imidazo-oxazole (OPC-67683)	0.006–0.024	Inhibition of cell wall synthesis	Phase 1, 2	Otsuka Pharmaceuticals
Diamine analog (SQ-109)	0.16–0.64	Inhibition of cell wall synthesis	Phase 1	Sequella
Pyrrole LL-3858	0.12	Inhibition of cell wall synthesis??	Phase 1	Lupin

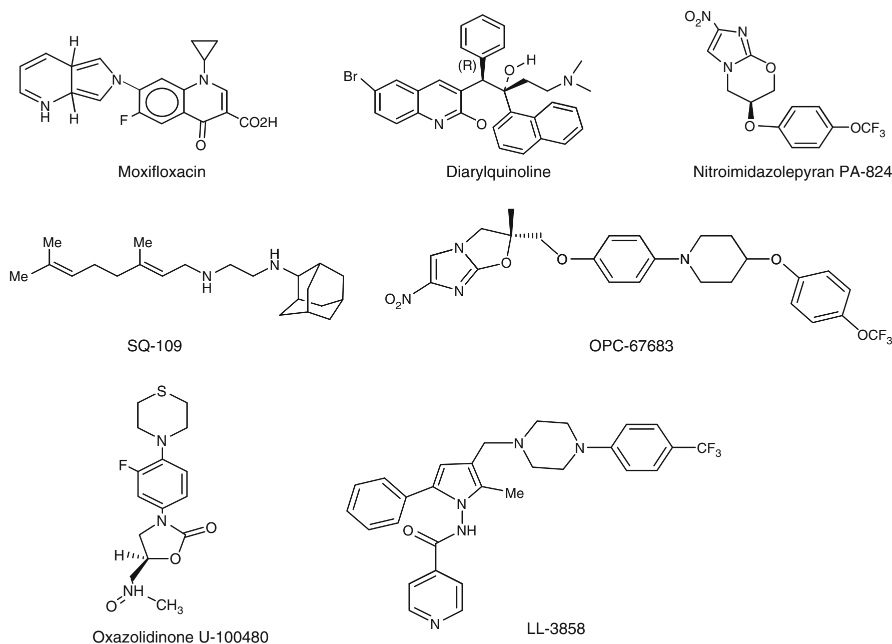

Fig. 22.2 Structures of promising TB drug candidates in clinical trials

replacement of ethambutol by MXF in the 2-month intensive phase of treatment did not affect the sputum conversion rate [17]. Nevertheless, a subsequent study demonstrated that replacing ethambutol by MXF in the 2-month intensive phase of treatment led to a statistically significant culture negativity in Brazilian patients [27]. In a separate study, replacing ethambutol by MXF or GFX but not ofloxacin in the 2-month intensive treatment showed improved sputum culture negativity [110]. A potentially exciting finding is that MXF in combination with RIF and PZA was more active than the INH + RIF + PZA regimen in the mouse model [93]. The higher activity of MXF + RIF + PZA than INH + RIF + PZA raises the hope that MXF may replace INH to shorten the TB therapy in humans. However, in a more recent human study, replacing INH with MXF in the 2-month intensive phase of treatment showed a small but insignificant increase in culture negativity [39]. More clinical trials of MXF and GFX in combination with existing drugs are ongoing with the aim to shorten the TB therapy.

Diarylquinoline. Diarylquinoline (TMC207) is a highly active, promising TB drug candidate that can shorten the therapy in the mouse model [3]. Diarylquinoline R207910 (J compound) preferentially inhibits the mycobacterial F1F0 proton ATP synthase [3] over mammalian homologs [54], and represents a new drug target for mycobacteria. The J compound is active against both growing and nongrowing persister bacterial populations [71], and is also active against MDR-TB strains in vitro and in mice [78]. The J compound was more active than INH and RIF and could shorten TB therapy from 4 months to 2 months in mice [3]. When used alone, the J

compound was more active than the standard RIF + INH + PZA regimen given for 2 months in the mouse model [78]. Of particular interest is the synergy between diarylquinoline and PZA and this is the most effective drug combination in sterilizing the infected organs [3]. This finding is consistent with the previous observation that *N,N*'-dicyclohexyl carbodiimide (DCCD), which inhibits the same F1F0 ATPase as diarylquinoline, synergized with PZA against *M. tuberculosis* [155]. A recent study has confirmed the superior antituberculosis activity of the combination of diarylquinoline and PZA, which is unmatched by any other drug combination in the mouse model of TB infection [62]. J compound in combination with second-line drugs was more active than the currently recommended regimen for MDR-TB AMK-ETH-MXF-PZA, with culture negativity for both the lungs and spleen after 2 months of treatment [78]. In a recent study aimed at evaluating the sterilizing activity of diarylquinoline, it was found that a 4-month treatment with diarylquinoline in combination with INH + RIF + PZA was equivalent to a 6-month INH + RIF + PZA in terms of culture negativity and relapse rate, and was more effective than a 4-month treatment with moxifloxacin + RIF + PZA in the mouse model [61]. The J compound had excellent early and late bactericidal activity in the mouse model, good pharmacokinetic and pharmacodynamic profiles, [111] with long half-life, and absence of significant toxicity in mice and in preliminary human safety testing, raising the hope that diarylquinoline may shorten the TB treatment in humans. In a 7-day early bactericidal activity (EBA) study in humans, the J compound at 400 mg per day dose was found to have a delayed onset of bactericidal activity starting at day 4 onward but its killing activity was not as active as INH (300 mg) and RIF (600 mg) included as positive controls at day 7 [110]. It was also found in this study that the serum concentration of the J compound showed concentration-dependent increases from a dose of 25–400 mg and that it was safe and well tolerated in the 7-day EBA study [110]. Due to its long half-life, the J compound has recently been shown to have higher activity than another long half-life TB drug rifapentine in a once-weekly regimen of murine TB in an 8-week treatment experiment [137]. Moreover, the J compound + rifapentine + pyrazinamide regimen given once weekly was more active than the current standard regimen of INH + RIF + PZA given five times a week [137], raising hope that it may be possible to develop an intermittent once-weekly regimen for TB treatment. Phase 2 clinical testing of the J compound for treatment of MDR-TB indicated that adding J compound (TMC207) to the standard five-drug therapy reduced the time of conversion of negative sputum culture and increased the proportion of patients with conversion of sputum culture by 48% compared with 9% in the standard control regimen [34]. Side effects were mild to moderate with nausea being a significant adverse event [34]. However, in this TMC207 Phase 2 trial for MDR-TB, the role of PZA resistance in affecting the trial outcome was not addressed. This is important since PZA plays an important role in the treatment regimen with TMC207 and comparable distribution of PZA-resistant strains in the TMC207 containing group and the control group is necessary to ensure a reliable trial result.

Nitroimidazopyran (PA-824) and OPC-67683. Nitroimidazopyran PA-824 is highly active against both growing and nongrowing *M. tuberculosis*. PA-824 was

initially thought to inhibit cell wall lipid biosynthesis [127]. More recently, PA-824 was shown to be a prodrug that is activated by a deazaflavin-dependent nitroreductase (Ddn)(Rv3547) to form three primary metabolites, with the major one being the des-nitroimidazole (des-nitro) [122]. The des-nitro compound–generated reactive nitrogen species, including nitric oxide (NO), is responsible for the anaerobic activity of these compounds, and may synergize the mycobacterial killing with the host-derived NO produced by macrophages. Interestingly, NO scavengers protected the bacilli from the lethal effects of the drug [122]. PA-824 was also active against MDR-TB strains, suggesting that it inhibits a new target in tubercle bacilli. PA-824 had considerable bactericidal activity against RIF-tolerant persisters in a 100-day-old culture at 10 μg/ml or higher, an activity greater than MXF, but had little activity at 1.25 μg/ml [59]. It was speculated that since PA-824 is 94% plasma bound, PA-824 may not reach sufficiently high concentrations to show bactericidal activity in cavitary lung lesions [59]. However, a more recent Phase I study suggested PA-824 was well tolerated following oral doses once daily for up to 7 days with serum concentrations of 3 μg/ml (1500 mg dose) in the single-dose study and 3.8 μg/ml (600 mg dose) in the multiple-dose study and an elimination half-life of 16–20 h [51]. PA-824 was as active as INH in animal models of TB infection [127]. In the mouse model, PA-824's minimal effective dose was 12.5 mg/kg/day and minimal bactericidal dose was 100 mg/kg/day [133]. PA-824 had bactericidal activity in the initial and continuation phase of treatment in mice, confirming its activity against both growing and nonreplicating bacilli [133]. Although PA-824 in combination with RIF and PZA led to higher sterilizing activity compared with the standard INH+RIF+PZA during the first 2 months of treatment in mice, PA-824 did not do any better than the standard INH+RIF+PZA to shorten the 6-month therapy [94]. In a more recent study, it was found that addition of PA-824 at 12.5 and 25 mg/kg/day did not increase the activity of RIF+PZA, but the addition of PA-824 at 50 and 100 mg/kg/day increased the activity in a dose-dependent manner. Mice treated with PA-824 (100 mg/kg)+RIF+PZA became culture negative after 2 months and free of relapse after 4 months of treatment. In contrast, some mice receiving the standard regimen of INH+RIF+PZA remained culture positive and 15% relapsed after completing 4 months of treatment [130]. In particular, the combination of PA-824 and PZA had synergistic activity equivalent to that of the standard first-line regimen [130]. In further experiments, it was shown that PA-824+MXF+PZA cured mice more quickly than the standard RIF + INH + PZA; however, substitution of PA-824 for MXF or PZA was detrimental but could replace RIF in the RIF + MXF + PZA regimen. However, PA-824 when added to the RIF + MXF + PZA regimen did not demonstrate any shortening of the treatment compared with RIF + MXF + PZA [92]. These findings support the evaluation of regimens based on the combination of PA-824+RIF+PZA or PA-824+MXF+PZA in phase II clinical trials. However, it remains to be seen if the dosage of 100 mg/kg PA-824 in the mouse study can be tolerated in humans.

A related nitroimidazo compound OPC-67683 is being developed by Otsuka [82] (Table 22.2). OPC-67683 inhibits mycolic acid synthesis and has a minimal effective dose of 0.625 mg/kg, which appears to be 20 times more active than PA-824.

OPC-67683 has activity for non-replicating persister bacilli in a drug exposure assay [112]. OPC-67683 in combination with RIF and PZA seemed to be more active than INH+RIF+PZA+EMB in a 6-month treatment in mice [82]. OPC-67683 is currently being evaluated in Phase I and Phase II EBA study.

Oxazolidinones. Oxazolidinones, discovered originally at Dupont in the 1970s and later developed at Pharmacia Upjohn, represent a new class of compounds that are active against a variety of Gram-positive bacteria. The oxazolidinone linezolid (Zyvox) is approved by FDA to treat singly or multiply resistant Gram-positive bacterial infections. Linezolid inhibits protein synthesis by binding to 23 S rRNA of the 50 S ribosomal subunit. Oxazolidinones had significant activity against *M. tuberculosis* with an MIC of 2–4 μg/ml and were also active against tubercle bacilli in mice [4,30]. A derivative of linezolid PNU-100480 showed significant better antimycobacterial activity than linezolid [6,30]. In a recent mouse study, PNU-100480 (100 mg/kg/day) when added to the standard daily regimen of INH+RIF+PZA resulted in an additional 2 log reduction in lung CFU counts during the first 2 months of treatment [144]. Furthermore, PNU-100480+moxifloxacin+PZA was more active than INH+RIF+PZA [144]. It will be of interest to determine if PNU-100480 in combination with moxifloxacin and PZA can shorten the TB treatment. The promising anti-TB activity of PNU-100480 has led Pfizer to plan a Phase I clinical trial for humans. In recent clinical studies, most MDR-TB or XDR-TB patients were successfully treated with linezolid in combination with other drugs [28,99,138]. Long-term therapy (up to 28 months) was well tolerated in most patients in one study [28]. But significant toxicity including anemia, neutropenia and peripheral neuropathy could occur [28,99,138]. While oxazolidinones have promising potential for the treatment of MDR-TB and XDR-TB, more extensive clinical studies are needed to evaluate the optimal dose, toxicity, and the degree of mycobacterial resistance development.

SQ109. SQ109 (N-geranyl-N‘-(2-adamantyl)ethane-1,2-diamine) is a new diamine antituberculous compound that was derived from high-throughput screening of EMB analogs [73]. SQ109 has good activity against *M. tuberculosis* with MIC of 0.5 μg/ml compared with its parent EMB with MIC of 5 μg/ml. SQ109 was also more active against *M. tuberculosis* than EMB and had comparable activity with INH in a macrophage model [64]. However, in mice the activity of SQ109 is not as high as INH but was about ten times more active than EMB [64]. In the mouse model of TB infection [64], SQ109 caused significant reduction of bacterial burden in the lung and spleen with favorable PK/PD properties. SQ109 in combination with INH, RIF, and PZA was more active than the EMB-containing regimen [91]. SQ109 inhibits cell wall synthesis and is active against both drug susceptible, EMB-resistant and MDR-TB strains. SQ109 is currently in Phase 1 clinical trial.

Pyrrole LL-3858. Pyrrole derivatives are known to have antimicrobial (fungal and bacterial) activity [22]. Pyrrole activity for *M. tuberculosis* was recently recognized [33]. LL-3858 (Sudoterb) is a pyrrole derivative that is under development by Lupin Ltd, in India [47]. LL-3858, an INH analog (Fig. 22.2), has anti-TB activity in vitro

with an MIC of 0.12 μg/ml [47]. In the mouse model, a 12-week treatment with 12.5 mg/kg LL-3858 showed good efficacy and clearance from lung and spleen. No relapse was observed up to 2 months following treatment. PK in mice was reported to be better than with INH in terms of half-life, Cmax, and AUC [47]. LL-3858 is currently being evaluated as a TB drug candidate in Phase 1 study.

Other drug candidates. Drugs used for treatment of other diseases, such as antifungal azoles [129, 19], phenothiazines (chlorpromazine and thioridazine) [2], and riminophenazine derivatives such as clofazimine [79], have good antituberculosis activity and could be candidates for further evaluation for treatment of TB. Rifalazil (RLZ) (KRM1648), a long half-life rifamycin, is highly active against a range of intracellular bacteria including *M. tuberculosis*, *M*ycobacterium *avium*, *Chlamydia trachomatis*, *Chlamydia pneumoniae*, and *H. pylori* [109]. RLZ is more active than RIF and rifabutin against *M. tuberculosis* both in vitro and in mice [121]. A preliminary safety study in humans showed that although once weekly RLZ at 10 and 25 mg was safe, RLZ at a dose of >100 mg produced flu-like symptoms and a transient dose-dependent decrease in white blood cell and platelet counts and did not show any better efficacy than RIF [36]. Although RLZ was evaluated in Phase II trial for treatment of infections caused by *Chlamydia*, *Clostridium difficile*, and *H. pylori*, no further information is available on the clinical trial of RLZ for treatment of mycobacterial infections.

22.6 Perspectives

Increasing emergence of MDR/XDR-TB along with the HIV pandemic present major challenges for effective control of TB. The currently used TB drugs were developed over 40 years ago, and there is an urgent need for new TB drugs to respond to increasing drug resistance. Current efforts involve whole cell screens to identify new drugs, drug target identification, structure-based drug design, as well as testing of new drugs or derivatives of existing drugs, evaluation of novel drug combinations with new drugs and existing drugs, development of novel slow-release drug delivery systems, with aim to find better and more effective drugs and therapies that shorten treatment and treat MDR/XDR-TB.

Although recent advances in mycobacterial genomics and molecular biology have made it possible to identify essential drug targets, the limitation of target-based approach is increasingly recognized [103,149]. Inhibitors are identified based on in vitro target-based screens, and there may still be problems with compound penetration and activity against whole organisms. Other issues in drug development include in vivo PK/PD profiles, toxicity, and in vivo activity, and in particular activity against persisters. Besides target-based screens, whole cell–based screens, especially persister screens under conditions that mimic in vivo environments, such as hypoxia, acidic pH, starvation and aging conditions, are being tested as models for

identifying compounds that are active against persisters [20, 25, 59]. However, the relevance of various in vitro persister models is hard to predict and the hits have to be validated in animal models. Despite all these efforts using in vitro persister models to mimic in vivo conditions, another layer of complexity that is not considered is the presence of persister organisms in vivo as in L-forms and in biofilms, which underlie persistent and latent infections and relapse. It is conceivable that drug screens against these more relevant forms may be equally important if not more important than the currently used models based on planktonic bacteria (persister and growing bacteria) for developing drugs that address persistence. Systems approaches like drug combinations or synthetic lethality screens in persister models and elucidation of critical networks in persisters may be used to identify much needed drugs which sterilize persisters in the host.

Several promising drug candidates are currently in clinical trials. There is hope that the current 6-month TB therapy may be shortened to some extent with the addition of new fluoroquinolones MXF or GFX or with rifapentine. There are good prospects that some new TB drugs may enter clinical treatment in the next few years. While new drugs that act on new targets can be useful for treatment of MDR/XDR-TB, not all of them can be expected to shorten the TB therapy, which is more difficult to achieve. Lack of appropriate in vitro persister models and screens and inadequate understanding of persister biology make the identification of new drugs that shorten the therapy less predictable. Meanwhile, creative use of existing drugs, optimal combinations of existing drugs and new drug candidates, and optimal dosing frequency should be evaluated in animal models and in clinical trials.

Although TB is a curable disease and DOTS expansion is important, more effective TB control goes beyond DOTS expansion and BCG vaccination and requires new thinking and novel approaches. We need more rapid diagnostic tools for early detection of disease and drug resistance, more effective vaccines, a targeted preventive treatment of LTBI with high risk to develop active disease, and a much more effective and shorter therapy. At the basic research and development level, improved understanding of the relevant biology of tubercle bacillus and its interaction with the host immune system, especially the problem of persistence mechanisms (and L-forms), are required to provide novel targets for drug development. Besides developing drugs that target the organisms, drugs that modulate or enhance the immune system to prevent reactivation of LTBI or kill persisters are also needed. TB is a complex social disease whose expression is dependent on bacterial, host, and socioeconomic factors. We need to tackle the TB problem from all these levels. Development of new TB drugs that shorten TB therapy and are active for MDR/XDR-TB will greatly facilitate control of TB. However, more effective drugs and therapy cannot be achieved without adequate support from governments, funding agencies, philanthropists, and public–private partnerships.

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Part VII
Antibiotic Discovery

Chapter 23
Resistance Trends and Susceptibility Profiles in the US Among Prevalent Clinical Pathogens: Lessons from Surveillance

Chris Pillar and Dan Sahm

23.1 The Era of Resistance

Since the advent of their discovery in the 1930s, antibiotics have served with great success on the front lines in the war against bacterial infection. A consequence of this success and widespread use was the inevitable evolution and emergence of resistance among bacteria from the resulting selective pressure. In the Golden Age of antibiotics (1940s–1960s), discovery of various new classes of antimicrobial agents outpaced the development of resistance by delivering new agents with different mechanisms of action that were effective against the resistant organisms of the day. In contrast, during the following period, there was a lack of development of novel classes of agents in favor of the chemical and structural modification of agents within established classes. While the development of novel classes or agents with activity against resistant organisms has slowed, the emergence and spread of resistance among bacteria has continued.

The degree to which antimicrobial stewardship and infection control is practiced varies greatly from region to region and has great influence on bacterial resistance. There has been a call for decreased and prudent use of antibiotics, although even in situations where antibiotics have stopped being used for human therapy, subsequent increases in susceptibility have been slow to develop [1]. Furthermore, the increasing amount of over the counter antibiotics and free/low-cost generics used throughout many parts of the developing world and the potential impact on resistance is also a concern.

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The continued use of available antibiotics from a limited number of classes, the decrease in the discovery of new classes, and the lack of development and approval of novel agents have contributed to the current "era of resistance." Infections caused by pathogens once considered easy to treat have become more challenging, and the emergence of multi-drug resistance further complicates antimicrobial therapy. While much attention has been given to the prevalence of drug-resistance among Gram-positive cocci (e.g., MRSA, VRE, MDRSP), the impact of infections caused by resistant Gram-negatives (e.g., *P. aeruginosa*, *Acinetobacter* spp., beta-lactam resistant Enterobacteriaceae) has become increasingly worrisome, due to limited therapeutic options. In such an environment, it is important to understand the current activity and trends in resistance of antibiotics across the available classes among the most frequently encountered pathogens to better select appropriate therapies and to help identify situations where there is an urgent need for new therapeutics.

23.2 Tracking Resistance Through Surveillance

Surveillance initiatives to evaluate the current activity profiles of antibiotics against target pathogens are commonly undertaken by hospitals, governmental agencies, and the pharmaceutical industry. Data from surveillance are most often used to track resistance to currently utilized agents and classes, to evaluate the success of existing infection control procedures and/or determine the need for improved infection control practices, to screen for the utility of developmental compounds, and to monitor for the emergence of resistance to newly developed agents.

Trends in resistance and activity profiles can vary as a result of the type of infection, regional variations in prescribing practice and infection control, the penetration of genetic mechanisms of resistance, and patient population (Fig. 23.1). Data from large scale geographically dispersed surveillance initiatives allow for subpopulation analysis of the impact of these factors on the activity of a given agent, while local surveillance data is useful for the guidance of empiric therapy within a given hospital or region.

Several reports have highlighted the overall trend towards increased antibiotic resistance among frequently encountered Gram-positive and Gram-negative pathogens today [1–7]. Recent trends of concern include the following: the prevalence of MRSA both in hospitals and in the community (CA-MRSA), *S. aureus* with reduced susceptibility to glycopeptides (e.g., VISA/VRSA), multi-drug resistance (MDR) among prevalent gram-positive pathogens (e.g., *S. aureus* and *S. pneumoniae*) and challenging gram-negative pathogens (e.g., *P. aeruginosa* and *Acinetobacter* spp.), and the spread of beta-lactamase resistance among Enterobacteriaceae (e.g., extended spectrum beta-lactamase [ESBL], metallo-beta-lactamase [MBL] and more recently serine carbapenemase [KPC] producers).

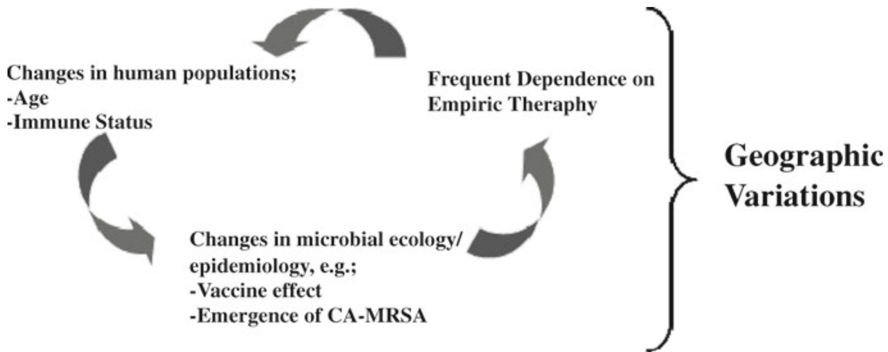


Fig. 23.1 Dynamics which highlight the need for antimicrobial surveillance

23.3 Evaluating Resistance and Antimicrobial Activity in the US

Aspects of drug development are frequently driven by the indications for which treatments are ultimately intended. Indication specific treatment guidelines ultimately take into account both current resistance patterns among target pathogens as well as the potential for resistance associated with certain patient comorbidities (e.g., age, hospitalization status, etc.). As a result, it is important for the drug development industry, both during development and post-approval, to take into consideration what pathogens are relevant to any sought indications, and to understand what important resistance phenotypes are necessary to cover among pathogens associated with a given indication and what impact if any select patient comorbidities may have on outcome.

This chapter will present surveillance data primarily from two large US based surveillance programs: The Surveillance Network (TSN; an electronic based surveillance in service at Eurofins Medinet (formerly Focus BioInova) since 1995, which collects susceptibility profiles and demographics on >1 million isolates per year as reported by clinical laboratories throughout the US) and TRUST 12 2008 surveillance (Tracking Resistance in the US Today; multi-year centralized surveillance sponsored by Ortho McNeil Pharmaceuticals). Surveillance data on beta-hemolytic streptococci and enterococci are in part from TSN and also from a separate 2008 Surveillance in the US sponsored by Targanta Therapeutics (acquired in 2009 by The Medicines Company). The overall intent of the data presented in the sections below is to achieve the following:

1. Provide an understanding of what pathogens are most common today.
2. Illustrate the extent of changes in resistance over time.
3. Understand the current in vitro activity of agents from varied classes and the impact of relevant resistance on activity profile.
4. Evaluate potential variation in resistance among specific subpopulations (e.g., patient age, infection type, geographic origin, etc.).

Data from TSN has previously been utilized by both the Food and Drug Administration and pharmaceutical industry for the evaluation of resistance trends and activity profiles of a variety of agents against target organisms [3, 8–16]. TSN consists of a database of laboratory test results across a variety of methodologies (CLSI methods and commercial systems e.g., VITEK and Microscan) uploaded from >150 participating clinical laboratories across the US. Data from TRUST surveillance has been used to analyze resistance trends and to generate local and regional antibiograms for a range of antimicrobials against common pathogens [14, 17–22]. Data on enterococci and beta-streptococci collected as part of a separate global surveillance program sponsored by Targanta Therapeutics (acquired in 2009 by The Medicines Company) has also been reported [23] and is utilized in part herein. MIC and susceptibility data from both TRUST and Targanta were generated via centralized broth microdilution. In brief, evaluated isolates consisting of non-duplicate, non-consecutive clinical isolates from a variety of specimen sources (skin/wound, urine, blood, lower/upper respiratory tract) were submitted from a large number of sites (>100) distributed across the US Bureau of Census regions to a central laboratory (Eurofins Medinet, Chantilly, VA) for confirmatory identification and susceptibility testing by broth microdilution in accordance with guidelines established by the Clinical Laboratory Standards Institute (CLSI) [24, 25].

23.4 Prevalent Pathogens by Specimen Type

Target pathogens and the prevalence of target pathogens associated with a particular infection vary based on the type of disease. There exist a wide variety of disease indications for which the pharmaceutical industry currently evaluates investigational agents, the most common of which include skin and skin structure infections (SSSI), community-acquired pneumonia (CAP), hospital-acquired pneumonia (HAP) and ventilator-associated pneumonia (VAP), urinary tract infections (UTI), and intra-abdominal infections (IAI). There are a number of available sources from which to identify target pathogens and their prevalence within a given indication including reviews [26–31], established treatment guidelines [32–35], data from targeted surveillance (e.g., NNIS [36]), and data collected as part of large clinical trials [37–41].

Surveillance data when analyzed by specimen source (e.g., urine, blood, etc.) is useful for determining the prevalence of organisms based on the frequency in which they are encountered and tested (with the important caveat that pathogens treated successfully through empiric therapy can be underrepresented in such analysis as they are less frequently cultured or tested). In Table 23.1, data compiled from TSN was used to determine the top ten pathogens tested in clinical labs across the US by

Table 23.1 Epidemiology of infections: top ten pathogens tested clinically across the US by specimen[a]

Blood (n = 130701)		Lower respiratory tract (*n* = 196886)		Skin/wound (*n* = 137832)		Urine (*n* = 1000446)	
Organism	% tested	Organism	% Tested	Organism %	Tested	Organism %	Tested
CoNS	28.8	*S. aureus*	28.8	*S. aureus*	53.6	*E. coli*	54.4
S. aureus	18.0	*P. aeruginosa*	27.7	*Enterococcus* spp.	9.3	*Enterococcus* spp.	11.3
E. coli	13.6	*K. pneumoniae*	5.9	*E. coli*	7.4	*K. pneumoniae*	9.6
Enterococcus spp.	9.1	*E. coli*	4.5	*P. aeruginosa*	5.5	*P. mirabilis*	5.3
S. pneumoniae	3.2	*S. pneumoniae*	4.4	CoNS	5.3	*P. aeruginosa*	4.2
P. aeruginosa	2.5	*S. maltophila*	4.3	*K. pneumoniae*	2.6	CoNS	2.8
VGS	2.1	*Acinetobacter* spp.	3.9	*P. mirabilis*	2.4	*S. aureus*	2.7
E. cloacae	1.9	*S. marcescens*	3.3	*E. cloacae*	1.9	*E. cloacae*	1.6
β-streptococci	1.9	*E. cloacae*	3.1	β-streptococci	1.2	*K. oxytoca*	1.2
P. mirabilis	1.5	*H. influenzae*	1.9	*Acinetobacter* spp.	1.1	*C. freundii*	1.1

CoNS coagulase-negative staphylococci, VGS viridans group streptococci
[a]Data collected from TSN 2007–2008

specimen type as an indication of what type of pathogens are most frequently encountered and tested among indications associated with select specimen types in the US today.

23.5 Resistance Trends and Antibiograms Among Prevalent Pathogens in the US

The sections below focus on each prevalent pathogen individually highlighting the following: noteworthy trends in resistance, longitudinal analysis of resistance to commonly tested and utilized antimicrobials over the past 10 years, the activity profile of these agents currently both overall and against resistant populations, and the prevalence of important resistance phenotypes among select subpopulations (e.g., elderly, blood isolates, ICU patients, etc.).

It is important to note that resistance patterns can vary considerably in other parts of the world based on variations in antibiotic usage and infection control practices, the penetration of particular resistance elements (e.g., CTX-M ESBLs among Enterobacteriaceae in parts of Europe [42, 43] and metallo-β-lactamases among *P. aeruginosa* in Asia and parts of Europe and Latin America [44, 45]), and differences in interpretive criteria (e.g., CLSI vs EUCAST). These factors resulting in regional variation highlight the need for multi-regional and international surveillance.

23.6 Staphylococci

23.6.1 S. aureus

S. aureus is a prevalent cause of a wide variety of infections. It is recognized as the primary pathogen for skin and skin structure infections (SSSI), but is also commonly encountered among bloodstream infections (BSI), and among surgical site infections (SSI) [46]. It is a prevalent pathogen of HAP/VAP though less common relative to Gram-negative pathogens [47], and with the emergence of CA-MRSA, its prevalence among patients with CAP has increased [48, 49]. Historically, resistance to penicillins was quick to develop and spread resulting in the development of penicillinase-resistant penicillins such as methicillin. However, methicillin resistance became established shortly thereafter, first in the hospital environment (HA-MRSA, e.g., USA100 PFGE genotype) and more recently among the community (CA-MRSA, e.g., USA300 PFGE genotype) [50, 51]. CA-MRSA are generally considered to be more susceptible than their HA-MRSA counterparts, which tend to have a higher degree of multi-drug resistance (a distinction likely to eventually disappear as CA-MRSA spread into the hospital and HA-MRSA into the community) [50–52].

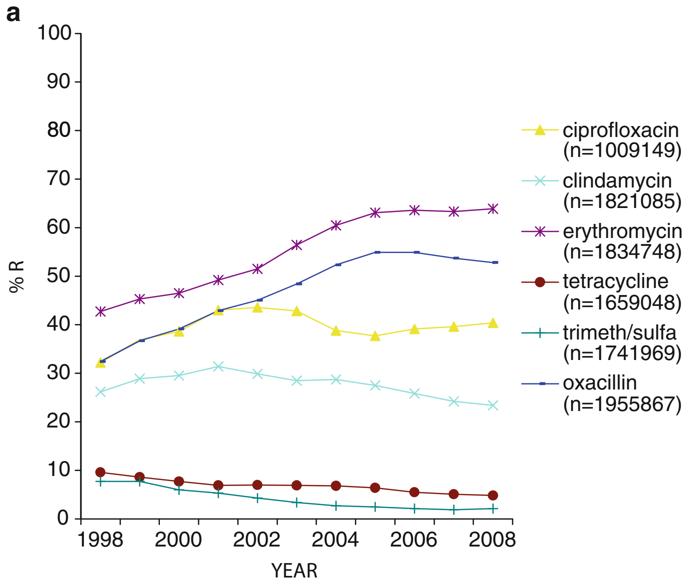
The prevalence of MRSA and the high degree of resistance among HA-MRSA to other available classes (macrolides, lincosamides, fluoroquinolones) spurred the development and use of glycopeptides (e.g., vancomycin) and more recently oxazolidinones (e.g., linezolid) and cyclic lipopeptides (e.g., daptomycin). Though decreased susceptibility to vancomycin, linezolid, and daptomycin has recently been detected among *S. aureus* [53–58], the frequency of resistant isolates has remained low, despite the therapeutic reliance on these agents due to lack of development and/or approval of new active gram-positive drugs. Nonetheless, the fact that resistance has emerged to any degree among these widely utilized agents is worth noting. Another topic of recent concern are reports of "MIC creep" for vancomycin combined with the reported increase in the isolation of vancomycin-intermediate *S. aureus* (VISA) [59–61] and the poor clinical outcomes associated with infection by VISA when treated with vancomycin [62, 63]. However, whether the reported increase in frequency of these strains is an issue of localized outbreaks versus widespread dissemination continues to be a topic of debate. MIC creep was not apparent in reports from large disseminated surveillance [64, 65].

As shown in Fig. 23.2 below, the overall rate of MRSA (as detected by oxacillin resistance) has increased from 32% to 53% over the past 10 years. The profiles of MRSA relative to *S. aureus* overall are different in that nearly all (>90%) MRSA are erythromycin resistant and prior to 2001 a substantial proportion of MRSA were also resistant to ciprofloxacin and clindamycin (decreases in resistance are presumed to coincide with the increasing prevalence of CA-MRSA which are generally susceptible to clindamycin and ciprofloxacin [50, 66]). Isolates non-susceptible to linezolid, daptomycin, and vancomycin were not readily detected (≤0.1%) over this period.

The current activity profile of commonly evaluated agents against *S. aureus,* as determined through TRUST 12 surveillance is shown in Table 23.2. Notably, the activity of linezolid, daptomycin, vancomycin, and tigecycline remains at or near 100%, regardless of whether the isolate is MRSA or multi-drug resistant (MDR), while susceptibility to erythromycin, clindamycin, and ciprofloxacin was diminished against MRSA and MDR isolates.

The current prevalence of MRSA and MDR based on specimen type, hospitalization status, and patient age is shown in Table 23.3. The %MRSA and %MDR was higher among inpatients relative to outpatients, and the prevalence increased with increasing patient age. MRSA rates were high across all evaluated specimen sources, with MDR highest among isolates from LRTI and urine.

There was regional variation within the US in the prevalence of both MRSA and MDR (Fig. 23.3), among skin and wound isolates of *S. aureus*. However, MRSA rates exceeded 65% in 5/9 US Bureau of Census regions and MDR exceeded 35% in 4/9 regions.



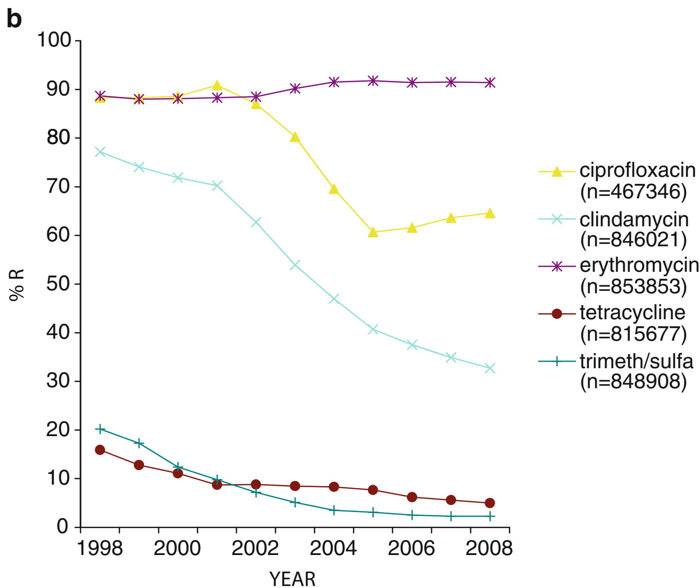


Fig. 23.2 Overall resistance trends in (**a**) *S. aureus* and (**b**) MRSA[a]

Table 23.2 Current activity profile against MSSA and MRSA[a]

Antimicrobial	MSSA (N=555)				MRSA (N=1086)				MDR[b] (N=759)			
Agent	MIC_{50}[c]	MIC_{90}[c]	%S[d]	%R[d]	MIC_{50}	MIC_{90}	%S	%R	MIC_{50}	MIC_{90}	%S	%R
Ciprofloxacin	0.5	16	84.3	13.9	16	>16	30.5	67.2	>16	>16	1.8	97.6
Erythromycin	0.5	>8	60.7	37.3	>8	>8	5.2	94.5	>8	>8	0.7	99.2
Clindamycin	0.12	0.25	92.8	6.7	0.12	>4	70.3	29.6	0.12	>4	53.6	46.0
Imipenem	0.03	0.06	100.0	0.0	0.5	4	90.1	7.3	0.5	16	86.3	10.1
Daptomycin	0.5	1	100.0	–[e]	0.5	1	99.9	–	0.5	1	99.9	–
Tigecycline	0.12	0.5	100.0	–	0.12	0.5	100.0	–	0.12	0.5	100.0	–
Trimeth/Sulfa	≤0.25	≤0.25	99.1	0.9	≤0.25	≤0.25	98.3	1.7	≤0.25	≤0.25	97.2	2.8
Gentamicin	0.25	0.5	99.1	0.9	0.5	0.5	97.3	2.7	0.5	1	95.9	4.1
Linezolid	1	2	100.0	–	1	2	100.0	–	1	2	100.0	
Vancomycin	1	1	100.0	0.0	1	1	100.0	0.0	1	1	100.0	

[a]Data from TRUST 12 surveillance (2008)
[b]MDR, multi-drug resistant defined as resistant to ≥3 of the following agents: ciprofloxacin, clindamycin, erythromycin, gentamicin, trimeth/sulfa and oxacillin
[c]MIC_{50} and MIC_{90} are reported as μg/mL
[d]Results interpreted in accordance with CLSI/FDA breakpoints as appropriate
[e]Dashed lines indicate CLSI/FDA breakpoints are unavailable for interpretation of susceptible (*S*), intermediate (*I*), and/or resistant (*R*)

Table 23.3 Variation in MRSA and MDR *S. aureus* by specimen, patient location, and patient age[a]

	N[b]	%MRSA	N[c]	%MDR[d]
Specimen				
Blood	18333	50.5	5038	41.5
Urine	18524	53.8	3612	52.6
LRTI	38620	52.2	11295	51.8
Skin/wound	54710	58.2	15438	30.2
Patient location				
Outpatient	171700	50.1	53055	29.5
Inpatient	80278	57.2	25636	43.5
ICU	18610	50.6	6481	43.1
Patient Age				
≤17 years	50447	48.6	15122	23.5
18–64years	143627	52.5	42701	32.1
≥65 years	68155	55.8	20642	49.5

[a]Data collected from TSN (2008–2009)
[b]Isolates with oxacillin results
[c]Isolates concurrently tested against oxacillin, clindamycin, erythromycin, trimethoprim-sulfamethoxazole, ciprofloxacin, and gentamicin
[d]Resistance to ≥3 of the drugs listed in footnote c

23.7 Coagulase-Negative Staphylococci (CoNS)

Although CoNS are common components of the skin microflora, they have also been implicated as pathogens in bloodstream infections, ocular infections, UTI (e.g., *S. saprophyticus*) and endocarditis [67]. Though CoNS are less virulent than *S. aureus*, resistance is more common among CoNS relative to *S. aureus*, with methicillin resistance exceeding 60% over the past 10 year period (Fig. 23.4). Similar to *S. aureus*, these strains remain nearly 100% susceptible to daptomycin, linezolid, and vancomycin, although non-susceptibility to linezolid was apparent among TRUST surveillance isolates of both methicillin susceptible (1.1%) and methicillin resistant (2.5%) CoNS (Table 23.4).

23.8 Enterococci

Enterococci are regarded primarily as commensals and are components of the intestinal microflora. However, enterococci are recognized as pathogens of bacteremia, UTI, and endocarditis and are less frequently associated with skin and wound infections (primarily as part of mixed infections or surgical site infections) [68, 69]. *E. faecalis* is the most common species associated with infections followed by *E. faecium*. Though *E. faecalis* is more prevalent, it is also more likely to be susceptible to agents such as vancomycin and ampicillin [68–70]. In contrast, *E. faecium*

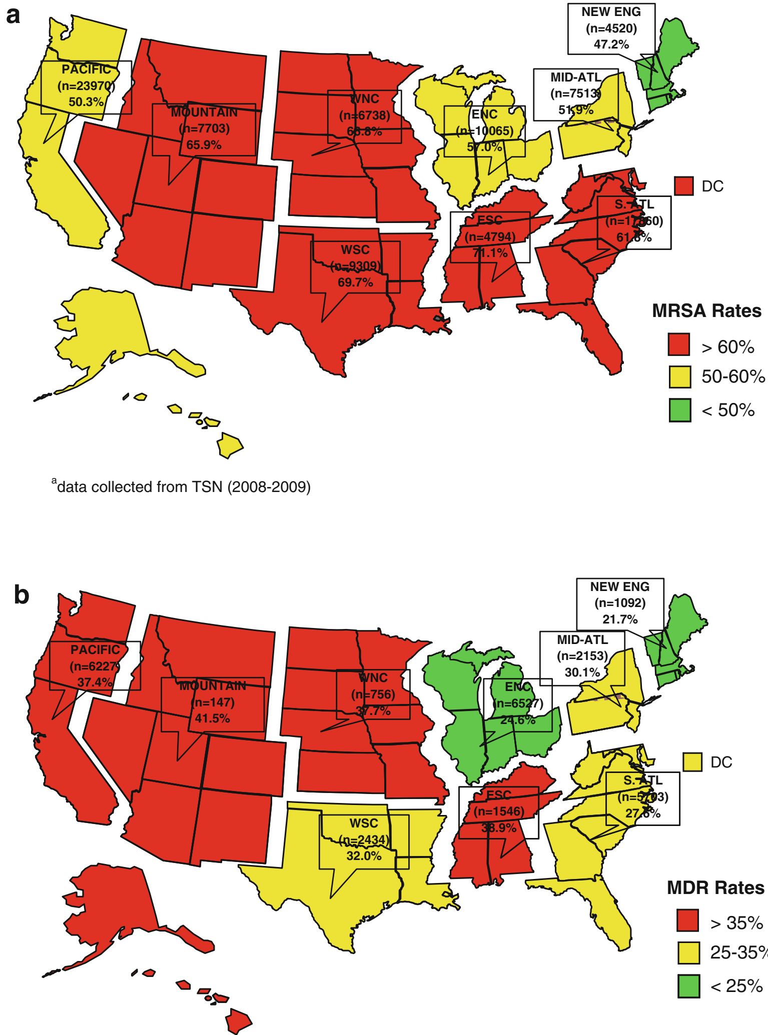


Fig. 23.3 Regional variation in (**a**) MRSA and (**b**) MDR *S. aureus* among skin/wound isolates[a]

tends to be more resistant, with vancomycin resistance well established within *E. faecium* in the US [68–70].

Figure 23.5 shows trends in resistance among both *E. faecalis* and *E. faecium* throughout the US over the past 10 years. Vancomycin resistance ranged from 3–4% in *E. faecalis* and rose from 68% to 77% among *E. faecium* over this period. *E. faecium* remained largely susceptible to quinupristin/dalfopristin over this period with resistance not exceeding 3% from 2006–2008. Resistance to daptomycin and linezolid did not exceed 1% for *E. faecalis* and was 1.6% and 3.5% for *E. faecium*, respectively, in 2008.

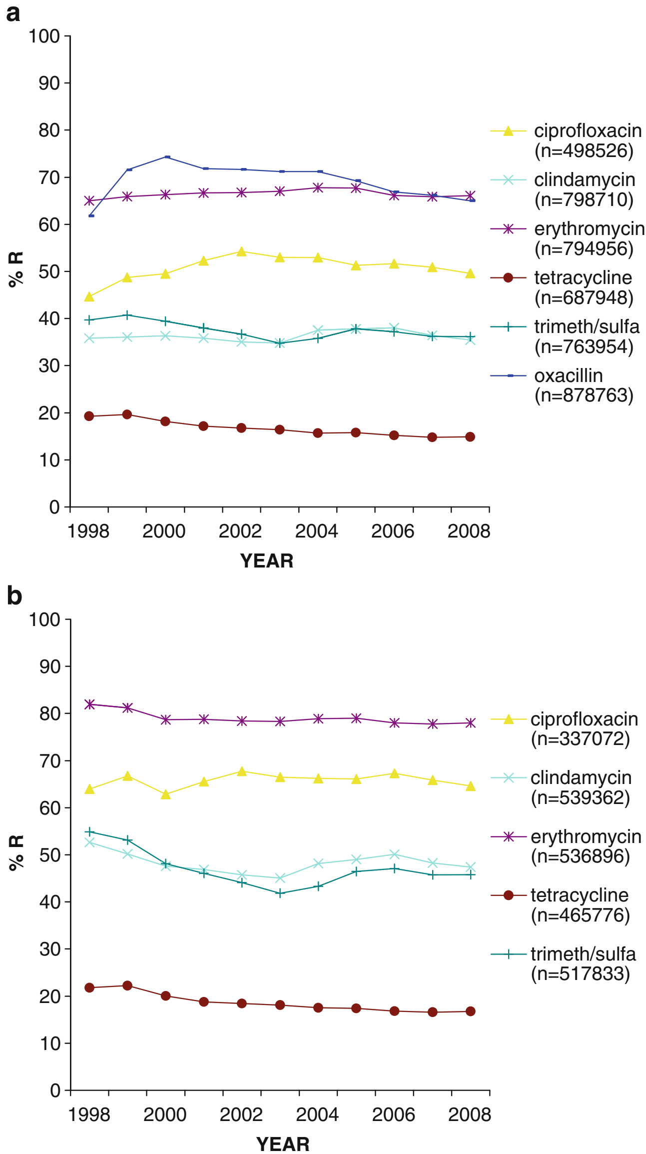


Fig. 23.4 Overall resistance trends in (**a**) CoNS and (**b**) MRCoNS[a]

Table 23.4 Current activity profile against MSCoNS and MRCoNS[a]

Antimicrobial	MSCoNS ($N=87$)				MRCoNS ($N=161$)			
Agent	MIC_{50}[b]	MIC_{90}[b]	%S[c]	%R[c]	MIC_{50}	MIC_{90}	%S	%R
Ciprofloxacin	0.25	>16	72.4	26.4	>16	>16	28.0	70.2
Erythromycin	0.25	>8	58.6	37.9	>8	>8	26.7	70.2
Clindamycin	0.06	4	89.7	10.3	0.12	>4	57.8	41.0
Imipenem	≤0.015	0.03	100.0	0.0	0.25	32	77.0	20.5
Daptomycin	0.5	1	100.0	–[d]	0.5	1	99.4	–
Tigecycline	0.25	0.5	97.7	–	0.25	0.5	98.8	–
Trimeth/Sulfa	≤0.25	>4	81.6	18.4	1	>4	60.9	39.1
Gentamicin	≤0.06	0.25	92.0	8.0	0.25	>16	60.9	28.0
Linezolid	1	1	98.9	–	0.5	1	97.5	–
Vancomycin	1	2	100.0	0.0	2	2	100.0	0.0

[a]Data from TRUST 12 surveillance (2008)
[b]MIC_{50} and MIC_{90} are reported as μg/mL
[c]Results interpreted in accordance with CLSI/FDA breakpoints as appropriate
[d]Dashed lines indicate CLSI/FDA breakpoints are unavailable for interpretation of susceptible (*S*), intermediate (*I*), and/or resistant (*R*)

The current activity profile of antimicrobials tested against *E. faecalis* and *E. faecium* collected in the US in 2008 (published in part by Arhin et al. 2009 [23]), is shown in Tables 23.5 and 23.6 below.

For both *E. faecalis* and *E. faecium*, the percentage of vancomycin resistance was elevated among the inpatient and ICU population relative to outpatient isolates, and vancomycin resistance was less prevalent among pediatric patients relative to adults and the elderly (Table 23.7). Vancomycin resistance was high for *E. faecium* across relevant specimen sources.

23.9 Streptococci

23.9.1 Beta-Hemolytic Streptococci

Beta-hemolytic streptococci (*S. pyogenes*, *S. agalactiae*, Group C/F/G streptococci) are common pathogens of a variety of skin and skin structure infections (primarily uncomplicated infections) and other diseases, including bacteremia, pharyngitis, and meningitis [68, 71, 72]. Beta-hemolytic streptococci remain nearly 100% susceptible to penicillins and other beta-lactam antibiotics [68, 71, 72].

Resistance to erythromycin and clindamycin among beta-hemolytic streptococci overall in the US has increased over time from 18% to 34% and from 10% to 24%, respectively (1998–2008; Fig. 23.6). Erythromycin and tetracycline resistance was more prevalent among *S. agalactiae* than among *S. pyogenes,* as determined in a recent US surveillance (Table 23.8), a trend consistent with other reports [73]. Isolates were 100% susceptible to penicillin, and also were 100% susceptible to linezolid, daptomycin, and vancomycin (Table 23.8).

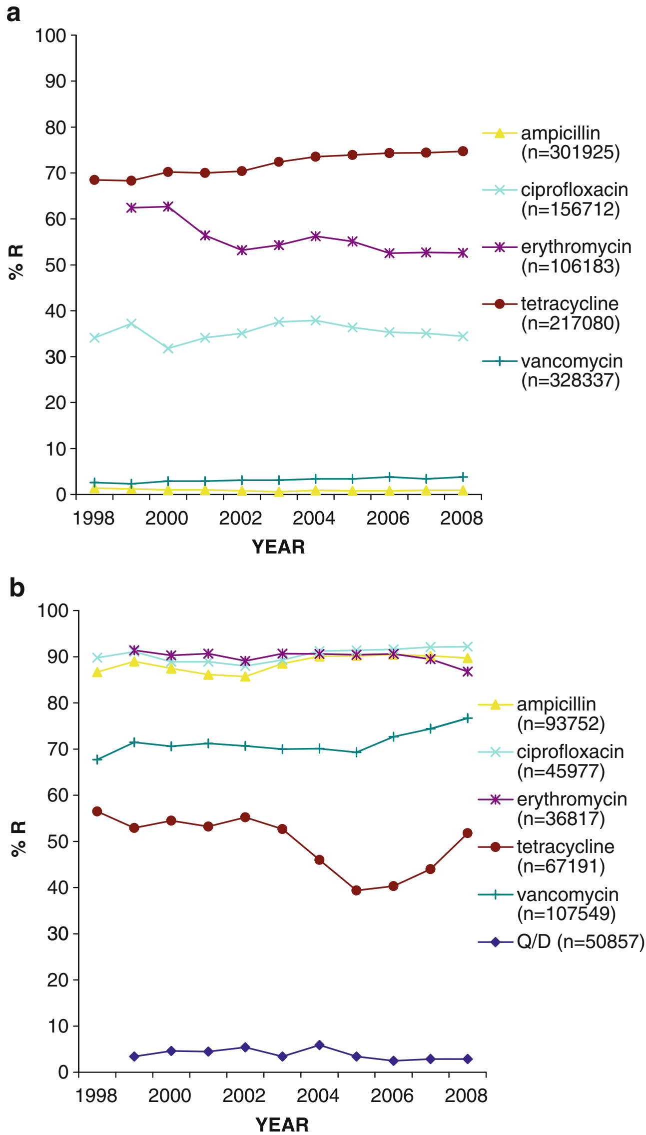


Fig. 23.5 Overall resistance trends in (**a**) *E. faecalis* and (**b**) *E. faecium*[a]

Table 23.5 Current activity profile against *E. faecalis* including vancomycin resistant isolates (VREfc)[a]

Antimicrobial	Overall (*N*=474)				VREfc (*N*=21)			
Agent	MIC_{50}[b]	MIC_{90}[b]	%S[c]	%R[c]	MIC_{50}	MIC_{90}	%S	%R
Levofloxacin	1	>4	64.6	35.2	>4	>4	0.0	100.0
Ampicillin	1	1	100.0	0.0	1	1	100.0	0.0
Daptomycin	1	2	100.0	–[d]	1	2	100.0	–
Tetracycline	32	64	25.9	72.8	32	32	33.3	61.9
Linezolid	1	2	100.0	0.0	1	1	100.0	0.0

**24.7% and 76.2% of VSEfc and VREfc, respectively, were resistant to high level gentamicin (>500 μg/mL)
[a]Data from Targanta US surveillance (2008)
[b]MIC_{50} and MIC_{90} are reported as μg/mL
[c]Results interpreted in accordance with CLSI/FDA breakpoints as appropriate
[d]Dashed lines indicate CLSI/FDA breakpoints are unavailable for interpretation of susceptible (*S*), intermediate (*I*), and/or resistant (*R*)

Table 23.6 Current activity profile against *E. faecium* including vancomycin resistant isolates (VREfm)[a]

Antimicrobial	Overall (*N*=199)				VREfm (*N*=156)			
Agent	MIC_{50}[b]	MIC_{90}[b]	%S[c]	%R[c]	MIC_{50}	MIC_{90}	%S	%R
Levofloxacin	>4	>4	9.5	89.4	>4	>4	0.0	100.0
Ampicillin	128	>128	10.1	89.9	128	>128	0.0	100.0
Daptomycin	2	4	100.0	–[d]	2	4	100.0	–
Tetracycline	0.25	>64	57.8	39.7	≤0.12	>64	58.3	39.7
Linezolid	1	2	100.0	0.0	1	1	100.0	0.0
Q/D	0.5	1	93.0	0.5	0.5	0.5	96.2	0.6

**7.0% and 23.7% of VSEfm and VREfm, respectively, were resistant to high level gentamicin (>500 μg/mL)
[a]Data from Targanta US surveillance (2008)
[b]MIC_{50} and MIC_{90} are reported as μg/mL
[c]Results interpreted in accordance with CLSI/FDA breakpoints as appropriate
[d]Dashed lines indicate CLSI/FDA breakpoints are unavailable for interpretation of susceptible (*S*), intermediate (*I*), and/or resistant (*R*)

Table 23.7 Variation in vancomycin resistance among *E. faecalis* and *E. faecium* by specimen, patient location, and patient age[a]

	N[b]	%VREfc	*N*[b]	%VREfm
Specimen				
Blood	5390	5.2	2757	73.1
Urine	24827	3.2	7370	77.0
Skin/wound	2716	5.4	2492	81.5
Patient location				
Outpatient	19876	2.6	3861	67.9
Inpatient	15567	4.8	9537	79.0
ICU	3141	6.1	2406	83.3
Patient age				
≤17 years	2534	0.6	500	51.0
18–64 years	14074	4.2	6891	80.0
≥65 years	19887	4.6	6474	77.6

[a]Data collected from TSN (2008–2009)
[b]Isolates with vancomycin results

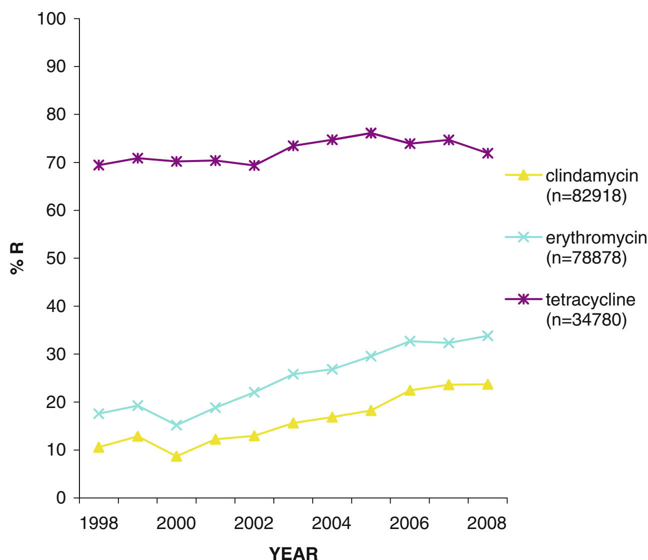


Fig. 23.6 Overall resistance trends in beta-hemolytic streptococci[a]

Table 23.8 Current activity profile against *S. pyogenes* and *S. agalactiae*[a]

Antimicrobial	*S. pyogenes* (*N*=399)				*S. agalactiae* (*N*=60)			
Agent	MIC_{50}[b]	MIC_{90}[b]	%S[c]	%R[c]	MIC_{50}	MIC_{90}	%S	%R
Penicillin	≤0.015	≤0.015	100.0	–[d]	≤0.008	0.015	100.0	–
Erythromycin	0.03	2	87.5	12.5	0.06	>2	50.0	50.0
Clindamycin	0.06	0.06	98.5	1.3	0.06	>1	71.7	28.3
Levofloxacin	0.5	0.5	100.0	0.0	1	1	100.0	0.0
Daptomycin	≤0.03	0.06	100.0	–	0.5	0.5	100.0	–
Tetracycline	0.12	16	86.5	13.0	32	32	13.3	86.7
Trimeth/sulfa	≤0.06	0.12	–	–	≤0.06	0.12	–	–
Linezolid	0.5	1	100.0	–	1	1	100.0	–
Vancomycin	0.25	0.25	100.0	–	0.25	0.5	100.0	–

[a]Data from Targanta US surveillance (2008)
[b]MIC_{50} and MIC_{90} are reported as µg/mL
[c]Results interpreted in accordance with CLSI/FDA breakpoints as appropriate
[d]Dashed lines indicate CLSI/FDA breakpoints are unavailable for interpretation of susceptible (*S*), intermediate (*I*), and/or resistant (*R*)

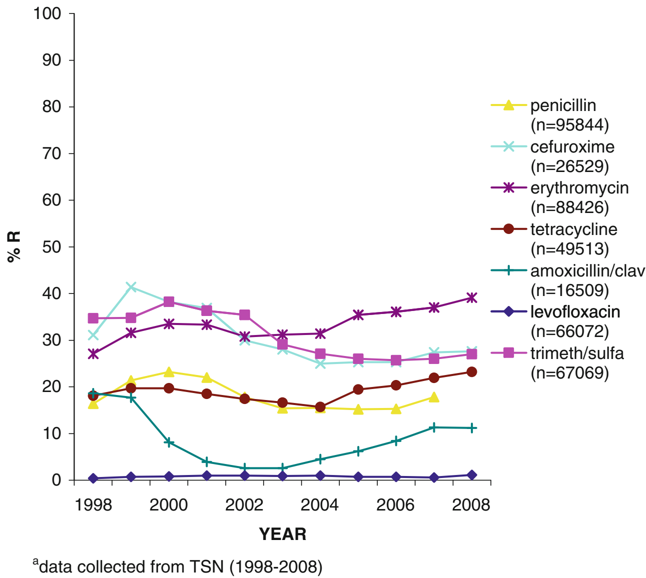


Fig. 23.7 Overall resistance trends among *S. pneumoniae*[a]

23.9.2 S. pneumoniae

S. pneumoniae is a prevalent cause of community acquired pneumonia (CAP), sinusitis, otitis media, and meningitis [74]. Pneumococci that remain susceptible to penicillin are largely susceptible to other agents widely used in the empiric treatment of respiratory infections. However, levels of penicillin resistance, increasing resistance to macrolides, and the prevalence of multi-drug resistance have complicated the selection of an appropriate initial empiric therapy [21, 75, 76]. Resistance to penicillin in the US has varied between 15% and 23% over the past 10 years (Fig. 23.7) while resistance to erythromycin nationally has climbed to 39% in 2008 from 27% in 1998.

During 2008, pneumococci collected as part of TRUST surveillance were 15% resistant to penicillin and 33% resistant to azithromycin (Table 23.9). A large proportion of isolates (28%) were also multi-drug resistant. As shown in Table 23.9, resistance to tetracycline, trimethoprim/sulfamethoxazole, amoxicillin/clavulanate and azithromycin was high among penicillin and multi-drug resistant isolates. These resistant isolates remained largely susceptible to levofloxacin, ceftriaxone, telithromycin, linezolid, and vancomycin.

There is a large degree of regional variation in the prevalence of penicillin resistance (Fig. 23.8a), high level azithromycin resistance (azithromycin MIC ≥ 16 μg/mL; Fig. 23.8b), and multi-drug resistance (Fig. 23.8c) with higher rates across the south and southeastern US. Furthermore, higher resistance rates for macrolides and

Table 23.9 Current activity profile against *S. pneumoniae* including penicillin resistant (PEN R) and MDR isolates[a]

Antimicrobial	Overall (N=2858)				PEN R (N=449)				MDR[b](N=795)			
Agent	MIC_{50}[c]	MIC_{90}[c]	%S[d]	%R[d]	MIC_{50}	MIC_{90}	%S	%R	MIC_{50}	MIC_{90}	%S	%R
Penicillin (oral)	≤0.015	4	62.7	15.7	4	4	0.0	100.0	2	4	6.5	56.2
Amoxicillin/clav	0.03	8	86.7	10.2	8	8	20.9	64.1	2	8	53.7	36.5
Imipenem	≤0.015	0.5	80.7	9.4	1	1	0.9	58.6	0.25	1	36.6	33.6
Ceftriaxone	0.03	1	94.0	1.4	1	2	61.9	8.7	1	2	78.4	4.9
Cefuroxime (oral)	0.06	8	77.5	19.2	8	16	0.2	98.2	4	16	29.2	63.9
Azithromycin	0.12	>128	66.5	33.4	>128	>128	10.0	89.8	>128	>128	7.2	92.7
Telithromycin	0.015	0.5	99.9	0.1	0.5	0.5	99.8	0.2	0.25	0.5	99.7	0.3
Levofloxacin	1	1	99.4	0.6	1	1	99.1	0.9	1	1	99.1	0.9
Tetracycline	0.25	32	79.8	19.9	32	32	27.4	72.4	32	>32	31.1	68.8
Linezolid	0.5	1	100.0	–[e]	0.5	1	100.0	–	0.5	1	100.0	–
Trimeth/Sulfa	0.25	4	73.7	18.4	4	8	16.5	73.7	4	8	27.7	60.6
Vancomycin	0.5	0.5	100.0	–	0.5	0.5	100.0	–	0.5	0.5	100.0	–

[a]Data from TRUST 12 surveillance (2008)
[b]MDR, multi-drug resistant defined as resistant to ≥2 of the following agents: penicillin, azithromycin, cefuroxime, tetracycline, and trimethoprim-sulfamethoxazole
[c]MIC_{50} and MIC_{90} are reported as μg/mL
[d]Results interpreted in accordance with CLSI/FDA breakpoints as appropriate
[e]Dashed lines indicate CLSI/FDA breakpoints are unavailable for interpretation of susceptible (*S*), intermediate (*I*), and/or resistant (*R*)

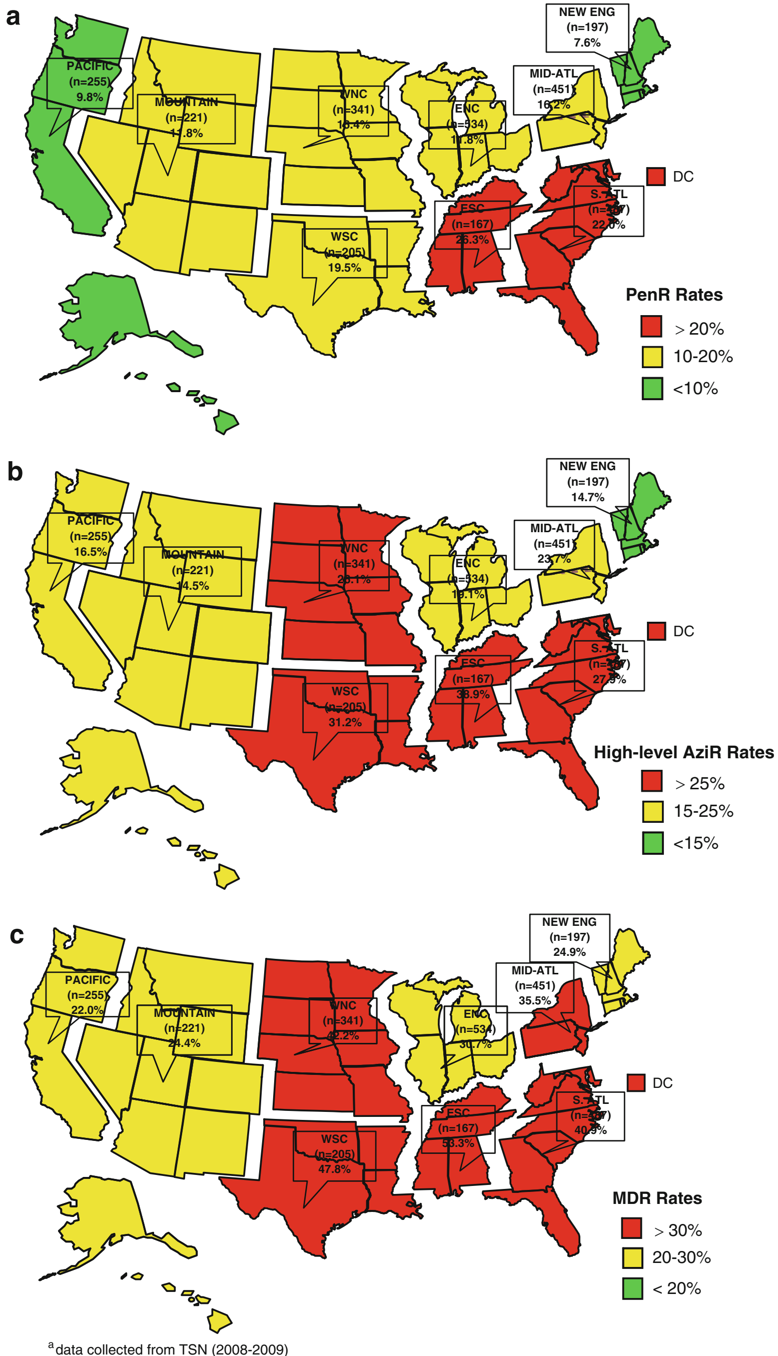


Fig. 23.8 Regional variation in (**a**) penicillin resistance, (**b**) MDR, and (**c**) high-level azithromycin (>16 mg/mL) resistance among *S. pneumoniae*[a]

Table 23.10 Variation in penicillin and multi-drug resistance among *S. pneumoniae* by specimen, patient location, and patient age[a]

	N	% PEN R	% MDR[b]
Specimen			
Blood	849	10.5	19.5
LRTI	1220	15.1	28.4
Sinus	107	17.8	33.6
Patient location			
Outpatient	1132	19.5	32.0
Inpatient	1127	13.5	25.1
ICU	436	11.7	22.5
Patient age			
≤17 Years	716	25.3	36.7
18–64 years	1289	12.3	25.4
≥65 years	832	12.7	23.6

[a]Data from TRUST 12 surveillance (2008)
[b]Isolate resistant to ≥2 of the following: penicillin, cefuroxime, macrolide, tetracycline, trimeth/sulfa

beta-lactams have been reported in Asia relative to Europe and the US [75]. Such variation further highlights the need for understanding the local antibiogram of pneumococci when considering the selection of an empiric therapy.

Co-morbidities also play a role in empiric therapy selection for the treatment of respiratory disease, in particular CAP [33]. Interestingly, when analyzing the impact of both age and hospitalization status on the prevalence of resistance among pneumococci in the US, more penicillin and multi-drug resistance is encountered among the outpatient population and pediatric population relative to the inpatient and adult/elderly populations (Table 23.10). Though the reason for this trend is not entirely clear, the increased carriage of resistant strains among the pediatric population as a result of increased exposure to beta-lactams or vaccination effect (replacement of vaccine serotypes with drug resistant non-vaccine serotypes) has been noted [77, 78].

23.9.3 Haemophilus influenzae

H. influenzae causes a similar spectrum of disease attributed to *S. pneumoniae* consisting of respiratory infections (sinusitis and pneumonia), meningitis, otitis media, and conjunctivitis [79]. Unlike *S. pneumoniae*, *H. influenzae* remain largely susceptible to the majority of agents utilized to treat the aforementioned infections. Resistance to penicillins (e.g., ampicillin and amoxicillin) via beta-lactamase production and trimethoprim/sulfamethoxazole are common [21, 75]. Table 23.11 illustrates the high degree of susceptibility of isolates encountered as part of TRUST surveillance in 2008 to commonly utilized therapeutics.

Table 23.11 Current activity profile against *H. influenzae*[a]

	Overall (N=716)			
Antimicrobial agent	MIC_{50}[b]	MIC_{90}[b]	% S[c]	%R[c]
Ampicillin	0.25	>8	76.8	22.9
Amoxicillin/clav	0.5	2	100.0	0.0
Meropenem	0.06	0.12	99.3	–[d]
Ceftriaxone	≤0.015	≤0.015	100.0	–
Cefuroxime (oral)	1	2	100.0	0.0
Azithromycin	2	2	98.9	–
Telithromycin	2	4	98.5	0.7
Levofloxacin	0.015	0.03	99.9	0.6
Tetracycline	0.5	1	98.5	0.8
Trimeth/sulfa	0.12	>4	79.1	16.8

[a]Data from TRUST 12 surveillance (2008)
[b]MIC_{50} and MIC_{90} are reported as µg/mL
[c]Results interpreted in accordance with CLSI/FDA breakpoints as appropriate
[d]Dashed lines indicate CLSI/FDA breakpoints are unavailable for interpretation of susceptible (*S*), intermediate (*I*), and/or resistant (*R*)

23.10 Enterobacteriaceae

The Enterobacteriaceae family of organisms contains a variety of clinically important genera and species including: *E. coli*, *K. pneumoniae* and other *Klebsiella* spp., *Proteus* spp., *Enterobacter* spp., *Citrobacter* spp., *Morganella morgannii*, *Salmonella* spp., *Shigella* spp., and *Serratia marcescens*. As would be expected of such a diverse group of organisms, the spectrum of disease associated with the Enterobacteriaceae is equally diverse. They are the most commonly isolated pathogens from the urine, and are commonly isolated from the bloodstream and lower respiratory tract [5, 42, 80]. *E. coli* is a prevalent cause of urinary tract infection and gastroenteritis, but also is a common agent of bacteremia and meningitis. *K. pneumoniae* is also a common uropathogen and a prevalent cause of pneumonia among hospitalized/ventilated patients. *Proteus* spp., are common UTI pathogens. Species of *Enterobacter* and *Citrobacter* rarely cause disease among the healthy population, but are common nosocomial pathogens, in particular among the ICU where they can cause a variety of infections including skin and wound infections, pneumonia, and UTI.

The emergence of resistance to advanced generation cephalosporins soon followed their introduction to use as Enterobacteriaceae capable of producing beta-lactamases were encountered. To date, the variety of beta-lactamases capable of hydrolyzing advanced generation cephalosporins, or extended-spectrum beta-lactamases (ESBL), has grown to represent a large group of enzymes subdivided into classes based on their spectrum and mechanism of action [81]. Though ESBLs largely have not been active against carbapenems, recently serine carbapenemases (e.g., KPCs) have emerged among Enterobacteriaceae in the US [82–84]. In Europe and elsewhere, metallo-β-lactamases (e.g., VIM) have conferred resistance to carbapenems among

these species [83, 85, 86]. A further complication to the spread of beta-lactamases among Enterobacteriaceae is that organisms with ESBLs (class A [e.g., TEM, SHV] among *E. coli*, *K. pneumoniae* and class C [e.g., AmpC] among species of *Enterobacter* and *Citrobacter*) are often resistant to other first line therapies such as quinolones, aminoglycosides, and trimethoprim-sulfamethoxazole [87–89], leaving few therapeutic options. To extend the utility of cephalosporins and other beta-lactams in the face of these resistant organisms, beta-lactamase inhibitors are being developed in combination with both approved and investigational beta-lactams.

Figure 23.9 shows trends in resistance among key members of Enterobacteriaceae based on TSN data from 1998 to 2008. Of particular interest is the increase in fluoroquinolone resistance amoung *E. coli* (2% in 1998 to 20% in 2008), and the emergence of imipenem resistance among *K. pneumoniae* (0% through 2004 to 0.3% in 2005 to 1.8% in 2008). Ceftazidime resistance, a marker of ESBL production, was most common among other Enterobacteriaceae (e.g., *Enterobacter* spp. and *Citrobacter* spp.) and *K. pneumoniae* relative to *E. coli* and *P. mirabilis* across the evaluated 10 year period.

The current overall antibiogram of Enterobacteriaceae as determined by TRUST surveillance in 2008 is shown in Table 23.12, while Tables 23.13, 23.14, 23.15, 23.16, 23.17, 23.18 show antibiograms by species, highlighting those with important resistant phenotypes (e.g., ESBL, derepressed AmpC). ESBL isolates were detected in 4.5% of *E. coli* and 7.8% of *K. pneumoniae*. 1.9% of *K. pneumoniae* were imipenem resistant. Derepressed AmpC production was detected in 13.4% of *Enterobacter* spp., and 13.3% of *Citrobacter* spp.. Excluding ESBL *K. pneumoniae*, resistant isolates remained largely susceptible to evaluated carbapenems and tigecycline.

As shown in Table 23.19, resistance to ceftazidime among Enterobacteriaceae was most commonly observed among isolates from LRTI and isolates from the ICU. Imipenem resistance among *K. pneumoniae* was also primarily associated with LRTI and ICU isolates. Minimal imipenem resistance was apparent among other Enterobacteriaceae species excluding *P. mirabilis* where imipenem has been documented to have low potency relative to other carbapenems (e.g., meropenem).

Among LRTI *K. pneumoniae* in the US, imipenem resistance was most commonly encountered among isolates from the Mid-Atlantic and East North Central regions (Fig. 23.10), findings consistent with the initial reports regarding the emergence and spread of KPCs throughout these areas [84].

23.10.1 P. aeruginosa

P. aeruginosa is an opportunistic pathogen causing infections in hospitalized patients whose immune defenses are somehow compromised. It is implicated as a primary pathogen in patients with burns and chronically infects the lungs of patients suffering from cystic fibrosis [90–92]. The ability of *P. aeruginosa* to survive and persist throughout the nosocomial environment has also made it a prevalent pathogen of hospital-acquired and ventilator-associated pneumonia, nosocomial UTI and bacteremia [90–92].

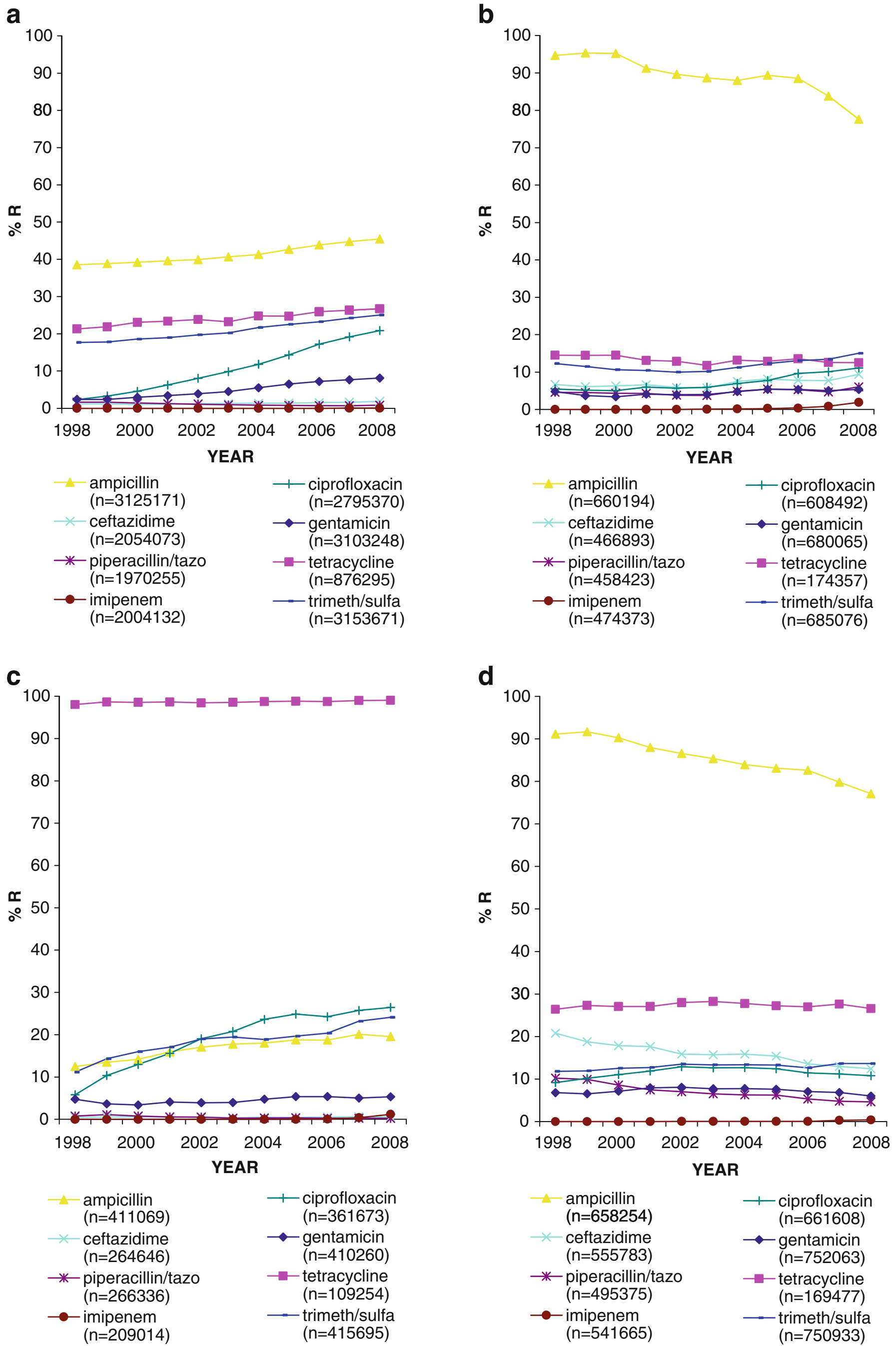


Fig. 23.9 Overall resistance trends in (**a**) *E. coli*, (**b**) *K. pneumoniae*, (**c**) *P. mirabilis*

Table 23.12 Current activity profile against Enterobacteriaceae[a]

Antimicrobial agent	Overall (N=5342)			
	MIC_{50}[b]	MIC_{90}[b]	% S[c]	%R[c]
Ampicillin	32	>32	34.9	56.7
Ceftazidime	0.12	1	93.0	6.2
Cefotaxime	0.06	1	92.3	5.3
Ceftriaxone	0.03	1	92.4	5.9
Cefepime	0.03	0.25	97.2	2.4
Piperacillin/tazobactam	2	8	93.9	3.4
Imipenem	0.25	2	99.2	0.6
Meropenem	0.03	0.12	99.2	0.7
Ciprofloxacin	0.03	32	82.9	15.8
Amikacin	2	4	98.8	0.3
Gentamicin	0.5	2	92.1	6.8
Trimeth/sulfa	≤0.25	>32	81.3	18.7
Tigecycline	0.5	2	97.4	0.3

[a]Data from TRUST 12 surveillance (2008)
[b]MIC_{50} and MIC_{90} are reported as μg/mL
[c]Results interpreted in accordance with CLSI/FDA breakpoints as appropriate

Table 23.13 Current activity profile against *Enterobacteriaceae* by species including resistant isolates[a]

Antimicrobial	Overall (*N*=1723)				ESBL[b] (*N*=77)			
Agent	MIC_{50}[c]	MIC_{90}[c]	% S[d]	% R[d]	MIC_{50}	MIC_{90}	% S	% R
Ampicillin	8	>32	51.6	48.2	>32	>32	1.3	98.7
Ceftazidime	0.25	0.5	96.3	2.7	16	64	44.2	40.3
Cefotaxime	0.06	0.25	95.2	3.8	>32	>32	1.3	98.7
Ceftriaxone	0.03	0.12	94.9	3.9	16	64	44.2	40.3
Cefepime	0.03	0.12	97.2	2.6	32	>128	37.7	55.8
Piperacillin/tazobactam	2	8	96.1	1.6	8	>128	72.7	11.7
Imipenem	0.25	0.25	99.8	0.1	0.25	0.5	97.4	1.3
Meropenem	≤0.015	0.03	99.8	0.1	0.03	0.06	97.4	1.3
Ciprofloxacin	0.015	64	75.0	25.0	64	>128	14.3	85.7
Amikacin	2	4	99.7	0.1	4	16	94.8	2.6
Gentamicin	1	16	89.4	10.3	4	>32	51.9	45.5
Trimeth/sulfa	≤0.25	>32	73.0	27.0	>32	>32	39.0	61.0
Tigecycline	0.25	0.5	100.0	0.0	0.25	0.5	100.0	0.0

[a]Data from TRUST 12 surveillance (2008)
[b]ESBL = phenotypically confirmed (reduction of ceftazidime/cefotaxime MIC when combined with clavulanic acid)
[c]MIC_{50} and MIC_{50} are reported as μg/mL
[d]Results interpreted in accordance with CLSI/FDA breakpoints as appropriate

Table 23.14 Current activity profile against *K. pneumoniae*[a]

Antimicrobial agent	Overall (N=1540)				ESBL[b] (N=120)			
	MIC_{50}[c]	MIC_{90}[c]	% S[d]	% R[d]	MIC_{50}	MIC_{90}	% S	% R
Ampicillin	32	>32	5.1	78.4	>32	>32	0.0	100.0
Ceftazidime	0.12	2	91.0	8.6	>128	>128	15.0	80.0
Cefotaxime	0.03	0.5	92.0	6.0	64	>64	20.0	61.7
Ceftriaxone	0.03	0.25	91.9	6.7	>64	>64	18.3	70.0
Cefepime	0.03	0.25	95.1	4.2	8	>128	51.7	40.8
Piperacillin/tazobactam	>128	>128	26.8	58.0	64	>128	35.0	49.2
Imipenem	0.25	0.5	97.5	1.9	0.25	16	83.3	12.5
Meropenem	0.03	0.06	97.7	1.9	0.06	16	84.2	12.5
Ciprofloxacin	0.03	2	89.6	9.7	64	128	24.2	72.5
Amikacin	1	2	96.5	0.7	16	32	67.5	6.7
Gentamicin	0.25	1	93.5	4.9	8	>32	45.0	41.7
Trimeth/sulfa	≤0.25	>32	85.9	14.1	>32	>32	23.3	76.7
Tigecycline	0.5	1	99.6	0.1	0.5	2	99.2	0.0

[a]Data from TRUST 12 surveillance (2008)
[b]ESBL = phenotypically confirmed (reduction of ceftazidime/cefotaxime MIC when combined with clavulanic acid)
[c]MIC_{50} and MIC_{90} are reported as μg/mL
[d]Results interpreted in accordance with CLSI/FDA breakpoints as appropriate

Table 23.15 Current activity profile against *P. mirabilis*[a]

	Overall (N=814)			
Antimicrobial agent	MIC_{50}[b]	MIC_{90}[b]	% S[c]	% R[c]
Ampicillin	≤1	>32	77.6	21.3
Ceftazidime	0.06	0.12	99.6	0.4
Cefotaxime	≤0.015	0.03	98.9	0.9
Ceftriaxone	≤0.015	≤0.015	98.6	1.1
Cefepime	0.03	0.12	99.3	0.7
Piperacillin/tazobactam	1	1	99.9	0.1
Imipenem	1	2	100.0	0.0
Meropenem	0.06	0.12	100.0	0.0
Ciprofloxacin	0.03	32	72.4	24.1
Amikacin	4	8	99.5	0.1
Gentamicin	1	4	90.8	7.1
Trimeth/sulfa	≤0.25	>32	76.4	23.6
Tigecycline	1	4	84.8	2.0

[a]Data from TRUST 1 2 surveillance (2008)
[b]MIC_{50} and MIC_{90} are reported as μg/mL
[c]Results interpreted in accordance with CLSI/FDA breakpoints as appropriate

Table 23.16 Current activity profile against *E. cloacae*[a]

	Overall (*N*=455)				Derepressed Amp C[b] (*N*=61)			
Antimicrobial agent	MIC_{50}[c]	MIC_{90}[c]	% S[d]	% R[d]	MIC_{50}	MIC_{90}	% S	% R
Ampicillin	>32	>32	18.9	71.2	>32	>32	0.0	100.0
Ceftazidime	0.5	64	78.5	19.1	64	128	0.0	100.0
Cefotaxime	0.25	>64	75.4	18.7	>64	>64	0.0	95.1
Ceftriaxone	0.25	>64	76.0	18.7	>64	>64	0.0	95.1
Cefepime	0.06	2	97.8	1.5	2	8	100.0	0.0
Piperacillin/tazobactam	4	64	82.6	9.5	128	>128	6.6	52.5
Imipenem	0.5	1	99.8	0.2	0.5	1	100.0	0.0
Meropenem	0.03	0.12	99.8	0.2	0.12	0.25	100.0	0.0
Ciprofloxacin	0.015	0.5	91.4	7.5	0.03	4	83.6	13.1
Amikacin	1	2	99.8	0.0	1	4	98.4	0.0
Gentamicin	0.5	1	94.7	4.2	0.5	8	86.9	6.6
Trimeth/sulfa	≤0.25	16	88.1	11.9	≤0.25	1	90.2	9.8
Tigecycline	0.5	1	98.9	0.0	0.5	1	100.0	0.0

[a]Data from TRUST 12 surveillance (2008)
[b]Phenotypically defined as resistance to cefoxitin and ceftazidime, ceftazidime MIC not affected by clavulanic acid, cefepime and carbapenem susceptible
[c]MIC_{50} and MIC_{90} are reported as μg/mL
[d]Results interpreted in accordance with CLSI/FDA breakpoints as appropriate

Table 23.17 Current activity profile against *Citrobacter* spp.[a]

	Overall (*N*=369)				Derepressed Amp C[b] (*N*=49)			
Antimicrobial agent	MIC_{50}[c]	MIC_{90}[c]	% S[d]	% R[d]	MIC_{50}	MIC_{90}	% S	% R
Ampicillin	16	>32	36.6	45.8	>32	>32	0.0	100.0
Ceftazidime	0.5	32	84.0	14.1	64	128	0.0	98.0
Cefotaxime	0.12	32	83.7	5.7	32	>64	4.1	42.9
Ceftriaxone	0.12	64	84.6	10.0	64	>64	4.1	71.4
Cefepime	0.03	0.5	99.7	0.0	1	2	100.0	0.0
Piperacillin/tazobactam	2	32	88.3	4.6	64	128	26.5	24.5
Imipenem	1	2	99.7	0.0	1	2	100.0	0.0
Meropenem	0.03	0.06	99.7	0.0	0.06	0.12	100.0	0.0
Ciprofloxacin	0.03	1	93.0	4.3	0.12	4	77.6	12.2
Amikacin	1	2	100.0	0.0	1	2	100.0	0.0
Gentamicin	0.5	1	93.5	6.0	0.5	32	87.8	12.2
Trimeth/sulfa	≤0.25	>32	83.5	16.5	≤0.25	>32	75.5	24.5
Tigecycline	0.25	1	99.5	0.0	0.5	1	100.0	0.0

[a]Data from TRUST 12 surveillance (2008)
[b]Phenotypically defined as resistance to cefoxitin and ceftazidime, ceftazidime MIC not affected by clavulanic acid, cefepime and carbapenem susceptible
[c]MIC_{50} and MIC_{90} are reported as μg/mL
[d]Results interpreted in accordance with CLSI/FDA breakpoints as appropriate

Table 23.18 Current activity profile against *S. marcescens*[a]

Antimicrobial agent	Overall (N=441)			
	MIC_{50}[b]	MIC_{90}[b]	% S[c]	% R[c]
Ampicillin	>32	>32	10.4	74.1
Ceftazidime	0.12	0.5	97.1	2.5
Cefotaxime	0.25	2	95.0	2.9
Ceftriaxone	0.12	1	95.9	2.9
Cefepime	0.06	0.25	98.6	0.9
Piperacillin/tazobactam	1	4	97.3	1.4
Imipenem	1	2	99.5	0.5
Meropenem	0.06	0.12	99.5	0.5
Ciprofloxacin	0.12	1	93.2	3.6
Amikacin	2	4	99.5	0.2
Gentamicin	0.5	2	96.8	2.5
Trimeth/sulfa	≤0.25	1	97.7	2.3
Tigecycline	1	1	99.1	0.0

[a]Data from TRUST 12 surveillance (2008)
[b]MIC_{50} and MIC_{90} are reported as μg/mL
[c]Results interpreted in accordance with CLSI/FDA breakpoints as appropriate

Initial treatment of *P. aeruginosa* is managed largely on an institutional basis, due to the large degree of resistance and multi-drug resistance among *P. aeruginosa* and the variation in resistance patterns within a given institution. Initial therapy, which can often consist of combination therapy to maximize initial chance for success, is usually adjusted after susceptibility testing of the patient's isolate as needed.

Common agents used to treat *P. aeruginosa* infections include anti-pseudomonal cephalosporins (e.g., ceftazidime/cefepime), carbapenems (imipenem/meropenem/doripenem), piperacillin/tazobactam, and aztreonam. Aminoglycosides (e.g., amikacin/tobramycin) are also commonly a part of therapy in particular if beta-lactamase resistance is an issue within a given institution. Multi-drug resistance among *P. aerguinosa* is a substantial concern. Resistance to beta-lactams is common, due to the prevalence of chromosomal beta-lactamases (e.g., AmpC) and ESBLs (including some with carbapenemase activity (e.g., metallo-β-lactamases [VIM/IMP], and OXA)), efflux pumps, and porin mutation (OprD) [81, 83, 90–92]. Drug efflux can also impact the activity of fluoroquinolones and aminoglycosides in addition to mutation of the target gens and acquisition of genes capable of modifying the target, contributing to the phenomenon of multi-drug resistance among these organisms. Limited therapeutic options are available in the context of multi-drug resistant *P. aeruginosa* infection.

Resistance among *P. aeruginosa* to common agents has been relatively stable over the past 10 years (Fig. 23.11), though susceptiblity to many commonly utilized agents currently is at or below 90%, excluding amikacin which remains the most active agent evaluated as part of TRUST surveillance (Table 23.20). In 2008, 13.4% of isolates were resistant to imipenem and 9.3% of isolates were multi-drug resistant. Among multi-drug resistant isolates, the only evaluated agent with any appreciable

Table 23.19 Variation in ceftazidime (CAZ) and imipenem (IPM) resistance among Enterobacteriaceae by specimen, patient location, and patient age[a]

	E. coli				*K. pneumoniae*				*P. mirabilis*				*Other Enterobacteriaceae*			
	N	%CAZ R	*N*	%IPM^R	*N*	%CAZ R	*N*	%IPM^R	*N*	%CAZ R	*N*	%IPM^R	N	%CAZ R	*N*	%IPM^R
Specimen																
Blood	10897	3.4	11994	0.1	4710	15.2	5074	3.7	1143	1.0	702	1.1	4214	15.2	4336	0.5
LRTI	5373	7.4	5637	0.4	6730	20.3	7180	5.3	1502	1.3	930	1.6	10529	16.8	10885	0.7
Urine	320331	1.6	316081	0.0	55885	7.4	57327	1.3	32087	0.5	16239	1.4	35930	11.6	34370	0.2
Skin/wound	6237	3.1	6610	0.1	2134	13.4	2268	4.1	2088	0.5	1154	1.0	4030	12.7	4112	0.8
Patient location																
Outpatient	260614	1.3	253949	0.0	42039	4.7	42713	0.9	26077	0.4	13690	1.3	33915	7.9	32873	0.2
Inpatient	65992	3.4	69286	0.1	22421	14.4	23759	3.3	10897	1.0	6464	1.3	22843	16.2	22862	0.5
ICU	11490	4.8	11788	04.81	6615	20.3	6877	4.5	1980	1.1	1112	1.3	8691	20.7	8744	0.9
Patient age																
≤17 years	34681	1.1	32036	0.1	4423	414	4140	0.5	2893	0.5	1666	1.6	7406	15.4	6832	0.4
18–64 years	155829	1.6	148380	0.0	27483	10.5	27832	2.1	14898	0.6	8332	1.2	27884	12.0	27217	0.5
≥65 years	126325	2.9	129094	0.0	36740	10.9	38311	2.6	22387	0.6	11320	1.4	23887	12.8	27909	0.3

[a]Data collected from TSN (2008–2009)

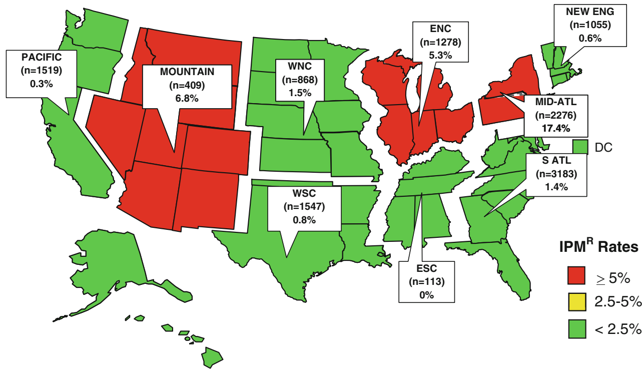


Fig. 23.10 Regional variation in imipenem resistance among LRTI *K. pneumoniae*[a]

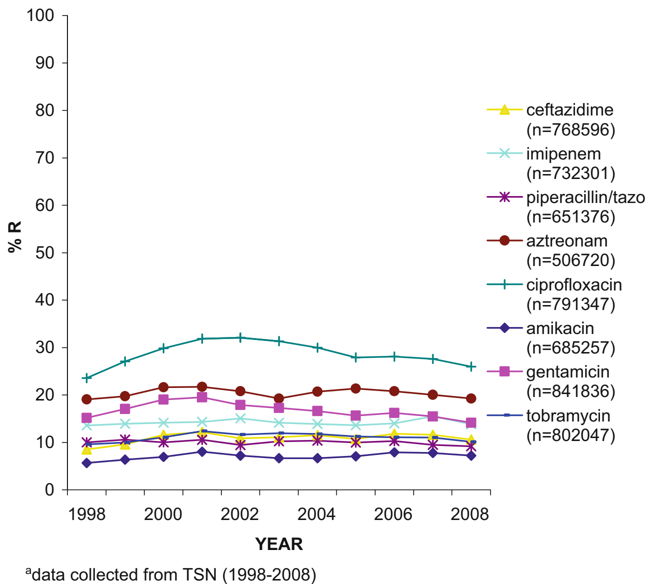


Fig. 23.11 Overall resistance trends in *P. aeruginosaa*

activity was amikacin (87% susceptible), while susceptibility to other evaluated agents was below 50%. Though not evaluated as part of TRUST surveillance, it is important to note that both colistin and polymixin B are also highly active against resistant *P. aeruginosa* isolates [93, 94].

Resistance to imipenem and multi-drug resistance in the US was more prevalent among LRTI isolates of *P. aeruginosa* than isolates recovered from other specimens (Table 23.21). Furthermore, imipenem and multi-drug resistance was more common among patients in the ICU and in the adult population (Table 23.21).

Table 23.20 Current activity profile against *P. aeruginosa* including resistant isolates[a]

Antimicrobial	All (N=1,533)				IPM NS[b] (N=291)				MDR[c](N=142)			
Agent	MIC_{50}[d]	MIC_{90}[d]	%S[e]	%R[e]	MIC_{50}	MIC_{90}	%S	%R	MIC_{50}	MIC_{90}	%S	%R
Ceftazidime	2	16	86.0	9.7	4	64	66.0	29.2	32	128	23.9	73.2
Cefepime	2	16	88.0	5.5	8	32	66.7	20.3	16	32	23.9	44.4
Imipenem	2	16	81.0	13.4	16	32	0.0	70.4	16	32	20.4	73.2
Pip/tazo	8	64	90.0	10.0	16	>128	69.8	30.2	>128	>128	22.5	77.5
Aztreonam	4	32	71.6	15.5	8	64	50.2	33.0	32	64	14.8	68.3
Ciprofloxacin	0.25	32	69.1	24.7	8	64	35.4	58.4	16	64	4.2	91.5
Tigecycline	8	16	–[f]	–	8	16	–	–	8	32	–	–
Tobramycin	0.5	2	91.5	7.3	1	>32	73.5	23.7	16	>32	45.1	51.4
Gentamicin	2	8	86.0	9.1	4	>32	63.6	27.1	32	>32	33.8	56.3
Amikacin	4	8	97.8	1.4	4	16	94.2	4.1	8	32	87.3	9.9
Trimeth/sulfa	8	>32	18.7	213	16	>32	13.1	86.9	32	>32	9.9	90.1

[a]Data from TRUST 12 surveillance (2008)
[b]Imipenem non-susceptible
[c]MDR, multi-drug resistant defined as resistant to ≥3 of the following agents: amikacin, ceftazidime, ciprofloxacin, gentamicin, imipenem and pip/tazo
[d]MIC_{50} and MIC_{90} are reported as μg/mL
[e]Results interpreted in accordance with CLSI/FDA breakpoints as appropriate
[f]Dashed lines indicate CLSI/FDA breakpoints are unavailable for interpretation of susceptible (*S*), intermediate (*I*), and/or resistant (*R*)

Table 23.21 Variation in imipenem resistance (IPM R) and MDR among *P. aeruginosa* by specimen, patient location, and patient age[a]

	N[b]	%IPM R	N[c]	%MDR[d]
Specimen				
Blood	2754	13.3	1861	10.0
Urine	24814	9.0	18170	7.5
LRTI	29476	22.4	20219	18.3
Skin/wound	4603	11.2	3325	8.9
Patient location				
Inpatient	30451	15.9	22910	12.2
ICU	11063	23.7	27232	18.9
Patient age				
≤17 years	8137	8.8	5806	6.0
18–64years	34223	18.3	25501	15.2
≥65 years	34381	11.5	24414	9.1

[a]Data collected from TSN (2008–2009)
[b]Isolates with imipenem (IPM) results
[c]Isolates concurrently tested against ceftazidime, piperacillin/tazobactam, imipenem, ciprofloxacin, gentamicin, amikacin
[d]Resistance to ≥3 of the drugs listed in footnote c

23.10.2 Acinetobacter spp.

Acinetobacter spp., like *P. aeruginosa*, are another example of opportunistic pathogens, which have become problematic due to high degrees of drug resistance and multi-drug resistance. Among *Acinetobacter* spp., the *A. calcoaceticus*-*A. baumannii* complex accounts for the majority of clinical disease [91, 92, 95]. Infection is largely confined to the hospitalized population where, similar to *P. aeruginosa*, it can cause pneumonia, UTI, bacteremia, and skin and wound infections among immuno-compromised patients [91, 92, 95]. Among *Acinetobacter* spp., there is an overall trend of increasing resistance to common agents over time, as shown by Fig. 23.12. Large increases in resistance to amikacin, imipenem, and piperacillin/tazobactam were apparent from 2006 to 2008. Data from TSN for colistin and polymyxin B is limited, but from 2007 to 2009, *Acinetobacter* spp. were 97% susceptible to colistin (n = 1,729) and 89% susceptible to polymyxin B (n = 1,030).

Against *A. baumannii* in 2008 (Table 23.22), 28% of isolates were resistant to imipenem and 45% of isolates were multi-drug resistant. Low percent susceptibility was observed for the evaluated agents against *A. baumannii* overall, presumably due to the high degree of multi-drug resistance and the limited activity of these agents against multi-drug resistant isolates.

Imipenem resistance and multi-drug resistance were most common among *Acinetobacter* spp., from LRTI relative to other specimen types (Table 23.23). Multi-drug resistance was very high among isolates from patients confined to the ICU (71%), and among the elderly population (60%).

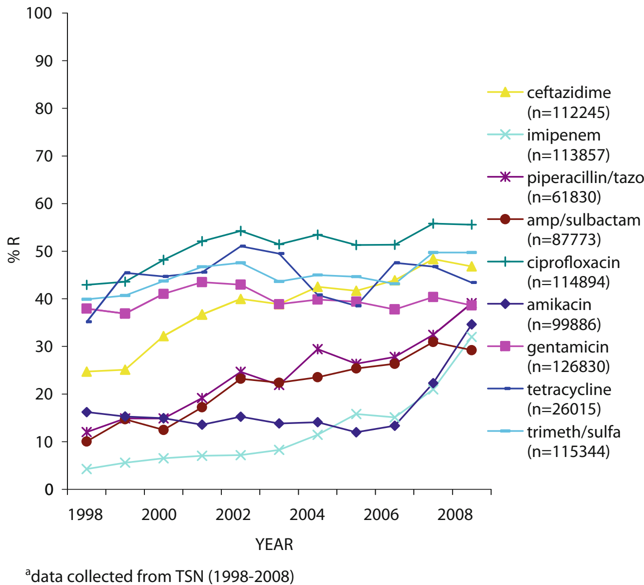


Fig. 23.12 Overall resistance trends in *Acinetobacter* spp.[a]

23.11 Challenges Ahead and the Need for Continued Vigilance

Many challenges for the treatment of bacterial disease exist in the era of resistance. The societal impact of the spread of drug resistance among commonly encountered pathogens is apparent with respect to the increased mortality and morbidity associated with treating resistant infections [1, 2, 96–99]. The current situation is ominous when one considers both the emergence and prevalence of multi-drug resistant pathogens and the lack of development and approval for use of novel antimicrobials refractory to existing mechanisms of resistance. The approval of new antibiotics in the US has steadily decreased over the past decade as reported by the IDSA [100].

Susceptibility trends among bacteria over the past decade, not solely in the US but globally, have, in many instances, been towards that of increasing resistance and multi-drug resistance. The current need for new agents is highlighted by the prevalence of resistance among "ESKAPE" pathogens today [2, 101]: vancomycin resistant Enterococci, methicillin resistant and multi-drug resistant *S. aureus*, beta-lactamase and carbapenemase producing *K. pneumoniae*, multi-drug resistant *Acinetobacter* spp., and *P. aeruginosa*, and beta-lactamase producing *Enterobacter* spp.. With limited therapeutic options, there is the likelihood of an increased reliance on drugs of last resort (e.g., colistin) and/or the few drugs that maintain activity against these organisms, the result being an increased risk in the emergence of resistance to the few therapeutic options available.

Table 23.22 Current activity profile against *A. baumannii* including resistant isolates[a]

Antimicrobial	All (N=349)				IPM NS[b] (N=120)				MDR[c] (N=157)			
Agent	MIC_{50}[d]	MIC_{90}[d]	%S[e]	%R[e]	MIC_{50}	MIC_{90}	%S	%R	MIC_{50}	MIC_{90}	%S	%R
Ceftazidime	8	>128	53.0	44.7	128	>128	6.7	91.7	64	>128	3.8	94.9
Cefepime	8	128	56.7	26.9	32	>128	10.8	60.0	32	>128	10.2	58.0
Imipenem	0.5	>32	65.6	28.1	32	>32	0.0	81.7	16	>32	24.8	62.4
Pip/tazo	16	>128	51.3	37.8	>128	>128	7.5	83.3	>128	>128	7.0	80.3
Ciprofloxacin	16	128	47.3	51.3	64	>128	1.7	98.3	64	>128	0.0	100.0
Tigecycline	0.5	2	–[f]	–	1	2	–	–	1	2	–	–
Tobramycin	1	>32	67.0	27.8	>32	>32	32.5	61.7	32	>32	37.6	55.4
Gentamicin	2	>32	57.0	39.8	>32	>32	13.3	82.5	>32	>32	15.3	80.3
Amikacin	4	>64	72.5	23.2	>64	>64	33.3	60.0	64	>64	42.7	51.0
Trimeth/sulfa	1	>32	53.6	46.4	>32	>32	13.3	86.7	>32	>32	13.4	86.6

[a]Data from TRUST 12 surveillance (2008)
[b]Imipenem non-susceptible
[c]MDR, multi-drug resistant defined as resistant to ≥3 of the following agents: amikacin, ceftazidime, ciprofloxacin, gentamicin, imipenem and pip/tazo
[d]MIC_{50} and MIC_{90} are reported as μg/mL
[e]Results interpreted in accordance with CLSI/FDA breakpoints as appropriate
[f]Dashed lines indicate CLSI/FDA breakpoints are unavailable for interpretation of susceptible (*S*), intermediate (*I*), and/or resistant (*R*)

Table 23.23 Variation in imipenem resistance (IPM R) and MDR among *Acinetobacter* spp. by specimen, patient location, and patient age[a]

	N[b]	%IPM R	N[c]	%MDR[d]
Specimen				
Blood	1223	27.0	269	28.6
LRTI	4470	45.2	874	68.6
Skin/wound	823	31.7	145	46.9
Patient location				
Inpatient	4401	36.9	1054	57.5
ICU	2975	46.6	635	71.2
Patient age				
≤17 years	942	6.4	178	5.1
18–64years	5784	32.9	1351	49.4
≥65 years	4194	41.2	871	60.0

[a]Data collected from TSN (2008–2009)
[b]Isolates with imipenem (IPM) results
[c]Isolates concurrently tested against ceftazidime, piperacillin/tazobactam, imipenem, ciprofloxacin, gentamicin, amikacin
[d]Resistance to ≥3 of the drugs listed in footnote c

The changing population in the world and its impact on the frequency of resistant infections must also be considered. The US Census Bureau estimates large increases in the proportion of elderly over the coming decades. From 2010 to 2050, there will be an estimated increase in people age 65–84 from 34 million (11% of the total population) to 66 million (16% of the total population) with an estimated increase in people age 85 and up from 4 million (2% of the total population) to 21 million (5% of the total population) [102]. Coinciding with this increase will undoubtedly be an increase in the amount of hospitalized patients or patients confined to long-term care facilities, where infections with antibiotic resistant organisms are much more likely to occur.

The general trend towards an overall increase in antibiotic resistance seen over the past decade, the decrease in drug development and approval, and the increasing proportion of elderly among the population emphasize the need for improved infection control practices and antimicrobial stewardship to better preserve the utility of the drugs currently available for use, and increased discovery, development, and approval of novel agents with activity against resistant organisms. These developments also underscore a sustained need to remain vigilant and aware of changes in these trends both globally and locally through surveillance initiatives designed to detect the spread of resistance, the emergence of new resistances, and to help guide local empiric therapy and infection control through the monitoring of *in vitro* activity profiles.

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Chapter 24
Chemical Properties of Antimicrobials and Their Uniqueness

Mark J. Macielag

Abbreviations

ClogP	Calculated logarithm of the partition coefficient
CMC	Comprehensive Medicinal Chemistry
DOS	Diversity-oriented synthesis
LogP	Logarithm of the partition coefficient
MRSA	Methicillin-resistant *Staphylococcus aureus*
TPSA	Total polar surface area

24.1 Introduction

The modern drug discovery paradigm emphasizes the use of synthetic compound libraries coupled with target-based high-throughput screens to identify new chemical entities for development. Despite the advances made in bacterial genomics and robotic screening technologies, the output of new drugs for regulatory approval in the antibacterial arena has been disappointingly low. Only two novel classes of antibacterial agents have been approved by the Food and Drug Administration in the last 40 years – the oxazolidinone class, of which linezolid is the prototype, and the lipopeptide class, represented by daptomycin. Several authors have speculated on the reasons for this lack of success [43, 1], but only recently have the chemical properties of the screening libraries been cited as a potential shortcoming in the discovery process [2–5]. Corporate compound libraries supply the raw materials for

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high-throughput screening campaigns and the resulting lead compounds that are optimized by medicinal chemists. Thus, the composition of the compound collection can have a direct bearing on the outcome of an antibacterial screening campaign and ultimately on the success or failure of antibacterial drug discovery programs.

It is instructive to review the history of a typical corporate compound screening collection to better understand why the yield of viable drug candidates from antibacterial screening campaigns has been extremely low. Historically, the corporate compound library was a repository for molecules synthesized during earlier drug discovery programs. Chemists would also submit intermediates to the collection to enable the serendipitous discovery of screening hits, to provide a supply of building blocks for future synthesis campaigns, and to boost their compound submission rate. A significant percentage of these intermediates contained reactive functionalities that were decidedly not 'drug-like.' Nevertheless, many of these compounds were included in assay plates for antibacterial target screens. With the advent of high-throughput screening technology, the scientific leadership at most pharmaceutical research companies realized that the number of compounds in the screening collection must increase dramatically to justify the investment in robotics. As a result, chemicals were procured from external vendors or brokers to supplement the collection of in-house synthesized compounds. Many of these purchased chemicals had been synthesized for purposes other than drug discovery and some came from synthetic compound collections of academic research labs, both in the U.S. and overseas. At about the same time combinatorial chemistry was hitting its stride, and vast numbers of randomly synthesized chemical libraries were being pumped into the corporate compound collections as feedstock for high-throughput screening campaigns with little attention paid to chemical properties.

By the late 1990s, pharmaceutical scientists began to realize the low percentage of viable lead compounds emanating from high-throughput screens might be traced to some extent to the nature of the chemical libraries themselves. An attempt was made to incorporate structural features frequently found in molecules that interact with target proteins. Typically, newly synthesized libraries were designed to promote interaction with G-protein coupled receptors, kinases, or proteases, for example. Rarely, if ever, were screening libraries biased toward antibacterial targets. At about the same time, pharmaceutical companies began to curtail their natural products research efforts for a number of reasons, including the perception this approach was slow, methodical, and incompatible with the timelines of modern drug discovery. The termination of natural products research at big pharma effectively eliminated the source of new molecular templates that had provided 75% of the existing antibacterial armamentarium.

As a result, antibacterial screening campaigns employed libraries of mostly lipophilic compounds with limited structural diversity, reflecting the output of combinatorial chemistry as well as the properties of the compounds purchased from external vendors. Most of the molecules were highly flexible with few features commonly found in antibacterial agents such as polar functional groups, complex ring systems, and chiral centers. Because most of the legacy programs at pharmaceutical companies

were directed at human health targets, the compounds were biased toward eukaryotic not prokaryotic targets. Given the nature of these screening libraries, the typical outcome of an antibacterial screening campaign would be the identification of surface-active agents and compounds with detergent-like properties that affected the integrity of bacterial cell membranes. When a biochemical assay was used as the primary screen, inhibitors of the target protein could frequently be discovered but generally these compounds had little or no whole cell activity. Rarely were compounds identified with activity against Gram-negative pathogens due to the excessive lipophilicity of the screening library. And even when hits were uncovered with promising antibacterial properties, the compounds had been designed to interact with eukaryotic targets. Thus, there were built-in toxicities and off-target activities that had to be overcome. A convincing argument can be made that the failure of antibacterial, target-based discovery programs over the last two decades is due, in large part, to the use of compound screening libraries that did not reflect the unique physicochemical property space occupied by the known antibacterial drugs.

24.2 Physicochemical Properties and Drug Action

Physicochemical properties are the drivers of the biological activity of drugs, influencing the absorption, distribution, metabolism, and excretion of the drug within the host, the penetration of the drug into the bacterium, and the interaction of the drug with its receptor protein. Some of the more pertinent physicochemical properties include the following:

- Molecular weight – Smaller molecules will diffuse more rapidly across cellular membranes and are more likely to undergo paracellular transit at epithelial cell barriers than high molecular weight substances. Drugs with molecular weights over 500 daltons (Da) are also prone to biliary excretion. High molecular weight compounds tend to bind with lower efficiency to target receptor proteins on a per atom basis, and as such are inferior starting points for chemistry lead optimization efforts.
- LogP – The logarithm of the partition coefficient (P) between water and octanol is a measure of the relative affinity of a drug for lipid versus water, or its liophilicity. A number of algorithms are available for the prediction of logP, including the chemical fragment-based method (clogP) of Leo and Hansch [6]. The lipophilicity of a drug is a basic determinant of the nature of enzyme/inhibitor as well as receptor/ligand interactions and is critically important in facilitating the passage of molecules through cell membranes and to distribute throughout the body. If a molecule has great affinity for the aqueous compartment as opposed to the lipid bilayer, it will not be able to enter cell membranes and will not be absorbed efficiently. Conversely, if a molecule is too lipophilic, it will not diffuse back out of the cell membrane and into the aqueous phase to access the site of action. Lipophilic drugs (i.e., those with a high logP value) are more prone to oxidative metabolism, hepatic extraction, and non-specific binding to plasma proteins.

- Total polar surface area (TPSA) – TPSA is defined as the area of the molecular surface of a drug, in square angstroms, occupied by electronegative oxygen and nitrogen atoms together with the attached hydrogen atoms. This parameter is a surrogate measure of the hydrogen-bonding capacity of a drug and appears to be inversely related to absorption from the GI tract and blood brain barrier penetration [7, 8].
- The number of hydrogen bond donor and acceptor atoms – The number of hydrogen bond donors in a drug molecule refers to the total number of NH and OH groups, whereas the number of hydrogen bond acceptors is the total number of N and O atoms. Both parameters reflect the ability of a drug to form hydrogen bonds, which may be a critical feature for productive enzyme/inhibitor or receptor/ligand interaction. In a molecule with a large number of hydrogen-bond donors or acceptors, a significant amount of energy is required to desolvate or break hydrogen bonds to water to facilitate passage through the lipid bilayer. Thus, highly polar molecules will have a difficult time passing through lipophilic barriers such as cellular membranes.
- The number of rotatable bonds – Rotatable bonds are single bonds in a molecule not in a ring that are bound to a non-terminal, heavy (i.e., non-hydrogen) atom. Amide C-N bonds are generally not included in the count due to their high rotational energy barrier. Reduced molecular flexibility due to a low number of rotatable bonds has been shown to be an important predictor of good oral bioavailablity [9]. The reasons for this are not completely clear, although it is thought that more rigid drug molecules are less prone to oxidative metabolism and P-glycoprotein mediated efflux than their more conformationally flexible counterparts [10, 11].

24.3 Physicochemical Properties of Antibacterial Drugs

The molecules that comprise the class of antibacterial agents and antibiotics are structurally diverse, consisting of low molecular weight synthetic drugs such as the fluoroquinolones, sulfa drugs, and anti-tuberculosis agents as well as oral drugs derived from natural products through semi-synthesis such as the macrolides and the oral cephalosporins. In contrast to drugs from other therapeutic areas, a large proportion of antimicrobial agents are administered parenterally in the acute care setting. Among these are a number of natural product-based antibiotics of significant structural complexity, including members of the aminoglycoside, tetracycline, glycopeptide, and streptogramin classes (Fig. 24.1).

Few attempts have been made to characterize or define the physicochemical properties that distinguish the antibacterial drugs and that are ultimately responsible for the unique pharmacological niche occupied by this class of compounds. Leeson and Davis compared the physicochemical properties of oral drugs that were launched between 1983 and 2002 by therapeutic area. They found that oral anti-infective drugs have the highest molecular weight, lowest lipophilicity, greatest number of oxygen and nitrogen atoms, the greatest number of hydrogen bond

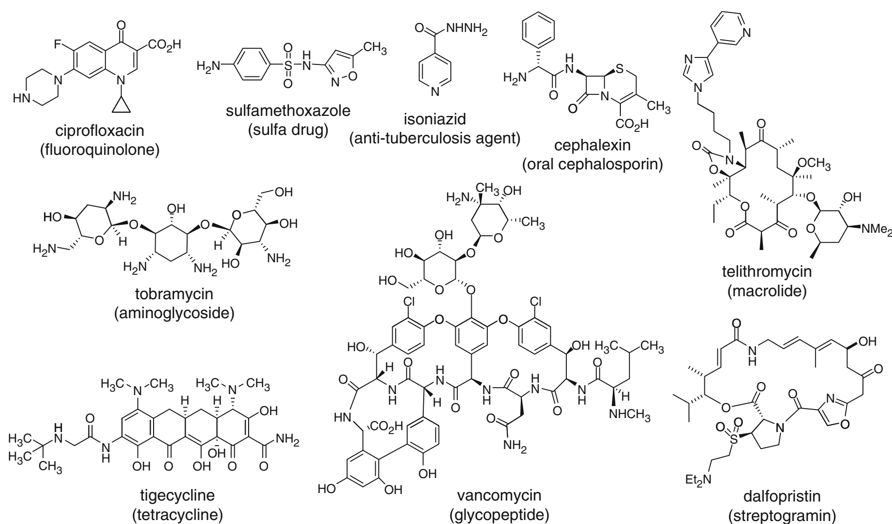


Fig. 24.1 Structural diversity of antibacterial drugs

acceptors, and the largest number of rings [12]. Their analysis did not discriminate among antibacterial, antifungal, antimalarial, antiparasitic, and antiviral drugs, however. An analysis of the physicochemical properties of parenteral drugs by Vieth and co-workers is especially germane to a discussion of antimicrobial agents, in light of the large number of drugs formulated for intravenous and intramuscular administration [13]. Compared to oral drugs, injectables have significantly higher molecular weights, greater polarity, and are more flexible.

More recently, O'Shea and Mozer analyzed the physicochemical properties of marketed and investigational antibacterial agents by their spectrum of activity [3]. Compared to a set of reference drugs, compounds with Gram-positive only activity had higher mean molecular weights, a larger number of hydrogen bond donors and acceptors, and greater total polar surface area. Compounds with Gram-negative activity had much higher relative polar surface areas (i.e., TPSA divided by total surface area of a molecule) than drugs from other classes. They were generally charged at physiological pH, and they had molecular weights less than 600 Da. In a similar vein, Gualtieri and colleagues showed that marketed antibacterial agents occupy a distinct region of physicochemical property space when compared to other drugs or the average chemical screening library [5]. Antibacterial agents were larger, with a greater number of rings, chiral centers, and hydrogen bond donors. Notably, they were more hydrophilic than most drugs and synthetic compounds. The authors stressed that such chemical properties are important to avoid excessive protein binding and to achieve the pharmacokinetic properties required for acceptable clinical efficacy.

At the time of this writing, a total of 80 systemic antibacterial agents and antibiotics with clinically useful activity against Gram-positive or Gram-negative pathogens are marketed in the United States or are in Phase III clinical trials (Table 24.1). Among these are compounds from 11 chemical classes, most highly represented by

Table 24.1 Antimicrobial agents used in the calculation of physicochemical properties

Agent	Oral gram-positive	Parenteral gram-positive	Oral gram-negative	Parenteral gram-negative
Aminoglycosides				
Amikacin		X		X
Gentamicin		X		X
Kanamycin		X		X
Tobramycin		X		X
Ansamycins				
Rifampicin	X			
Rifapentine	X			
β-lactams				
Amoxicillin	X		X	
Ampicillin		X		X
Aztreonam				X
Cefaclor	X		X	
Cefadroxil	X		X	
Cefazolin		X		X
Cefdinir	X		X	
Cefditoren		X		X
Cefepime		X		X
Cefixime	X		X	
Cefoperazone		X		X
Cefotaxime		X		X
Cefotetan		X		X
Cefoxitin		X		X
Cefpodoxime		X		X
Cefprozil	X		X	
Ceftazidime		X		X
Ceftibuten	X		X	
Ceftizoxime		X		X
Ceftaroline		X		X
Ceftobiprole		X		X
Ceftriaxone		X		X
Cefuroxime		X		X
Cephalexin	X		X	
Cephalothin		X		X
Dicloxacillin	X			
Doripenem		X		X
Ertapenem		X		X
Imipenem		X		X
Loracarbef	X	X	X	X
Meropenem		X		X
Nafcillin		X		
Oxacillin		X		
Penicillin G		X		
Penicillin V	X			
Piperacillin		X		X
Ticarcillin		X		X

(continued)

Table 24.1 (continued)

Agent	Oral gram-positive	Parenteral gram-positive	Oral gram-negative	Parenteral gram-negative
Diaminopyrimidines				
Iclaprim	X	X		
Trimethoprim	X		X	
Fluoroquinolones				
Ciprofloxacin	X	X	X	X
Delafloxacin	X	X	X	X
Gatifloxacin	X	X	X	X
Gemifloxacin	X		X	
Levofloxacin	X	X	X	X
Moxifloxacin	X	X	X	X
Trovafloxacin	X	X	X	X
Ulifloxacin		X		X
Glycopeptides				
Dalbavancin		X		
Oritavancin		X		
Telavancin		X		
Vancomycin		X		
Lincosamides				
Lincomycin		X		
Clindamycin	X	X		
Macrolides				
Azithromycin	X	X		
Cethromycin	X			
Clarithromycin	X			
Dirithromycin	X			
Erythromycin	X	X		
Telithromycin	X			
Streptogramins				
Dalfopristin		X		
Quinupristin		X		
Sulfonamides				
Sulfadiazine	X		X	
Sulfamethoxazole	X		X	
Sulfisoxazole	X		X	
Tetracyclines				
Doxycycline	X	X	X	X
Minocycline	X		X	
Tetracycline	X		X	
Tigecycline		X		X
Miscellaneous				
Chloramphenicol	X		X	
Colistin				X
Daptomycin		X		
Fosfomycin	X		X	
Linezolid	X	X		
Nitrofurantoin	X		X	

the β-lactams and fluoroquinolones, but aminoglycosides, ansamycin antibiotics, diaminopyrimidines, glycopeptides, lincosamides, macrolide antibiotics, streptogramins, sulfa drugs, tetracylines, and a small group of miscellaneous agents are also represented. The physicochemical properties of such a chemically diverse set of compounds can most effectively be discerned by grouping them by route of administration, given the distinct solubility and permeability requirements of intravenous and oral drugs. For purposes of the following analysis, an oral agent was considered to be one with a marketed or investigational oral dosage form whereas a parenteral agent was defined as a marketed or investigational drug with a formulation for parenteral injection. Classification according to this scheme furnishes 54 parenteral agents and 39 oral agents, including 13 compounds with both parenteral and oral dosage forms (Table 24.1).

To enable a relevant comparison of physicochemical properties, a reference set of drugs from other therapeutic areas was constructed from the MDL® Comprehensive Medicinal Chemistry (CMC) database (version 2006.1), which contains over 7,500 compounds from all therapeutic categories that have been approved for human therapy or have undergone clinical trials. Compounds from the CMC database that are classified as buffers, topical drugs, radiopaque and contrast agents, diagnostics, chelating agents, and the like, were not included in the property analysis. Physicochemical descriptors were computed in Pipeline Pilot™ (version 6.1, SciTegic, Inc.) using the default settings.

The unique chemical property space occupied by the antibacterial drugs can be readily illustrated by plotting the percentage of compounds from both the parenteral and oral categories as a function of the value of a particular physicochemical parameter and comparing the resulting distribution to drugs from other classes as represented by the CMC data set (Fig. 24.2a–f). The majority of drugs from the general reference set have molecular weights below 550 Da, with the largest percentage below 350 (Fig. 24.2a). In contrast, the molecular weight distribution of oral antibacterial agents is bimodal, with a substantial percentage between 350 and 450 Da, but with another group in the 700–900 molecular weight range. The latter are the macrolide and ansamycin antibiotics, compounds that achieve good oral bioavailability through carrier mediated transport mechanisms and passive membrane diffusion [14]. The latter process is facilitated by intramolecular hydrogen bonding interactions that serve to reduce polarity and minimize the desolvation energy at the lipid membrane interface. The molecular weights of the parenteral antibacterial agents tend to cluster around 350–450 Da, but the distribution is shifted to higher values compared to the CMC data set. In addition, a number of parenteral antibacterial agents have molecular weights greater than 1,000, including the glycopeptide antibiotics, the lipopeptide agent, daptomycin, the streptogramin derivative, quinupristin, and the peptide antibiotic, colistin.

A distinction between antibacterial agents and drugs from other therapeutic areas is apparent in the plot of clogP values (Fig. 24.2b). Whereas clogP values of the reference set demonstrate an approximately normal distribution centered around 2.5–3.5, both oral and parenteral antibacterial agents are considerably less lipophilic, with most clogP values clustering near 0 (i.e., equal affinity for lipid and water).

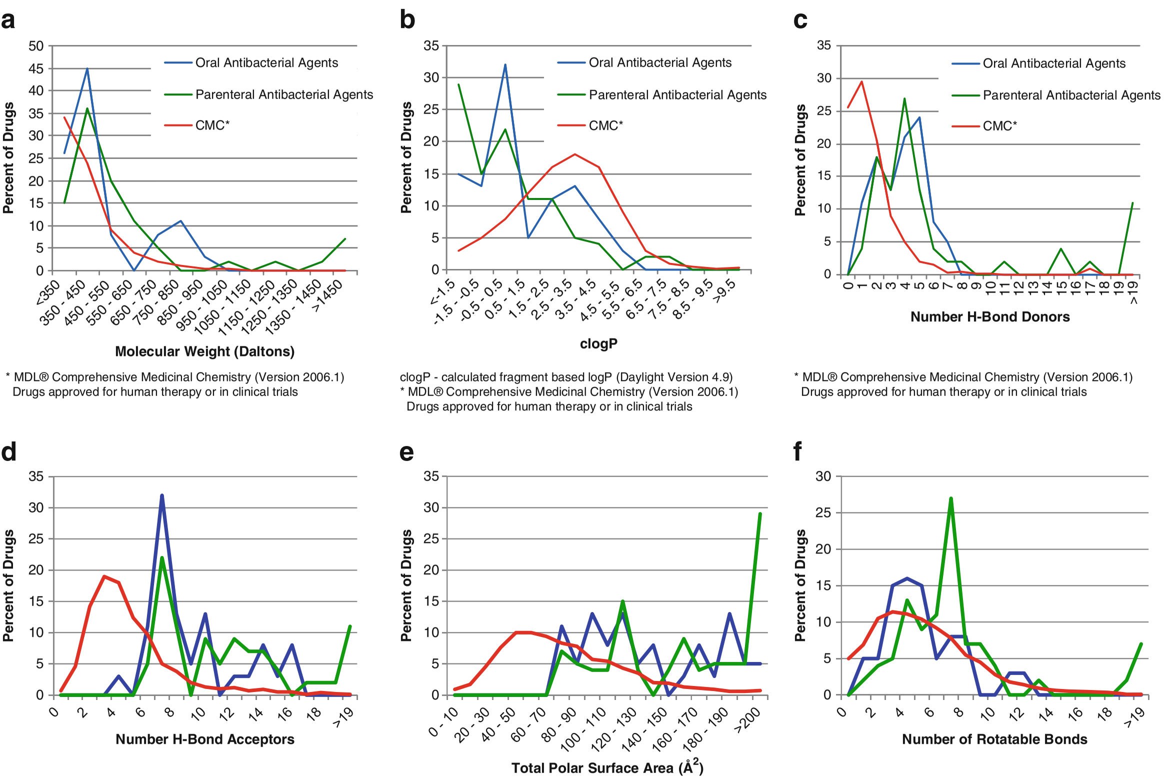


Fig. 24.2 Physicochemical property distribution of oral antibacterial agents, parenteral antibacterial agents and drugs from other therapeutic categories (CMC) based on (**a**) molecular weight, (**b**) clogP, (**c**) number of hydrogen bond donors, (**d**) number of hydrogen bond acceptors, (**e**) total polar surface area, and (**f**) number of rotatable bonds

This fact has major implications for the design of anti-infective screening libraries and for the chemical strategies employed to optimize hits from high-throughput screens, as it implies the judicious placement of polar functional groups to enhance antibacterial activity rather than the traditional reliance on the hydrophobic effect to boost potency. A bimodal distribution of clogP values is evident for the oral antibacterial agents, with a small subset of compounds closer to the reference set. Oral drugs, with high logP values again include the ansamycin and macrolide antibiotics as well as a group of more hydrophobic synthetic antibacterial agents (iclaprim, delafloxacin) and semi-synthetic antibiotics (dicloxacillin, penicillin V, clindamycin).

Oral and parenteral antibacterial agents consistently have a greater number of hydrogen bond donor (Fig. 24.2c) and hydrogen bond acceptor (Fig. 24.2d) groups than drugs from other therapeutic areas. Especially noteworthy are the large number of antibacterial agents, with more than ten hydrogen bond acceptors and the high percentage of parenteral antibacterial agents, with more than ten hydrogen bond donors. In fact, this is not particularly surprising in view of the peptidic and/or glycosidic framework of many antibiotics. Each peptide linkage contributes a minimum of one hydrogen bond donor and two hydrogen bond acceptor groups whereas most sugars carry four or five donors and acceptors. Thus, drugs with cyclic peptide or depsipeptide backbones such as the glycopeptide, lipopeptide, and streptogramin B antibiotics, and those with repeating sugar units such as the aminoglycoside, glycopeptide, and macrolide antibiotics, contain anywhere from 4 to 25 hydrogen bond donors and 14–43 hydrogen bond acceptors.

Consistent with the data in Figures 24.2c, d, the mean total polar surface area of oral and parenteral antibacterial agents is significantly greater than for most other drugs and the distribution is skewed toward much higher values (Fig. 24.2e). The relative percentage of electronegative nitrogen and oxygen atoms is quite high in antibiotics consisting of sugar, aminosugar, and peptide building blocks, and this is reflected in total polar surface area values of 180–700 $Å^2$.

An interesting distinction between parenteral antibacterial agents and other drugs, including the oral antibacterial agents, is apparent in the rotatable bond distribution plot (Fig. 24.2f). Parenteral antibacterial agents tend to be more flexible than drugs from the CMC reference set, as nearly 50% of the drugs in this class contain between 7 and 10 rotatable bonds. In contrast, oral antibacterial agents have a similar rotatable bond count distribution as the general drug set. In comparing the number of rotatable bonds of oral and parenteral β-lactam antibiotics, for example, 91% of oral β-lactams have less than seven rotatable bonds, whereas 63% of parenteral β-lactams have greater than seven rotatable bonds, primarily due to the bulky, flexible substituents appended to the heterocyclic core structure in the latter group.

Thus, the prototypical oral antibacterial agent has a similar molecular weight and rotatable bond count as most drugs approved for human therapy; however it has reduced lipophilicity and greater total polar surface area due to a larger number of hydrogen bond donor and acceptor functionalities. Notable exceptions to this composite profile include the macrolide and ansamycin antibiotics, which have high molecular weights and more customary logP values. The prototypical parenteral antibacterial agent, on the other hand, is unconventional by nearly every

Table 24.2 Physicochemical properties of antibacterial agents by source

Property	Synthetic	Natural product	CMC[a]
Molecular weight	356	616	339
clogP	–0.3	0.0	2.8
TPSA (Å)^2	108	206	68
# H-Bond donors	3.1	6.6	1.7
# H-Bond acceptors	7.7	13.7	5.0
# Rotatable bonds	4.1	8.2	4.1

[a]MDL® Comprehensive Medicinal Chemistry (Version 2006.1). Drugs approved for human therapy or in clinical trials

physicochemical measure, with a higher molecular weight, a lower logP value, a greater total polar surface area, more hydrogen-bond donors and acceptors, and greater flexibility than drugs from other therapeutic areas.

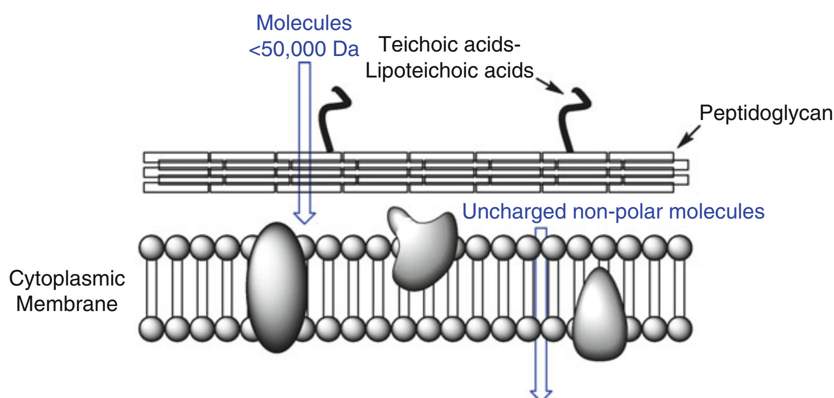
Antibacterial drug discovery has for many years been reliant on natural product research as a source of new chemotypes for lead optimization and development. In fact, 61% of the oral agents and 76% of the parenterals in Table 24.1 are either natural products themselves or derived from a natural product scaffold through semisynthesis. It is conceivable, therefore, that the physicochemical properties of antibacterial agents may be more a reflection of the chemical space occupied by microbial secondary metabolites than the chemical property requirements imposed by the nature of antimicrobial therapy. Examination of the physicochemical properties of antimicrobial agents by source, however, tells a different story (Table 24.2). Antibacterial agents derived from natural products are indeed larger, more polar, and more flexible than conventional drugs. They are distinguished by the high density of functionality and macrocyclic molecular skeletons typical of the products of polyketide synthases and non-ribosomal peptide synthetases. Although synthetic antibacterial agents have lower molecular weights and rotatable bond counts than natural product-based templates, they too are more polar than compounds in the CMC data set, with decreased lipophilicity and a larger number of hydrogen bonding functional groups. Therefore, the unique chemical characteristics of antibacterial agents are, to a large extent, an intrinsic property of an effective chemotherapeutic agent.

Why are the chemical properties of antibacterial agents so different from other drugs? It has generally been conceded that the preferred bacterial protein or membrane targets should bear little or no sequence or structural homology to mammalian gene products to improve the odds of identifying drugs with selective toxicity. Thus, an inhibitor or antagonist is more likely to possess unique pharmacophoric features in order to complement the unusual molecular architecture of the prokaryotic target. Hand in hand with the concept of target dissimilarity and ligand uniqueness is the requirement that an antibacterial agent avoid interaction with potential target proteins of the host. A satisfactory safety margin must be achieved in the face of the high blood levels required to inhibit the growth of target organisms in the infected tissue. As others have pointed out, antibacterial therapy is a ‘killing discipline’ wherein complete inhibition of several targets in multiple disparate

organisms is considered the norm, rather than partial modulation of a single target in one organism, as with most other medical conditions in which drugs play a treatment role [1, 15]. Such conditions impose unique constraints on the molecular architecture of antibacterial drugs.

The pharmacodynamic characteristics of bacterial killing dictate that the free, unbound antimicrobial agent be present at high enough concentrations and/or for a sufficient period of time to be effective in eradicating the infection. This places strict limits on the degree of plasma protein binding. Lipophilic drugs generally bind with greater avidity than hydrophilic molecules to serum proteins, such as albumin, α-1-acid glycoprotein and γ-globulin. Consequently, antibacterial agents must be sufficiently polar to reach free concentrations above the minimum inhibitory concentration of the infecting organism(s). At the same time, the drug must be sufficiently lipophilic to avoid rapid clearance mechanisms such as glomerular filtration that would reduce systemic exposure. Therefore, a delicate balance of the physicochemical properties must be maintained in an antibacterial drug in order to achieve optimal clinical efficacy [5].

Another important influence on the chemical properties of antibacterial agents is the nature of the membrane barriers that restrict access to the bacterial cytoplasm. Passage through the bacterial cell wall, and in the case of Gram-negative pathogens, the outer membrane, imposes critical structure and property limits on molecules that have designs on bacterial cell entry [3, 4]. For an antibacterial agent to access a protein target in the cytoplasm of a Gram-positive pathogen, the molecule must traverse the thick peptidoglycan layer, followed by passage through the lipophilic cytoplasmic membrane (Fig. 24.3). To penetrate the Gram-positive cell envelope the drug must exist in an uncharged or neutral form, must be of relatively low polarity, and must have limited flexibility. If, however, the drug target is located in the peptidoglycan matrix or on the outside of the cytoplasmic membrane, the requirement for penetration through the lipid bilayer is eliminated and the molecule can be more hydrophilic with greater conformational flexibility. The impact of the Gram-positive membrane barrier is evident in the physicochemical properties of the drugs with activity against these microorganisms (Table 24.3). Although larger and more polar than most conventional drugs, the property value means and ranges of oral Gram-positive compounds are substantially lower and narrower, respectively, than for parenteral Gram-positive antibacterial agents. The chemical properties required for facile diffusion through the GI epithelium and the bacterial cytoplasmic membrane are quite similar. Therefore, it is not surprising, that the mechanism of action of over 70% of the orally bioavailable Gram-positive drugs involves inhibition of targets located in the bacterial cytoplasm. The oral compounds that bind to extracellular targets (β-lactam antibiotics) are generally more hydrophilic than those that penetrate into the cytosol. Whereas the mean and range of clogP values for drugs that inhibit cytosolic targets are 1.06 and −2.95–5.41, respectively, the corresponding values for compounds that inhibit extracellular targets are −0.61 and −2.51–2.98. The reduced lipophilicity of the latter group is consistent with the extracellular mode of action of the β-lactams and the fact that these compounds undergo facilitated transport rather than passive diffusion at the GI epithelial barrier.



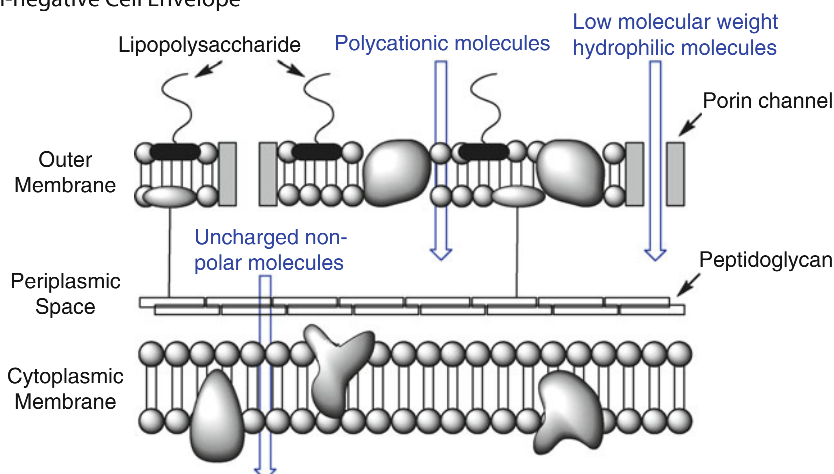


Fig. 24.3 Effect of route of entry on the chemical properties (in *blue*) of antibacterial agents

Parenteral Gram-positive drugs span a wide range of physicochemical properties and include compounds that stretch the limits of what would normally be considered drug-like properties (Table 24.3). For example, the lipopeptide antibiotic, daptomycin, has a molecular weight of 1621 Da, TPSA of 702 Å^2, and a clogP value of −2.43, with 25 or more hydrogen bond acceptors, hydrogen bond donors, and rotatable bonds. Sixty percent of the parenteral Gram-positive agents have an extracellular mechanism of action, with most binding to the peptidoglycan matrix or inhibiting enzymes involved in its biosynthesis. Therefore, the stringent property requirements imposed by dual passage through the GI epithelium and the bacterial cytoplasmic membrane are lifted for many compounds of this class. The high aqueous solubility necessitated by the route of administration is reflected in the lower clogP value (−0.37) than for the oral Gram-positive agents (0.59) (Table 24.3).

Table 24.3 Mean and (range) property values of antibacterial agents based on spectrum of activity and route of administration

Property	Gram-positive oral	Gram-positive parenteral	Gram-negative oral	Gram-negative parenteral[a]
Molecular weight	450 (138–877)	589 (299–1817)	356 (138–457)	453 (299–646)
clogP	0.59 (−2.95–5.41)	−0.37 (−6.67–7.21)	−0.53 (−2.95–1.95)	−1.4 (−6.7–2.0)
TPSA (Å)^2	132 (70–217)	195 (71–702)	120 (70–185)	160 (73–332)
# H-bond donors	3.7 (1–7)	6.3 (1–25)	3.8 (1–7)	5.0 (1–17)
# H-bond acceptors	9.2 (4–16)	13.1 (6–43)	7.8 (4–12)	10.7 (6–18)
# Rotatable bonds	4.8 (1–12)	7.7 (1–35)	3.8 (1–8)	6.0 (1–10)

[a]Does not include colistin (MW = 1169), the mechanism of action of which involves disruption of the Gram-negative outer membrane

Drugs directed at Gram-negative targets have a more restricted range of physicochemical properties due to the unique characteristics of the Gram-negative cell wall and the ubiquitous presence of multidrug efflux pumps (Table 24.3) [3, 4, 16]. The formidable outer membrane consists of proteins, phospholipids, and lipopolysaccharide, which provides a permeability barrier to hydrophilic substances (Fig. 24.3). The principal entry into the bacterium is through the channels formed by the polar amino acid side chains of the porin proteins, which restrict passage of lipophilic substances. The constitutive efflux pumps of the Enterobacteriaceae and pseudomonads have broad substrate specificity with a general preference for amphiphilic compounds that reside at the cytoplasmic membrane interface [17]. Examination of the range of physicochemical property values for Gram-negative agents shows an upper limit cutoff of 650 Da for molecular weight and 2 for the clogP value (Table 24.3), consistent with the normal physiologic role of porins to regulate movement of small hydrophilic nutrients through the outer membrane. Thus, in order to penetrate the Gram-negative cell envelope, the molecule must be of relatively low molecular weight and lipophilicity. The sole exception to this rule is colistin (a mixture of polymyxin derivatives) with a molecular weight of 1169, which functions to disrupt the structural integrity of the outer membrane by displacing bridging divalent cations on the outer leaflet.

An additional criterion is applicable for Gram-negative drugs that are administered orally or that must diffuse through the inner cytoplasmic membrane to reach their targets [3, 18]. Such molecules must be polar, weakly charged or zwitterionic to pass through the porin channels, but they must also exist in an uncharged state with sufficient lipophilicity to cross the GI epithelium and/or to penetrate the cytoplasmic membrane. Only a few compound classes have the requisite balance of hydrophobic/hydrophilic properties, those being the diaminopyrimidines, the fluoroquinolones, the sulfa drugs, the tetracyclines, chloramphenicol, and nitrofurantoin (Table 24.1). Although the aminoglycosides and fosfomycin affect intracellular targets, both types of drug take advantage of solute-specific transport systems to enter the bacterial cell cytoplasm [4]. The fluoroquinolones are unique in that they generally contain both acidic and basic functional groups with pK_a values close to physiological pH. Consequently, neutral, zwitterionic, and charged forms of the molecule are in equilibrium, permitting passive transport through the various permeability barriers of the pathogen and host. This important structural characteristic has also enabled the facile development of oral and parenteral dosage forms in contrast to most other drugs with Gram-negative activity.

The β-lactams are the sole class of antibiotics that bind to target proteins located in the periplasmic space of Gram-negative bacteria. These drugs tend to be more polar on average than compounds that inhibit intracellular targets, as penetration of the cytoplasmic membrane is not required for their mechanism of action. Nevertheless, the same size restriction applies as for other agents in order to successfully navigate the porin channels. Analogous to the Gram-positive agents, parenteral Gram-negative compounds have lower mean clogP values than their oral counterparts due to the aqueous solubility requirements imposed by the route of administration (Table 24.3).

24.4 The Impact of Physicochemical Property Filters on the Outcome of Antibacterial Screens

The pharmaceutical industry has been under intense pressure to reduce the time required to discover and develop new therapeutics. As a result, the medicinal chemist has shifted his focus from solely improving *in vitro* potency to simultaneous optimization of multiple parameters such as potency, efficacy, safety, and ADME properties. Although combinatorial chemistry, parallel synthesis, and high-throughput screening have enabled the preparation and evaluation of huge numbers of compounds, these techniques alone do not provide the means to faster drug discovery. The chemical structures of the compounds in the library and the associated physicochemical properties are now recognized as critical elements in a successful drug discovery program.

In the late 1990s and early 2000s, the quality of corporate compound screening collections began to suffer, due to the addition of excessively lipophilic combinatorial libraries and purchased chemicals. In response, computational chemists proposed a series of physicochemical property filters or 'rules of thumb' for chemical properties favoring oral bioavailability of drugs. The objective was to weed out compounds with poor physicochemical properties early in the discovery process, before a significant amount of time, resources, and money were invested in a molecule with potentially fatal flaws. The most widely quoted set of guidelines is Lipinski's pioneering 'Rule-of-Five' [19], which states that poor absorption or permeation is more likely when at least two out of the following four parameters are out of the range:

- There are more than five hydrogen bond donors
- The molecular weight is over 500
- The clogP is over 5
- There are more than ten hydrogen-bond acceptors

Subsequently, Egan et al., developed a predictive model of human passive intestinal absorption using compounds reported in the literature and applying multivariate statistical methods [20]. Two parameters in particular, PSA and AlogP [21], were sufficient to predict with 95% confidence that a test compound would have low (<30%) or high (90%) intestinal absorption in humans. For compounds with high oral absorption, the upper limits of PSA and AlogP were 131.6 Å^2 and 5.88, respectively.

Veber et al., published a simple set of rules that emerged from analysis of rat pharmacokinetic data acquired by GlaxoSmithKline [9]. They found that good oral bioavailability is more likely when there are ten or fewer rotatable bonds in a molecule and the total polar surface area is less than or eq 140 Å (or the number of hydrogen bond donors and acceptors is 12 or fewer).

More recently, Martin proposed a more refined set of criteria that take into account the ionization state of a drug at physiological pH [22]. The author determined that oral bioavailability greater than 10% in the rat is more likely for anionic

molecules, like carboxylic acids, for example, when the total polar surface area is less than or equal to 75 Å^2. For neutral, zwitterionic, or cationic molecules, the Lipinski 'Rule-of-Five' applies.

The influence of these or similar rules for drug-like properties has been pervasive throughout the drug discovery process, since their formulation a decade ago. In particular, such rules have been used in supplementing corporate compound collections by guiding the design of compound libraries from parallel synthesis and aiding in the selection of compounds for purchase from external vendors. Property filters have also been used in the assembly of focused libraries for screening, including against antibacterial targets. Once compounds had been identified from high-throughput screens, property filters were used to prioritize the hits for optimization by medicinal chemistry and to eliminate compounds that lie outside the range. Finally, rules for drug-like properties have been used to direct medicinal chemistry lead optimization efforts. For example, a score, based on whether a compound conforms to these rules, may be reported back to the chemist who registers the compound in the corporate database.

In determining the potential impact of physicochemical property filters on the outcome of antibacterial screening campaigns, it is important to examine some of the assumptions used in their design. First, it was assumed that a drug would be administered orally. Intravenous and non-systemic agents were generally not included in the basis set of compounds used to determine drug-like properties. As mentioned previously, the majority of antibacterial agents are administered by injection, including most drugs for serious nosocomial infections. Parenteral drugs are more polar and generally contain ionizable functional groups that enhance aqueous solubility. Thus, the properties of many of these compounds would be expected to lie outside the designated range.

Second, it was assumed that orally administered drugs would undergo passive absorption from the GI tract. Compounds that are substrates for transporter proteins have structure-activity relationships that deviate from the norm, which tend to confound analysis of physicochemical properties. As a result, drugs that were known or suspected to undergo carrier-mediated transport were typically not included in the training set. Although many oral antibacterial agents are absorbed passively through the GI epithelium such as sparfloxacin and chloramphenicol [23, 24], a large percentage are substrates for biological transporters. Most oral cephalosporins are well known to be substrates for dipeptide transporter proteins such as the PepT1 transporter, which enables these drugs to pass through epithelial cells and into the systemic circulation [25]. A distinct active transport system has also been shown to aid the absorption of certain members of the fluoroquinolone class, including levofloxacin and grepafloxacin [28]. Similarly, a carrier with limited capacity in the intestine appears to facilitate absorption of members of the tetracycline class of antibiotics [26].

Finally, in formulating the rules for drug-likeness, no attempt was made to distinguish among the various therapeutic classes of drugs. The application of a uniform physicochemical property filter across all targets, as has been done at most pharmaceutical companies, implies that compounds from all therapeutic categories behave similarly and have comparable properties; the evidence would suggest otherwise.

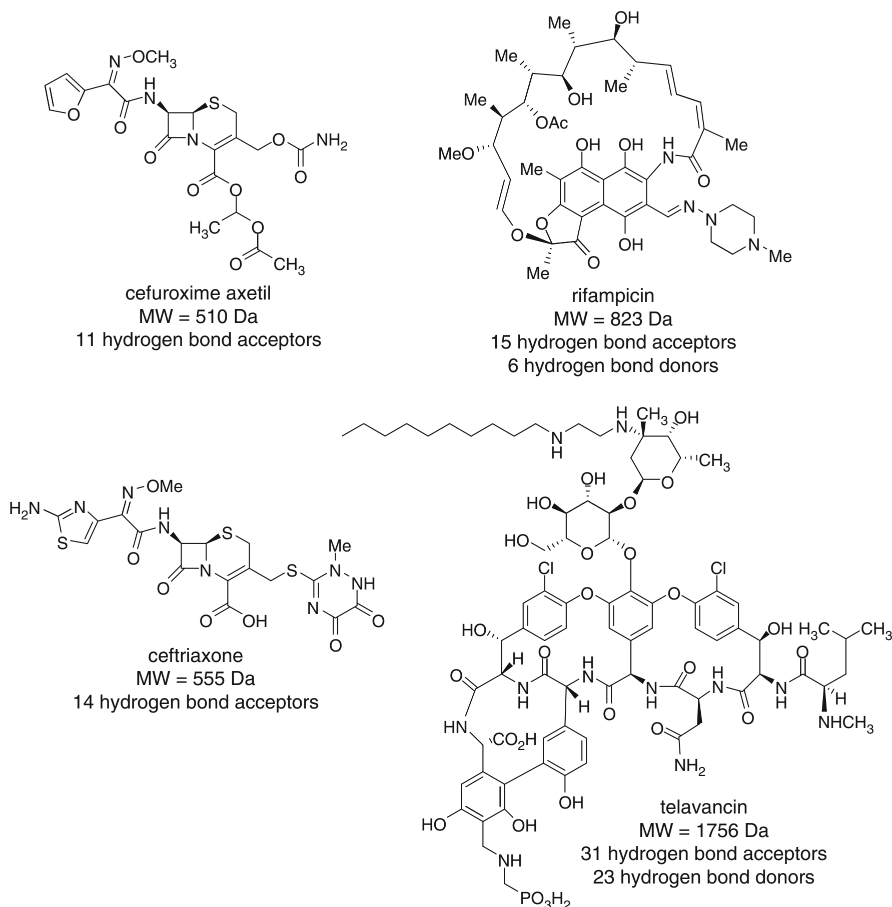


Fig. 24.4 Structures of antibacterial agents that violate the 'Rule-of-Five'

As noted in Sect. 3 of this chapter, antibacterial drugs have unique physicochemical properties and must be analyzed differently than most drugs. Moreover, Lipinski had already observed in 1997 that drugs from certain categories tended to be outliers in physicochemical property space, including antibiotics, antifungals, vitamins, and cardiac glycosides [19]. Many oral and parenteral antibacterial agents violate the 'Rule-of-Five,' such as the oral and parenteral cephalosporins, the glycopeptide antibiotics, and the ansamycin antibiotics (Fig. 24.4).

The above physicochemical property guidelines have demonstrated their value when applied in the proper context, such as for aiding the design of synthetic libraries directed at human health targets. However, the rules are inconsistent with the properties of most of the existing antibacterial armamentarium. It follows that compound libraries that conform to these rules may not be suitable for antibacterial screens. In retrospect, the lack of success in recent years in identifying novel antibacterial leads from corporate compound collections is not surprising.

24.5 New (and Old) Strategies for the Design of Antibacterial Screening Libraries

Chemical screening libraries designed to interact with bacterial targets must reflect the unusual chemical properties of antimicrobial agents to improve the success rate of identifying new chemical entities for infectious disease. The 'one-size-fits-all' approach that has been used in the assembly of compound libraries for screening against targets from all therapeutic areas has severely handicapped the discovery of novel lead compounds for antibacterial drug discovery. In view of the above mentioned physicochemical property requirements, chemical libraries for an antibacterial screening campaign should incorporate both natural products and compounds derived from small molecule synthetic chemistry.

Despite the perceived shortcomings of natural products research in the modern drug discovery paradigm, compounds from natural sources have distinct advantages that cannot be overlooked. Billions of years of natural selection have optimized these structures for protein interaction and antibacterial activity. Natural products may be considered privileged structures with core scaffolds that have been biosynthesized to interact with a limited number of existing protein folds, explaining why hit rates from high-throughput screens are frequently much higher than for small molecules [27]. In addition, the density of functionality inherent in many natural product templates enables the efficient inhibition of multiple protein targets simultaneously [15]. The complex structural frameworks of many natural products generally make them poor substrates for metabolizing enzymes, which endows them with superior pharmacokinetic and pharmacodynamic properties compared to small molecules. An additional attractive feature of natural product antibiotics is their evolved ability to access cytoplasmic targets, either through passive diffusion or active transport mechanisms [4], a property difficult to engineer into synthetic small molecules. The value of natural products, as a source of antimicrobial agents, is clearly evident from the fact that three-quarters of marketed antibacterial agents are natural products or semi-synthetic derivatives of natural products, a higher fraction than for drugs from other therapeutic areas (Table 24.1). This trend has continued in the last decade, with six of seven antibacterial drugs recently marketed or submitted for regulatory review in the United States being of natural product origin. (Natural product-based: doripenem, ceftobiprole, dalbavancin, telavancin, oritavancin, cethromycin; synthetic: iclaprim).

Natural products research has been viewed as the domain of specialists and has been unfairly tarred with the reputation of being time-consuming, costly, and incompatible with high-throughput screening approaches. However, recent advances in instrumentation, robotics, and screening methodology have dramatically improved productivity [29, 30]. Although much of the low hanging fruit has already been picked, only a small fraction of the bioactive compounds from common antibiotic-producing organisms, such as the actinomycetes, is estimated to have been discovered thus far [31, 32]. Furthermore, recent advances in newer methods for antibiotic discovery such as the use of uncultivated microbes [33], genome mining [34], and

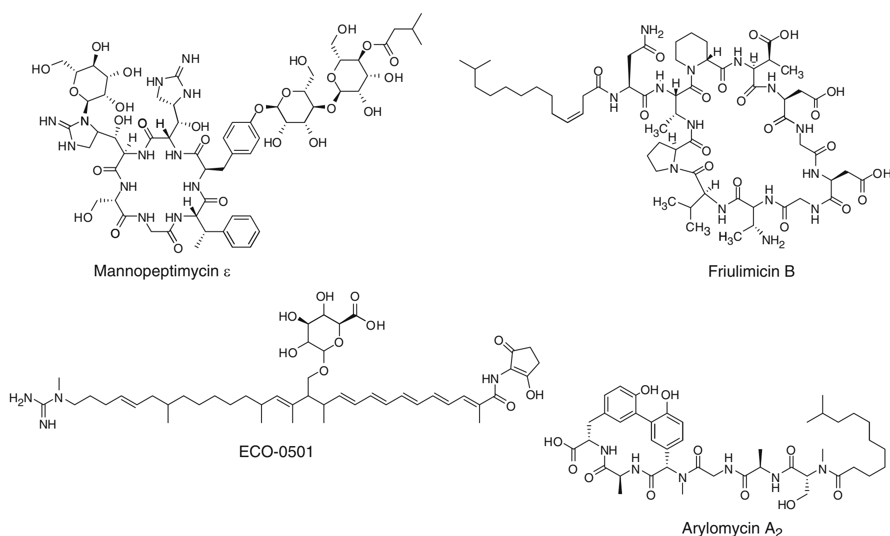


Fig. 24.5 Structures of recently identified antibiotics

metagenomic libraries [38], are likely to yield novel compounds with increasing regularity, supporting the argument that natural products chemistry should be integrated into the routine antibacterial discovery flowchart. Production of 'unnatural' natural products through genetic manipulation of polyketide synthases offers yet another means to interrogate previously inaccessible regions of chemical property space [39].

In the last decade, several antibiotics have been identified which have been underexploited by medicinal chemistry due to the contemporary shift of resources toward the generation of small molecule screening libraries. Among the antibiotic classes that have been reported recently are the following:

- The mannopeptimycins, from *Streptomyces hygroscopicus*, that interfere with late stage cell wall synthesis by binding to lipid II and inhibiting transglycosylation [35]
- Friulimicin B, a lipopeptide in clinical development that also targets late-stage cell wall synthesis by inhibiting the synthesis of the lipid I precursor [36]
- ECO-0501, the discovery of which from the vancomycin-producer *Amycolatopsis orientalis* exemplified the use of genome scanning strategies for the identification of new antibiotics [40]
- The arylomycin antibiotics, which exert their antibacterial activity by inhibiting the Type I signal peptidase, an essential protein for bacterial viability and growth [41]

The structures of these antibiotics highlight a degree of chemical diversity and density of functionality that is not readily accessible through conventional synthetic chemistry (Fig. 24.5).

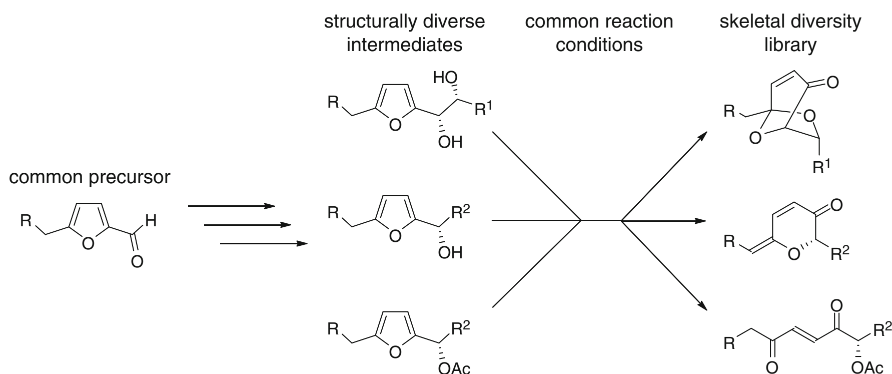
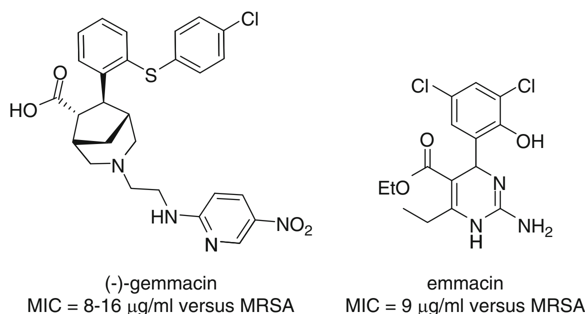


Fig. 24.6 An example of the skeletal diversity accessible by diversity-oriented synthesis [43]

Small molecule synthetic chemicals also deserve a place in a standard antibacterial screening library. The structural diversity of synthetic chemicals is limited only by the imagination of the medicinal chemist. Small molecules provide the ideal platform for iterative approaches to the design of inhibitors of a specific bacterial target protein, either through structure-based drug design or fragment-based approaches using X-ray crystallography, mass spectrometry, or nuclear magnetic resonance. Nevertheless, it is important that the chemical properties of these molecules be in a range that is suitable for antibacterial lead optimization. Given the similarities between the molecular property values of natural products and marketed antibacterial drugs, synthetic compound libraries for antibacterial drug discovery should be designed with an eye toward emulating the chemical properties and diversity of natural products. The focus must shift from chemical strategies driven largely by availability of reagents and ease of synthesis to novel chemistries that incorporate some of the complex structural features of natural products, including polycyclic carbon skeletons, multiple chiral centers, and a higher degree of oxygenation.

Of the various chemical strategies proposed to enhance the biological relevance of synthetic compounds, diversity-oriented synthesis (DOS) is particularly well-suited to the design of compound libraries directed at antibacterial targets [42, 44]. In contrast to the standard combinatorial library that samples a small region of chemical property space, a small molecule collection generated by DOS will typically span a much broader region of bioactive chemical space, including areas inhabited by natural products. Such libraries are both structurally diverse and structurally complex, making it more likely to discover compounds with biological activity, particularly in whole cell antibacterial screens where the full complement of targets is available. In a compound library produced by DOS, structural diversity may result from variations in the nature of the building blocks, the molecular skeleton, the appended functional groups, or the relative orientation of substituents. Skeletal diversity is widely recognized as the most effective way to interrogate large regions of chemical property space (Fig. 24.6). Consequently, most of the emphasis

Fig. 24.7 Structures of (−)-gemmacin and emmacin



in DOS has been on the development of new methods for the production of multiple scaffold libraries [44].

DOS libraries designed specifically for screening in whole cell antibacterial assays have been used to identify compounds with activity against methicillin-resistant *Staphylococcus aureus* (MRSA) (Fig. 24.7). In particular, the mechanism of action of (−)-gemmacin, a molecule with a 3-azabicyclo[3.2.1]octane skeleton, involves selective disruption of the bacterial cell membrane [45]. The dihydropyrimidine derivative, emmacin, was shown to be an uncompetitive inhibitor of bacterial dihydrofolate reductase, with an IC_{50} value of 5.4 μM [46]. Both compounds represent a potential starting point for chemistry optimization to identify more potent and selective antibacterial agents for development.

Recent years have witnessed an increase in the number of compound libraries built around a specific core structure (particularly complex ring systems) from natural products [47]. The rationale for this approach is based on the hypothesis that the core scaffolds of natural products have evolved to engage with a limited number of common tertiary structural elements of target proteins. Thus, these scaffolds serve as biologically validated starting points for the construction of chemical libraries with a greater probability of producing a desired biological effect [48]. The objective of such a 'biology-oriented synthesis,' however, is to identify compounds with biological activities distinct from the original parent molecule by varying the functionality in the periphery of the scaffold.

One of the early successes in the identification of novel antibacterial agents using this strategy resulted from high-throughput antibacterial screening of a 10,000-member compound library of 2,2-dimethylbenzopyran derivatives [49]. The 2,2-dimethylbenzopyran motif was originally selected as the library scaffold, due to its ubiquitous presence in natural products, such as robustic acid, as well as in a multitude of compounds with a range of biological activities [50]. Within this screening library, a number of benzopyran-derived cyanostilbene analogs were identified as active against an abbreviated panel of Gram-positive bacteria. Further optimization of the screening hits led to a novel compound with activity comparable to vancomycin against several MRSA strains (Fig. 24.8).

A more recent example of 'biology-oriented synthesis' is illustrative of its application to the identification bacterial enzyme inhibitors [51, 52]. The macrolines are

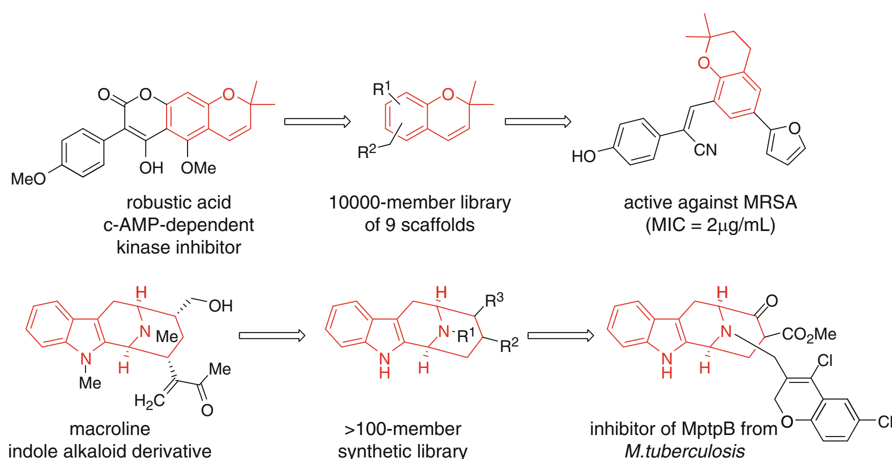
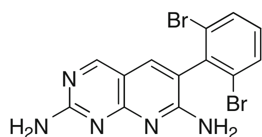


Fig. 24.8 The discovery of novel antibacterial agents by 'biology-oriented synthesis' (core structures highlighted in *red*)

a family of indole alkaloid derivatives with a range of biological activities, including antiamoebic, antiplasmodic, and antihypertensive properties. Inspired by the tetracyclic cycloocta[*b*]indole framework of these natural products, a small library of more than 100 enantiomerically pure macroline analogues was synthesized and screened against a number of eukaryotic and prokaryotic protein phosphatases [53]. Included among the enzymes in the screening panel were MptpA and MptpB, two tyrosine phosphatases secreted by *Mycobacterium tuberculosis* to disrupt the host defense mechanism by dephosphorylation of proteins involved in the interferon-signaling pathway. A number of selective inhibitors of MptpB were identified with IC_{50} values in the low micromolar range (Fig. 24.8). Extensive NMR spectroscopy studies revealed that these inhibitors bind to the enzyme at a site distant from the active site. The authors concluded that the cycloocta[b]indole scaffold may represent a promising starting point for the development of novel antimycobacterial agents. Although largely underexploited for the identification of novel antibacterial agents, these successful examples of 'biology-oriented synthesis' should encourage its broader application to antibacterial discovery in the future.

A new approach to identification of novel antibacterial agents, termed 'library repurposing,' takes advantage of the inherent eukaryotic bias in pharmaceutical compound collections by focusing on antibacterial targets with high sequence and/or structural homology to human drug targets [54]. Screening of the Pfizer compound collection against a membrane-compromised, efflux-pump deficient strain of *Escherichia coli* identified several pyridopyrimidines with whole cell activity (Fig. 24.9). Although poorly active against wild type *E. coli*, *Pseudomonas aeruginosa*, and Gram-positive bacteria, a number of analogues showed good activity against fastidious Gram-negative organisms, including *Haemophilus influenzae* and *Moraxella catarrhalis*. Interestingly, structurally related pyridopyrimidines were pre-



pyridopyrimidine
MIC = 0.125 μg/ml versus
Haemophilus influenzae

Fig. 24.9 Structure of a pyridopyrimidine inhibitor of the Gram-negative biotin carboxylase subunit of acetyl-CoA-carboxylase

viously shown to be potent ATP-competitive inhibitors of eukaryotic protein kinases. Reverse genetics experiments, biochemical assays, and X-ray crystallography were used to unambiguously demonstrate that the pyridopyrimidines bind to the ATP-binding site of the biotin carboxylase subunit of acetyl-CoA-carboxylase, which catalyzes the first committed step in fatty acid biosynthesis. Co-crystal structures of numerous pyridopyrimidine analogues bound to biotin carboxylase revealed a distinct binding mode compared to eukaryotic kinases, suggesting the potential for target selectivity. In fact, follow-up screening against mammalian acetyl-CoA carboxylase and a panel of serine/threonine and tyrosine kinases showed that the most potent inhibitors were selective for bacterial biotin carboxylase. Fragment-based drug design approaches were used to expand the number of chemotypes with physicochemical properties suitable for lead optimization [55]. An analogous 'repurposing' strategy has been proposed for identifying novel antibacterial agents that function by antagonizing ion channels or inhibiting prenyltransferases [56]. Such a strategy effectively constitutes a shift in antibacterial target space rather than the chemical property space of the screening library.

24.6 Perspective

The inexorable rise in drug resistance, particularly among the Gram-negative bacteria, has signaled an urgent need for new antibacterial agents with novel mechanisms of action. The bacterial genomics revolution of the last 15 years has provided a wealth of essential targets for exploitation. Ironically, during the same period of time, the quality and relevance of pharmaceutical screening libraries was badly neglected, making it impractical to take advantage of this emerging bacterial target space. The paucity of attractive lead compounds resulting from high-throughput screens has made it evident that microbiology and chemistry must work in concert to improve the probability of successfully developing new antibacterial drugs.

Antimicrobial agents occupy an unusual sector of chemical property space, due to requirements for cellular penetration and high free drug concentrations in the plasma. Compound libraries intended for whole cell or biochemical screens should be designed to conform to these unique property requirements. In constructing the library, the chemist should take into account the route of administration, the desired spectrum of

activity, and the cellular location of the target within the bacterium, as each of these factors may dramatically influence the appropriate chemical properties.

The process of medicinal chemistry lead optimization has traditionally increased molecular weight and logP of the chemical series under investigation, owing to a fixation with potency and target affinity. In the case of antibacterial chemistry, however, potency considerations alone are unlikely to produce effective drugs. Attention must also be directed toward molecular properties affecting phenomena that are less easily quantified, such as drug transport and non-specific binding. The analysis of physicochemical properties earlier in this chapter suggests that proper placement of polar functionalities rather than lipophilic groups may represent a more effective strategy for advancing a novel series of inhibitors by increasing intracellular concentrations of drug or reducing protein binding.

Natural products continue to represent an ideal platform for antibacterial drug discovery, due to their structural complexity, functional group density, and the evolved ability to penetrate the bacterial cell envelope. The challenges associated with dereplication, isolation, structure elucidation, and chemical synthesis of analogues are often cited as impediments to the reintroduction of natural products as a practical source of antibacterial compounds. However, advances in technologies for purification and characterization of structurally complex molecules as well as new methods for identification and production of previously unknown antibiotics make this an indispensible source of molecular diversity.

Knowledge of the factors influencing bacterial cell penetration, transport, cellular efflux, and protein binding of antibacterial drugs is increasing rapidly, and it is likely that chemical property filters, analogous to the ‘Rule of Five’ will be developed in the future to help guide antibacterial drug design. Until that time, scientists involved in antibacterial chemistry would be well advised to study the properties of the compounds in the existing antibacterial armamentarium and to apply those lessons to their research.

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Chapter 25
Natural Products in the 21st Century

Sheo B. Singh

25.1 Introduction

Medical preparations from plants and plant extracts have been used for the treatment of human diseases for thousands of years in the eastern cultures and are still practiced there in the form of Ayurvedic and Traditional Chinese Medicine. The follow-up of traditional medical know-how led to the chemical isolation, characterization, and biological testing of active natural product principles leading to the discovery of aspirin, morphine, and quinine (Fig. 25.1). These discoveries constituted the beginning of the current western medicinal practice [1–3]. Many of the preparations from plants were also used as antiseptics and for the treatment of infections. However, the discovery of penicillin, from *Penicillium notatum,* and its use as antibiotic for treatment of bacterial infections was the first example of a purified and structurally characterized natural product used as a single agent. This discovery revolutionized medical practice, saving both human and animal lives. The discovery of penicillin allowed for the establishment of new disciplines of fermentation microbiology and microbial chemistry. The success of penicillin led to unparalleled efforts by government, academia, and the pharmaceutical industry to discover new compounds from natural sources for the treatment of bacterial infections, resulting in the discovery of nearly all classes of natural product antibiotic scaffolds by 1962. These compounds were discovered simply by measuring zones of inhibition of bacterial strains on agar plates after applying whole broth or extracts obtained from microbial fermentations. Despite major technological advances, the approach to identify novel antibiotics in the twenty first century is generally very similar to that used by Fleming in the last century. Mining of the bacterial genome is providing new avenues to the identification

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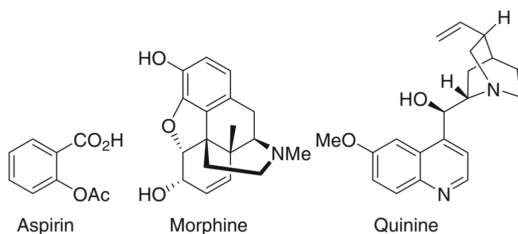

Fig. 25.1 Structures of aspirin, morphine, and quinine

of essential bacterial targets that can be used for the discovery of antibacterial agents with reduced side effects [4–9]. Unfortunately, this approach has yet to provide new drugs.

Target based screening for drug leads using high-throughput screening (HTS) of compound collections with enzyme and receptor based cell free assays has been extremely successful for mammalian targets, but it has failed miserably in yielding similar results against antibacterial targets for various reasons, the most important being lack of cell penetration. The GlaxoSmithKline (GSK) group recently published their experience for such an effort where screening against 70 antibacterial targets provided disappointing results and no measurable success [10]. The frustration displayed by Payne et al., is not unique to GSK, as perhaps every corporation had similar results from their HTS campaigns.

Even in the twenty first century, natural products remain the major sources for drug leads and for the development of antibiotics [2]. Significant advances in microbial isolation, fermentation, natural products chemistry, and synthetic biology techniques have recently occurred that undoubtedly provided tremendous opportunities to apply these tools to the discovery of novel antibiotics. This chapter reviews the current antibiotic leads and drugs, their mode of action, and highlights emerging tools that have the potential to revolutionize the discovery of new antibiotics; the chapter is also an extension and an update of a previous publication [11].

25.2 Sources for Antibiotics

Drug discovery heavily relies on screening for identification of leads that serve as starting points for further chemical optimization, leading to development of clinical agents. There are two major sources for screening for antibiotic leads: natural products and synthetic compounds. Natural products have been the major sources for discovery of novel chemical scaffolds for many drugs [2] as well as early leads that were chemically modified and developed as antibacterial agents. In fact, natural products account for all but three classes of clinically used antibacterial agents (e.g., oxazolidinone, quinolones, and sulfa drugs, Fig. 25.2). The rational drug design approach is a third source for drug leads particularly when the 3D structure of the

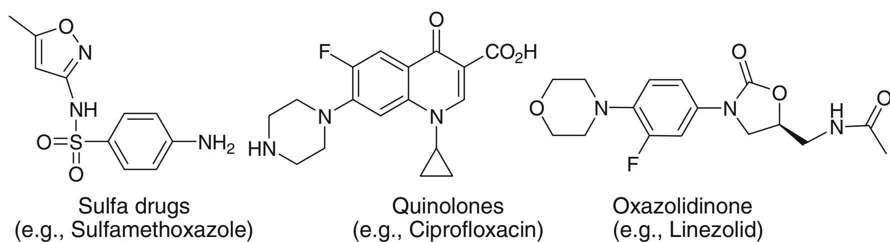

Fig. 25.2 Three classes of antibacterial agents of synthetic origin

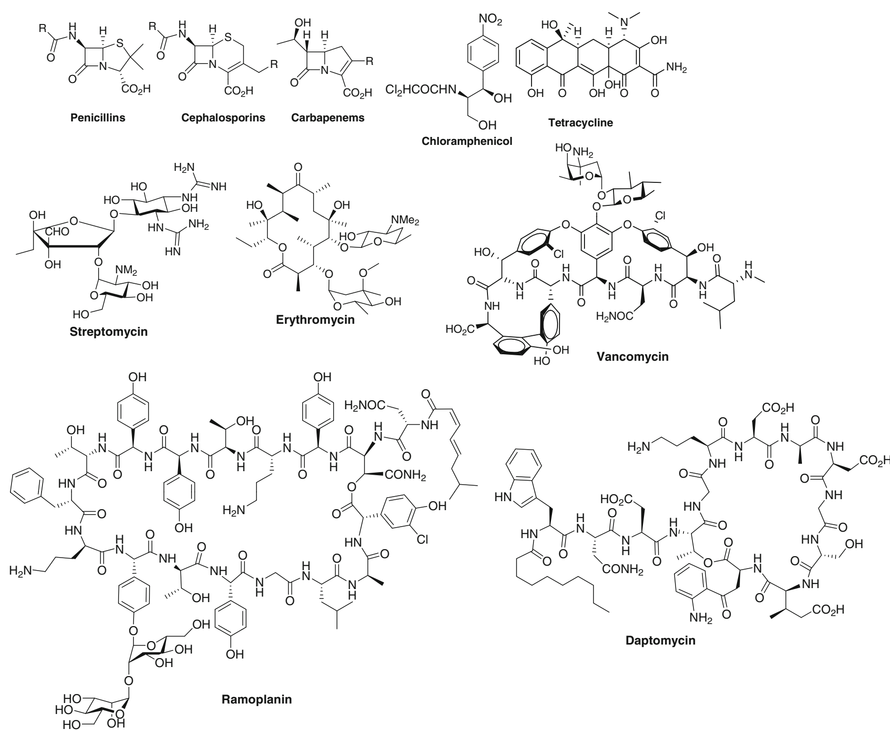

Fig. 25.3 Representative classes of antibacterial agents of natural product origin

biological target is known. This approach has not yet been very effective, however, significant progress is being made. Discovery of ATP site-directed inhibitors of Gyrase B and ParE were recently reported from this approach [12].

The discovery of penicillin paved the way for the development of a plethora of broad-spectrum natural antibiotics and led to the "Golden age" (1940–1962) of antibiotic discovery. Most classes of novel natural product antibiotics were discovered (Fig. 25.3) in this period, which, for five decades, provided scaffolds for semi synthesis and which continues to play a significant role even today. These classes of

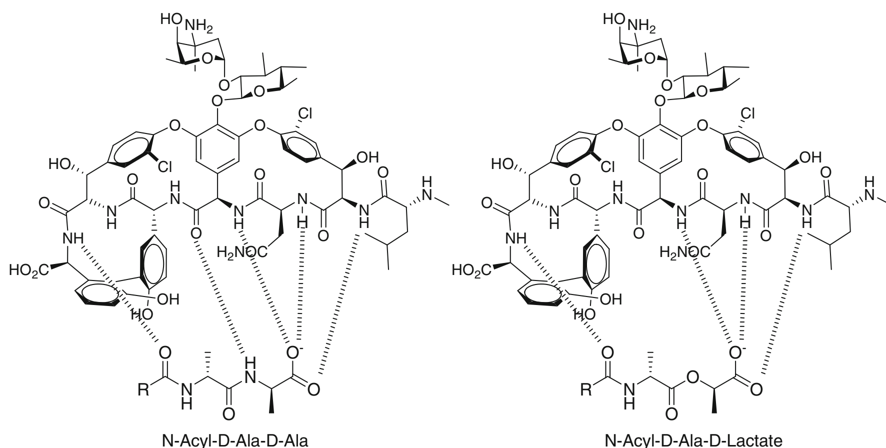

Fig. 25.4 Interaction of vancomycin to the active site D-Ala-D-Ala of peptidoglycan (*left panel*) and D-Ala-D-Lactate (*right panel*)

natural products include the phenyl propanoids (chloramphenicol), polyketides (tetracycline), aminoglycosides (streptomycin, gentamicin), macrolides (erythromycin), glycopeptides (vancomycin, ramoplanin), streptogramins (quinopristin and dalfopristin), and β-lactams (penicillins, cephalosporins, carbapenems and monobactams). The three classes of β-lactam antibiotics are distinguished by differences in the second non-β-lactam ring motif. Penicillins contain a five-membered sulfur heterocycle, cephalosporins a six-membered sulfur heterocycle, and carbapenems a five-membered carbocycle (Fig. 25.3).

Antibiotics from natural sources range from compounds with small molecular size (e.g., thienamycin) to large peptides (e.g., ramoplanin). They generally possess complex architectural scaffolds and densely deployed functional groups, allowing for maximal number of interactions with molecular targets, often leading to exquisite selectivity for pathogen targets *versus* the host. This is nicely illustrated by the interaction of vancomycin to its target. Vancomycin has five hydrogen bond contacts with the D-Ala-D-Ala terminal end of peptidoglycan. The D-Ala-D-Ala motif is modified in Van^{R} resistant organisms to D-Ala-D-lactate leading to the loss of one of the hydrogen bonds resulting in a 1000-fold drop in binding affinity and complete loss of antibiotic activity (e.g., Fig. 25.4) [13].

25.3 Mechanism-of-Action (MOA) of Antibiotics

All antibiotics used in the clinic were discovered by empirical methods. The mode of action (MOA) for all of them was determined significantly after their discovery and often after their clinical development and regulatory approval as a clinical agent.

The natural antibiotics are known to hit most of the major biological pathways, but the majority of them impart their antibacterial effect by inhibition of bacterial cell wall and protein synthesis. Exceptions include the rifamycin class of natural products that inhibit RNA synthesis. The key point is that for the discovery of antibiotics one does not need to know precise MOA before one begins the discovery process. Since *in vitro* cell free target based discovery has been mostly unsuccessful for antibacterial lead discovery by HTS screening of synthetic collections, empirical screening of natural products against whole cell bacteria remains a viable discovery strategy particularly when combined with new technologies for isolation and detection. Successful discovery of novel chemotypes could be significantly enhanced if the bacterial cells were engineered so that targets are either sensitized or overexpressed allowing for differentiation of compounds by MOA based inhibition of bacterial growth from among general cellular poisons. This would minimize rediscovery of known antibacterial agents that has hampered the field by resulting in diminishing return.

25.4 Traditional and Contemporaneous Discovery Approaches

The antibacterial discovery approach that resulted in the discovery of penicillin was also used for the discovery of other antibiotics, and it continues to be useful even today; as such there is nothing wrong with the method. The process involves growing bacterial strains impregnated on agar plates and applying fermentation broths or broth extracts of natural products to the agar 'lawn.' After incubation for a predefined time period, the zone of bacterial clearance is measured. A broth or extract that exhibits a zone of clearance is then subjected to bioassay-guided chromatographic fractionation, leading to the isolation of active natural product(s), followed by structural elucidation of the active compound. The original discovery of penicillin was made from a fungal source; however, prokaryotic organisms produce most of the other natural antibacterial agents.

As illustrated earlier, the empiric whole cell screening method has delivered all of the clinically useful antibiotics, but it suffers from significant drawbacks. Because of the lack of MOA knowledge, it cannot discriminate real antibiotics from general poisons, and therefore provides an unacceptably high hit rate (20–30%). This high hit rate was not a problem in the mid-century, since not many compounds were known and most of the hits led to the discovery of new compounds. However, due to the very large number of known antibiotics produced in varying amounts with varying degree of activities, cumulative discovery of compounds (and lack of their differentiation at the screening stage) has manifested a considerable challenge in differentiation of known antibiotics from new antibiotics, even with the application of state of the art analytical methods. *In vitro* cell-free screening was also used post the molecular biology revolution, but the success of the discovery of new meaningful natural antibiotics was dismal, analogous to that reported by Payne et al., for the

screening of corporate synthetic libraries [10]. A target based whole cell approach for discovery is the most viable approach for discovery of antibiotics. Certainly, a number of whole cell target specific discovery approaches have been used for the screening of natural products; however, no systematic approaches have been reported that could be applied for most of the essential new targets, until the recent report of an antisense based approach (vide infra) [14].

25.5 Antibiotic Resistance and Contemporary Strategies to Overcome Resistance

After introduction of a large number of antibiotics to the clinic, during and immediately after the 'Golden Age' of antibiotics, complacency started setting in among the experts, and many thought that treatment of bacterial infections has been essentially conquered. No sooner had these discussions started to take place than antibiotic resistance began to emerge, becoming rampant in certain instances and resulting in vancomycin becoming the antibiotic of last resort for the treatment of nosocomial Gram-positive bacterial infections. After a number of years of use, emergence of nosocomial vancomycin-intermediate *Staphylococcus aureus* (VISA) and *Enterococcus faecalis* resistant (VRE) strains have become a common occurrence [15, 16]. It is now accepted that resistance is inevitable, and that resistance management will be part of the process for all new antibiotics.

For whatever reason, the post 'Golden Age' discovery of novel antibiotic scaffolds has been unsuccessful. Lack of discovery of new scaffolds and increased experience of the chemistry of existing scaffolds has prompted researchers to adopt semi synthesis strategies of existing leads to produce antibiotics with incremental improvement that gained activity against resistant bacterial pathogens. This led to the development of multiple generations of penicillins, cephalosporins, carbapenems, aminoglycosides, tetracyclines, macrolides, glycopeptides, quinolones, and an oxazolidinone. Alternate strategies involved targeting the mechanism of antibiotic resistance to reverse the loss of antibiotic potency. This led to the development of β-lactamase inhibitors (e.g., clavulanic acid, sulbactam, tazobactam) that are co-administered with a β-lactam antibiotic (e.g., amoxicillin), leading to combination products such as Augmentin® [17]. Similarly, strategies were adapted to block antibiotic efflux, leading to the discovery and development of tigecycline (Fig. 25.5), in which key chemical modifications of the tetracycline core led to reduction of

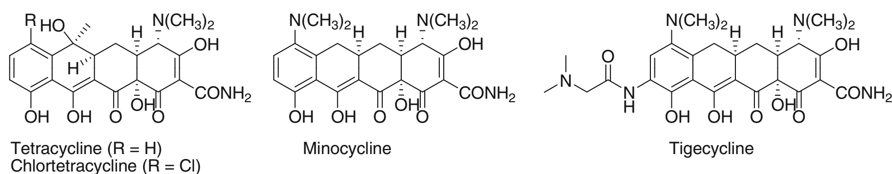

Fig. 25.5 Chemical structures of tetracycline and its derivatives

efflux and gain of susceptibility. Specifically, antibiotic efflux was blocked when the aminoglycyl group was added to the ring A of minocycline.

25.6 The Impact of Molecular Biology on Antibiotic Discovery

The impact of molecular biology on drug discovery has been nothing but revolutionary, and perhaps single handedly contributed to the growth of the pharmaceutical industry in the 1980s and early 1990s. Molecular biology has revolutionized the ability for target identification and protein expression facilitating *in vitro* screening and leading identification in all disease targets including antibacterial agents. This screening method has been highly successful in many areas of drug discovery and has allowed for identification of leads from screening that were optimized and developed into clinical products. Unfortunately the antibiotic field has not experienced similar success. The process has allowed for the discovery of *in vitro* synthetic enzyme inhibitors for a number of targets, but those inhibitors could not be turned into antibacterial agents, due to lack of cell penetration, which could not be overcome by chemical modifications[10]. Inhibitors of t-RNA synthetase [18], FabH [19], and carbapenamase [20] are a few exceptions. To the best of my knowledge, no natural product inhibitor of any significance was reported using the cell free screening approaches. Synthetic gyrase inhibitors, the benzimidazole, and indazole nuclei [21] are more successful examples, which were apparently discovered by computer- aided design through computer modeling of enzyme and virtual docking. Optimization of these synthetic inhibitors led to molecules that are not only potent inhibitors of bacterial gyrase but that also have *in vitro* and *in vivo* antibiotic activity.

25.7 Genome Sequencing and Broad Spectrum Antibacterial Targets

Haemophilus influenzae was the first pathogenic bacterium whose genome was fully sequenced in 1995. Subsequently, full genome sequences of a number of other bacterial species were performed. DNA sequence comparison of the genomes of a series of Gram-negative (*H. influenzae* and *Moraxella catarrhalis)* and Gram-positive (*Streptococcus pneumoniae*, *Staphylococcus aureus,* and *Enterococcus faecalis)* pathogenic bacteria revealed genes that were highly conserved and suggested a treasure trove of new broad-spectrum targets. It was predicted that *S. aureus* contains ~265–350 essential genes [14, 22]. Currently marketed drugs target approximately 15–25 of these essential gene products. *A priori* prediction of druggable targets from these unexploited targets is an extremely difficult task, and as a result is subject to considerable debate. Pharmacological validation of target is one of the key processes for drug development and requires identification of meaningful leads.

25.8 Natural Product-Based Antibiotic Discovery

Natural product based drug discovery programs involve highly interactive and synergistic interactions of at least three distinct yet related scientific areas: (1) sensitive and robust biological targets/assays; (2) sources of natural products; (3) isolation and structural characterization of natural products (Fig. 25.6).

25.8.1 Sensitive and Robust Biological Assays: Differential Sensitivity Screening Approach

Technologies exist to regulate expression of a particular gene, rendering the organism sensitive to an antibiotic affecting the same target. Differential sensitivity, using wild-type and resistant pairs, is a widely used method for the discovery and for the profiling of leads, as exemplified by the discovery of philipimycin [23]. While wild-type/resistant pair screening is an effective screening approach it is inherently less sensitive. Sensitivity is a hallmark of a successful screen. Target specific antisense approach is a highly sensitive approach for screening, and it is applicable for all essential genes; therefore, all targets can be screened using the same technology [14]. This assay can be performed in liquid or agar based assay formats as needed [14, 24].

The basic concept of antisense screening is that novel antibiotic leads can be detected from the same sources that previously produced no leads, owing to the increased sensitivity of the antisense assays. This detection is possible due to the lower expression of the gene product in an antisense knockout strain (usually regulated by an exogeneous inducer of the antisense RNA) compared to its isogenic wild type strain leading to hypersensitivity of the target specific inhibitors. In agar based antisense assays, when a zone of clearance from a natural product broth is compared between antisense and wild-type plates, a larger zone of inhibition on the antisense plate and smaller zone of inhibition on the wild-type plate indicates a mechanism-based inhibitor against the target gene with reduced expression [24]. Thus, one is able to identify specific mechanism-based inhibitors among many other antibiotics

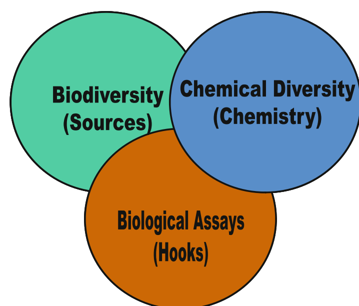

Fig. 25.6 Effective paradigm of natural product discovery

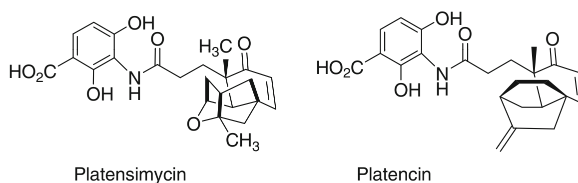

Fig. 25.7 Chemical structures of platensimycin and platencin

and non-specific toxins. This method allowed discovery of platensimycin [25, 26] and platencin [27, 28], novel inhibitors of elongation condensing enzymes of fatty acid synthesis (Fig. 25.7), and a number of other novel inhibitors that putatively interact with the RPSD (S4) protein of protein synthesis machinery [29–31]. While a number of technologies exist that allow for specific target specific sensitization (e.g., promoter replacement technology [32], and multi-copy cloning [33]), to the best of my knowledge, perhaps with the exception of *E. coli* based controlled promoter technology [34], the antisense technology is perhaps the only one for creating target specific "dial downs" for all essential targets. This critical feature was utilized and antisense strains were prepared for all essential genes. After significant optimization, an assay was designed and implemented in an array format that allowed for screening and profiling of all essential (~250) targets simultaneously [35].

This method allowed for identification and differentiation of antibiotics in crude extracts based on target specific strain depletion, and resistance and was one of the most powerful tools in dereplication of natural products at the extract stage. When this approach was applied in combination with highly sensitive high resolution Fourier Transform mass spectrometry (HRFTMS), the dereplication of known antibiotics became almost foolproof, particularly if that antibiotic or class of antibiotics had been seen in the assay before and was part of the database [35]. The critical importance of sensitive and robust whole cell target based screening assays for discovery of novel antibiotics, particularly natural product antibiotics, could not be over emphasized.

25.8.2 Sources for Novel Natural Products

Novel natural products with unprecedented structural diversity and complexity with biological activities are found in almost all biological sources including terrestrial plants, lichens, and marine macro organisms (656 new compounds in 2003 [36]). Marine organisms continue to be highly productive sources for novel drug leads for cancer and many other disease targets, and a number of these natural product derivatives have entered clinical development [37–39]. However, they are less popular and not as well explored as antibiotic leads. Similarly plant sources have not been popular for antibiotic discovery and have not delivered clinically relevant antibiotics; however, plant derived natural products have been shown to have anti-staphylococcal activities (Gibbons [40]). Microbial sources have been the most prolific sources for antibiotics and were readily adopted by industry.

25.8.3 Microbial Sourcing, Isolation and Strain Dereplication

Sourcing for a microbiological material involves the collection of substrates that possess the microbial organism such as soil and environment samples (e.g., leaf litter, animal dung, etc.) from diverse geographical areas but most importantly from a variety of habitats. This seemingly simple and mundane task happens to be one of the most critical aspects for the contemporary discovery of novel natural products. While the process for collecting the organisms is similar as it has been done historically, this process has become significantly much more complex after the Rio De Janeiro treaty of "Convention on Biological Diversity" [41], which requires the meticulous documentation of collection records of the sample and the painstaking tracking of disposition of the sample. This treaty is very important and ensures the rights of countries that provide biological substrates to be properly rewarded. Unfortunately, the treaty has led to significant delays in access to the materials, resulting in a slow progression of natural products discovery, due to the significant and often ill-defined bureaucratic hurdles, additional expense, and legal requirements for well-intentioned researchers. There is an urgent need to establish a global process and an open access global database that clearly identifies the names of individuals and government departments responsible for helping set up such agreements between the interested parties. Perhaps this could be created and maintained under the auspices of the United Nations. This open policy should allow for smoother and more efficient communications, reduce the time and energy required to perform paperwork, and result in a faster discovery of natural products thus harnessing and unleashing the power of compounds of natural origin for treating potentially untreatable diseases to the benefit of mankind. Fortunately, the biosynthesis and production of compounds are not exclusive and limited to unique biota and same compounds are produced by different genera distributed throughout the world [42–44], allowing the discovery of new compounds to continue.

Sources for the isolation of microorganisms from natural habitats include soil, leaf litters, dung, marine habitats, deep water, and other environmental niches. Since even a small amount of the natural habitat contains millions of organisms, the isolation of these organisms to single strain is a challenging and painstaking task. Classical methods for the recovery of producer organisms have favored fast growing organisms and have been reasonably successful in the identification of both novel producing strains and novel compounds. Specifically, the classical approach involved plating microorganisms on an agar surface with or without antibiotics (antibiotics are used to selectively prevent overgrowth by a major grower) to detect and isolate the microorganism. The isolated organisms are subsequently fermented for assessment of their ability to produce a bioactive compound. This assessment has classically been by zones of clearing of spent-media broths and/or extracts of the fermented culture. The key to success has been to suppress the overgrowth of fast growing microorganism, which would otherwise prevent the slower growing strains from reaching a critical mass to present as a potential producer strain that can be harvested. It is relatively easy to isolate hundreds or even thousands of strains of fungi,

actinomycetes, and other bacteria in a relatively short time by using indirect methods [45, 46]. Moreover, semi-automated platforms for microbial isolation have been described recently [46].

Recently, significant improvements in microbial isolation techniques have been made that use high dilution extinction culture conditions, allowing the isolation of single strains regardless of their growth rates. This conceptually simple, but innovative protocol, has facilitated the isolation and fermentation of novel organisms [47]. Similar in strategy to the approach of limited dilutions in monoclonal hybridoma procedures, 96 well microtiter plates can be used for the isolation of microorganisms in high dilution conditions; however, statistically only ~13% of wells contain microorganisms while the remaining wells are empty. The lower percentage recovery is expected, as the principle is to dilute until one or less than one microorganism (just as with monoclonal-producing hybridomas) are in each unique well. Modifications have been made to circumvent this problem of low recovery by applying a bead capture technology to this limited dilution protocol in which captured individual organisms can be sorted by flow cytometry. This process allows the capturing of beads containing organisms in individual wells of 96-well plates and discard the beads that do not contain any microorganisms [48]. While high dilution extinction culturing in 96-well was originally reported for prokaryotic organisms it was recently adopted for eukaryotic organisms [49].

After the microorganism isolation, the next step is dereplication, which is the process of identification and characterization of the isolated microbial strain, and subsequent matching to a pre-characterized genus/species, or naming of a new genus/species. The most common methods for dereplication of fungal species are morphological characteristics. Dereplication of prokaryotic organisms based on morphology is more challenging, due to the lack of clear morphological characteristics and requires growth of these organisms in select media followed by measurement of size and growth pattern. The more precise dereplication of *Actinoplanes* and *Streptomyces* requires significant work, involving fatty acid analysis (FAME) and 16 S rRNA analysis, which is both time consuming and faces the constant challenge of harvesting redundant isolates. More advanced efforts have included the use of Fourier Transform Infra Red (FTIR) to dereplicate microbial isolates [48], which has increased throughput but has yet to be proven successful [48]. The early elimination of redundant organisms is critical for success and cost effective discovery of natural products. The earlier it can be achieved with precision the better it is for the discovery process. A new method like the Gram test used for typing of Gram-positive and Gram-negative organisms can be very beneficial if it can distinguish the producing organisms directly at the earliest steps of organism recovery.

25.8.3.1 Genomics-Guided Natural Products Discovery

The current dereplication methods allow for comparison of organism similarity but do not provide evidence whether these organisms produce new compounds, variants of known compounds, or known compounds. They compare the overall organisms

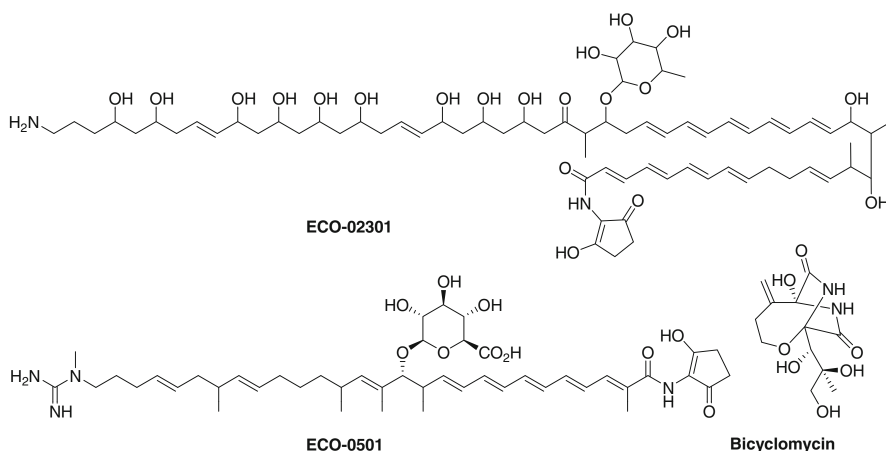

Fig. 25.8 Chemical structures of ECO-02301, ECO-0501 and bicyclomycin

but do not specifically compare their biosynthetic machinery, which is what is important for secondary metabolite production. Full genome sequencing is one of the easiest methods that allows for comparison of the biosynthetic machinery leading to an ultimate dereplication. Application of this method is not feasible at this moment, due to high cost. However with technological efficiency speed of genome sequencing is improving leading to an exponential reduction in cost [50]. Genome scanning technology, developed by Ecopia Biosciences, Inc., is an alternative approach to full genome sequencing, which is also reasonably effective in achieving the same goal [51]. Both of these methods have already demonstrated a significant benefit in the discovery of new natural products from known producing organisms. Farnet et al. applied genome scanning technology to study 60 well known microbial strains that were known to produce 65 natural products [51]. They discovered that these organisms encoded >600 gene clusters capable of producing >600 natural products including 65 gene clusters for 65 known compounds. For example, this technology allowed for the discovery of ECO-02301 (Fig. 25.8) (antifungal) from *Streptomyces aizunensis* NRRL B-02311, a well known producer of bicyclomycin [52] and ECO-0501 from *Amycolatopsis orientalis*, a well known producer of vancomycin [53]. Surprisingly, before the application of genome scanning technology, ECO-0501 was not discovered by traditional methods, despite having antibacterial activity similar to vancomycin. In both cases, the structural diversity between the newly discovered compounds compared to the known compounds was tremendous. Full genome sequencing methods provided similar results of yielding more gene clusters than number of known compounds previously recognized that were produced by these strains. For example, more than 20 gene clusters were discovered from full genome sequencing of *S. coelicolor* [54] and *S. avermitilis* [55] encoding for the synthesis of polyketides and non-ribosomal peptides, which is substantially more than the number of compounds known from these organisms. As evident from above discussions, these methods not only can be useful in the dereplication of new organisms but also for the discovery of new compounds from known and well

studied organisms. Needless to say the twenty first century will see tremendous improvement of the technology that would allow for a meaningful growth of this area of natural products research. With improved genome sequencing and analytical methods this century undoubtedly would see tremendous growth for the discovery of secondary metabolites from known and new organisms. It is expected that these technologies will be easily accessible and should revolutionize the discovery of a large number of novel natural products. The gene clusters predict structures of the new metabolites with significant accuracy, making structure elucidation a significantly less onerous task.

25.8.3.2 High Throughput Microbial Cultivation

Whether it is new organisms or known organisms, experience suggests that the secondary metabolism of a growing culture of microorganisms frequently leads to the production of secondary metabolites that are often made by the producer organism to protect its existence in a given environment. As such, different environments (e.g., different media or growth conditions) can alter growth and production of secondary metabolites – and the artificial manipulation of the growth conditions can enhance the production and diversity of secondary metabolites. One clever example was reported recently by Duetz's group that allowed for the optimization of growth media in high-throughput (HT) in microtiter plates [56–58]. Using the HT procedure, a single microbial isolate could be grown simultaneously in dozens of varied micro-environments on a miniaturized 1 mL scale in 96 well plates [56–58]. However, there is no true 'model' of secondary metabolite production that guides the research labs to invest in increasing unique isolates or an increased variety of growth conditions. This approach was successfully validated in the industrial setting by Bills et al., where they studied the production of fungal secondary metabolites, which were then applied for antibiotic general screening [49]. In this approach, Bills et al. arrayed multiple medium and measured the number of metabolites produced by individual strains leading to a consensus for a maximal number of growth media that provided the optimal cost effective and balanced results. Unfortunately, the study of natural product production remains a 'brute-force,' a tedious and somewhat unsophisticated science, which has contributed to the movement away from natural products as a lead-seeking paradigm.

Various people have postulated that antibiotics are secondary metabolites that are produced by microorganisms as defense mechanisms from either co-existing life forms or environmental predators [59]. The changing environmental conditions, whether *in situ* in nature or artificial in the laboratory, select for the fittest microorganism(s) that produce new antibiotics. This in turn may select for microorganisms that produce yet another set of metabolites to fight these newer 'predators' microorganisms that have a growth advantage by virtue of their ability to survive better in the environment. Furthermore, it has already been postulated that antibiotic producers adopt different self-defense mechanisms to avoid their own suicide (when producing antibiotics), protecting themselves against their own produced and secreted extracellular 'drugs' [60]. Among these protection mechanisms are the

following: inactivating their antibiotic products; modifying the antibiotic target sites (such as enzymes or ribosomes); or blocking the entrance of the active compounds into the cell similar to antibiotic resistance mechanisms [59, 60].

It is predicted that less than 1% of prokaryotic and ~7% of fungal strains have been isolated and cultured, thus these technologies provide tremendous potential for the discovery of novel natural products and antibacterial agents from as yet untapped sources [61]. This discovery has been evident by studies towards access to new sources and finding newer growth condition to harvest new organisms. For example, recently significant advances have been made for successful recovery of samples from deep sea water, isolation, and fermentation of marine microorganisms [62, 63]. Despite a significant decline in industrial natural products programs, many thousands of novel natural products are reported each year [64]. The study of secondary metabolites from marine microorganisms remains a nascent field and significant contributions are expected from these sources in twenty first century.

25.8.3.3 Environmental Metagenome (EDNA) Approach

It has been suggested that a majority of environmental organisms could not be cultured in the laboratory. Therefore various approaches have been recently attempted to develop methods to access the biosynthetic potential of these organisms without fully culturing them. One of the approaches involved to access 'uncultured' organism is to extract total DNA from environmental samples. The DNA is then digested and ligated into a vector and introduced into a surrogate or heterologous host. These strategies are based on the idea that genes involved in the biosynthesis of secondary metabolites are usually arrayed in clusters, physically contiguous in the microbial chromosome. Thus, large fragments of DNA can be cloned that allow for the access to metagenomic DNA or environmental DNA (eDNA) that in principle is capable of expressing partial or complete natural biosynthetic pathways in heterologous hosts that are known not to produce secondary metabolites [65–68]. *Escherichia coli* was used as a heterologous host in one of the first approaches [69, 70] whereas *Streptomyces lividans* was used in the second approach [71]. A series of novel N-hydroxy amides called terragines were isolated from soil eDNA expressed into a *Streptomyces lividans* host [71], and a series of compounds listed here were isolated from an *E. coli* host. These are long-chain N-acyl amino acids [69] and its synthases [72], violacein [70], long-chain acyl phenols [73], long-chain fatty acid enol esters [74], palmitoylputrescine [75], N-acyl arginine and tryptophan derivatives [76], isocyanide [77, 78], and antibiotic tubromycins [79]. This approach has the potential to represent the true Holy Grail of natural products discovery, but has yet to yield compounds of any significance and, so far, has been rather disappointing. At present, this approach is mainly in the realm of academic laboratories and industry is waiting for initial successes before it will jump in. It is expected that in the upcoming years of the twenty first century discovery of new hosts and the other technological advances will make this or similar approaches productive for discovery of novel natural products.

A related transgenic approach has been reported for the cultivation of slow-growing fungi, in which cosmid-size genomic DNA isolated from individual fungal colonies was cloned and introduced into an *Aspergillus nidulans* host. The resultant transgenic strains were fermented and analyzed for secondary metabolites, leading to identification of two compounds that were not produced by the host control [80]. Many eukaryotes are not amenable to be grown in sufficient scale in artificial culture conditions, but it is feasible to get enough biomass for DNA extraction from very small colonies cultured in the laboratory (as in the case described above), or directly from the field, (e.g., as fruiting bodies) (e.g., mycorrhizogenous basidiomycetes) or lichens (in the case of lichenized ascomycetes). Thus, this approach presents in principle an attractive method to exploit the metabolic potential of fungi refractory to current cultivation techniques.

In any case, much work needs to be done to validate these approaches and to re-engineer them into industrial-scale platforms if they are to become real novel natural product discovery engines.

25.8.3.4 New Natural Products by Biotransformation

Biotransformation is one of the highly effective methods capable of transforming a natural product into new natural product analogs regardless of structural complexity. Microorganisms are capable of performing various reactions that include oxidation, halogenations, acylations, alkylation, cyclizations, and others. Biological oxidations are perhaps one of the most prominent reactions that oxidize inactivated CH sigma bonds of the natural products, which is critical for further derivatization of compounds that lack functional groups. The new oxidized natural products are then used for chemical modifications by semi synthesis to produce compounds with better potency and pharmacokinetic properties for eventual drug development. The oxidation by biotransformation of compactin to pravastatin is one of the examples of this method. Pravastatin was developed as a clinical agent for treatment of hypercholesterolemia [81, 82].

25.8.3.5 New Natural Products by Mutation of Organisms

Random mutation (e.g., by chemical mutagenesis) of microorganisms has been one of the proven methods for altering the genetics of microorganisms and altering their capabilities for the production of new natural products. This procedure has been time tested and a proven method for the improvement of the production level of natural products in industrial settings. Repeated mutagenesis followed by strain selection allows not only the increased titer of natural products of interest, but it also allows for the suppression of unwanted natural products in the mixture. The high titer and suppression of the unwanted minor natural products helps in purification efficiency of the major natural products and leads to significant cost reduction at industrial scale. Mutagenesis can also reverse the ratio of major-minor natural products in

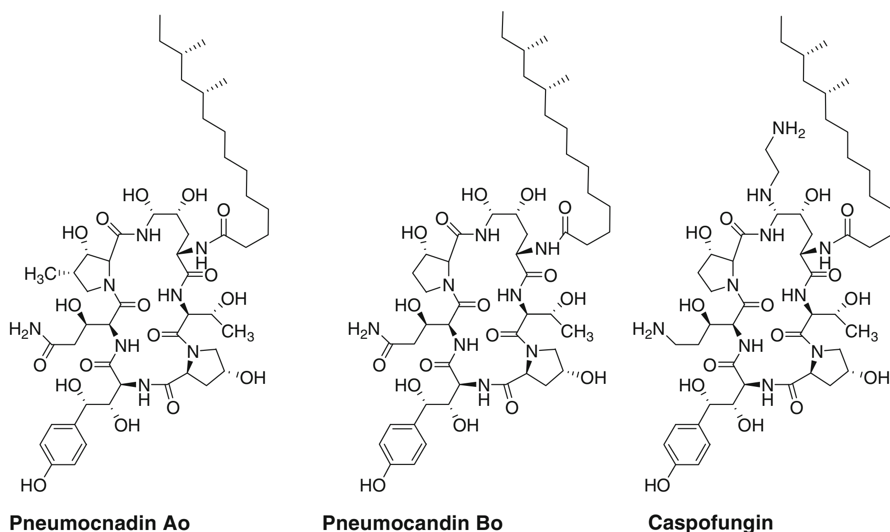

Fig. 25.9 Chemical structures of pneumocandins and caspofungin

favor of higher production of minor compound and significant reduction of original major compound. This process was very effectively demonstrated in almost exclusive production of pneumocandin Bo by suppression of the original major product pneumocandin Ao [83]. Semisynthesis of pnuemocandin Bo led to caspofungin, a highly successful FDA approved antifungal agent (Fig. 25.9). Optimization of media and growth conditions with new organisms play critical roles in the improvement of titer and biosynthesis of new compounds. Knowledge of biosynthesis helps both in the titer improvement and biosynthetic preparation of new compounds. Once biosynthesis and the biosynthetic precursors are known, rational substitution of the precursors helps in the production of new designer natural products. This process is called precursor directed biosynthesis. Petersen et al. again exemplified this, during production optimization of pneumocandin Bo. They isolated six new analogs of pnuemocandins from the high producing strain of pneumocandin Bo by substitution of the amino acids [84].

25.8.3.6 Discovery of New Natural Products by Synthetic Biology (Combinatorial Biosynthesis, Pathway Engineering)

Genome sequencing and genetic methods have made possible the understanding of the finer details of biosynthetic pathways of natural products at molecular and genetic levels. This knowledge has allowed for potential biosynthesis of new 'non-natural natural products' by gene swaps. This is particularly true for the classes of natural products that are synthesized by genes present in the form of contiguous gene clusters such as polyketide synthase (PKS) and non-ribosomal peptide

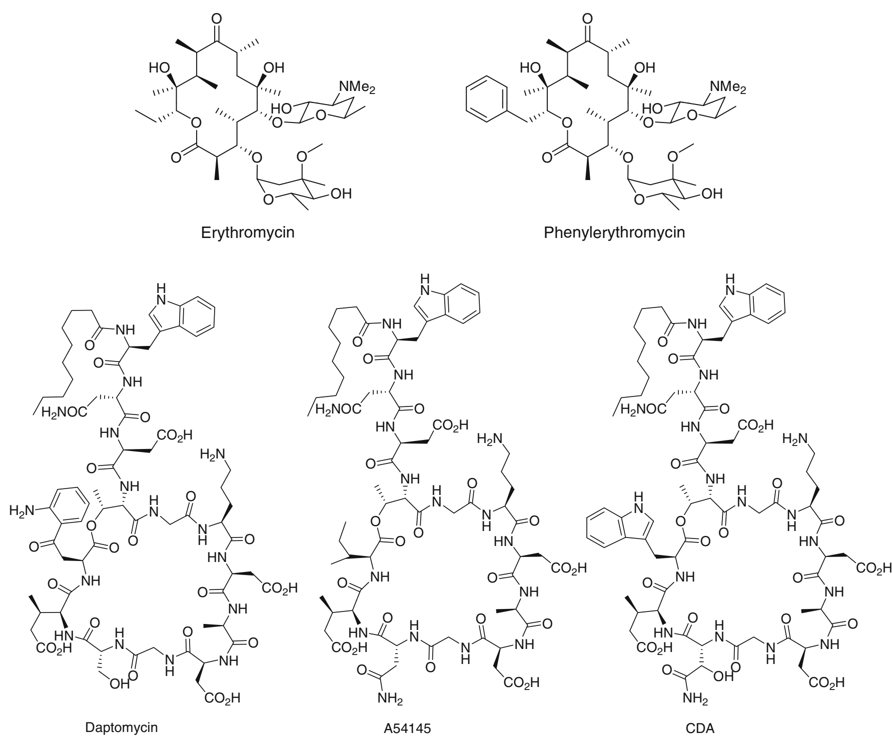

Fig. 25.10 Chemical structures of new natural products by synthetic biology

synthases (NRPS) [85]. The substitution of individual domains or modification of genes leads to biosynthesis of new natural products. While this cannot be done on demand and requires much effort, domain swaps have been accomplished allowing for broader substrate specificity leading to acceptance of modified starter units. Two examples of synthetic biology approaches are the following: (1) combinatorial biosynthesis of the engineered PKS of erythromycin by module exchanges found in nature or modules that were designed by genetic engineering for substrate promiscuity allowed for the biosynthesis of unnatural erythromycin analogs (e.g., phenylerythromycin biosynthesized by exchange of loading domain by 2-phenylacetyl CoA, Fig. 25.10) [86, 87]; (2) similar combinatorial biosynthetic approaches in which recombination of single or multiple module exchanges of the daptomycin NRPS gene cluster allowed for amino acid substitutions that would not be easily achieved by other methods (Fig. 25.10) [88, 89]. Synthetic biology approaches are only applicable to clustered biosynthetic genes and to the un-clustered biosynthetic genes such as genes biosynthesizing aminoglycosides. In last few years, significant progress has been made in the synthetic biology approaches to make designer unnatural products by PKS and NRPS. However, more progress needs to occur before this method parallels the chemical synthesis. The synthetic biology method is advanced enough to complement the chemical semisynthetic approaches for producing new

compounds. This is a particularly valuable tool in augmenting semi synthesis where the chemistry is challenging due to lack of chemical selectivity and reactivity or where no chemical handles are present.

25.9 Natural Products Chemistry

Natural products chemistry is the third spoke of the wheel of natural products based drug discovery and focuses on the isolation of active secondary metabolites from biologically active fermentations consisting of the complex mixture. The compound(s) responsible for the antibiotic activity is often present in trace amounts, against a background of large amounts of related biosynthetic and structurally unrelated metabolites, making the process challenging. Purification of biologically active natural products involves bioassay-guided fractionation in an iterative approach where each chromatographic step is followed by testing fractions in an appropriate biological assay, generally the one that detected the activity. Obviously, the isolation of the active entity is most effective when accomplished in the least number of steps, mostly orthogonal chromatographic steps, producing the least number of fractions [42, 43, 90–92]. When effectively implemented, the pure compound can be easily isolated in two iterations and in less than 2 weeks. However this process can be extremely challenging in the event that the active compounds are present in trace amounts and in the presence of large amounts of related compounds as was the case in the isolation of thiazomycin, which was present in less than 1 mg/L in the presence of related compounds in the amounts 200–300 mg/L. However, by application of innovative isolation approaches, it was isolated efficiently in two steps [93]. Once pure compound is isolated, its structure can be determined by spectroscopic methods, including mass and NMR spectroscopic studies. Both of these methods are significantly advanced allowing for structure elucidation of complex molecules in the amounts of less than 1 mg. Ultraviolet and infrared spectral data provide information about chromophore and functional groups. However, if the molecule is extremely complex and/or does not contain sufficient hydrogen in the molecule, structure elucidation using only these methods, is extremely challenging. Final structural proof can be obtained from X-ray crystallographic analysis, if the compounds can be crystallized. Until recently the presence of a heavy atom (e.g., bromine, iodine or occasionally chlorine), whether already present in the natural product or chemically added, was required for X-ray methods to allow for determination of absolute configuration. Now the technology has advanced that the presence of a heavy atom is not a requirement for such analysis. Alternatively relative configuration could be elucidated by NMR methods and absolute configurations by chemical derivatization (e.g., Mosher ester) followed by NMR analysis.

In recent years, significant improvements have been made in the isolation methods, including discovery and development of chromatographic supports (both normal and reversed phase), allowing much finer chromatographic separations. However, most important improvements have been made in automation. Parallel

chromatographic (i.e., at least ten channels) systems and liquid handling systems now allow high throughput fractionations on a moderate scale. Combining these new methods and equipment for purification of natural products has led to significant gains in throughput. Small scale fractionations, particularly when only modest resolutions are desired, can now be performed efficiently in 96-well formats packed with reversed phase resins. This is cost and time effective for extract profiling, prioritization, and modest purification. When the titer of the active principle is low, (e.g., less than 1 mg/L) and the mixture is highly complex, isolation can be very challenging and may require a series of purification steps. A number of other purification techniques (e.g., counter current chromatography (CCC), supercritical fluid chromatography (SFC), and capillary electrophoresis (CE)) are now often used for the purification of natural products.

Structure elucidation techniques have also improved significantly in recent years, especially LCMS and NMR methods. The LC-ICR/FTMS (Fourier transform ion cyclotron resonance mass spectrometer coupled with liquid chromatogram) [94], is highly sensitive, accurate and capable of measuring molecular mass with exceptional accuracy leading to the generation of molecular formulas with sub-microgram quantities of natural products. While mass spectral data is critical for molecular formula determination, NMR plays a much bigger role in the determination of the structure of natural products. Recent introduction of capillary and cryoprobes has made the structure elucidation of natural products with 5–10 μg of material a reality. However, while structure elucidation from this low amount of material has been demonstrated, this is neither routine nor useful in drug discovery, particularly in antibacterial discovery where biological assays require significantly more material. Structure elucidation of a reasonable natural product is most efficient and time and cost effective when ~3–5 mg material is used for NMR analysis, allowing the collection of a full range of high quality, high resolution NMR data in less than 48–72 h. Interpretation of the data to a structure remains a highly manual and expertise dependent undertaking leading to highly variable efficiencies. With sufficient expertise greater than 90% of novel structures can be elucidated in less than 2 weeks. Attempts are being made to automate structure elucidation, but this remains in its infancy and, as a result, lacks wide acceptance. The automatic extraction of the raw data from the NMR spectrometer remains challenging, compounded by the lack of a perfect data set, higher order of structural complexity, and overlapping or poorly resolved signals. Technological advances in the field of automated structure elucidation would provide a significant boon to natural product research, and we can look forward to the day when integrated data collections and automated structure elucidation will be routine and operator dependent limitations would no longer exist.

25.9.1 Chemical Dereplication

A large number of natural products with various degrees of antibiotic activities have been reported during the last century and the number continues to grow. That poses

a significant challenge in the differentiation of known natural products from novel natural products and thus hampers the discovery of novel natural product antibiotics. Therefore, a key aspect of natural product antibiotic discovery is the development of an algorithm that allows the efficient and accurate elimination of known compounds so that efforts can be directed to the discovery of novel active compounds. This is an arduous task and has been one of the causes for the de-emphasis of many industrial natural product efforts. It is currently possible to profile chemical extracts by comparing the major components, often identifying them pre- or post-screening, with LCMS. This helps in the prioritization and grouping of extracts. However, extract profiling should not be confused with dereplication. Dereplication is a process that allows the linkage of a compound structure with biological activity. This process becomes extremely challenging when the sensitivity of the biological assays is higher than that of the analytical methods. One way to overcome this difficulty is by making a list of the compounds that have been found active against the given target and use these to search by single ion plot of high-resolution mass ion, a process called *targeted dereplication* [42]. This process can be automated and extended for known compound classes that may be expected to show up as positives in a particular assay. For this process, a list of compounds with adjusted high-resolution mass ion values can be generated that can then be used for single ion plot comparison and searching of the observed mass spectral data set generated by LC-ICR-FTMS. After isolation of a new active compound, the list is appended. This is very effective tool for dereplication of specifically targeted compounds. This process has been successfully applied in our antibacterial screening operations at Merck (Singh et al. 2003).

25.10 Lead Optimization of Natural Products

Microorganisms make secondary metabolites for their own utility and not for human use. While they do possess starting properties often better than early synthetic leads, these compounds often do not possess optimal pharmacokinetic and physical properties needed for human drugs, though a number of natural products have become drugs without any modifications. To convert natural products into drugs, requires systematic chemical structural modifications by a process called lead optimization, which has become routine regardless of the lead source (i.e., synthetic or natural product). While the process is not trivial, the approach generally uses standard chemical reactions to modify a lead compound, relying on biological assays to measure relevant biological parameters (e.g., *in vitro* target inhibition, MICs, *in vivo* efficacy, cytotoxicity, PK, etc.). Natural products identified as antibacterial leads typically have bacterial permeability (i.e., access to the target) and thus avoid the need for engineering in bacterial membrane and cell wall permeability, a situation often encountered with synthetic leads. Natural product lead optimization involves

derivatization of the existing natural product structure that requires the production of larger amounts (10–1,000 g) of the natural product. It is therefore imperative to have access to resources (biological material, capacity for large scale fermentation and chemical purification) that allow for large scale production of natural products. The addition of new and milder chemical reactions such as ring closing metathesis (RCM) and Suzuki reactions etc., allow for chemical modification of natural products that was not possible in the past. Continued discovery of newer and milder chemical reactions would make natural products chemistry easier not only by semi synthesis but also by total synthesis, and will alleviate today's apprehension by some to undertake semi synthesis of natural products.

25.11 Future of Antibiotic Discovery

With the emergence of bacterial resistance to existing antibiotic chemotypes discovered in the 'Golden Age' of antibiotic drug discovery, it is becoming extremely challenging to discover and develop new antibiotics with better drug profiles by chemical modification of existing scaffolds. These conditions demand more investment for discovery of new chemotypes. Natural products would be key sources, which are unfortunately being significantly deemphasized by industry. The de-emphasis of natural products as potential drug scaffolds for medicinal chemistry is a serious threat for new antibiotic discovery. The shortcomings of natural products could be overcome by improvement of technology and use of innovative approaches. Some of those have been discussed earlier. The discussion that 'low-hanging fruits' have been found already does not hold true when one looks at various recent discoveries (e.g., platensimycin and platencin) [25, 27].

Discovery of new scaffolds that can be used by medicinal chemists to understand structure-activity relationship and produce novel antibiotics for human use are critical. Natural products continue to provide abundant scaffold diversity, which when combined with 'purposeful design,' is a winning combination of discovery and development of antibiotics. Natural products are often produced by microorganisms, as a defense from predators; therefore, it is reasoned that these ecological defense systems, produced to combat competing microbial life forms, would have some antimicrobial activity that gives the producing organism an advantage and, as such, are antimicrobials to begin with [59–61, 95]. In the search for novel antibiotics, it would be difficult to imagine a more specific source of naturally occurring antimicrobials than in nature itself. In addition, there is little value placed in the knowledge that the far majority of natural product leads have both target-based inhibitory activity and are antimicrobial to begin with, versus the majority of nanomolar *in vitro* cell free inhibitors identified from screening library collections that have limited or no antimicrobial activity, and for which there is no known SAR to build in cell permeability [10].

25.12 Conclusions and Perspectives

Bacteria continue to evade existing antibiotics, and we continue to fail to discover new antibiotics. At this rate, there is a high probability that the bacteria could win. Unfortunately, there was shortsightedness by many corporations to overemphasize screening synthetic sample collections to discover antibiotics at the expense of natural products. While there is no *a priori* reason to expect non-antimicrobial chemicals that reside in chemical warehouses to have inherent antibacterial activity, there is good reason for the secondary metabolites produced in nature by microorganisms competing for an ecological niche to have inherent antimicrobial activity. So why have we virtually abandoned natural products as a starting point for antibacterial/antibiotic? Every corporation has corporate specific reasons, but it may be due to confusion between the lack of effort by most industrial discovery organizations and the lack of successful execution of the process of natural product discovery. Historical analysis suggests that natural products contributed almost all antimicrobial scaffolds over the past 60 years, yet the dearth of quality, novel natural product leads combined with the wave of 'me-too' semi-synthetic antibiotics has lessened the value of searching for novel antibiotic scaffolds. Unfortunately, rediscovery of known compounds contributes significantly towards the cost at a natural product's operation and the yield of discovery of novel natural product antibiotics continue to go down, particularly when operated in traditional way. However, this can be overcome by application of newer techniques at all phases of natural products discovery, miniaturization of processes, use of full automation, and, most importantly, use of clearly differentiating biological assays, as applied for the discovery of platensimycin. With the advent of faster and cheaper gene sequencing technology, the day is not too far away when dereplication can be achieved by comparison of the biosynthetic gene clusters of the organisms which will provide clear and convincing comparison of compounds produced by them. This should be the ultimate method to select organisms for further studies and improvement of productivity of novel natural products as demonstrated by Ecopia Biosciences for the discovery of ECO-0501. If the process is reasonably funded, then the twenty first century should see improvement in synthetic biology techniques where the new unnatural products could be designed and produced on demand. When this method is coupled with concomitant advances of chemical reactions, current apprehension, and limitation of structural modification of densely functionalized and highly complex natural product would become a thing of the past. However, these could be only possible if increased emphasis is placed on the discovery of new natural product by increased innovation in sourcing, altering the genetics of the exiting organisms, systematic modification of biosynthetic gene clusters by mutagenesis, and other yet undiscovered method that could allow production of dramatically different natural product chemotypes.

The natural products have been sources for numerous life saving drugs such as cholesterol lowering agents (e.g., compactin and lovastatin), immunosupressants (e.g., cyclosporin and FK506), CCK receptor antagonists (e.g., asperlicin), and antiparasitics agent (e.g., avermectin [2]). Society will lose significantly if the natural

product reservoir is completely removed from the toolbox. With new targets and advances in natural product–based technologies, the time is ripe to re-focus efforts on natural products and to revitalize antibiotics discovery. Bacterial genomic data together with innovative natural products approaches have the potential to recreate a second "Golden Age" of antibacterial agents, both addressing bacterial resistance and commercial limitations of current approaches [96, 97].

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Chapter 26
Permeability of Bacteria to Antibacterial Agents

Wright W. Nichols

26.1 Introduction

The processes of the permeation of compounds into and the efflux of compounds out of bacteria are important for understanding the physiology of bacteria in their natural habitats [54]. They are also important for understanding clinical resistance to antibacterial drugs [38, 55, 56, 99, 100]. We now recognize that the permeation of compounds into and their efflux out of bacteria are phenomena that significantly affect the discovery of new antibacterial drugs [61, 62, 94, 114]. Lomovskaya and Watkins [61] point out that many classes of antibiotics that are narrow-spectrum would be broad-spectrum were it not for promiscuous Gram-negative efflux pumps: lincosamides, streptogramins, rifamycins, fusidic acid, oxazolidinones, many macrolides, and many β-lactams. Therein lies a major difficulty for target-based antibacterial drug discovery: compounds that inhibit the growth of Gram-positive bacteria can be designed and created with less difficulty than compounds that inhibit the growth of Gram-negative bacteria (e.g., the antibacterial GyrB inhibitors described in [5]). Although efflux pumps are not discussed extensively in the present review, the general point is worth noting that their variability and redundancy present a formidable challenge to medicinal chemistry in the design of antibacterial agents that will be effective against Gram-negative bacteria.

Recent years have witnessed an explosion in the literature of outflow pumps being responsible for intrinsic low susceptibility [98] and clinical resistance [131] to antimicrobial agents (and see [100], in this volume). Outflow pumps can be both specific and promiscuous [55, 56, 63, 99, 100]. They exist in Gram-positive bacteria (e.g., [32]) and Gram-negative bacteria [82]. However, prior to the recognition of the importance of promiscuous outflow pumps, particularly in Gram-negative bacteria

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[57, 58], more emphasis was put on the 'permeability' of bacteria to antibacterial agents, where that term was used in the sense of attainment, or lack of attainment, of an inhibitory concentration of a compound at a target site in the cytoplasm [24, 27, 74]. The term 'permeability' has the assumption or connotation of a compound's permeation being driven by passive diffusion down a gradient of concentration [59] and has been used in that sense for microorganisms (e.g., [8, 16, 113]). However, for bacteria, the term also has been used, quite often, to encompass all processes that affect the concentration in the cell of an externally added compound (i.e., without specifying mechanism [20, 123]). That is, 'impermeability' might occur as a result of active processes such as efflux. In the present review, the term 'permeability' is used in the Lieb and Stein [59] sense of passive, diffusive, permeability.

Bacterial permeability has been reviewed previously [49, 51, 83, 114, 123]. However, owing to experimental difficulties, there have been few studies that attempt to measure kinetics and kinetic constants and to fit those values into dynamic models of the overall cellular action of antibacterial agents. Thus reviewers have rarely explored that topic (see [74, 90]; and especially [60]; for exceptions). On the other hand, molecular [13, 40, 53, 64, 92, 107, 122] and structural [1, 36, 46, 70, 101] studies of bacterial efflux have advanced rapidly over the past 20 years, leading to an elegant structure-based biochemical model for the AcrAB-TolC efflux process [71]. Nonetheless, even in that highly-active field of research, measurements of the kinetics of efflux (that is, net outflow kinetics after correcting for inflow rates) are lacking except for two recent studies [60, 72]. The present review attempts to summarize studies of the kinetics of diffusion and passive permeation across bacterial cell envelopes with the following aims:

1. To provide a framework for understanding the cellular dynamic basis of the MIC [60, 74].
2. To stimulate further measurement of permeation and efflux kinetics and kinetic constants of compounds of importance in the discovery of new antibacterial agents.

As briefly stated above, in this review I shall restrict the term "permeability" of bacteria to that of passive permeability: thus excluding, say, the active transport of compounds into bacteria (e.g., [123]). This is complicated because mechanistically passive processes can result in a higher concentration of a compound in the cytoplasm than in the external medium. An example of such a compound would be a lipid-soluble, and thus membrane-permeant, cation. The trans-cytoplasmic-membrane electric potential drives membrane-permeant cations to a higher concentration in the cytoplasm than in the external aqueous medium (see [67], and references therein). In the context of drug discovery, for a designed inhibitor of a bacterial enzyme, the most likely mechanism of entry of the bacterial cell is likely to be diffusion across the envelope layers [5]. This means that better understanding of passive permeability, and efflux per se rather than net efflux, via assays that measure permeation and efflux independently should reveal more useful structure-activity-relationships (SAR) than do the minimum inhibitory concentration (MIC) of a compound, or its MIC in pump-deficient mutants. This would be similar to the studies

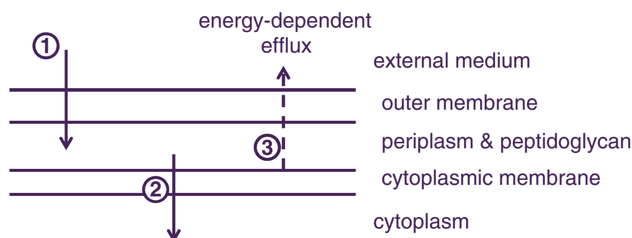

Fig. 26.1 Conceptual framework for analyzing the overall processes of inflow and outflow across the Gram-negative bacterial cell envelope. *Process 1*. Diffusion across the Gram-negative bacterial outer membrane. *Process 2*. Diffusion across the cytoplasmic membrane. *Process 3*. Active outwardly-directed pumping across the envelope into the external medium, coupled with influx of H^+ ions across the cytoplasmic membrane (i.e., driven by the proton-motive force). Diffusion across the periplasm is assumed to be a rapid process for small molecules (molecular weight <700 g mol^{-1}) compared with diffusion across the outer and cytoplasmic membranes. Capsules are not displayed because calculations show that they are also not rate-limiting for the diffusion of molecules of the size of antibiotics [44, 115]. The envelope of the Gram-positive bacterial cell is conceptualized similarly but without the outer membrane; and with efflux processes occurring across the cytoplasmic membrane only

that have led to improved medicinal chemistry understanding of the component parts of drug absorption and elimination (e.g., [124]; and see [62] and references therein for more recent advances). The corollary is that better understanding of passive inflow processes and their kinetics should also lead to better understanding of mechanisms of antibacterial drug resistance.

For reference to the layers that determine the net rate of flow of an externally added compound into the cytoplasm, see the simplified cartoon in Fig. 26.1. In Fig. 26.1, flows 1 and 2 are the major passive diffusion processes discussed throughout this chapter (assuming a relatively high permeability coefficient for diffusion across the periplasm; see below for comments on rates of permeation through Gram-negative and Gram-positive murein). Flow 3 is an energy-dependent outwardly directed flow, and it is assumed that its rate is determined by the concentration of that solute in the periplasm, as used, for example, by Nagano and Nikaido [72]. In the case of macrolides in wild-type *E. coli* for instance, flow 3 is mostly represented by the activity of AcrAB-TolC [68].

26.2 Permeability of the Different Layers of Bacterial Cell Envelopes

Compounds in solution move from regions of high concentration to ones of low concentration by the process of diffusion, familiar to readers of this book (see [10, 44, 118] for extended treatments). Passive penetration of novel compounds into the cytoplasm of bacterial cells is governed by the same principles, but with the added kinetic influences of crossing the various layers of the cell envelope, active efflux,

and specific and non-specific binding to 'sinks' on the diffusion pathway. In the special case of compounds that are subject to a chemical reaction on the diffusion pathway (e.g., β-lactam compounds in the presence of β-lactamases), the process to be considered is that of reaction-diffusion, rather than just that of diffusion [75].

For the purposes of analysis, permeation through the different layers of the cell envelope will be considered in turn, followed by a simplified integration of several of the processes in order to better understand their interplay in governing the overall rate of penetration into intact cells. The sequence of topics below starts at the cytoplasmic membrane and considers each cellular layer in turn, moving outwards (Fig. 26.1). The approach is to describe general principles with examples, rather than attempting to present a comprehensive review of supporting experimental data.

26.2.1 Cytoplasmic Membrane

26.2.1.1 Structure of the Cytoplasmic Membrane as the Basis of its Permeability Properties

Bacterial cytoplasmic membranes are unit membranes consisting of a phospholipid bilayer and proteins [11, 42, 47], with proteins constituting about 60% of the membrane [69]. The basic structure is thus one of polar headgroups at the outer surface, a bimolecular hydrophobic phase, and another layer of polar headgroups at the cytoplasmic surface; similar to the cytoplasmic and certain organelle membranes of eukaryotic cells. Clearly the details of the chemical structure of the phospholipids and the nature of embedded and surface-attached proteins are different from those of eukaryotic cells, and likewise bacteria differ between species; although partly owing to technical reasons, details of envelope proteomes remain to be fully elucidated (e.g., [34, 132]). For example, bacterial cytoplasmic membranes contain cardiolipin (1,3-bis(*sn*-3′-phosphatidyl)-*sn*-glycerol), which in mammals is found in the mitochondrial inner membrane (e.g., [42]). Mammalian cytoplasmic membranes contain sterols, which are not generally found in bacteria. Even in one bacterial strain, changes in membrane composition occur in response to changes in environmental conditions (e.g., [42, 112]). Nevertheless findings of the permeability properties of more-easily studied lipid bilayer membranes can be applied in principle to the permeability properties of bacterial cytoplasmic membranes [69, 89, 113]. Where specific studies have been made of the passive permeation of compounds through bacterial cytoplasmic membranes, the findings have been consistent with findings for phospholipid bilayer membranes generally: for example this was the view expressed in the review of Cronan et al. [11] and in the analysis by Nikaido and Thanassi [89] of the permeation of tetracyclines and fluoroquinolones into the bacterial cytoplasm. The present author is not aware of any counter-hypotheses to this well-established, general, phospholipid-bilayer model of the bacterial cytoplasmic membrane.

26.2.1.2 Permeability Coefficient and the Quantitative Basis of Permeability

The kinetics of diffusion of a compound from the aqueous phase on one side of a membrane to the aqueous phase at the other side is analyzed quantitatively in terms of the 'permeability coefficient,' P, which has the dimensions of distance × time^{-1}. It can be understood intuitively from the following relationship: net flow of compound across unit area of the membrane = $P(c_a - c_b)$, where c_a and c_b are the concentrations of the compound in the aqueous phases at the two sides of the membrane (eqn S1 of [78]; and for more detail see [119], pp 41–42). Examples of the use of permeability coefficient in understanding passive bacterial permeability are provided below.

26.2.1.3 Properties of Solutes that Determine the Rapidity with Which They Diffuse Across Lipid Bilayer Membranes

Collander made early experimental measurements of permeability coefficients for the passive diffusion of compounds across the cytoplasmic membranes of cells of *Nitella mucronata* [8]. The conclusions were clear: the permeability coefficient of a compound was higher with higher hydrocarbon/water partition coefficient (roughly to a power of 1.5) and lower with increasing molecular mass (to a power slightly higher than unity). Collander also hypothesized that shape might explain some of the deviation from the quantitative model-based only on partition coefficient and mass.

The seminal text on the principles of the movement of compounds across lipid bilayers is that of Stein [118]. The basal permeability of membranes is believed to stem from the properties of the lipid bilayer [59]. As a generalization, the more hydrophilic a compound, the greater is the energy barrier to its entry (i.e., partition, into the hydrophobic membrane interior). Quantitatively, the partition coefficient that is found best to explain the effect of partition on membrane permeability is the one for n-hexadecane-water as opposed, for example, to the one for n-octanol-water. Lieb and Stein [59] show how individual functional groups such as hydroxyl (when replacing hydrogen) make their partial contribution to the membrane's permeability to a given compound. In other words, the part of the membrane that determines permeability through partition is the interior hydrophobic milieu not the polar membrane surface. The higher energy barrier for a strongly hydrated, hydrophilic compound implies a lower probability of its passively entering, and thus diffusing across, the hydrophobic part of the membrane; which in turn is associated with slower kinetics and a concomitantly lower permeability coefficient of that membrane for that compound ([118], pp 34–35). This is understood well in the art of medicinal chemistry for designing drugs that are more highly permeant through mammalian cell membranes and other lipid barriers: for example, for gut absorption (e.g., [2]), or distribution of compounds into brain interstitial fluid [26].

The other major determinant of a membrane's non-protein-mediated; passive permeability for a given compound is the diffusion coefficient within the hydrophobic membrane interior. The property that raises or lowers this diffusion coefficient

most when comparing compounds is molecular volume, which is directly related to molecular weight. An important observation is that the relationship between molecular volume of the diffusing compound and its intra-membrane diffusion coefficient (and hence the membrane permeability) is steep compared with the relationship between molecular volume and the aqueous diffusion coefficient. This steep relationship is similar to that which is seen for diffusion within polymers as opposed to that seen for diffusion in water. From a medicinal chemistry design viewpoint, this means that increasing the molecular weight within a compound series, other things being equal, will decrease the membrane permeability more steeply than might be thought intuitively on the basis of molecular size. Lieb and Stein [59] review data on the interplay between partitioning and molecular volume in determining a membrane's permeability to a given compound: the findings are consistent with relative permeabilities being described by just those two properties. As a final point, for a given molecular weight and partition coefficient, a compact compound diffuses faster than an extended (e.g., cigar-shaped) compound, resulting in a higher permeability of biological membranes to the more compact compound [59].

As a recent example of medicinal chemistry attempts to understand and exploit the structural determinants that afford more rapid trans-membrane permeation of compounds, Kuhn et al. [48] have analyzed the effects of intra-molecular hydrogen bonding. While holding other structural features constant, it would appear that introducing an internal hydrogen bond into a compound could result in a higher permeability coefficient without a concomitant increase in lipophilicity and decrease in aqueous solubility [48]. This is an elegant medicinal chemistry idea, but whether it can be used practically in the design of antibacterial agents remains to be seen.

The understanding that the passive permeability of biological membranes is a function of the lipid bilayer has resulted in many studies measuring permeability coefficients of artificial lipid bilayers, with the assumption that in the absence of specific transporters or channels for the particular solute these will be good guides to the permeability coefficients of biological membranes of similar lipid composition (for specific examples, see [3, 6, 7, 14, 129]; and references therein). Note that the permeability of membranes to water must be interpreted carefully, owing to the presence in natural membranes of protein-based water-specific pores, as described by Lieb and Stein ([59]; and see [108], for a bacterial example).

26.2.1.4 Permeation of Antibacterial Agents Across Bacterial Cytoplasmic Membranes

What examples are there of antibacterial compounds whose cytoplasmic-membrane-penetration behavior has been analyzed in terms of the above theoretical picture? Nikaido and Thanassi [89] elegantly analyzed the permeation of tetracyclines and fluoroquinolones across bacterial cytoplasmic membranes. In summary, the rate of transmembrane diffusion is so much faster for the non-ionized, uncharged, form than the rates of the various electrically-charged, ionized species that the non-ionized form can be said to be the entity that diffuses across the cytoplasmic membrane,

while the various ionized, electrically-charged forms effectively do not. Moreover, this difference in rate of diffusion results in a final equilibrium distribution such that (in the absence of active pumping processes) the permeant, non-ionized, molecule is at the same concentration in the aqueous compartments on either side of the membrane. Thus the sum of the concentrations of the ionized and non-ionized forms of weakly acidic compounds such as tetracycline is higher in the aqueous compartment on the side of the membrane that is at higher pH (more alkaline). A calculation of the different ionized species at extracellular and cytoplasmic pHs shows that tetracycline inside *E. coli* is predicted at equilibrium typically to be about double its concentration in the external aqueous phase [89]. Similar calculations predict that a weakly-basic group, such as that found in piperazine-containing compounds, will have the opposite effect of reducing concentration in the more alkaline compartment (in infections, usually the bacterial cytoplasm). Clearly, when a compound possesses both weakly acidic and weakly basic groups, the two effects will compete. These predictions fit with the experimental data [89], giving weight to the theoretical picture founded on the membrane properties described above. That is, the permeability of the *E. coli* cytoplasmic membrane to fluoroquinolones and tetracyclines is consistent with partition into, and diffusion across, the phospholipid bilayer part of the membrane as described in [59]. In a separate study, which was also consistent, Sigler et al. [113] found that the overall permeability coefficient of the cell envelope of *E. coli* for tetracycline (where the cytoplasmic membrane was rate-limiting) was very close to the permeability coefficient for the same compound diffusing into phospholipid liposomes.

Cations in which the charge on the ionized atom is delocalized or screened by hydrophobic substituents are more rapidly permeant through synthetic and natural membranes than are naked cations (see [67, 111]). In one study, berberine moved rapidly across the cytoplasmic membrane of *S. aureus* and across a planar phospholipid film, in both cases behaving as an electrogenic passively permeating cation [111]. However, in that study permeability coefficients were not measured.

26.2.1.5 Designing Antibacterial Agents that Must Cross the Cytoplasmic Membrane to Reach Their Target Sites

Increasing the Passive Permeation of a Solute by Derivatization

In the case of negligibly slow cytoplasmic-membrane-penetrating inhibitors of the *Salmonella typhimurium* (as a test Gram-negative bacterium) enzyme, 3-deoxy-D-*manno*-octulosonate cytidylyltransferase (CMP-KDO synthase), derivatizing the inhibitor with a cleavable hydrophobic group converted a whole-cell-inactive compound to a growth-inhibitory one [93]. This pro-drug approach showed that knowledge of the permeability behavior of the cytoplasmic membrane can, in principle, be used in the design of antibacterial agents, even though that particular compound series did not generate an antibacterial compound for advanced clinical studies.

Increasing the Permeation of a Solute by Converting it to a Substrate of a Transporter

In the present review, relevant to antibiotics and antibacterial drug discovery, I have purposefully omitted consideration of protein-mediated transport of antibacterial agents, as this has been reviewed elsewhere [123]. Moreover, in the design of novel antibacterial compounds that act at cytoplasmic targets, designing a compound that would cross the membrane through an existing protein-mediated transporter or channel is too limiting to enable a useful drug to be discovered in a realistic time frame. The reason for this is that the specificities for spatial distribution of functional groups and size of the substrates of such transporters or channels would constrain design flexibility in the face of the need for optimization of all other drug-related properties such as target-binding potency, outer membrane penetration, evasion of efflux, drug safety, etc., (see [5]). The one strategy that was successful in designing inhibitors that crossed the cytoplasmic membrane via a transporter utilized the degeneracy of the oligopeptide transporter in enteric bacteria [24, 27].

Lower Limit of the Permeability Coefficient for a Bacterial Growth Inhibitor that Acts in the Cytoplasm

If we accept that the passive permeability properties of the bacterial cytoplasmic membrane do approximate those of mammalian cell membranes, then, as pointed out above, medicinal chemistry does have an extensive literature on designing compounds for rapid passive trans-membrane permeation, while achieving the other necessary properties of a drug. In quantitative terms, the theoretical analysis (Fig. 26.2; see the supplementary material; [78]; for the derivations that provide these results) shows that to ensure that a compound can reasonably readily enter bacterial cells by passive permeation, the lipid bilayer permeability coefficient needs to be higher than about 10^{-8} cm s^{-1}. This should be enough for designing inhibitors of the growth of Gram-positive bacteria (because peptidoglycan is not a significant barrier to diffusion: see below), starting with an inhibitor of a target in the bacterial cytoplasm.

26.2.1.6 Efflux Across the Cytoplasmic Membrane

Efflux is an additional barrier in Gram-positive bacteria, but is not as important for antibacterial drug design as it is in Gram-negative bacteria (see below). For example oxazolidinones such as linezolid inhibit the growth of Gram-positive bacteria at concentrations of $\leq$1 mg/L, but are less active by 100-fold or more against Gram-negative bacteria, as a consequence of efflux ([41]; although see [52], for oxazolidinones that display MICs against *Haemophilus influenzae* and *Moraxella catarrhalis* in the range 0.25–8 mg/L). Poole [100] also points out that Gram-negative efflux pumps are largely responsible for the lack of activity against Gram-negative bacteria of compounds that are typically low-concentration growth-inhibitors of Gram-positive bacteria.

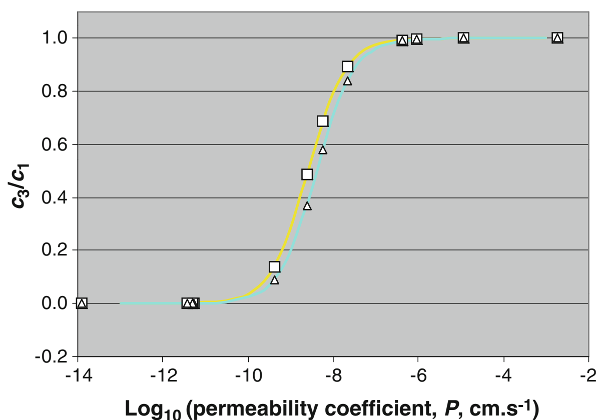

Fig. 26.2 Theoretical ratio between external (c_1) and cytoplasmic (c_3) concentrations of solutes at steady state in exponentially-growing cells of a typical Gram-negative and a typical Gram-positive bacterium as a function of permeability coefficient (*P*). As stated in the text (and see [78, 119], pp 41–42), the kinetics of diffusion of a compound from the aqueous phase on one side of a membrane to that at the other side is expressed quantitatively in terms of the permeability coefficient, *P* (dimensions of distance × time^{-1}). Thus net flow of the compound across unit area of the membrane being considered = $P(c_a - c_b)$, where c_a and c_b are the concentrations of the compound in the aqueous phases at the two sides of the membrane (eqn S1 of [78]). For the *curves* shown here, the dynamic pseudo-steady-state was constructed such that influx of compound exactly balanced dilution through exponential growth. See the supplementary material ([78]; eqn S15) for details. The *curves* are colored *yellow* for Gram-positive and *light blue* for Gram-negative bacteria. The steady-state concentration ratios (□, Gram-positive; Δ, Gram-negative) for a variety of compounds or ions of known permeability coefficient are shown (compounds and permeability coefficients listed below). This illustrates how closely the internal concentration would approach the external one at steady state under the defined ideal conditions for a range of familiar compounds. The permeability coefficients of the example compounds, with a brief statement of the membrane for which the permeability coefficient was measured, are as follows. 1.9×10^{-3} cm s^{-1}: water at 25°C (egg lecithin planar bilayer membranes; [134]). 1.2×10^{-5} cm s^{-1}: p-aminobenzoate at 30°C, pH 7.0 (planar bilayer membranes prepared from brain phospholipids; [3]). 9.4×10^{-7} cm s^{-1}: 2′-deoxyadenosine at 25°C (egg lecithin planar bilayer membranes; [134]). 4.3×10^{-7} cm s^{-1}: erythromycin in the unprotonated form at 37°C, pH 7.8 (*Staphylococcus epidermidis* cytoplasmic membrane; calculated from uptake data of [22]). 2.1×10^{-8} cm s^{-1}: erythromycin (ionized plus non-ionized) at 37°C, pH 7.8 (*S. epidermidis* cytoplasmic membrane; calculated from uptake data of [22]). The pKa of the dimethylamino group of erythromycin is 9.1 [23]. 5.6×10^{-9} cm s^{-1}: tetracycline (ionized plus non-ionized) at 22°C, pH 7.5 (intact *E. coli*; [113]). 2.4×10^{-9} cm s^{-1}: tetracycline (ionized plus non-ionized) at 22°C, pH 7.8 (phospholiposomes; [113]). 4.1×10^{-10} cm s^{-1}: tryptophan at 22°C, pH 6.0 (egg phosphatidyl choline liposomes; [6]). 5.3×10^{-12} cm s^{-1}: glycine at 20–22°C, pH 7.0 (egg phosphatidyl choline liposomes; [6]). 5.0×10^{-12} cm s^{-1}: phosphate monoanion at 20–22°C, pH 4.0 (egg phosphatidyl choline liposomes; [6]). 3.7×10^{-12} cm s^{-1}: lysine at 20–22°C, pH 7.0 (egg phosphatidyl choline liposomes; [6]). 1.2×10^{-14} cm s^{-1}: Na^+ at 4°C; (egg lecithin vesicles: [33]; reviewed by [14]). For structures of several of these examples, see the supplementary material [78]

26.2.1.7 Understanding the Permeation Across the Cytoplasmic Membrane in the Context of Whole-Cell Activity of Antibacterial Agents that Act Against Cytoplasm Targets

It is worth noting that the idea that "a compound doesn't get into the cell" is not a very useful concept on which to base an analysis of structure-activity relationships between related compounds. The permeability coefficient is a continuum, at the low extreme of which any material that does enter the cell will only attain a low concentration balanced against the dilution provided by growth [78]. In effect, such compounds can be regarded for practical purposes as being "impermeant": however, that all-or-none way of considering bacterial permeability obscures the continuously variable nature of the permeability coefficient. The idea of 'non-permeant' is misleading when dealing with permeability coefficients in the steep part of the curve (shown in Fig. 26.2 i.e., in the region of 10^{-9}–10^{-7} cm s^{-1}). As is shown in the supplementary material [78] and discussed further below, the continuous (as opposed to a binary permeant/non-permeant) nature of the permeability coefficient becomes important when trying to understand the effects of structural changes in a compound on net cell permeation when the passive inward diffusion is opposed by active efflux.

26.2.1.8 Exceptional Permeation of Some Polycations

Multiply-positively-charged compounds such as cationic peptides appear to provide an exception to the general rules outlined above. Some polycations are believed to directly alter the lateral domain structure of lipid bilayer membranes and thus directly increase their permeability [19]. Whether this can be exploited outside the area of cationic peptides and peptoids, is currently unclear.

26.2.2 Different Permeability Barriers Between Gram-Positive and Gram-Negative Bacteria

In contrast to Gram-positive bacteria, there are additional challenges in designing inhibitors of the growth of Gram-negative bacteria, starting with a lead inhibitor of a cytoplasmic target. Peptidoglycan is not one of these because, for the sizes of compounds typical in target-based drug discovery (molecular weight < 700 g mol^{-1}, approx.), peptidoglycan is not a significant diffusion barrier (see below). However, there are two further obstacles to the permeation of antibacterial agents into Gram-negative bacteria compared with Gram-positive bacteria: these are the outer membrane and the efflux pumps (Fig. 26.1). The permeability of the outer membrane is considered below. The properties of efflux pumps in conferring resistance to antibacterial agents are reviewed elsewhere in this book [100]. In conclusion, the challenge in designing compounds to inhibit the growth of Gram-negative bacteria is to understand the medicinal chemistry 'rules' not only for rapid permeation across phospholipid bilayer membranes, but also those for high rates of penetration across

the outer membrane and avoidance of efflux. Note that such ‘rules’ will always need to be combined with the parallel medicinal chemistry ‘rules’ for all other requisite drug-like properties, such as analyzed by Gualtieri et al. [25]. Macielag [62] in the present volume describes the special properties of antibacterial compounds compared with those of other drugs.

26.2.3 Peptidoglycan

In the context of passive bacterial cell permeability to antibacterial molecules of typical sizes used in antibacterial chemotherapy (for example with molecular weights (g mol^{-1}) linezolid, 337; tobramycin, 468; ceftazidime, 546; azithromycin, 749; rifampicin, 823; vancomycin, 1,486; daptomycin, 1,620; teicoplanin, 1,885), the peptidoglycan sacculus is not thought to be a significant general permeability barrier [44, 51]. Space prevents a fuller treatment here, but diffusion calculations based on similar kinetic modeling to that provided in the supplementary material [78] are consistent with this view, except for one example, which is the diffusion of vancomycin across the peptidoglycan layer of vancomycin-intermediately-susceptible *Staphylococcus aureus* (VISA) [12]. In the case of VISA, insufficient data were available for the kinetic modeling to distinguish clearly between penetration of vancomycin being limiting or non-limiting in determining the magnitude of the MIC (Nichols, 2009 unpublished). It is relevant in this context that [39] hypothesize that the diffusion distance for vancomycin in VISA is not simply that across the usually-considered surface peptidoglycan layer. They propose rather that it is the distance from outside the sacculus to the centre of the division septum, which could be almost the cell radius shortly before division. The consequent increase in diffusion time has not been estimated.

26.2.4 The Outer Membrane of Gram-Negative Bacteria

The permeability properties of Gram-negative bacterial outer membranes have been reviewed extensively, as seen by the following: [17, 28, 29, 49, 73, 81, 83, 85, 90, 123, 126, 133]; however, the present work is restricted mostly to describing general principles.

26.2.4.1 The Lipid Bilayer

With one exception, that of relatively low alkyl chain mobility, the basis of the general barrier to the diffusion of hydrophilic compounds across the outer membrane is the same as that of the cytoplasmic membrane. That is, the membrane is fundamentally a lipid bilayer that presents a hydrophobic shell in which hydrophilic compounds are of low solubility, and hence its permeability coefficient for hydrophilic compounds is low. The lipid structure of Gram-negative outer membranes is understood to consist

Table 26.1 Permeability coefficients for the passive diffusion of compounds across Gram-negative bacterial outer membranes or artificial lipid bilayers

Compound	Experimental system	Permeability coefficient (cm s^{-1})	Permeability parameter (mL (s cm^2 lipid)$^{-1}$)	Reference
Pregnene-triol	Wild-type *S. typhimurium*: whole cells	4.5×10^{-6}		Plésiat and Nikaido [96]
	LPS[a]-deficient *S. typhimurium*: whole cells	$5\text{–}12 \times 10^{-5}$		Plésiat and Nikaido [96]
Cephaloridine	LPS[a] liposomes + Mg^{++}		$2\text{–}5 \times 10^{-9}$	Snyder and McIntosh [117]
	Phospholipid liposomes + Mg^{++}		4.6×10^{-7}	
Nitrocefin	LPS[a] liposomes + Mg^{++}		$1\text{–}2 \times 10^{-10}$	Snyder and McIntosh [117]
	Phospholipid liposomes + Mg^{++}		2×10^{-8}	

[a]*LPS* lipopolysaccharide

of an inside, periplasmic-facing, layer of phospholipid, and an outside, externally facing layer of lipopolysaccharide (LPS) (see [83], for a description of the key experimental evidence). Unfortunately, there do not exist good measurements of the kinetics of passive permeation of hydrophilic compounds across the lipid portions of outer membranes in intact cells (but see below for measurements with hydrophobic probe compounds) nor in model in vitro systems. The outer lipid leaflet of Gram-negative outer membranes has been modeled experimentally using liposomes formed from isolated lipopolysaccharide, and such liposomes have been used to measure permeability coefficients in vitro [117]. Snyder and McIntosh [117] measured permeability coefficients of the liposomes for two compounds: nitrocefin and cephaloridine. Those two compounds are regarded as fairly hydrophobic β-lactams, despite bearing one negative and one negative and one positive charge at neutral pH, respectively (for structures, see the supplementary material; [78]). Inferences about permeabilities to hydrophilic compounds must be made based on the prior knowledge about lipid bilayers generally (see Sect. 26.2.1 , above). In summary, the passive permeability of the phospholipid-lipopolysaccharide bilayer of the outer membrane to hydrophilic compounds is believed to be the same as that of a phospholipid bilayer, with one major difference, which is described below:

The molecules that form the outer membrane result in one major difference in passive permeability compared with the passive permeability of phospholipid bilayer membranes. The passive permeability of the outer membrane's lipid bilayer even to hydrophobic compounds is relatively low. As shown in Table 26.1, the permeability parameters (proportional to the permeability coefficient) of lipopolysaccharide liposomes for the two compounds, nitrocefin and cephaloridine, were tens- to hundreds-of-fold lower than those for liposomes made from isolated bacterial phospholipids [117]. The most reliable knowledge of passive permeability of outer membranes to lipophilic compounds in intact cells is based on direct measurement of the permeation of steroids through the cell envelope of several Gram-negative bacteria [96, 97].

Unlike the early measurements of permeation rates across the outer membranes of intact cells (e.g., [81, 91, 135]), these measurements were made after the discovery of efflux pumps, and the authors conducted control experiments to show that efflux was not introducing an error into the measurements of permeation rates. First, the permeation rates were measured under respiration-inhibited conditions, which would be expected to decrease the trans-cytoplasmic-membrane protonmotive force, the driving force for efflux. Secondly, the over-expression of efflux pumps did not change the measured permeation rates. Thirdly, the addition of uncoupler, carbonyl cyanide m-chlorophenylhydrazone (CCCP), which would also collapse any remaining protonmotive force did not change the permeation rates [97]. The permeability coefficients of hydrophobic probe compounds for crossing intact outer membranes of *S. typhimurium* were about tenfold lower than the equivalent permeability coefficients obtained with 'deep rough' mutants ([96]; one example, pregnene-triol is shown in Table 26.1). The significance of this is that the deep rough mutants can only synthesize truncated LPS, and consequently phospholipid appears in the outer layer of the outer membrane, making it more similar to a phospholipid-bilayer membrane. Thus for hydrophobic compounds, the permeability of membranes composed of LPS was about tenfold lower than that of membranes containing phospholipid. This difference is not as large as was once thought, before it was realized that measurements of rates of permeation across the outer membranes of (energy sufficient) intact cells was subject to errors caused by then-unsuspected efflux pumping (reviewed in [83]).

What is the explanation for the low permeability of the LPS layer of the outer membrane to hydrophobic compounds compared with the permeabilities of phospholipid bilayer membranes? It is most likely low fluidity, compared with that of phospholipid membranes, as a consequence of the close-packing of the lipid tails of lipopolysaccharide, especially when the LPS is cross-bridged by Mg^{2+} ions between the anionic sugar phosphates. The evidence that supports this hypothesis consists of the following: (i) the high thermal transition temperatures of LPS-containing membranes, and (ii) X-ray diffraction data that reveal a highly-ordered gel-like state (reviewed in detail in [83]). It had formerly been thought that the long polysaccharide chains of the externally-facing outer membrane surface present a layer of 'structured water' that is an energetic barrier to the permeation of hydrophobic compounds that cannot form and break hydrogen bonds, and thus cannot substitute solvent in the hydrated closely-packed oligosaccharide-water matrix. This idea of 'structured water' had been consistent with the increased permeability of outer membranes of "deep rough" mutants, as summarized above. However, Snyder and McIntosh [117] showed that the degree of truncation did not alter the permeability of LPS liposomes (i.e., in the absence of phospholipid), contradicting the water-diffusion-barrier hypothesis. As mentioned above, the higher permeability of the outer membrane in 'deep rough' mutants that contain truncated LPS can be explained by the content of phospholipid in the outer leaflet. Thus the LPS liposome data of Snyder and McIntosh [117] were consistent with truncated LPS, leading to raised outer membrane permeability in whole cells (Table 26.1) not because of the permeability of membranes composed of truncated LPS, but because of the changed structure of the outer membrane by virtue of its content of phospholipid in the outer leaflet.

Early observations about the sensitivity of Gram-negative bacteria to antibacterial agents of known hydrophilicity [81] are consistent with the above interpretations. The DNA gyrase inhibitor, novobiocin, is a hydrophobic compound whose MIC is high vs wild-type *S. typhimurium* (128 mg/L; [96]). However, its MIC is 2 mg/L in “deep rough” mutants that possess severely truncated lipopolysaccharides in the outer membrane and in which the outer leaflet is believed to contain a fraction of phospholipids [116].

26.2.4.2 Water-Filled Channels that Traverse the Gram-Negative Bacterial Outer Membrane

The passive permeability of the outer membrane to compounds that are not strongly hydrophobic is dominated by transmembrane water-filled channels formed by pore-forming proteins [83]. The word “porin” was coined to describe a protein that formed a non-specific channel in the outer membrane of *S. typhimurium*, and Nikaido [83] has advised for clarity that its use be restricted to proteins that form non-specific transmembrane diffusion channels. However, other workers have used the term for proteins that form relatively solute-specific channels. One even sees “porin” being used interchangeably for “pore” (e.g., “diffusion through porins”), which also detracts from the precision of the term. Here I follow Nikaido’s [83] convention, using the term “porin” only for proteins that form non-specific, water-filled, pores.

On a broader biological level, while perhaps not relevant to antibiotic penetration into bacteria per se, it is interesting to note that the proteins that conduct solutes across the outer envelopes of mitochondria and plant plastids such as chloroplasts are evolutionarily- and structurally-related to the Gram-negative bacterial outer membrane pore-forming proteins [17]. This is consistent with the theory of the evolutionary origins of mitochondria and plant plastids being ancient prokaryotic organisms related to the progenitors of contemporary Gram-negative bacteria [17].

Some pore-forming proteins form relatively solute-selective pores (e.g., the sugar-selective pore formed by LamB in the outer membrane of *E. coli* [130] or the basic-amino-acid- (and imipenem-) specific pore formed by OprD in the outer membrane of *P. aeruginosa* [30, 125]. Others form pores that can pass hydrophilic molecules based on size and/or charge. For example, PhoE forms an anion-selective pore [45]. With regard to the passive permeation of novel antibacterial compounds into Gram-negative bacteria, understanding of diffusion through protein-mediated aqueous pores has benefited from measurements of fluxes of β-lactam compounds of various molecular weights and hydrophilicities into both proteoliposomes [135] and intact cells [91]: although it is important to recall that studies with intact cells before the early 1990s were subject to an unknown efflux that would have introduced errors into the measurements. Understanding has also come from studies of non-antibiotic compounds (e.g., oligo- and polysaccharide probes) the general findings from which, such as pore sizes and their molecular weight cut-offs, can be applied to the passive permeation of antibacterial agents [15, 87]. For example, the outer membrane exclusion limit of *E. coli* is approximately 500 g mol^{-1} [87], based on the molecular weight of

raffinose: 504 g mol^{-1}; and that of *H. influenzae* is approximately 1,400 g mol^{-1} [127]; that of *P. aeruginosa* is roughly 1,200 g mol^{-1}, based on an OprF-mediated pore radius of 0.78 nm [4]; however, these molecular size cut-off values must be interpreted loosely. Both those values, and the inferred pore radii, are usually based on the permeating solute being spherical and being of a particular density whereas not all compounds have their mass distributed spherically. In summary, for compounds that can pass through water-filled pores in the outer membrane, the rate of flow of the compound depends on its size in relation to the size of the pore (as governed by the Renkin equation; [104]), and it also depends on its hydrophobicity and number and sign of electric charges carried [88]. Indeed each bacterial species can contain several outer membrane pores, each with its size limit, electric charge preference, and hydrophobicity selectivity, that change with growth medium, so there is no single answer to the question of "what is the molecular weight cut-off of the outer membrane aqueous diffusion channels?" (e.g., see the data of [88]). The molecular weight cut-off is a guide only, but nevertheless it is a potentially useful guide to the medicinal chemist.

Observations with outer-membrane, pore-forming proteins in intact cells, liposomes and black-lipid membranes (artificial lipid bilayer membranes across which the movement of ions can be readily measured by means of electrodes) can now be explained in terms of their crystallographically-determined 3-dimensional structures and the structures of the transmembrane pores formed [109, 110]. One general point relevant to the passive permeation of hydrophilic vs. hydrophobic compounds through general porin-mediated pores is that the constriction in the pore (the "eyelet") appears to be hydrophilic-compound-selective, owing to charged amino acid residues that create an electric field making penetration by low dielectric, non-polar, compounds energetically unfavorable [109, 110]. Also, as would be predicted from diffusion theory, the size of this constriction is a determinant of the rate of diffusion through the pore [110]. Electrophysiology studies and modeling also lead to the idea that even for 'non-specific' diffusion through pores formed by porin proteins (e.g., OmpF) there is an obligatory binding step on the diffusion pathway [95]. This binding requirement slows the diffusion of solutes such as antibiotics by several orders of magnitude compared with free diffusion through a channel of similar size in the absence of the binding requirement [95]. This also means that diffusion through porin-mediated pores across the outer membrane can be saturable [95], but whether this is important at the micromolar and tens-of-micromolar concentrations typical of clinically useful MICs is currently unclear.

A powerful means of understanding the physiology of bacteria, including with respect to the processes of permeation of compounds that inhibit bacterial growth, is the characterization of mutants that are either less- or more-susceptible to such compounds. There is a large literature describing examples from both in vitro selection experiments and observations of clinical isolates of reduced antibiotic-susceptibility (e.g., [31, 103]). The subject is briefly summarized by Nikaido [83, 84]. However, note that reduction in passive influx alone can rarely explain an increase in MIC. That would only be the case if the rate of growth were able to balance the reduced rate of influx such that the intracellular concentration of the noxious compound would not rise to the level inhibitory to wild-type cells. The permeability coefficient would have to be reduced to balance permeation against growth rate. A reduction in

influx usually means a reduction in the steady internal concentration that is poised at a steady value representing the balance between influx and either efflux (multi-drug efflux pumps: see [84, 100]), or metabolism (e.g., β-lactamase-catalyzed hydrolysis in the periplasm: see [74, 86]), or both of those phenomena. Rarely would intracellular trapping of antibacterial agent to its target be responsible for a major fraction of the internal balance against influx (e.g., [36]).

Other types of mutants that add to the understanding of the processes of influx and efflux are those with either inactive or reduced expression of components of the efflux pumps (for example the MIC of novobiocin was 1 mg/L in an *acr* mutant of *S. typhimurium*, as opposed to 64 mg/L in the parent strain: [92]). The antithesis of this is that over-expression of efflux pumps can re-set the balanced internal concentration of antibacterial agent to a lower value at any given external concentration, resulting in a higher MIC (continuing the same example: the MIC of novobiocin increased from 64 to > 256 mg/L in an over-producer of the AcrAB efflux pump: [92]). This phenomenon is an important contributing factor to clinical resistance to multiple drugs in bacteria isolated from patients [55, 56, 84, 100].

The principles above apply to novel antibacterial agents created in target-based drug discovery programs. The SAR of both permeation and efflux need to be understood in order to advance compound design to a similar level to that used in the design of absorption and pharmacokinetic properties. Rates of efflux and permeation must always be considered together (see the supplementary material, [78], for a basic kinetic analysis that demonstrates this connectedness); however, it is important to measure each one independently, because changes in some chemical properties might increase or decrease permeability coefficients while having a different effect on the rate coefficients for efflux. Understanding the quantitative influences in each direction would be expected to be useful in the design-make-test approach to target-based antibacterial drug discovery.

26.2.4.3 Disruption of the Outer Membrane Permeability Barrier

The balance between influx and either efflux or internal metabolism of an antibacterial agent can be shifted to higher internal concentration when the outer membrane is permeabilized, as in deep rough lipopolysaccharide mutants analyzed above or through the addition of agents that permeabilize the outer membrane [126]. The phenomenon of increased permeability caused by the addition of other compounds is not analyzed further here, but again it serves to illustrate that the concept of the kinetics of passive influx being balanced against either efflux or internal metabolism represents a robust explanatory model.

26.2.4.4 Efflux Across the Outer Membrane

The understanding of the passive permeability of the outer membranes of Gram-negative bacteria was largely worked out in the 1970s and 1980s using both inferences based on antibiotic sensitivities and on kinetic measurement of permeation of

probe compounds into the periplasm and into the cytoplasm. Interestingly, that was before anyone realized that many of the test compounds were also being subject to active extrusion by pumps that accept hydrophilic and hydrophobic substrates from the periplasm and from the outer leaflet of the cytoplasmic membrane and expel them to the aqueous phase external to the outer membrane [18, 55, 56, 71, 84, 100]. Examples of such pumps are the AcrA-AcrB-TolC complex of *E. coli* and the similar MexA-MexB-OprM complex of *P. aeruginosa*. In other words, in many of the pioneering and subsequent broadening studies, there was a hidden process that created unknown errors in measurements. Fortunately, this did not prevent the general conclusions being drawn that appear still to be valid some 20 years after the understanding that such errors might have been made. However, it does mean that specific measurements made in the studies pre-1990 (and some later than that) cannot be taken at face value. Rather the methods of those studies must first be examined and a conclusion drawn about whether efflux would or could have played a significant part in the measurements, thus creating significant errors in the absolute values of, say, permeability coefficients and periplasmic concentrations of compounds. For example, one would predict that abolishing the protonmotive force, which is the driver for efflux by many multidrug efflux pumps, would result in a higher periplasmic concentration balanced, say, against β-lactamase-catalyzed hydrolysis for a β-lactam compound. Nagano and Nikaido [72] tested this hypothesis with confirmatory results. The addition of CCCP to intact cells of *E. coli* hydrolyzing nitrocefin caused a stimulation of the hydrolysis rate, qualitatively consistent with loss of efflux that had been maintaining a lower, hydrolysis-rate-determining, periplasmic concentration [72]. As a control for that test of the hypothesis, no such stimulation was observed in a Δ*acrAB* strain [72]. It is important to understand this point about active efflux causing potential errors in the measurement of passive permeation rates because it means that one cannot take permeability coefficient measurements from the literature and use them in modeling without checking the likely errors as outlined above. On the other hand, this note about errors in the estimation of permeation rates and outer membrane permeability coefficients is simply a word of caution. The research on outer membrane permeability from the pre-efflux era represented great achievements: from understanding the structural basis of passive permeability through the phospholipid-lipopolysaccharide asymmetric bilayer, and through the aqueous channels formed by porin proteins; to being able to construct a kinetic model of the basis of the β-lactam MIC in Gram-negative bacteria that agreed reasonably well with experiments [74, 86, 128]. It also enabled the testing of hypotheses such as that of the "trapping" of β-lactam antibiotics by periplasmic β-lactamases as a mechanism of resistance in strains that express β-lactamase at a higher level than do more-susceptible strains [35].

In an advance over neglecting active efflux, two studies have now simultaneously measured rates of passive influx and active efflux in Gram-negative bacteria [60, 72]. The work of Nagano and Nikaido [72] was designed to measure the kinetics of the efflux process catalyzed by AcrAB-TolC in *E. coli*. However, they also measured for the first time permeability coefficients clearly shown to be in the absence of efflux (achieved, as mentioned above, by the addition of carbonylcyanid-m-chlorophenylhydrazone, CCCP, to collapse the trans-cytoplasmic-membrane protonmo-

tive force). We now understand, for example, that for hydrophobic compounds such as nitrocefin, the outer membrane permeability coefficient had been underestimated. However for hydrophilic compounds (e.g., cefazolin) efflux can be negligible, so the permeability coefficients of such compounds measured previously were free of the error that might have resulted from the then-unknown efflux process.

26.2.5 Capsules and Exopolysaccharides

Bacterial capsules are discrete, tightly bound layers, and generally composed of carbohydrate that lie external to the peptidoglycan or outer membrane of Gram-positive or Gram-negative bacterial cells, respectively [105]. Calculations show that capsules are not rate limiting for the diffusion of molecules of the size of antibiotics [44, 115].

26.3 Multiple Cells: Permeability of Biofilms and Colonies

The permeability properties of biofilms and microcolonies constitute too large a subject to include in the present review. Certainly at one time, the hypothesis of general 'impermeability' was put forward to explain the observed lower susceptibility to antibacterial agents of bacteria in biofilms and microcolonies than the same bacteria in dispersed suspension [9]. This hypothesis as a general explanation was challenged on the basis of experimental data and related modeling [75, 77, 79, 80]. It is now understood that permeation of antibacterial agents into biofilms and microcolonies can be restricted, especially if a detoxifying chemical reaction occurs on the diffusion path throughout the matrix (such as β-lactamase-catalyzed hydrolysis), even if the rate of that reaction would be considered very low in a conventional dispersed suspension [76]. However, the hypothesis of a general permeability barrier has been refuted [77, 80, 121]. Stewart has provided an excellent review of the principles of diffusion into and within biofilms for readers who wish to explore the topic further.

26.4 Permeation into Intact Bacterial Cells

26.4.1 Defining 'Impermeable': Passive Permeation Balanced Against Bacterial Growth

'Permeability' is a continuum. The passive permeability of an individual bacterial cell to different compounds can range from very high (consider water or a gas molecule) to very low (consider a multiply-charged ion). Thus, it is interesting to ask the

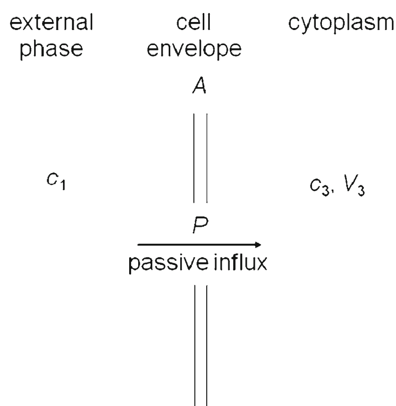

Fig. 26.3 Conceptual model used for kinetic analysis of the passive penetration of a solute into bacterial cells in the absence of efflux: applicable to an antibacterial compound that acts at a target within the cytoplasm. Each bacterial cell is represented by a single compartment with a single surface (area designated by *A*), across which passive diffusion occurs, governed by a single conventional permeability coefficient (*P*). This permeability coefficient incorporates the diffusion gradient between the bulk aqueous solution and the cell surface (i.e., the 'unstirred layer'), and it incorporates the diffusion across the peptidoglycan layer. Exponential expansion (i.e., growth) is modeled by assuming that the total volume (specific volume, V_3, multiplied by mass of cells) and surface area (specific area, *A*, multiplied by mass of cells) of the population of cells in the the test system increase exponentially with time at a constant growth rate. That is, the change in shape that occurs between each cell division is ignored so that each cell is modeled as having an average volume and surface area. Symbols referring to cytoplasmic parameters are assigned the subscript "3" in order to be consistent with other kinetic models in which subscript "2" refers to the periplasm. Thus c_3 and c_1 are the cytoplasmic and external concentrations, respectively, of the solute

question of whether there is a threshold permeability coefficient, below which, in the absence of outwardly-directed pumping, a compound can be considered to be non-permeable (i.e., excluded from the cell). For the sake of clarity, let us consider a compound that is not affected by the ΔpH or Δψ across the cytoplasmic membrane. An approach to this question is summarized here: See Fig. 26.3 for the conceptual model and the supplementary material [78] for the resulting kinetic equations.

As can be seen from Fig. 26.2, when the permeability coefficient is lower than about 10^{-10} cm s^{-1}, the concentration of a compound inside the cell will not rise to more than about 2–4% of the external concentration. This is a rough estimate because a compound that slowed cell growth significantly at that concentration would diffuse in to a higher steady state concentration depending on by how much the growth rate had slowed down. The midpoint, neglecting an effect on growth rate, was at a permeability coefficient of about 3×10^{-9} cm s^{-1}. If the permeability coefficient is higher than that, then by passive diffusion, the concentration in the cytoplasm can be expected to rise to more than 50% of the external concentration before it is balanced by cell growth. Of course, for antibacterial compounds at external concentrations typical of MICs, a 50% concentration in the cytoplasm would be expected to be growth-inhibitory (to an extent dependent on the detailed mechanism

of action), so growth would slow and hence the cytoplasmic concentration at the balance point would rise. That is the above permeability coefficient of about 3×10^{-9} cm s^{-1} would likely yield a higher steady-state concentration than half the external concentrations even under these very theoretical conditions because of the neglecting of the effect of the compound in the cytoplasm on the growth rate. Suffice to say that in the region of permeability coefficient below about 10^{-10} cm s^{-1}, a compound can be said to be effectively impermeant. From a medicinal chemistry point of view, this is not a severe challenge. For example, among seven cytotoxic drugs, Stein [120] reported permeability coefficients between 5×10^{-8} and 1.2×10^{-3} cm s^{-1}. All of these are higher than the 3×10^{-9} cm s^{-1} midpoint discussed above; Fig. 26.2 also shows permeability coefficients of compounds familiar to readers of this chapter, including tetracycline and erythromycin. Neither of these antibiotics is in the region of 'non-permeant' permeability coefficients (as expected since these are both effective antibacterial drugs). However, it is also worth pointing out some of the other compounds that have high permeability coefficients such as p-aminobenzoate or the deoxyribose-sugar-containing compound, 2′-deoxyadenosine. One might have expected a compound with hydroxyl groups such as 2′-deoxyadenosine to have been nearer the region of non-permeant, owing to the hydrophilic nature of the hydroxyl groups. The curve displayed in Fig. 26.2 shows that this is not so.

26.4.2 Passive Permeation Balanced Against Efflux

From the above arguments, we are led to the general conclusion that not many antibacterial compounds made by medicinal chemists will approach the extreme of being non-permeant per se. What is clear is that a lack of efficacy of compounds that inhibit an essential biochemical target in the cytoplasm will, in many cases, be a consequence of reduced access to that site by virtue of outwardly directed pumping, efflux. This efflux counters the influx, which, paradoxically, leads to a different conclusion about the importance of the magnitude of the permeability coefficient from the above conclusion. Once an outwardly directed pump operates on an inwardly diffusing compound, the value of the permeability coefficient *does* become important. How can that be so? The basic principle is illustrated in Eq. (26.1) (identical to eqn S25 in the supplementary material, [78]):

$$\left(\frac{c_3}{c_1}\right)_{t \to \infty} \approx \frac{1}{1+\dfrac{k}{P}} \tag{26.1}$$

To derive this kinetic equation, the bacterium was modeled as a single compartment with a single boundary layer, similar to that shown in Fig. 26.3, across which only two processes operate: passive inward/outward diffusion and active efflux (see [78]). The equation describes the eventual ratio between internal (c_3) and external (c_1) concentrations, respectively, of a compound for which an efflux pump

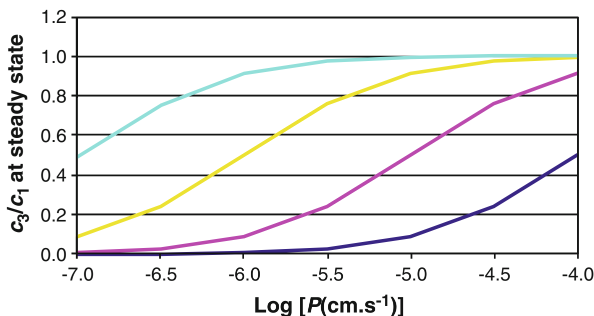

Fig. 26.4 The effect of the magnitude of the efflux kinetic constant on the steady-state ratio between internal and external concentrations of a compound poised against passive permeation through the cell envelope. The passive permeation is represented by a single overall permeability coefficient, P. c_1 represents the external concentrations of compound, c_3 the internal concentration. The ratio c_3/c_1 expresses the internal concentration as a fraction of the external concentration. The curves are the lines of eqn (1; see above), using cell values (area and volume per mg dry mass, etc.) typical of *E. coli*. Each line represents a different value of the efflux coefficient, k. *Dark blue*: $k=1\times10^{-4}$. *Magenta*: $k=1\times10^{-5}$. *Yellow*: $k=1\times10^{-6}$. *Light blue*: $k=1\times10^{-7}$. *Analagous curves* constructed using cell values typical of a Gram-positive coccus were very similar (*curves* not shown)

exists that can be described by an efflux coefficient, k, having the same units as the permeability coefficient, P. Figure 26.4 displays that eventual internal- to external-concentration ratio, c_3/c_1, when influx exactly balances efflux, as a function of permeability coefficient for different values of the efflux coefficient.

By inspection of Eq. (26.1), one can infer the following ideas: In the case of a compound for which permeability is high compared to k, the internal and external concentrations would eventually equilibrate. When efflux dominates and k is much higher than the permeability coefficient, the eventual steady state concentration ratio tends to P/k, the ratio between the coefficients. When the permeability and efflux coefficients are the same, the internal concentration would eventually poise at half the external concentration, as would be expected intuitively and as can be seen in Fig. 26.4.

From the theoretical analysis, the clear inference is that the magnitude of the permeability coefficient is important, even when a compound is subject to efflux, because, across a variety of pump arrangements, the efflux coefficient and the permeability coefficient for a given membrane always appeared in inverse relationship with each other in the derived equations: see Sect. 26.4.3.2 below, and Nichols [78] eqns (S27)–(S34).

Understanding that the effects of intracellular binding sinks (e.g., for compounds that act at the ribosome), or chemical reaction (e.g., β-lactamase-catalyzed hydrolysis) are neglected; this clearly shows the two situations where the permeability in a series of chemically-related antibiotics (e.g., as would be synthesized in a discovery project: [5]) is important. First in the region where permeability coefficient dominates the efflux coefficient, the eventual concentration in the periplasm or cytoplasm is predicted to attain between half and equal to the external concentration. In the

region where efflux dominates, the eventual concentration ratio that can be expected is directly proportional to the ratio between the permeability coefficient and the coefficient for efflux. That ratio might well be substantially lower than 0.5 (i.e., internal concentration much lower than half the external concentration), but might well still be effective for antibacterial action at the compound's target site. In that case, and, if there is no change in the efflux coefficient, changing the permeability coefficient will result in a proportionate increase or decrease in the eventual steady-state concentration to be expected in the internal compartment. In a way, this conclusion is counter-intuitive because it states that increasing the envelope permeability coefficient for a new antibacterial compound synthesized, as one of a series of related compounds is actually most critical when efflux dominates.

26.4.3 Assembling the Complete Picture: Interplay Between Permeation, Efflux, and Reaction

26.4.3.1 Mechanisms Other than Permeation and Efflux that Can Influence Internal Concentrations

Other mechanisms of reduced access to the target site do occur, including enzyme-catalyzed inactivation, as mentioned above, and, in special cases, binding to sites that remove the antibiotic from free solution (e.g., ribosomes or possibly peptidoglycan in the case of VISA, see above). However, as has also been pointed out by others (e.g., in the context of β-lactamase: [43, 66, 102]), these mechanisms act synergistically with the outwardly directed pumping to counter the inward diffusion of the antibacterial agent. Space prevents further developments of models here, but see Nichols [74], Nikaido and Normark [86], and Waley [128] for previous attempts at explicitly modeling the interplay between diffusion and reaction in their effects on the ultimate MIC. Those models were created before it was realized that efflux systems existed that could pump compounds out of the periplasm. Mazzariol et al. [66] have suggested that those models fit the data available at that time because the compounds that were used to test them were poor substrates of efflux pumps. In other words, neglecting the pumping did not introduce major errors. Lim and Nikaido [60] have now re-cast the earlier model [86] to include efflux pumping, and find excellent agreement between theoretical and measured MICs of β-lactam antibiotics.

26.4.3.2 Additive and Multiplicative Effects of More than One Efflux Pump

Lee et al. [53] argued on the basis of experimental evidence that an efflux pump that pumped compounds from the periplasm of Gram-negative bacteria could provide an additive or a multiplicative effect with a pump across the cytoplasmic membrane

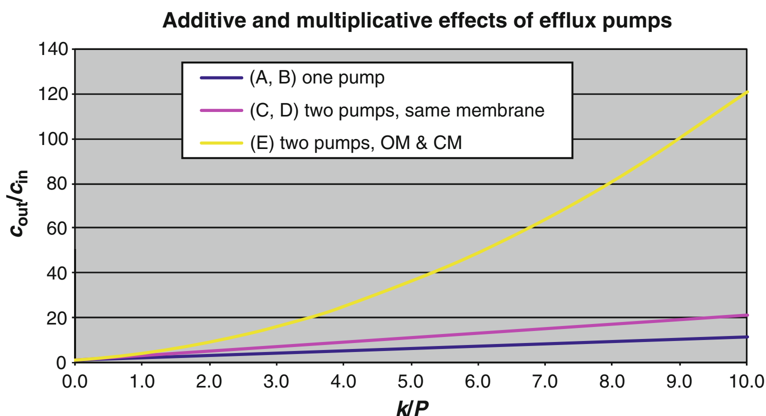

Fig. 26.5 Ratio at steady state between the concentrations of antibiotic in the external medium and in the cytoplasm for various configurations of efflux pumps in Gram-negative bacteria. The figure shows the plots of the three basic equations derived from five basic models listed as A–E in the supplementary material ([78], Sect. S4). (A, B) One active efflux system, which could cross either the outer or the cytoplasmic membrane. (C, D) Two active efflux systems, both crossing the same membrane; either the outer membrane or the cytoplasmic membrane. The pumps are of equal efficiency, that is the ratio k/P is the same for each pump. (E) Two active efflux systems in series: one across the outer membrane, and one across the cytoplasmic membrane. As for the *curve plotted* for models C and D, the two pumps are equally efficient, such that the ratio k/P is the same for each pump in its respective membrane. The greater power of the pumps-in-series arrangement is clear

that pumped the same compound out of the cytoplasm into the periplasm. Their modeling agreed qualitatively with the experimental data and was intuitively attractive, but did not explicitly show the relationship between the kinetic constants of permeation and efflux across the two barriers concerned. Figure 26.5 shows how the ratio between external and cytoplasmic concentrations of antibiotic vary with predominance of the efflux pump over permeation (k/P, the ratio between the coefficients) through the same membrane for the different arrangements of a single pump; two pumps in parallel, and two pumps in series (see supplementary material, [78], for details of the kinetic modeling). In that illustration, for the simple modeled system of both pumps being equally efficient, each with $k/P=10$; the single pump gives roughly tenfold protection, two pumps in the same membrane provide about 20-fold protection, but two pumps in series provide about 120-fold protection (Fig. 26.5).

26.4.3.3 The Concept of the MIC-Determining Concentration at the Target Site, C_{crit}

In all the above analyses, it is useful to bear in mind the concept of a particular concentration in the cytoplasm being a critical concentration, let us call it c_{crit}, that

determines the minimum inhibitory concentration, the MIC. In the case of periplasmic targets such as those of β-lactam compounds, the c_{crit} concept refers to the concentration in the periplasm. The MIC is a conventionally-agreed endpoint, which in liquid medium is defined as a lack of visible turbid growth after incubation for a defined period of time (typically 16–20 h) after initially suspending the bacteria in the solution of the antibacterial compound at a sparse *ca* 1×10^5 cfu mL^{-1}. The c_{crit} concept for target sites that are in the cytoplasm is applicable to either Gram-positive or Gram-negative bacteria. For targets that are external to the cytoplasmic membrane (e.g., penicillin-binding proteins or the targets of vancomycin), the best c_{crit} to assume would be the periplasmic concentration (using the concept of a Gram-positive periplasm as suggested by [65]). The c_{crit} is then the determinant of affinity with the target for a rapidly binding compound or it determines the rate of inactivation of a target if that is slow compared with other processes. Most target-inhibition processes that determine the MIC are likely to be approximately affinity-based or rate-of-inactivation-based, although other modes could be envisaged. As in all modeling, the c_{crit} concept is an over-simplification, but its value is that it can lead to an initial intuitive understanding of the physiological basis of the MIC [74] from which more-complex models can be built requiring fewer simplifying assumptions. Even simple models can also be a source of hypotheses that stimulate useful experimentation.

26.4.3.4 Introducing Efflux into Explanatory Models of the MIC

As mentioned above, the early explicit models of the β-lactam MIC did not take into account efflux [74, 86, 128]. Lakaye et al. [50] on the other hand created an explicit model of permeation balanced against hydrolysis by periplasmic β-lactamase that also took into account efflux pumping. That model was limited at the time by a lack of knowledge of the kinetic constants of any efflux pumps. With the elegant experimental work of Nagano and Nikaido [72] and Lim and Nikaido [60], curves are now available that relate rate of efflux to periplasmic concentration for several β-lactams in *E. coli*.

One of the compounds that Lakaye et al. [50] modeled was cephaloridine, for which efflux kinetic parameters have now been measured in *E. coli* by Nagano and Nikaido [72]. If one uses those efflux kinetic parameters (half-saturation, 288 μM; and V_{max} 1.82 nmol mg dry $mass^{-1}$ s^{-1}) in eqn (2) of Lakaye et al. [50], then one obtains the result that in the β-lactamase-negative strain at an MIC of 15 μM, the periplasmic concentration of cephaloridine would have been about 4.5 μM, rather than the 15 μM that the authors estimated. Then applying this critical periplasmic concentration to the β-lactamase-producing strain, 908R, one finds a predicted MIC of 1950 μM, which agrees better with the measured MIC of 1,600 μM than the value of 5700 μM, which had been predicted assuming negligible efflux. Thus in progressing from assumptions to measurements as more is known about efflux, predictions of physiologically modeled MICs can improve significantly.

26.4.3.5 The Influence of Efflux on the Potency of Cytotoxic Drugs in Mammalian Cells

Although this chapter is about permeability of bacteria to antibacterial agents, in the context of building models of the interplay between passive inward diffusion, active efflux, and other biochemical processes, the reader's attention is directed towards Stein's [120] instructive analysis of the impact of P-glycoprotein on the action of cytotoxic drugs against mammalian cells.

26.5 Epilogue

At one time, biochemical studies of 'permeability' and even the concept of permeability used in explanations of bacterial cellular phenomena had an academically low reputation as the last refuge of the biochemist who could not think of a better explanation [20]. This ideology prevailed despite there being outstanding physiologists such as Hill [37] and Roughton [106] who contributed permeability-based ideas to explain the kinetics of the penetration of oxygen into tissues and cells. To an extent, reduced permeability is still occasionally invoked as an implied catch-all mechanism of reduced susceptibility to antibacterial agents. For a number of years, decreased diffusion of antibacterial agents in biofilms was such a general hypothesis (see Sect. 26.3). Another more recent example of such a hypothesis is that of the decreased permeation of vancomycin across the murein sacculus of VISA, mentioned but not analyzed in detail above (Sect. 26.2.3). Gale et al. [21] provided another perspective on the notion of generalized permeability changes accounting for resistance. They proposed that it is unlikely that bacteria can reduce general permeability as a means of becoming less-susceptible to antibacterial agents, because that would raise many problems in explaining how such a "molecular overcoat" could reduce the influx of toxic compounds without affecting the influx of nutrients. Interestingly, however, we now understand that the up-regulation of efflux pumps does indeed represent that "molecular overcoat."

Notwithstanding the above, I would suggest that "permeability" is an important biochemical and physiological concept, subject to rigorous quantitative experimental and theoretical study. Not only is it scientifically respectable, but we ignore it at significant risk to understanding, given that diffusion is ultimately at the heart of growth and form in biology. Moreover, the physical nature of diffusion through various biological layers like bacterial cell envelopes, and the opposing phenomenon of transmembrane efflux; are also at the heart of a kinetic-based quantitative understanding of antibiotic susceptibility and resistance. Improving that quantitative understanding is expected to help in the creation of new antibacterial medicines derived from target-based drug discovery. This will be particularly important for the discovery of novel anti-Gram-negative bacterial agents.

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Chapter 27
Novel Antibacterial Targets/Identification of New Targets by Comparative Genomics

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27.1 Introduction

It is remarkable to reflect on the fact that the first complete genome sequence of a bacterium, *Haemophilus influenzae,* emerged only some 15 years ago. The pace of DNA sequencing, driven partly by new sequencing technologies, has resulted in an ever-increasing number of microbial sequences available for examination and study. This has had a profound impact on many areas of microbial study, including microbial physiology, genetics, and the emerging field of large-scale studies on gene regulation and systems biology. There have been several efforts to define the genes that are essential for microbial survival (at least in the laboratory), and to compare this irreplaceable "parts list" among microbial genera.

Very early on, antibiotic discovery groups were keenly interested in the identification of the subset of genes that encoded essential functions, as these were perceived to offer new target ideas for novel antimicrobials. Most existing antibiotic classes interacted with a relatively small number of processes in the bacterial cell. Genomic information offered the ability to survey across a large number of pathogens and identified common key enzymes in essential pathways. This information was employed to establish a broad range of high throughput screens to search for novel inhibitors among chemical libraries. Despite a significant effort by several groups, relatively few inhibitors were identified, and some have declared genomics as a source of novelty to be a failure [43]. However, the high expectations of an immediate payoff and the haste with which this work was pursued may have undermined the

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effort prematurely. In this chapter, we will review the work to date and point toward paths forward for genomic-based work on antimicrobial target identification. While genomics has extremely broad utility in the overall process of antibiotic drug discovery and development, this chapter will limit itself largely to the identification of potential targets for antimicrobials.

27.2 Bioinformatic Analysis of Microbial Genomes

The arrival of the *Haemophilus influenzae* genome in 1995 [19] heralded the extension of the shotgun sequencing strategy to a bacterial genome sequence, originally used by Sanger to sequence λ phage [49]. Prior to this event, it was believed by many that large genomes would require an orderly sequencing of overlapping, physically mapped gene segments (as was underway for *Escherichia coli* K-12 using the Kohara λ clone ordered library [10]). The ability to assemble the *H. influenzae* genome largely from small random "shotgun" sequenced fragments using computer assembly algorithms provided the proof that direct sequencing without prior construction of physical gene maps was possible. Of vital importance was the development of computational methods that could align and assemble the numerous short sequences into larger contiguous ("contigs") elements.

Genome analyses of bacteria (as well as other organisms) were initially focused on assembly and annotation of single genomes. Sequencing was performed using Sanger dideoxy fluorescent sequencing and capillary electrophoresis on banks of automated DNA sequencing machines [51]. The shotgun strategy consisted of shearing chromosomal DNA, separating the unselected, random fragments by size, and creating plasmid libraries (usually one with smaller size fragments [~2 kb] and another larger fragment library [~10 kb]). Both ends of many thousands of the plasmids in the library are sequenced (400–700 bp on average), to give "mate-pairs" which associate two areas of sequence at the end of each insert. Statistical treatments dictate how many times on average each region must be sequenced in the shotgun protocol, based on read length and size of the genome. In general, coverage is in the 8- to 16-fold region for the shotgun phase of the project. Through this multiple coverage of the genome by the many overlapping sequence fragments, it is possible to assemble these fragments by computer (using programs such as TIGR Assembler [45]) into larger sections of the genome termed "contigs", and eventually into larger scaffolds, which may still have gaps in the sequence. Some areas of the genome will not be represented and these may be "unclonable" in plasmids (perhaps due to toxicity or other properties of the region when replicating in the bacterial host), and gap filling in the laboratory using PCR extension and other techniques are part of the finishing steps to complete the finished genome assembly. Other features such as repetitive DNA (e.g., Box elements in pneumococci) and repetitive regions such as rRNA also contribute to the labor involved in the finishing problem.

The arrival of new technologies such as pyrosequencing (Roche/454), Sequencing by Synthesis (Illumina), non-optical semiconductor ion sequencing (Ion Torrent) and

DNA Ligase-mediated Sequencing (Applied Biosystems) has accelerated the rate at which genomic sequences can be obtained [38]. Many of the new sequencing technologies directly amplify the genome segments to be sequenced in vitro. These methods do not require the subcloning and amplification of short DNA fragments in bacteria, reducing the necessity of dealing with sequencing “unclonable” regions that do not show up after passage through a bacterial host. However, these technologies at present tend to generate somewhat shorter “reads” than capillary electrophoresis systems, and the challenge of sequence assembly with smaller regions has been discussed [46].

Concurrent with the genomic sequencing and assembly, much effort was expended in devising computational algorithms to parse and identify gene-coding regions in newly sequenced bacteria. Different software tools have been devised to scan genomic DNA sequence and identify protein coding regions, putative promoters, rRNA and t-RNA regions and many other features. Programs such as GeneMark and Glimmer have been employed to identify potential coding regions in the genome. GeneMark employs a Markov model that uses a fixed number of bases to statistically predict the likelihood that the region encodes a gene [4]. This program was employed in the *H. influenzae* gene identification. Glimmer is a follow on program that uses a more powerful interpolated Markov model (variable number of bases) to find coding regions in a microbial genome sequence [48].

After the identification of potential gene encoding regions in a genome sequence, functional predictions are attempted. Much of the early work on functional gene annotation relied heavily on the gene products that had been characterized experimentally in model organisms such as *E. coli, Salmonella typhimurium,* and *Bacillus subtilis*. The importance of decades of experimental bacterial genetics and physiology work that laid the basis for assigning gene functional roles cannot be overstated. Nevertheless, this still left a significant number of genes without documented physiological roles.

As new microbial genomes were sequenced, comparison of the translated amino acid sequences with programs such as those of the BLAST family (e.g., BLASTX, BLASTP, PSI-BLAST [2]) or FASTA [44] identified similarities with genes of established function. In the case of extensive similarities, the genes were considered to be orthologous and were derived from a common ancestral gene and encode proteins with the same function in different species. Coupled with this information was a continued effort by microbial physiologists to experimentally investigate some of the newly identified unknown function genes. Examples of such studies abound, and the reader is directed to a small number of examples to obtain a flavor of these efforts (see, e.g., [22, 36, 37]).

In subsequent years, an overwhelming number of computational programs have aimed at systematizing and extending the assignment of function to putative genes. Combined with increasing sequence information to draw upon, efforts such as TIGRFAMS and Clusters of Orthologous Genes (COGS) serve to identify families of similar function proteins, each using a different strategy. Programs such as EcoCyc for *E. coli* and HinCyc for *Haemophilus influenzae* attempt to rationalize the gene products into metabolic and regulatory pathways [30]. Suites of integrated programs such as the National Microbial Pathogen Database resource and the

Comprehensive Microbial Resource offer broad capabilities in analyzing microbial genomes [12, 40]. Comprehensive lists of available analysis programs on web sites for genome analysis have grown and are compiled in several publications (e.g., [17, 41]). Myriad tools exist to examine genomics information in different ways. Each January, an issue of Nucleic Acids Research is wholly devoted to short articles on new and updated programs for analysis of sequence information.

The volume of genomic information emerging has increased to the point that manual curation and annotation of sequences has been greatly supplemented by automated "pipeline" programs. An example is the Comprehensive Microbial Resource [12], which uses various programs such as Glimmer to identify putative genes, and then compares these to a "trusted" set of experimentally verified proteins in a database and also to protein families through TIGRFAM. The sequences are also automatically run in a BLAST search for similarity, and potential coding regions examined for features such as signal sequences, transmembrane helices and other well-established protein properties. Manual curation occurs subsequent to this step, and the data is released to GenBank after appropriate quality control has been applied. Overall, the numbers of sequenced bacterial genomes (approaching 1,000 at the time of this writing) and the improved quality of both the bioinformatic analyses and experimental work as a result of genomic sequences has had a broad impact in microbiology, including in the selection of potential antimicrobial targets and identifying antibiotic resistance mechanisms.

In terms of identifying gene functions that might make appropriate targets for novel antibiotic development, several criteria are applied to a bioinformatic analysis. Comparative genomics permits the identification and exploration of conserved genes among a set of pathogens. This indicates the potential range (e.g., broad spectrum versus Gram-negative only pathogens) of a selected target. Further, with the availability of human genome data, targets that might be highly conserved in humans and potentially prone to adverse toxicity as a result can be identified. Further informatics analysis can suggest functions for conserved genes, and can be helpful in designing high throughput screens to identify inhibitors. As detailed below, several genetic approaches have been employed to identify genes that are essential for survival and reproduction in bacterial pathogens. These genes are selected as targets in programs to identify chemical inhibitors that may be starting points for drug development.

27.3 Identifying New Targets for Antimicrobial Compounds

There are several approaches employed for verifying novel antimicrobial targets identified from bioinformatic analyses. Most of these methods aim to identify genes whose products are essential for bacterial survival under certain conditions. This is based on the rationale that compounds that interfere with the function of essential cellular processes will result in inhibition of growth or bacterial killing. Most existing antibacterials on the market target gene products that are required for growth on nutrient rich artificial media on the assumption that these targets will also be required

for growth in the host [9]. The fact that current clinical microbiology antibiotic susceptibility testing is based on growth in artificial media encourages development of antimicrobials that inhibit growth under similar conditions. Therefore, the following sections will discuss approaches to identify genes essential under such in vitro test conditions.

The recent explosion in the number of available genome sequences has allowed the construction and mapping of mutations at any chromosomal site in a number of bacterial pathogens. This has spurned the development of methods to evaluate gene essentiality on a genome-wide scale. There are two primary approaches to assess gene essentiality. One is by random mutagenesis and the other method is by targeted mutagenesis. We provide a few examples of both of these methods and how they can be used to characterize gene essentiality.

27.3.1 Random Mutagenesis for Essentiality Testing

27.3.1.1 Transposon Saturation Mutagenesis

Transposons constitute a large family of mobile genetic elements that can move between genetic loci. For the purpose of saturation mutagenesis, the type of transposons typically employed insert with little sequence specificity into the bacterial chromosome and carry antibiotic resistance markers that can be used to select for insertion events. Traditionally, transposons are delivered to the bacterial cell on a plasmid with a conditional replicon via transformation or conjugation and transposition onto the chromosome occurs inside the cell. More recently, methods of in vitro transposition have been employed (see below). Mutants carrying transposon insertions in essential genes will not survive; therefore, essential genes are identified based on a near zero frequency of transposon insertions. DNA sequencing of the flanking sequences and alignment with the published genomic sequence can map the location of the transposon on the chromosome. Some caveats are that distal insertions in a gene may not completely destroy gene function and some insertions can have polar affects and disrupt transcription of downstream genes, which may be essential (Fig. 27.1). These factors can lead to false conclusions about gene essentiality. Additionally, obtaining saturation of the chromosome by the transposon and mapping of the insertions can be arduous.

In response to some of the problems encountered with transposon mutagenesis, several variations have been developed. One of these methods is called GAMBIT (Genomic Analysis and Mapping by In vitro Transposition) [1], which works best with naturally transformable species such as *Streptococcus pneumoniae*, *H.influenzae* or *Neisseria gonorrhoeae* due to their ability to take up linear DNA for homologous recombination with the bacterial chromosome. The first step of this process involves highly saturated in vitro transposition mutagenesis and recombination onto the bacterial chromosome. The in vitro transposition is performed with a variant of the eukaryotic mariner transposon that has been engineered to carry an antibiotic

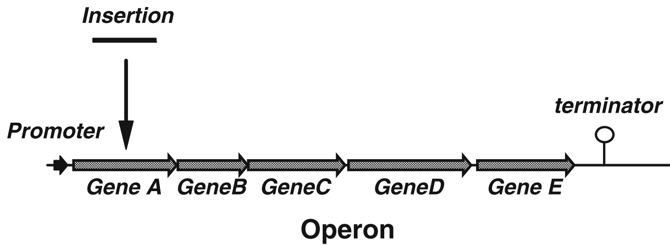

Fig. 27.1 The polar effect in an operon. This illustrates an operon, which is a set of genes that are under the control of a common, single promoter and have a transcriptional terminator at the end. In the case of a disruption of a promoter proximal gene by an insertion, there can be significant effects on transcription of downstream genes. In this example, disruption of Gene A could impair expression of downstream genes. Even if Gene A were non-essential, a downstream gene (e.g., Gene D) could be essential. Hence disruption of Gene A by insertion would erroneously indicate the essentiality of that gene, when in fact it is Gene D which is essential. Additional experiments would be necessary to clarify the true situation

resistance marker. The Himar1 transposon is typically used because only a single protein is required for the transposition reaction in vitro, and it has very little insertion site specificity. The targets for transposition are extended-length PCR products of selected regions of the bacterial chromosome, each approximately 10 Kb in length. This mutagenized pool of transposon-carrying PCR products is then introduced into the bacterial cell via transformation. The transposon will be inserted into the chromosome by homologous recombination between the chromosome and the DNA sequence surrounding the transposon. Successful uptake of the transposon and transfer to the chromosome is selected by plating on media containing the antibiotic for which the transposon encodes resistance. In the second step, the genomic location of each transposon is mapped by genetic footprinting. In this technique PCR off of the chromosomal DNA is performed with one primer that anneals to the mariner element and the opposite strand primer is specific for the chromosomal position from that particular mutagenized pool of mutants. These PCR products are analyzed by agarose gel electrophoresis. Each band on the gel represents a mariner element located at a given distance from the chromosomal primer site. The size of the gel bands indicates the location of the transposon relative to the chromosomal position of this locus specific primer. Because transposons that inactivate genes required for viability will not survive, regions on the gel that contain no transposon inserts (or blank spots) represent essential genes.

A similar technique to GAMBIT, called genome scanning has also been developed [47]. Mutagenesis is also performed in vitro; however in this case, the target of transposition is isolated *H. influenzae* chromosomal DNA. This DNA is subsequently transformed into *H. influenzae* and mutants are selected on media containing the antibiotic that has resistance encoded in the insertion element. The insertions are identified by Southern blots with a probe for the transposon. Sequencing the genomic DNA with primers that anneal to the transposon identifies the precise locations of the insertions. Regions where no insertions are found are presumed to contain essential gene(s).

Another derivative of the mariner transposon that has been employed for identifying essential genes called TnAraOut carries an antibiotic resistant marker as well as a promoter whose expression is induced by arabinose [29]. This promoter is placed at the end of the transposon such that it drives transcription outside of the insertion. If the TnAraOut element inserts in front of an essential gene, the arabinose promoter will drive expression of the downstream gene and viability of the cell will be dependent on the presence of arabinose. Although this system is adaptable to a variety of bacterial species, it was first applied to *Vibrio cholerae*. In this instance, the TnAraOut element was delivered to the chromosome in vivo in the presence of the selection antibiotic and low concentrations of arabinose. To identify essential genes, the smaller colonies that formed in the presence of low amounts of arabinose were selected and examined for growth in the presence of high arabinose and no arabinose. The transposon mutants that failed to grow in the absence of arabinose were presumed to be driving expression of adjacent essential genes. The DNA surrounding the transposon was then sequenced to identify the essential gene.

In all the examples described so far, the transposon insertions have been mapped individually using gene-specific PCR. This labor-intensive process limits the throughput of the genetic footprinting approach. Two techniques have been developed to overcome this bottleneck. One relies on microarray technology and the other on resequencing or parallel sequencing technology to locate transposon insertion sites. The microarray method, termed Transposon-Mediated Differential Hybridisation (TMDH), has been applied to a mariner transposon mutant library of *Staphylococcus aureus* as a screen to reduce the burden of the highly accurate but laborious PCR-based analysis [6]. In this process a library of *S. aureus* mutants is created with a form of mariner transposon that has a T7 promoter at the end of the element which directs transcription out toward the downstream chromosomal DNA in the presence of the T7 polymerase (which is not encoded by the bacteria). Genomic DNA from the library is extracted and digested with a restriction enzyme, followed by amplification with linker PCR. In vitro transcription is then induced from the transposon T7 promoter in the presence of the T7 polymerase and fluorescently labeled dNTPs. DNaseI is subsequently used to remove the DNA template. The labeled RNA run-offs are then hybridized to a microarray containing 60-mer oligonucleotide probes representing the *S. aureus* genome sequence. Regions that do not contain transposons show low signal intensities on the array and regions with transposons show higher intensities. However, the higher intensity regions display an irregular distribution of signal due to several factors such as the number of transposons present, their distance from the probes and local sequence environments that affect the efficiency of transcription from the T7 promoter. Software analysis of the microarray data is crucial for correct prediction of essential genes (areas with no transposons inserted) and in the case of *S. aureus*, higher specificity and sensitivity was found if the genomic DNA was digested separately with two different restriction enzymes. Each digested sample was then analyzed on microarray chips and the results were combined. This requirement is based on the fact that the RNA run off probe is defined by the restriction enzyme site, and not actual gene boundaries. For example, a probe that hybridizes within an essential gene may give a signal on the

microarray if it is downstream of a transposon that has integrated outside the gene. Therefore, not all probes are informative. To determine a list of candidate essential genes, the microarray data from informative probes overlapping each gene across all of the arrays using both restriction enzymes were combined. 274 candidate essential genes were identified by microarray and a further 235 candidates were chosen for examination by PCR and sequencing based on a manual inspection of the microarray data. One caveat of this method is that some of the smaller genes may be missed.

Two groups have exploited the relatively new massively parallel sequencing or re-sequencing technology to identify the position of transposon insertions in *Salmonella typhi* and *S. pneumoniae*. For the case of *S. typhi* the technique was termed TraDIS (transposon-directed insertion-site sequencing) [32], while the *S. pneumoniae* approach was called Tn-seq [55]. Both efforts rely on Illumina sequencing technology to sequence the transposon-chromosome junctions in a very large pool of mutants all at once. Illumina sequencing technology generates short sequence reads (up to 100 bases) from large numbers of DNA fragments that are immobilized on a flow cell surface. These millions of short sequencing reads can be assembled onto the genome sequence, allowing a whole bacterial genome to be re-sequenced in one experiment. During typical re-sequencing experiments, oligonucleotide linkers are ligated onto sheared genomic DNA fragments and all these fragments are amplified with universal PCR primers. Sequencing is then performed using complementary oligonucleotide primers. For mapping transposon insertions, the fragment pools are amplified using a universal primer and a primer complementary to the end of transposon. As with other analyses of transposon libraries, the lack of transposon insertions in a region leads to the presumption of gene essentiality. However, with re-sequencing technology, it is possible to analyze, on average, more than 80 inserts per gene, in theory ensuring a higher degree of saturation mutagenesis.

27.3.2 Targeted Gene Disruption Strategies

27.3.2.1 Plasmid Insertion Mutagenesis

While transposon insertion into the chromosome is random, genes can also be disrupted by targeted insertions. There are several methods for creating targeted gene disruptions. One such method is called plasmid insertion mutagenesis and has been applied to characterize essential genes in *B. subtilis* [31], *S. aureus* [57] and *S. pneumoniae* [53]. In this process PCR is used to amplify an internal portion of the coding sequence of the gene being targeted for disruption (approximately 300–500 bp in length), and this fragment is cloned into a plasmid suicide vector (Fig. 27.2). The suicide vector encodes for a selectable antibiotic resistance marker and cannot replicate in the target bacterial organism. Upon transfer to the host bacterium, the suicide plasmid integrates into the chromosome via a single crossover recombination event between the gene being targeted for disruption and the homologous sequence on the plasmid. The single crossover recombination results in a gene duplication

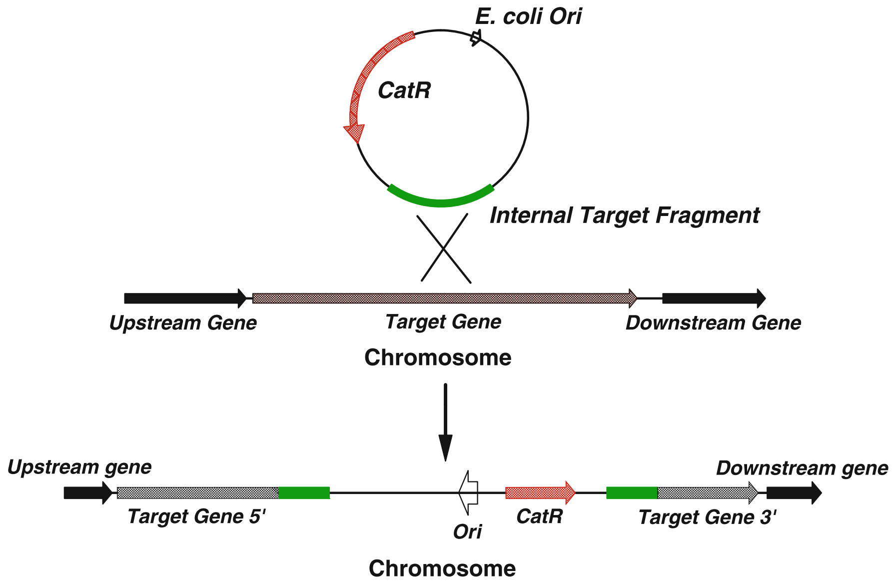

Fig. 27.2 Plasmid insertion mutagenesis. This illustrates the single crossover insertion of a non-replicating plasmid into the chromosome; guided by a small region of sequence homology. In this example, a small, internal region of DNA sequence that is identical with the central region of the target gene (*green*) is cloned into the plasmid in *E. coli*. The *E. coli* plasmid is transformed into a Gram-positive background (e.g., *Streptococcus pneumoniae*) where it is incapable of replication. By selecting for chloramphenicol resistance (CatR gene- *red*) in the Gram positive organism, one can identify the organisms in which the plasmid has recombined with the chromosome. By utilizing only the central portion of the target gene, two partial (non-functional) gene segments (*gray* and *green*) bracket the inserted plasmid

event; however, two partial copies of the gene are created on the chromosome because an internal fragment of the gene was used on the suicide plasmid. It is important to maintain antibiotic selective pressure, as it is possible for the plasmid to excise from the chromosome and restore the wild type gene in the absence of antibiotic. Insertions in essential genes would be lethal and antibiotic resistant recombinants would not be recovered. One of the limitations of this method is that it will not work for genes less than 300 bp long and there is a possibility that the plasmid insertion in the chromosome could have polar affects, changing the expression of neighboring downstream genes to cause lethality (Fig. 27.1).

27.3.2.2 Allelic Replacement

Another method of targeted gene disruption is called allelic exchange. Unlike plasmid or transposon insertion mutagenesis, in this method the targeted gene is deleted from the chromosome and may be replaced by an antibiotic resistance marker or an in-frame deletion. The advantage of this method is that if performed carefully with

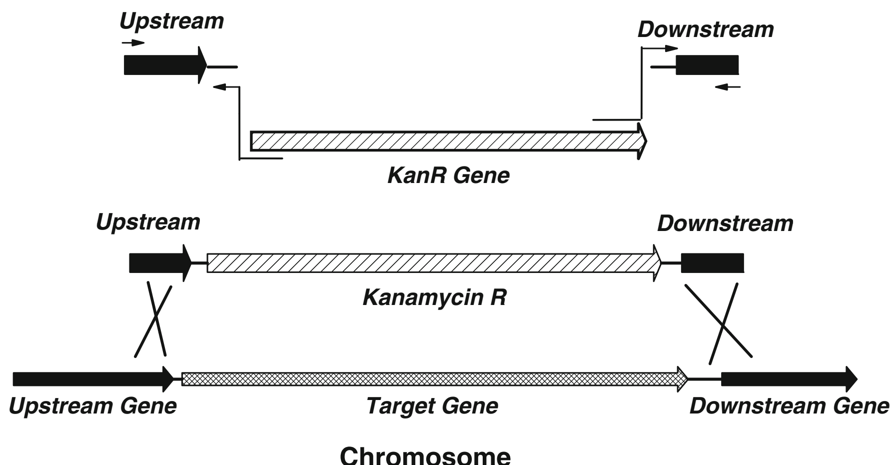

Fig. 27.3 Splice Overlap Extension PCR and allelic replacement. This technique uses PCR to generate an antibiotic resistance marker (e.g., kanamycin) flanked by the upstream and downstream chromosomal regions of the target gene intended for deletion. PCR is used to fuse the upstream and downstream sequences to the antibiotic resistance marker. Initially, three separate reactions are performed. Two reactions generate the upstream and downstream chromosomal regions with a small tail of identity to the resistance gene, and a third PCR generates the kanamycin resistance gene. Subsequently, the amplified upstream and downstream regions are PCR fused via the tails with the kanamycin cartridge, resulting in a single product containing the three regions. This fragment is then transformed into the target bacterial strain (usually a naturally transformable strain), and the homologous upstream and downstream regions promote recombination and integration into the chromosome at the target site. This results in deletion of the target gene and replacement by the selectable kanamycin resistance

regard to gene initiation and stop codon points, it is less likely to disrupt transcription of downstream genes. PCR is used to amplify the DNA upstream and downstream of the target gene as well as a gene encoding antibiotic resistance (Fig. 27.3). These three DNA fragments are then fused together in a method called Splice Overlap Extension PCR (SOE) that inserts the antibiotic resistance gene between the fragments flanking the targeted gene [25]. In organisms that have highly efficient transformation systems, such as *S. pneumoniae* or *H. influenzae*, it is possible to introduce this "spliced" PCR product directly into the cell via transformation followed by antibiotic selection. The antibiotic resistance marker will replace the target gene on the chromosome through a double crossover event between the chromosome and the two regions of homology on either side of the resistance marker. For organisms that are not naturally transformable with linear DNA, the "spliced" PCR product can be cloned into a suicide vector and then introduced into the host organism. Unlike plasmid insertion involving a single crossover event as described above, this is a stable mutation as the target gene is no longer on the chromosome and cannot be regenerated by excision. However, the frequency of double crossover events can be rare, therefore counter-selectable markers on the suicide vector can help eliminate single recombination events that merely integrate the suicide vector into the chromosome. The negative counter-selectable marker employed will kill the host bacterium if it is

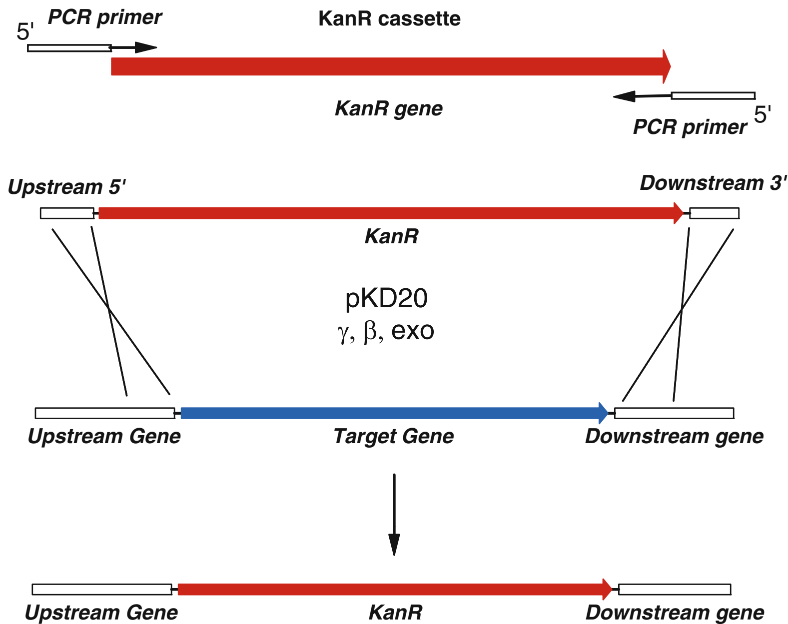

Fig. 27.4 Replacement of genes in *Escherichia coli* by the lambda red system. A resistance gene (e.g., kanamycin) is amplified employing two PCR primers that contain ~ 50 bases of identity flanking the region to be deleted. This product is introduced by electroporation into an *E. coli* strain expressing the lambda phage homologous recombination system (lambda red). The lambda genes, termed gamma, beta, and exo, are expressed from a plasmid (e.g., pKD20) under the control of the arabinose promoter. These gene products promote recombination of the linear PCR product into the chromosome via the small regions of identity. This results in deletion of the target gene and replacement by the selectable kanamycin resistance.

still present under certain growth conditions. "Spliced" PCR products can also be used to create unmarked, in-frame deletions on the bacterial chromosome. In this case, the PCR amplifications of the regions flanking the target gene are fused to one another by a two step PCR method. This fragment is then cloned into a suicide vector that encodes for both positive and negative selection markers for chromosomal integration and plasmid excision events. Successfully achieving double crossover recombination events can be laborious, making it not amenable to analysis of gene essentiality on a whole genome wide scale.

Under normal conditions, linear DNA cannot be introduced by transformation or electroporation into *E. coli* because it is degraded by the RecBC nuclease. As a result, for many years, construction of gene deletions on the *E. coli* chromosome was somewhat cumbersome. Many bacteriophages encode their own homologous recombination systems. Two groups have exploited such a system from λ phage to incorporate linear DNA fragments into the *E. coli* chromosome [11, 59]. In both of these methods the λ Red genes are transiently expressed from a regulated promoter to recombine a DNA fragment with the chromosome (Fig. 27.4). The λ Red system is comprised of three genes called *gam*, *bet*, and *exo*. Gam inhibits the RecBCD and

SbcCD nucleases from destroying the linear DNA and Exo and Beta promote recombination. Furthermore, with these recombination systems, gene targeting and replacement by an antibiotic resistance gene requires only short regions of homology with the regions immediately flanking the target gene (approximately 40 bases on each end). Therefore, the recombining linear DNA fragment can be created in a one-step PCR reaction using an antibiotic resistance gene cassette as a template with primers to the cassette containing 5′ tails homologous to the regions upstream and downstream of the gene being targeted for disruption. A group from Japan used this method to systematically create single-gene in frame deletions over the entire *E. coli* chromosome [3]. They targeted 4,288 genes for deletion and obtained 3,985 mutants. This collection of mutants, called the Keio collection, presumably represents the non-essential genes in *E. coli*. The λ Red system can also be exploited to easily introduce regulated promoters or gene reporters to the chromosome. In addition, there are reports of the λ Red system being adapted to other Gram negative pathogens, thereby extending its utility [13, 33, 58].

27.3.3 Gene Down-Regulation

The functions of essential genes are hard to examine genetically because knock out mutations in them are not viable. Down-regulating expression of a gene is another way to assess how the absence of that gene product will impact the cell. There are several ways of achieving this. Traditionally, an inducible promoter is inserted just upstream of the target gene thereby making expression of the target gene dependent on the presence of a small molecule or inducer. If the target gene is essential, the bacteria will not grow in the absence of the inducer. There are a wide variety of inducible promoters. Promoters regulated by IPTG (lactose), arabinose, or tetracycline are typically used in Gram-negative species while IPTG (lactose), tetracycline or xylose are often employed for Gram-positive bacteria. The level of expression of some inducible promoters can be modulated to obtain impaired growth by changing the inducer concentration such that there are just sufficient amounts to enable growth [14]. Strains with suboptimal expression were employed in a cell-based screen, on the theory that they were sensitized to inhibitors of the downregulated target, present in lower numbers. One caveat is that for some genes the basal level of expression in the absence of inducer can be sufficient for gene function. Another drawback is that replacing the natural promoter on the chromosome can have polar effects on downstream genes under the same promoter. To avoid the possibility of polar effects, the target gene can be put under the control of an inducible promoter and inserted into an ectopic chromosomal location or plasmid. A nonpolar deletion is than introduced into the wild-type copy of that gene.

Another method of downregulation involves a variation of the λ Red system. The recombining PCR product, instead of deleting the target gene, introduces an amber stop codon into the target gene along with a downstream antibiotic resistance gene [23]. This PCR product is co-electroporated into *E. coli* along with a plasmid encoding

for the amber suppressor tRNA under the control of the arabinose promoter. Selection for insertion of the amber stop codon is performed in the presence of arabinose such that the stop codon suppressor is expressed. Upon removal of arabinose from the growth media, expression of the amber stop codon suppressor is turned off and the target gene message will be truncated at the premature amber stop codon. This incomplete protein is presumably non functional. Subsequently, morphological changes can be monitored and the kinetics of cell death can be measured.

In *S. aureus*, down regulation of gene expression has also been achieved by expressing antisense RNA from an inducible promoter [20]. Antisense RNA inhibits expression by forming a RNA-RNA duplex between mRNA and the antisense RNA, thereby blocking translation of the message. Designing fragments that will make effective antisense RNAs for a particular target is not always easy. To get around this problem, in *S. aureus*, a shotgun antisense procedure was developed where random genomic fragments were cloned on a plasmid under the control of a regulated promoter. To identify genes essential for growth, this library was screened for fragments whose expression inhibits growth. The genes affected by antisense expression were identified by comparison of the DNA sequence of the plasmid insert to the published annotated genome sequence.

27.4 Overproduction of Target Gene Products for HTS and Structural Studies

Once bacterial targets are identified and validated, the potential exists to use these targets in the development of high-throughput screens (HTS) with the ultimate goal of discovering inhibitory compounds that can be advanced into the drug discovery pathway. Two important criteria must be satisfied prior to implementing an HTS that could produce thousands of data points per day of screening. First, some type of robust assay must be available and amenable to a high-throughput format. This assay could be enzymatic or by another approach such as binding affinity. Second, enough intact, functional gene product and any other necessary substrates, factors, etc. must be obtained to satisfy the reagent requirements for completion of the HTS. In particular, the availability of sufficient amounts of soluble, pure protein can be a limiting factor for screen initiation [27]. The first step in this process is the identification of target genes followed by cloning into expression vectors. The use of PCR in conjunction with the availability of bacterial genomic sequence data has allowed relatively straightforward cloning of desired target genes into a variety of bacterial expression vectors, most of which have been designed for use in *E. coli*.

One example of a family of commonly used expression vectors are the pET vectors marketed by EMD Biosciences/Novagen (www.emdchemicals.com) (Fig. 27.5). These pET plasmid vectors allow for cloning and expression of target genes in *E. coli* under the control of strong bacterial bacteriophage T7 transcription signals. Expression is induced when a source of T7 RNA polymerase is provided in the host

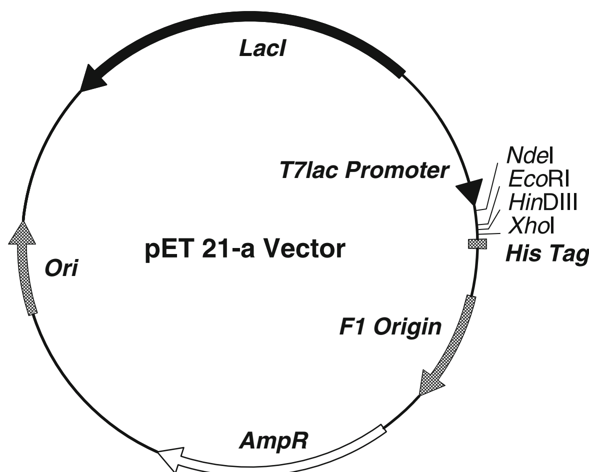

Fig. 27.5 Vector for high level protein expression Significant quantities of protein are required for purposes such as HTS and enzymatic characterization. Specialized vectors (in this example, pET-21a from EMD Biosciences/Novagen) have been devised that place the gene of interest under the control of highly expressing promoters, such as the T7/lac promoter. Genes of interest are amplified by PCR and inserted into the convenient restriction sites downstream of the promoter. The presence of the LacI repressor permits tight regulation of gene expression, minimizing the potential for toxic effects during cell growth. When cells have reached the desired density, expression can be induced to high levels with IPTG. The vector also offers the option of placing a hexa-histidine "tail" on the protein, which can be used to purify the recombinant protein on nickel resin columns, which preferentially bind the histidine tag region. Other similar vectors have additional features that can be employed to optimize protein production and purification.

cell resulting in a powerful expression response and large amounts of gene product following induction. Target genes are first cloned into host strains that do not contain a source of T7 RNA polymerase greatly reducing gene expression, an important property for gene products that may be inherently toxic or toxic when overexpressed. The expression plasmid then can be transformed into another *E. coli* host strain that contains a chromosomal copy of the T7 RNA polymerase gene under control of a *lac* promoter. Now expression can be induced with the addition of IPTG to the bacterial culture, "turning on" gene expression and protein production. Varying the amount of inducer, which is sometimes important in obtaining soluble protein, can control expression levels. T7 promoters are available with different stringencies that can be vital in obtaining good yields of toxic proteins and thus increasing the number of targets amenable to this approach. These pET vectors and accompanying reagents are available as kits from the manufacturer and are relatively easy to use.

The use of regulatable overexpression systems in bacteria often results in sufficient yields of protein to initiate HTS. However, this is not always the case. For example, membrane proteins tend to be more difficult to overexpress probably due to their extensive regions of hydrophobicity [8]. Occasionally, proteins are produced in an insoluble form such as in inclusion bodies [39]. This can sometimes be addressed by several techniques developed to recover soluble, functional protein

including reducing the growth temperature of recombinant bacterial cells [5, 39, 54]. For expression of such proteins with solubility issues, reducing the temperature to 30°C or even lower can often help dramatically improve yields of soluble protein. An example of a commercially available system for this purpose is the TaKaRa cold expression system offered by Clontech (www.clontech.com) with a choice of several cloning vectors. For those proteins where bacterial hosts are ultimately not successful, one can try alternative methods such as insect baculovirus or yeast expression systems such as *Pichia pastoris* [7, 28].

Once proteins are produced in sufficiently large quantities, further purification is often necessary. Affinity purification tags can be fused to any protein of interest and several are available from commercial vendors. These tags, which consist of small amino acid additions added to either the C-terminal or N-terminal positions on the recombinant protein, allow for rapid, high yields of purified proteins [34]. Fusion tags are often included in the expression plasmids to facilitate this process. Probably the most widely used affinity tag consists of polyhistidine (His-tag, usually hexahistidine) and offers several advantages for the researcher [24]. The tag is relatively small, non-immunogenic, and may not need to be removed to maintain protein activity. No specific protein structure is required to be effective and purification of insoluble proteins can occur under denaturing conditions. This tag binds to immobilized transition metals and Ni-NTA (Ni[II]-nitriloacetic acid) resin is most often used for affinity chromatography. Finally, there are numerous commercial vectors available from various sources (examples: www.qiagen.com, www.clontech.com, www.invitrogen.com, www.promega.com, etc.). Examples of other affinity tag systems include glutathione-S-transferase (GST) based on the strong affinity of GST for glutathione [51] and maltose-binding protein (MBP) that uses affinity for amylose resin [35]. These latter two tags are much larger than His-tags and require cleavage to regenerate active protein after purification. For further information on affinity tags see the review by Waugh [56]. Efforts continue in attempts to further improve methods to obtain large amounts of proteins with acceptable purity for screening and other purposes.

27.5 Mutant Isolation to Determine Mechanism of Action

After identification of hits from screens, it is desirable to identify or validate the target of inhibitors that are found to have antibacterial activity. Functional genomics studies play a key role in both target validation and mechanism of action determination [21]. One way to accomplish these objectives is to isolate mutants resistant to the inhibitor. If the target is known, as with strains employing regulated expression of a specific gene, one can obtain DNA sequence data for the target gene and look for mutations. If no mutations are discovered within the gene-coding region or if strains are used that do not use regulated expression, this method is not sufficient. With the current and continuously expanding microbial genome database and the impressive advances that have been made in DNA sequencing techniques, it is now possible to use whole genome sequence to analyze genomes [42]. One can even sequence multiple strains

and directly compare the data. A recent example of the use of whole genome sequencing employed this approach to compare three independent *Streptococcus pneumoniae* mutant strains resistant to linezolid [16]. Mutations in 23S RNA were found in all three mutants including one mutation previously reported to increase linezolid resistance. In addition, three new mutations were discovered and experimentally confirmed to be involved in antibiotic resistance.

Another approach is to construct screening strains that are designed to selectively identify inhibitors of the desired targets. Thus, one can obtain screening hits that presumably affect a known target or pathway making subsequent mechanism of action verification an easier process. An example of this approach was reported by Steidl and colleagues [52] where *lacZ* fusion strains were constructed using the promoters of five cell wall stress stimulon genes that were induced only in the presence of cell wall antibiotics. Induction was observed in all fusion strains by all cell wall inhibitors tested and not by other antibiotics that were not cell wall agents. Therefore, inhibitors identified in screens using these strains would also presumably target the bacterial cell wall. Another example of this approach was described by Shapiro and Baneyx where a *sulA-lacZ* fusion construct was used to detect inhibitors of DNA replication [50]. The application of this methodology to high-throughput screening was also demonstrated. A similar strategy was reported by Fischer et al. using a fatty-acid-pathway-specific reporter assay as an example [18]. Freiberg and colleagues [21] extended this approach to enable the identification of novel antibacterial compounds by compiling a database of expression profiles induced by 14 antibiotics through whole-genome microarray data analyses. One could match the expression profile of an unknown compound to one induced by previously characterized antibacterial compounds. They demonstrated the utility of their methodology by applying their approach to two novel antibiotics indicating that one was an inhibitor of phenylalanine-tRNA synthetase and the other was an inhibitor of the bacterial acetyl coenzyme A carboxylase.

Another chemical genomics strategy was recently reported where 245 *Staphylococcus aureus* antisense RNA strains were engineered for reduced expression of staphylococcal genes essential for growth [15]. These strains were now sensitized for inhibitors of the associated gene products thus providing an indication of the target of the inhibitor. The utility of this approach was demonstrated in an accompanying paper where two new peptidoglycan inhibitors that potentiated carbapenem activity against methicillin-resistant *S. aureus* were profiled [26]. See the Cell-based Screening (Chap. 28) in this book for more detailed discussions.

27.6 Conclusion

Bacterial genomic information has had a profound impact on the antibiotic discovery process, and presented an opportunity to dramatically expand the range of targets available for antimicrobial drug discovery. Initial bioinformatic efforts were focused on identifying genes in sequenced genomes, assigning function where possible, and

comparative genomics to compile a list of common, conserved genes. From this list, experimental approaches were devised to determine genes essential for cell survival.

While the gene disruption methods presented here can yield tremendous amounts of information, one should be cautious when interpreting data from experiments aimed at finding essential genes on a whole-genome wide scale. These experiments mostly rely on negative data, or the inability to isolate a genomic insertion or deletion. When gene essentiality is suspected, the mutant should be generated again in the presence of a complementing copy of the target gene at an ectopic site. This will confirm that the mutant could not be isolated due to essentiality of the deleted or disrupted gene.

Genomics information is also important in the downstream process after a target has been selected. The availability of gene sequences permits precise construction of over expression vectors that yield quantities of highly purified target protein. This capability is critical to large scale HTS campaigns aimed at identifying inhibitor compounds. Microbial genomics also has role to play in identifying mechanism of action for antimicrobial compounds in the discovery phase. It is still an open question whether any of the single gene targets will ultimately yield fundamentally new compounds (see Silver, Chap. 2 for a discussion). However, there is no question that genomics has altered our view of the microbial world, and expanded our understanding of antibiotic mechanisms of action and drug resistance.

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Chapter 28
Cell-Based Screening in Antibacterial Discovery

Scott D. Mills and Thomas J. Dougherty

28.1 Introduction

28.1.1 Beginnings

The use of intact bacterial cells to screen for the presence of growth inhibitors is at the foundations of early antibiotic research. In 1928, the clearing of bacterial growth around a fungal colony on an agar plate was the key observation by Fleming [1] that led to the discovery of penicillin. In the same era, Dubos, then a student of Selman Waksman, discovered gramicidin by using a cross-streaking agar plate method in which the inhibition of one organism by another could be readily discerned. Waksman's laboratory steadily experimented and improved screening methods over the years [2]. Indeed, virtually all of the earliest discoveries of antibiotics were by bacterial growth inhibitory activity.

One exception was Prontosil, the first synthetic antibacterial of the sulfonamide chemotherapeutic agents in 1935. It was Gerhard Domagk who first showed that Prontosil, inactive against *Streptococcus pyogenes* in vitro, cured mice infected by β-hemolytic streptococci. Later compounds in the same class did have in vitro activity, as it was found that Prontosil required metabolism to release the active sulfanilamide. The sulfonamides were the first antibacterials to be used on a large scale to treat a range of bacterial infections, particularly those caused by streptococci [3, 4]. These were most important during the early years of World War II until the introduction of penicillin; however even today sulfa compounds retain a place in antibacterial therapy.

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28.1.2 The Golden Age of Antibiotics

The first antibiotics were prescribed in the late 1930s, beginning a great era in discovery, development, and prescription. The discovery of the first naturally occurring antibiotics revolutionized medical practice due to their unprecedented success in preventing and curing infectious diseases. Penicillin was discovered by Fleming in 1928, and later isolated by Florey and Chain in 1939 [5]. In 1941, Moyer succeeded in optimizing the growth conditions of *Penicillium notatum* for mass production of penicillin, and in 1943 clinical trials were performed showing that penicillin was the most effective antibacterial agent available [6]. Penicillin production was quickly scaled up and made available to treat Allied soldiers wounded in World War II. Using a systematic method to discover new antibiotics by their effects on bacterial growth inhibition, Schatz and Waksman discovered streptomycin [7] in 1944. The activity of this compound against *Mycobacterium tuberculosis* was considered a major breakthrough [8]. With the end of World War II, many companies with fermentation backgrounds for the production of chemicals, such as Pfizer in producing citric acid, invested efforts in identifying new antibiotics from fermentation sources. Drug companies conducted intensive searches for new antibiotics that resulted in the discovery of most of the key antibacterial drug classes that are in use today. This exhaustive discovery effort was based largely on the screening of microbial fermentation broths for the presence of metabolites that kill pathogenic bacteria in vitro, and extracts which could subsequently cure infections in animal models in vivo. The "classic" bacterial cell-based screening approach was labor intensive, relatively low throughput, and relied predominantly on soil inhabiting Actinomycetes, especially *Streptomyces* species, as rich sources for new antibiotics. Soil samples from around the world were collected and screened for activities. In addition to streptomycin, other aminoglycosides were discovered such as kanamycin [9] and neomycin [10]. The tetracyclines [11, 12] chloramphenicol [13], cycloserine [14], and rifamycin [15] were all early antibiotic discoveries, and all of these used the cell-based screening methodology to identify the antibacterial activities in fermentations.

The primary technique used to identify antimicrobial activity was to cut small wells into agar plates which had first been seeded with a culture of various bacterial target pathogens. The wells were then filled with the test fermentation broth, and the plates placed into an incubator. Over time, the compounds in the wells diffused into the surrounding agar. After overnight incubation, antibacterial activity was manifested as zones of no growth (inhibition) surrounding the wells that contained fermentations with activity. A variation on this assay used paper discs soaked in the test fermentation broths instead of wells. The discs were placed on the surface of the pathogen-seeded agar plates, and again zones of growth inhibition indicated activity. During the early post–World War II years (1950–1970), major new classes of antibiotics were discovered using these relatively simple bacterial cell screening systems. An excellent review, published in 1961, details a symposium at which Eli Lilly scientists describe the painstaking process by which they discovered and developed the antibiotic vancomycin [16]. In addition to the paper disc process used to identify active cultures, this paper also illustrates the use of paper

chromatography combined with microbial growth inhibition detection to identify active fractions. This paper is also an excellent primer on how antibiotics were discovered and developed during the so-called "Golden Age." As new classes were discovered, it became more important to establish if an activity in a screen was truly novel; clever schemes were devised for dereplication, a process whereby the novelty of an activity was assessed. As might be anticipated, the earliest antibiotics identified were often also the most commonly produced. Dereplication schemes were essential to quickly discriminate common reisolations from the increasingly rare novel antimicrobial finds. Between 1944 and 1972, human life expectancy jumped by 8 years – an increase largely credited to the introduction of antibiotics. Bacterial infection as a leading cause of death plummeted.

28.1.3 Declining Discovery Efforts, Emergence of Resistance and Unmet Medical Need

Following the successes of the golden age, antibiotic discovery efforts declined, and by the end of the 1960s, new classes of antibiotics were not being developed. By 1970, the pace of novel compound discovery had slackened, and Lloyd Conover of Pfizer surveyed the pharmaceutical community as to what new directions were needed for antibiotic discovery to continue. Improvements in screening technology/detection methods as well as novel sources of antibiotic-producing cultures were cited as necessary [17]. On the other hand, there were some at that time who thought that the war against bacterial infections had been decisively "won" and questioned the need for continued antibiotics discovery efforts. However antibiotic resistance, while still relatively rare in that period, encouraged continued antibiotic research. At the same time, the industry shifted focus to some extent from novel discovery to concentrating on improving the activity and pharmacological properties of the existing antimicrobial compounds, a process that largely sustained antimicrobial research through the 1980s and even into the early 1990s. As a result, much of the limited discovery effort was focused on exploiting semi-synthetic approaches to chemically alter and thus improve the properties (e.g., revive drugs that were ineffective due to emergence of resistance, expand spectrum of activity by improving potency and selectivity, and optimize pharmaceutical properties to increase dosing or safety profiles) of existing classes. In this period, drug companies also began to focus more of their attention on other areas of medical need, where therapeutic and economic opportunities seemed greater. With a broad range of therapies available, programs were curtailed as the medical need for antibiotics was erroneously perceived to be fulfilled.

28.1.4 Resistance and Resurgent Interest

As is now readily apparent, with continued use over time antibiotic resistance increased dramatically and spread, rendering some classes of antibiotics increasingly

ineffective. By the 1990s, penicillin-resistant strains of one of the most common causes of pneumonia, *Streptococcus pneumoniae*, as well as drug-resistant gonococci spread around the world. Hospital associated resistant infections such as MRSA, VRE, and gram-negative organisms also were on the rise. Today, we are confronted with some organisms that are multidrug resistant and in some cases, few options remain for effective therapy. The Infectious Diseases Society of America issued its landmark paper "Bad Bugs, No Drugs" in 2004 in response to the alarming number of pathogens that are multidrug resistant.

> The Centers for Disease Control and Prevention (CDC) have compiled data that demonstrate that the rate of infection due to methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *E. faecium* (VRE), and fluoroquinolone-resistant *P. aeruginosa* have increased dramatically over the last decade. More people now die of MRSA infection in US hospitals than of HIV/AIDS and tuberculosis combined. Furthermore, panantibiotic-resistant infections now occur. Several highly resistant gram-negative pathogens – namely Acinetobacter species, multidrug-resistant (MDR) *P. aeruginosa*, and carbapenem-resistant Klebsiella species and *Escherichia coli* – are emerging as significant pathogens in both the United States and other parts of the world. Our therapeutic options for these pathogens are so extremely limited that clinicians are forced to use older, previously discarded drugs, such as colistin, that are associated with significant toxicity and for which there is a lack of robust data to guide selection of dosage regimen or duration of therapy.

28.2 Modern Antibiotic Discovery Programs

28.2.1 Target-Based Genetics/Genomics Antibiotic Discovery

Since the introduction of the quinolones in 1962, only two novel antibacterial classes have been approved for clinical use, linezolid in 2000 and daptomycin in 2004 [18]. In the 1990s, new technologies and innovations such as bacterial genome sequencing, high-throughput screening, molecular modeling, and combinatorial chemistry inspired a revival in antibiotic research. The integration of these novel technologies was meant to foster a new drug discovery paradigm based on high-throughput screening for inhibitors of enzyme targets essential for bacterial survival. The concept was that any novel whole-cell activity had been largely screened out of the existing natural product and synthetic compound libraries, and so high-throughput screening against the genomically selected intracellular targets would lead to an abundance of novel chemical starting points with intrinsic potency against these essential bacterial enzymes. These chemical leads could then be optimized by medicinal chemists to convert potent enzyme inhibitors into whole-cell active, broad-spectrum antibacterials capable of curing infected animals. Great effort was put into identifying potential new targets in a broad range of bacteria by genome sequence comparisons, with target proteins selected, purified, and screened for inhibitors in large high-throughput screening (HTS) campaigns.

In reality, this task has proved to be more difficult than anticipated. Part of the difficulty associated with the discovery of new antibacterial classes is that a high

bar was established by early antibiotics in terms of their safety and efficacy. An additional difficulty is the requirement for potent broad-spectrum activity due to the nature of empiric treatment by physicians who often treat clinical indications based on symptoms, sometimes never isolating the causative agent. In recent years, many large efforts in antibacterial discovery have been scaled back or stopped altogether based on the perceived lack of success of the genomics-driven target-based discovery paradigm for antibiotic research [19]. There are multiple aspects that contribute to the high attrition rate associated with target-based screening. One of the most difficult challenges of the target-based discovery paradigm is to transform a potent enzyme inhibitor into a compound with broad-spectrum antibacterial activity. The field may have grossly underestimated the difficulty in generating the structure activity relationships (SAR) required to overcome the cell membrane permeability barriers and to avoid the plethora of efflux pump mechanisms that are inherent in bacteria for survival in harsh nutrient-deprived environments. However, the target-based strategy has led to a few promising lead compounds that have made it to preclinical development for treatment of Gram-positive infections. As yet, this strategy has not delivered any late-stage clinical candidates (Phase II or higher), but given the fact that large-scale genomic information is a relatively recent development, this may not be surprising. On the other hand, effective treatments of serious Gram-negative multidrug-resistant infections have remained elusive with only a few less than optimal options [20]. Future research will need to focus more effort on these extremely challenging and problematic pathogens.

In fact, as outlined in several sections below, molecular biology and genomics has had a profound impact on the practice of bacterial cell-based screening, with several extremely clever approaches. Examples will include several ways to produce targeted alterations in gene expression levels in bacteria that are used to improve the sensitivity range for detection of compounds which inhibit specific targets. Several bacterial gene reporter systems to signal the presence of subinhibitory levels of antimicrobials have been devised, and there are several genetic approaches for the mechanism of action determinations.

28.2.2 Physicochemical Properties of Antibiotics

Before addressing cell-based screening techniques, another critical aspect of antibiotic screening requires brief discussion. This is the properties of the chemical collection used as the screening substrate. In recent years, many large pharmaceutical companies have attempted to optimize their compound libraries, but a question arises in whether a collection biased for human disease targets is also satisfactory for antibacterial screening.

A set of guidelines have been developed to ascertain the “drug-like” characteristics of molecules, in an attempt to predict the most effective directions to explore in chemical space. The original, highly cited “rules” were defined by Lipinski et al. in 1997 [21], and really derive from the properties of marketed (largely oral) products.

It is clear that the physicochemical properties of antibacterial drugs do not necessarily follow the guidelines established in other therapeutic areas, namely, Lipinski's rules [22, 23]. In fact, Lipinski explicitly stated that antibiotics (and a few other drug classes) by and large lie outside of the "rule of 5" parameters [21]. Macielag (see Chap. 24, this volume) and O'Shea and Moser [24] have analyzed the properties of antibiotic classes, and elaborated on specific attributes found in antibacterial compounds. It is clear that the antibiotics, both natural products and synthetics, occupy a chemical space that has properties distinct from compounds that target human drug receptors. Yet the libraries that have been screened within the industry are often optimized toward other therapeutic areas and are often biased to follow Lipinski's rules, thus the failure to identify new antibiotics in these collections may be expected to some degree.

28.2.3 A Comment on Natural Products

Part of the explanation for the difficulties with existing chemical libraries is undoubtedly that most antibiotics likely evolved as natural product secondary metabolites with advantageous biologically active properties (e.g., the ability to penetrate microbes and selectively inhibit target enzymes) that make them well suited for therapeutic uses despite their physical properties. The true function of antibiotics made by bacteria and fungi is still debated, with some arguing that these naturally occurring small molecules may have evolved for competitive purposes in their ecosystems. It has also been argued that natural product antibiotics may have evolved for modulating inter-microbial communication [25]. Such chemical communication would depend on the concentrations of these small molecules, with a given molecule acting as a messenger at low concentrations and exerting antibiotic activity at higher concentrations.

In the past, natural product extracts have played a significant role in the discovery of antibacterials. In recent years, these efforts have been deemphasized for a number of reasons, but one major argument has been diminishing returns and the rediscovery of known compounds placing a heavy burden on dereplication processes. A different philosophy has been developed by Baltz, who has recently argued that in order to identify novel antibiotics in fermentations from soil organisms, it is necessary to combine high-throughput cell-based screening tools with known antibiotics that inhibit the growth of more common forms of antibiotic-producing organisms, a process he terms "deep selection" [26]. It has been estimated that the pharmaceutical industry has screened roughly 10,000,000 microbes for natural products over the past 50 years. However, only a very small percentage of microbial species has been screened due to the tremendous diversity of soil microbes and the limitations of available screening techniques. He outlines a plan of attack to identify new antibiotics from soil organisms. Rather than classic flask fermentations, Baltz describes a system that encapsulates spores from soil samples in calcium alginate microbeads. These beads contain growth media and suppressing antibiotics, acting

as a self-contained microfermenter system. This miniaturized system yields the large numbers of new organisms he postulates are necessary to find novel natural products. In addition, he describes the genetic construction and deployment of an *E. coli* screening strain that is resistant to 15 common antibiotic classes, thereby increasing the probability that a hit which inhibits growth of this strain will be in a novel class. This screening strain would greatly reduce the dereplication burden of reisolating known classes of antibiotics. This is critically important to the effort to "deep select" for novel soil-derived antibiotics.

Likewise, programs to employ genomics-based approaches for screening and engineering of the antibiotic-producer organisms are another potential source of novelty. Many ingenious paths are being explored to identify or create new antibiotic pharmacophores from microorganisms. For a more detailed discussion of these efforts, see the chapter by Singh in this volume (Chap. 25).

28.3 Bacterial Cell-Based Screening Techniques

28.3.1 Whole-Cell Screens: Simple Microtiter Plate or Agar Plate-Based Phenotype Assays

As described above, most of the early antibiotic compounds were discovered using bacterial cell growth inhibition assays. Although one might anticipate that these types of assays are no longer in use, this form of screening campaign continues to the present day. An example would be the recent description of a liquid turbidimetric bacterial cell screen employing *E. coli* and *Pseudomonas aeruginosa* [27]. In this case, a compound library of some 150,000 small molecules was screened. This represents the "classic" bacterial cell-based screening approach. A variation of this type of screening has used colorimetric or fluorometric indicators to measure bacterial viability [28]. Metabolic activity can be assayed by the use of the tetrazolium salt MTT (3-(4,5 dimethylthiazyl-2-yl) 2,5 diphenyltetrazolium bromide). MTT is added at the end of an incubation of bacteria with potential inhibitors. The MTT is reduced to a colored product by microbial dehydrogenases, and measured spectrophotometrically. Growth inhibition leads to reduced product formation. Other growth detection methods include ATP production (luciferin-luciferase) as a measure of inhibition. Most of these screening assays are done in either 96 or 384 microtiter plate liquid formats.

A variation of growth inhibition as an indicator of antimicrobial activity employs an agar-based method. In this assay, agar is cooled to just above the point where it solidifies and is then seeded with a low inoculum of the indicator bacteria used for detection. Solubilized test compounds are applied either into wells cut into the agar, on paper discs soaked in the test compound solution, or the test compound solution is applied in small volumes directly to the surface of the agar and allowed to absorb. Multiple compounds can be tested on large plates. The plates are incubated after the

application of the test substances, and over several hours the bacterial growth will become evident. The test material diffuses away from the point of application, and a gradient is established. If the material has antibacterial activity, a circular zone lacking bacterial growth is observed around the active compound.

28.4 Selected Target Bacterial-Based Screening

28.4.1 Pathway Screens: Cell Wall

As screens which measured inhibition of bacterial growth cell began to yield diminishing returns due to reduced yields and dereplication becoming a major consideration, more sophisticated cell-based screening strategies were devised to target specific pathways or processes. Perhaps the most widespread examples of this approach were in phenotypic screens for cell wall synthesis inhibitors (Fig. 28.1). To this day a perennial favorite as a target for antibacterials exploitation, interest in this pathway was stimulated by the lack of toxicity and efficacy of the β-lactam antibiotics [29]. Several groups took different approaches to identify cell wall inhibitors. In addition to improved sensitivity permitting detection of low-abundance molecules in fermentations, these screens would also incorporate an element of target specificity. The screens were the result of improved understanding of the mechanism of action and physiological responses to cell wall antibiotics.

A number of mechanism-based screens were devised which detected cell wall inhibitors. These included relatively simple screens, such as employing two organisms, *Bacillus subtilis* and *Mycoplasma*, which relied on the fact that mycoplasmas do not have peptidoglycan, and therefore only *B. subtilis* would be inhibited. A secondary assay measured the incorporation of radiolabeled diaminopimelic acid into the cell wall fraction of Bacillus. Other cell wall screens employed organisms that were hypersusceptible to β-lactam compounds [30–32]. Hypersusceptible mutants to other cell wall antibiotics were also derived [33]. A different screening approach was employed by researchers at Merck (see L. Silver, Chap. 2, this volume).

Fig. 28.1 (continued) diaminopimelic acid (DAP-Gram negatives) or L-lys (predominates in Gram positives, but can be other amino acid substituents) on UDP-MurNac. The dipeptide D-alanyl-D-alanine is added using ATP to generate UDP-N-acetyl muramyl-pentapeptide (UDP-MurNac-pentapeptide). MraY next catalyzes the transfer of MurNac-pentapeptide from UDP to a C55 undecaprenol to form Lipid I. Addition of a N-acetyl-glucosamine (from the UDP carrier form) to the 4 position on the muramic acid moiety generates Lipid II, which is the complete peptidoglycan precursor bound to lipid. In some Gram-positive organisms, additional peptide bridge amino acids are added at this stage. The Lipid II form is then translocated across the cell membrane, and penicillin-binding proteins (PBPs) catalyze transglycosylation (of the disaccharide) and transpeptidation (of the peptide side chains represented by solid lines) of the disaccharide pentapeptide into the preexisting cell wall

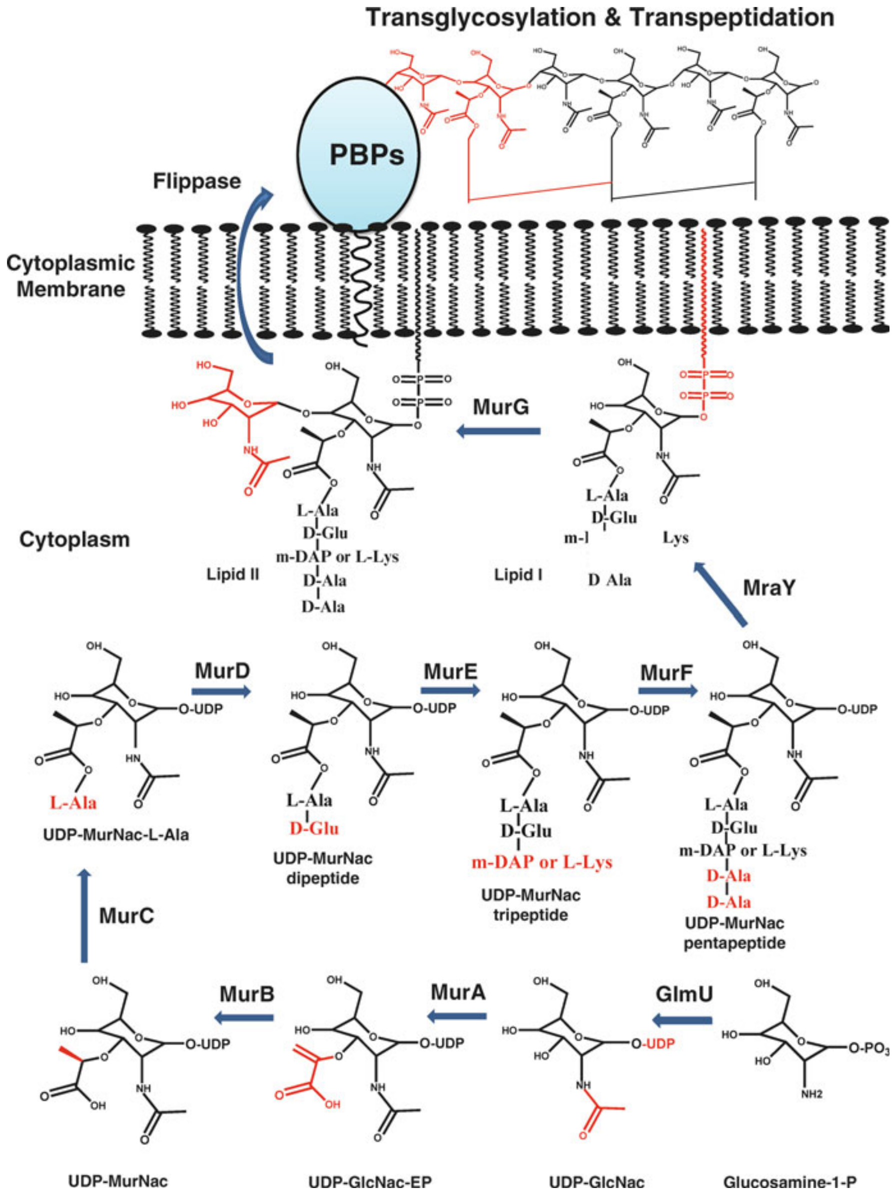

Fig. 28.1 Cell wall biosynthesis pathway in bacteria. The figure illustrates the steps, both intracellular and extracellular in constructing peptidoglycan with each step change shown in red. Beginning in the lower right, the enzyme GlmU adds an acetyl group (from acetyl-CoA) and a uridine diphosphate (from UTP) to glucosamine-1-phosphate. This yields UDP-N-acetyl-glucosamine. This in turn is modified by MurA to a 3-enolpyruvate form using phosphoenolpyruvate as the donor. MurB subsequently reduces the enolpyruvate to lactate with NADPH, generating UDP-N-acetyl muramic acid (UDP-MurNac). A set of sequential amino acid ligases (MurC, MurD, MurE), employing ATP as an energy source for the reactions, respectively, add L-alanine, D-glutamate, and either Meso

In the early 1960s, a program was devised by Hendlin and Dulaney to specifically target the cell wall pathway [34]. This assay measured the ability of compounds to produce spheroplasts from Gram-negative rod bacteria in hypertonic media. This was a heroic effort, as direct visualization of the spheroplasts under the microscope was employed as the screen. Fosfomycin (phosphonomycin) was discovered, which is an antibiotic that inhibits the activity of the first committed step in peptidoglycan synthesis, transfer of the enol pyruvate moiety onto UDP-N-acetyl-glucosamine, which is catalyzed by the protein MurA. A similar assay using *Proteus mirabilis* spheroplasts and inhibition of [^{14}C] diaminopimelic acid into the cell wall fraction of *E. coli* led to the discovery of fosfonochlorin. Finally, the spheroplast assay was also responsible for the discovery of carbapenems. The compound thienamycin was identified in a fermentation broth of *Streptomyces cattleya* and a more stable amidine derivative was commercialized [35]. This compound, imipenem (a combination of the amidine thienamycin and cilastatin, which inhibited kidney degradation of the antibiotic), was the first of several carbapenems on the market. These compounds continue to be used in serious infections to this day.

Imada et al. at Takeda used hypersusceptible mutants of *P. aeruginosa* and *E. coli* to discover β-lactam antibiotics [36] that were not produced by fungi, but rather products of bacterial cultures (in this case, *Pseudomonas acidophila*). These molecules were unique from other β-lactam antibiotics in that the β-lactam ring was not part of fused ring systems (thiazolidine and dihydrothiazine) but rather monocyclic β-lactams. At the same time, Richard Sykes' group at Squibb also discovered monocyclic β-lactams, which they christened monobactams [37, 38]. For their screen, they used a sensitive β-lactamase induction assay in *Bacillus licheniformis* to signal the presence of β-lactam containing molecules at very low concentrations. In fact, this assay was capable of detecting compounds at concentrations as low as 1 ng/mL. Their strategy also focused on screening nontraditional sources (e.g., bacterial cultures) for novel activities. One of the molecules was successfully commercialized as aztreonam (Azactam). Aztreonam has the unusual property of binding with a strong preference to PBP3 in Gram-negative rods, resulting in cell filamentation. The use of the β-lactamase induction phenotypic screen is one of the earliest instances of promoter-based screening responding to a specific signal molecule.

Another compound discovered by a β-lactam screening system is lactivicin. This was identified by Takeda scientists using the hypersensitive mutants described above. In this case, the compound is a cyclic dipeptide which does not have a β-lactam ring. The compound did bind and acylate PBPs, and in *B. subtilis* exhibited high affinity for PBPs 1, 2, and 4 [39, 40].

Other screens have been devised that discriminate bacteriostatic from bacteriolytic (autolytic) activity of antibacterial compounds [41] in *E. coli*. This phenotypic screen is an agar plate-based screen which relies on the fact that β-galactosidase is sequestered within bacteria cells. Cell wall lysis releases the β-galactosidase, which is detected on X-gal plates as a blue halo area surrounding the growth inhibition zone. A similar assay employing β-galactosidase in *B. subtilis* has also been described recently [42].

A microbial cell screen for glycopeptide compounds was described by O'Sullivan et al. [43]. Glycopeptides are also cell wall inhibitors working through a different

mechanism than β-lactams. Glycopeptides block cell wall synthesis by tight binding to the D-alanyl-D-alanine portion of the peptidoglycan pentapeptide, blocking cell wall assembly. The screen was a two-plate assay in which both plates were inoculated with *B. subtilis*. One of the two plates also contained cell walls purified from *Staphylococcus aureus*. The cell walls act to bind and lower the concentration of glycopeptide compounds. Hence the inhibition zone sizes of the *B. subtilis* indicator strain are reduced if the inhibitor compound is a glycopeptide in the cell wall containing plate versus the zone size from the corresponding well in the plate without cell walls. This assay led to the discovery of the compound lysobactin.

Another glycopeptides phenotypic screen along similar lines was the tripeptide reversal assay employed at Smith Kline and French. In this assay, the peptide analog diacyl-L-lysyl-D-alanyl-D-alanine antagonized the activity of compounds in complex fermentations against the *B. subtilis* indicator organism [44]. Using this assay, novel glycopeptides were discovered. A somewhat similar approach was also taken by Lepetit researchers. In this case, the fermentation broth was passed over columns of Sepharose to which D-alanyl-D-alanine had been coupled. The column would presumably retain glycopeptide-like molecules. In this case, the enriched eluent from the column after elution with aqueous ammonia was screened for bacterial cell inhibitory activity [45].

As the genes and pathway for bacterial cell wall synthesis were further defined, additional cell-based screens were devised. As an example, studies with microarrays revealed the presence of a cell wall stress stimulon, in which several genes were upregulated in the presence of cell wall antibiotics. These genes could be coupled to reporter systems to discover new cell wall inhibitors. Rothstein et al. [46] found that the regulated β-lactamase system from *Citrobacter freundii* could be used in *E. coli* to detect cell wall inhibitors. In this system, the AmpR regulator responds to the presence of increased concentrations of intracellular cell wall fragments which are recycled from cell wall degradation, inducing the synthesis of the AmpC β-lactamase. Inhibition of any step in the entire cell wall pathway, from synthesis of the UDP-muramic acid peptide precursors up to the transpeptidation or transglycosylation reaction products, will trigger the system to respond. Response can be monitored by the colorimetric indicator nitrocefin, which is broken down by β-lactamase.

28.4.2 Other Examples of Pathway Screens in Cells

Physiological processes other than cell wall inhibition have been the subject of whole-cell assays. One example is the anucleate blue cell assay, used to detect Type II topoisomerase inhibitors [47]. In this assay, topoisomerase inhibitors cause the generation of anucleate cells as a result of faulty chromosome partitioning. In the blue cell assay, a β-galactosidase gene (*lacZ*) is placed on a plasmid, and its expression is suppressed by a *lacI* repressor on the chromosome. The plasmid replication gene is under the control of a λ phage promoter P_R, and repressed by the λ phage *cI* gene on the chromosome. Failure of chromosome partitioning (in the presence of an inhibitor compound) causes the loss of control on plasmid numbers,

amplifying the plasmid, and leading to induction of the β-galactosidase as the *lacZ* copy number increases. A blue zone of hydrolyzed X-gal indicator around the growth inhibition zone on an agar plate constitutes a positive test. Interestingly, this assay can also pick up inhibitors of MreB, a cell division associated protein that acts to properly partition the dividing cell [48, 49].

Another example is a novel screen that will identify inhibitors of bacterial DNA synthesis inhibition recently described [50]. This screen takes advantage of a cold temperature-sensitive mutant of *E. coli* that has a mutant allele of the DNA replication initiation protein, DnaA. This allele causes excessive replication initiation at 30°C, leading to cell death. A DnaA inhibitor would inactivate the protein, removing the temperature-sensitive phenotype. In order to support chromosome replication in the absence of DnaA (complete inhibition), the *rnhA* gene is also deleted, which permits replication initiation by a secondary pathway via recombination enzymes.

28.4.3 Target-Based Bacterial Screening: Altered Target Activity

LpxC is a metallo-enzyme that carries out the second step in the synthesis of lipid A, an essential component of the Gram-negative outer membrane. Clements et al. reported on the screening of an *lpxC* mutant of *E. coli*, predicted to be hypersusceptible to LpxC inhibitors, with a collection of low molecular weight compounds containing metal chelating groups [51, 52]. Several compounds with MICs < 1 μg/mL were identified by this screen, including two sulfonamide derivatives of α-(*R*)-amino hydroxamic acid. Testing of the active compounds against a broader panel of pathogens revealed that the compounds were specifically active against Gram-negative pathogens with MICs versus wild-type *E. coli* of 1–2 μg/mL. In addition, the compounds had IC_{50}s ranging from 160 to 400 nM against *E. coli* LpxC enzyme activity. Further, selection for resistant *E. coli* mutants followed by sequencing genes involved in lipid A and fatty acid biosynthesis revealed mutations in *fabZ* and *lpxC*. This report validates the use of target-based whole-cell screening to identify target-specific inhibitors with antibacterial activity for further development.

Another interesting approach was the screening of the *E. coli* Keio collection of strains for changes in antibiotic susceptibility [53]. In the Keio set, almost 4,000 individual knockouts of nonessential genes have been constructed. Tamae et al. subjected each of these strains to seven antibiotics with diverse mechanisms of action at several subinhibitory concentrations. They were able to identify several previously unknown gene products which contributed to changes in susceptibility for one or more of the antibiotics tested. Some unexpected relationships emerged from the study, for example, the reduction in the MIC of vancomycin (not usually able to penetrate the outer membrane of *E. coli*) from 500 μg/mL in the isogenic parent strain to 4 μg/mL in a *surA* deletion strain. This may reflect the newly discovered role of *surA* as a chaperone involved in the proper assembly of key outer membrane components [54].

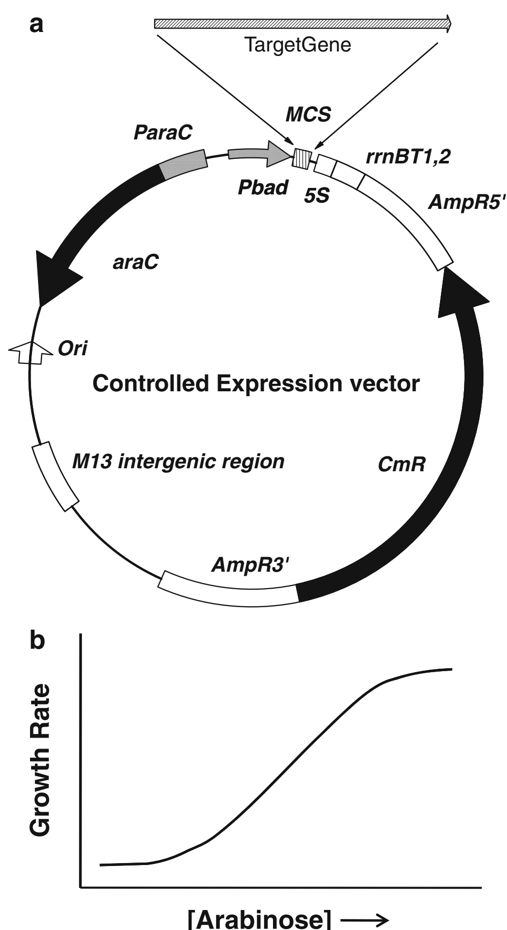

Fig. 28.2 Controlled expression of an essential target gene employing the arabinose promoter system. (**a**)The target gene of interest is placed under the control of the regulated arabinose promoter, and transformed into an appropriate *E. coli* strain. The corresponding gene in the bacterial chromosome is deleted. The growth of the cells becomes dependent on the presence of arabinose to drive the expression of the gene. (**b**) By manipulating the concentration of arabinose, the gene expression level can be adjusted to a point at which the amount of gene product becomes limiting for growth, slowing the overall growth rate. Cells in such a condition are sensitized to any inhibitor of that gene product, and can be employed to selectively screen for new inhibitors (see Ref. [55] for a detailed description of the system)

28.4.4 Target-Based Bacterial Screening: Gene Promoter Regulation

The advent of new molecular technologies and bacterial genome sequences offered an opportunity to precisely engineer bacteria to become exquisitely susceptible to compounds targeted to selected essential functions. An excellent illustration of such an approach is the work of DeVito et al. [55] in which an array of bacterial strains was engineered to become more sensitive to inhibitors of specific essential enzymes. Genes for specific selected targets were cloned on a plasmid with the highly regulated arabinose promoter, thus modulating expression levels of the gene via changing external arabinose concentrations (Fig. 28.2). The corresponding essential gene was then deleted from the chromosome, leaving the externally regulated copy as the sole source of target protein.

Reducing the expression of the essential gene target should render the cell hypersusceptible to an inhibitor of the target. It should, as well, reduce the growth rate of the cells, as the regulated step becomes a rate-limiting process in the cell. In their paper, DeVito et al. demonstrate this for the case of *fabI* (enoyl-acyl carrier protein reductase), *murA* (UDP-N-acetyl-glucosamine enolpyruvyl transferase enzyme), and *metG* (methionyl-tRNA synthetase gene) limited cultures. In the case of both MurA and FabI, where inhibitors (fosfomycin and triclosan, respectively) are available, susceptibility of the downregulated strains was increased tenfold over the parental strains. Importantly, the cells became selectively more susceptible only to antibiotics targeted against the downregulated gene targets. There was no impact on cell sensitivity to antibiotics that work through other targets. Perhaps surprisingly, the reduced expression of *murA* did not impact ampicillin susceptibility. An antisense approach (see below), which downregulated the next step in the pathway *murB*, did affect antibiotics targeting later steps in the cell wall pathway.

28.4.5 Bacterial Cell Target-Based Screening: Antisense Downregulation

Similar to the system described above, one strategy for cell-based screening is to sensitize the bacteria to sublethal concentrations of an inhibitor targeted at a particular gene or pathway. This strategy works particularly well when screening fermentation broths, where activities may be present in low concentrations or the inhibitory activity may be intrinsically weak. Antisense RNA has been successfully employed in *S. aureus* as both a target validation method as well as a screening method (Fig. 28.3). In this strategy, antisense RNA against the desired target gene is inducibly expressed using a regulated expression system. For target validation, expression is usually driven to a maximum level, resulting in inhibition of cell growth. For screening, levels of antisense expression are chosen such that the cells are relatively depleted for the protein being targeted. These cells are more susceptible to an inhibitor of the target protein in comparison to cells expressing the normal levels of the target protein.

Initial reports of antisense RNA experiments in *S. aureus* were from Ji et al. [56]. A plasmid with a tetracycline controllable promoter was employed along with sheared genomic DNA. DNA sizes in the 200–800 base-pair range were selectively inserted into the plasmid and transformed into *S. aureus*. From among the transformants with these random inserts were selected those with the ability to inhibit growth upon induction of the tetracycline expression system. Amplification of the inhibitory sequences by PCR followed by DNA sequencing identified antisense sequences in essential genes. This effort was aimed primarily at identifying essential genes, although increased sensitivity to known antibiotic inhibitors was demonstrated. A similar strategy was employed by Forsyth et al. [57] using a xylose-inducible expression system. Again, random small DNA fragments were expressed in *S. aureus*. A total of 658 genes were identified that were inhibited by antisense RNA in this screen. The researchers also demonstrated that cells could be sensitized

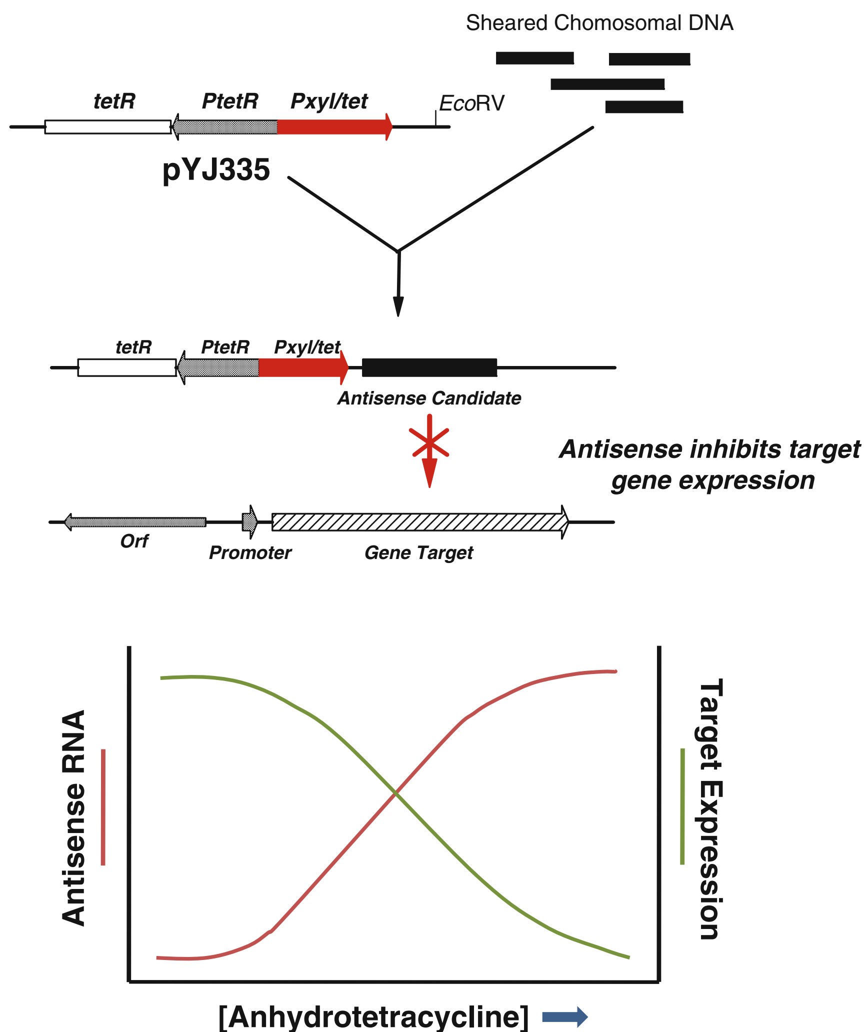

Fig. 28.3 Controlled expression of an essential target gene with antisense RNA. In this case, random gene fragments were used to screen for antisense RNA generating regions which suppressed bacterial growth. The effect of some of these antisense regions could be correlated with antisense expression on their cognate gene targets. The level of expression from the xyl/tet promoter could be controlled with anhydrotetracycline, and the resulting downregulation of gene expression used to sensitize organisms for screening purposes [56]. The xylose promoter has also been employed for antisense expression [57, 58]

(increased susceptibility) to the fatty acid biosynthesis inhibitor cerulenin by induction of antisense RNA to the *fabF* gene.

The application of this technology to antibiotic drug discovery was implemented at Merck. Natural product extracts were screened with a two-plate assay. Duplicate samples of extracts were arrayed on two agar plates, one of which had *S. aureus* expressing the wild-type levels of all cell enzymes and the other had a strain which downregulated the level of the *fabH* and *fabF* gene products by antisense [58]. Initially phomallenic acid A was identified with this technology. Subsequently, the natural products platensimycin [59] and the related platencin [60] were described. Both compounds were identified by employing the *fabF* antisense technology during screening. Platencin inhibited both the FabF and FabH condensing functions. The *fabF* gene encodes the essential step 3-oxoacyl-ACP synthase II (elongation-condensation) and the *fabH* is the β-ketoacyl-acyl carrier protein synthase III in the bacterial Type II fatty acid biosynthesis pathway [61]. In vitro activity of platencin against a range of Gram-positive pathogens was observed, and the compound also demonstrated activity in a mouse model of disseminated staphylococcal infection.

Others have employed antisense technology to validate targets, and downregulation of *metS* and *murB* expression levels in *B. anthracis* serve as an example. The expression of xylose promoter driven antisense RNA against the *metS* gene led to sensitization to an experimental compound known to be an inhibitor of this t-RNA synthetase. Likewise, antisense downregulation of the *murB* gene led to sensitization to inhibitors of the cell wall pathway, such as oxacillin and cloxacillin [62], and downregulation of the *murA1* gene increased susceptibility to fosfomycin [63]. Another application has been for mechanisms of action screening studies. In this case, an array of the *S. aureus* antisense strains generated a library of 245 essential genes that have been attenuated in expression. The individual strains were binned into pools as to similar levels of response to the antisense inducer (xylose) on their individual antisense-regulated genes. Strains with similar growth responses were placed in the same cluster and the cluster exposed to test antibiotics in a microtiter plate-based screen. This sets up a competitive growth situation, and any strain whose antisense-downregulated gene places that strain at a disadvantage under the influence of the antibiotic (i.e., is hypersensitized due to the downregulated gene) is eliminated from that pool of strains [64]. Subsequent analysis yields patterns of individual attenuated genes that are collectively affected by particular antibiotics, and thus are associated with the mechanism of action of that compound.

28.5 Application of DNA Microarrays and Expression Profiling for Mode-of-Action Determination

28.5.1 DNA Microarrays

DNA microarrays provide a global snapshot view of the physiological state of a population of cells by looking at the levels of gene expression corresponding to the entire genome in a treated population of cells relative to an untreated control.

This powerful tool in conjunction with bioinformatics analyses can tell us how genes are regulated in response to stress induced by changes in the growth environment (e.g., nutrient status, temperature, and exposure to antibiotics). An expression profile is thus defined as the gene expression pattern (e.g., induced, unaffected, or repressed) of an organism under specified conditions of growth. Therefore, expression profiles are relative to other expression profiles. The differences between expression profiles may elaborate significant information useful for categorizing specific treatments. For example, expression profiles collected for bacteria treated with different antibiotics may provide clues regarding the mode-of-action. This information can be utilized to categorize antibiotics with specific mode-of-action, and in turn have potential to predict the mode-of-action of novel compounds with antibacterial activity. Ultimately, the data collected as expression profiles have potential applications in developing specific assays, such as designing biosensor strains, as described in Sect. 6.

28.5.2 Expression Profiles for Antibiotic-Treated Bacteria

Early studies looking at genome-wide expression profiling analyzed stress responses such as heat-shock [65]. These experiments paved the way for collecting and determining the best way to utilize these data. In 2001, Gmuender et al. examined the effects of two DNA gyrase inhibitors with different mechanisms of inhibition, novobiocin and ciprofloxacin, on global gene expression in *Haemophilus influenzae* [66]. In this early study, clear differences were observed between the two treatments. Novobiocin induced a pattern of gene expression suggesting that initiation of transcription had been affected. In contrast, ciprofloxacin induced the expression of genes that typically respond to DNA damage (e.g., SOS repair genes).

In a paper by Sabina et al., the authors monitor the transcriptional response of *E. coli* to four distinct classes of translation inhibitors [67]. Each of the four translation inhibitors has distinct mechanisms of inhibition. Azaleucine blocks activation of leucine by LeuRS, mupirocin blocks transfer of the activated leucine to tRNA, kasugamycin blocks initiation of polypeptide synthesis, and puromycin blocks elongation. Distinct profiles were observed for each inhibitor, for example, treatment with azaleucine resulted in a distinct heat-shock and generalized stress response, while puromycin treatment resulted in elevated expression of genes encoding ribosomal and translation-related proteins.

Shaw et al. monitored the global changes in gene expression upon treatment of *E. coli* with four distinct classes of bactericidal agents [68]. The agents included transcription inhibitor rifampin (targeting RpoB), translation inhibitor kanamycin (targeting the 30S ribosomal subunit), DNA replication inhibitor norfloxacin (targeting DNA gyrase), and cell wall biosynthesis inhibitor ampicillin (targeting penicillin-binding proteins). Norfloxacin induced expression of the SOS-response genes *recA*, *recN*, *sulA*, *sbmC*, as well as *tdcB* and *tdcC* similar to what was described above for ciprofloxacin. Kanamycin induced the expression of sigma 32-regulated genes involved in heat-shock response. Rifampin induced expression of *rpoB*, *rpoD* (sigma 70), nucleotide salvage, purine biosynthesis, nucleotide and nucleoside conversions, etc.

Ampicillin induced fewer genes expression but did include upregulation of transport genes including *fepA* (ferric enterobactin) and *acrF* (RND transport family).

Collectively, the studies described above and others like it formed the basis of a database describing the characteristic expression patterns for bacteria treated with specific classes of antibiotics. This information can be utilized to predict the mode-of-action for novel compounds with antibacterial activity where the target is unknown. In addition, this information is useful for identifying promoters that specifically respond to different classes of antibiotics in order to construct reporter fusions in bacterial biosensor strains, discussed in Sect. 6.

28.5.3 Application of Expression Profiling for MOA Determination

An example of using expression profiling for MOA determination can be found in a paper by Friedberg et al. [69]. In this case, they started with a compound with antibacterial activity but unknown mode-of-action. The authors first assembled a compendium of transcription profiles induced by 14 reference antibiotics with known targets. Friedberg et al. then compiled expression profiles for conditional mutants where specific targets were downregulated to mimic chemical inhibition. The downregulated target genes included *ileS*, *pheST*, *fabF*, and *accDA*. The authors then showed that the yet uncharacterized natural product moiramides induced expression of genes involved in fatty acid biosynthesis, and that the expression profile closely matched the one induced by the *accDA* conditional mutant. This study is a good example of applying new technologies to identify targets for antibacterial compounds with unknown mode-of-action.

Further, an example where antibiotic-induced promoters were utilized to develop a cell-based pathway-specific reporter system (bacterial biosensor – see next section) to screen for novel antibacterials is described in a paper by Fischer et al. [70]. In this example, the authors identified genes that were specifically induced by inhibitors of fatty acid biosynthesis in *B. subtilis.* The next step involved alignment of the conserved promoters that were regulated in response to inhibition of fatty acid biosynthesis. A promoter consensus sequence was determined, and this conserved promoter was fused with the firefly luciferase gene and transformed into *B. subtilis* to create a reporter strain for cell-based high-throughput screening.

28.6 Bacterial Biosensors as Tools for Antibacterial Discovery

28.6.1 Bacterial Biosensors

Bacteria sense and adapt to their constantly changing environment (e.g., chemical composition, temperature fluctuation, and nutrient availability) through elaborate signal transduction mechanisms that result in appropriate concurrent changes in

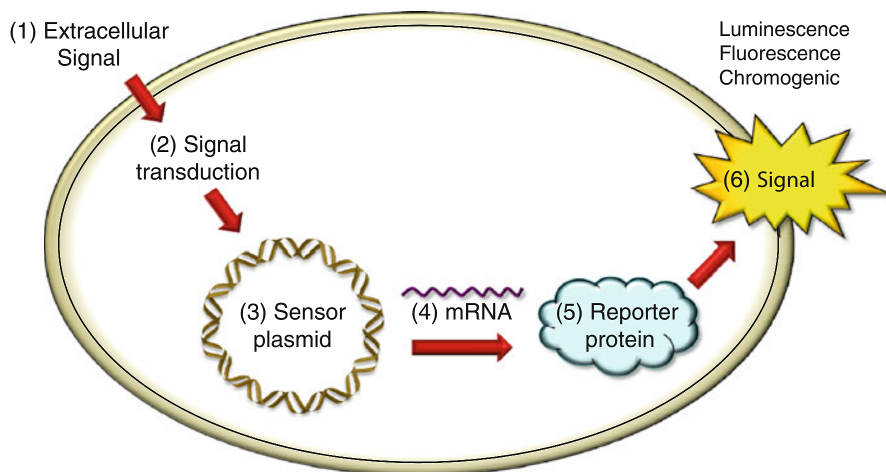

Fig. 28.4 Bacterial biosensor strain (*1*) senses an extracellular signal in its environment, and (*2*) transduces the signal to effect intracellular processes including induction of specific response genes. (*3*) A sensor plasmid contains a promoter, known to be induced in response to a specific signal, fused with a reporter gene. (*4*) The reporter gene is induced leading to expression of reporter mRNA, and (*5*) a specific reporter protein that transmits a measurable (*6*) signal in the form of luminescence (luciferase), fluorescence (GFP), or an enzyme (β-galactosidase) that converts a chromogenic substrate into a colorimetric signal

gene expression. In the context of this chapter, a biosensor is a recombinant bacterial cell that senses a small molecule signal and, by virtue of a supplied reporter gene fused with a stress-response promoter, signals its presence to the outside world in the form of luminescence, fluorescence, or enzymatic conversion of a colored dye (Fig. 28.4). Biosensors are particularly useful to detect environmental pollutants and genotoxic agents, by-product contaminants in food processing, and pharmacologically relevant small molecules such as antibiotics [71]. Bacterial biosensor reporter assays have been established to screen large compound collections for inhibitors of a specific target or a metabolic pathway to detect antibacterial compounds at subinhibitory concentrations [70, 72, 73]. Additionally, several research groups have elaborated bacterial biosensor assays for determining the target pathway for antibacterial compounds with unknown mode-of-action [74–77].

28.6.2 *Bacillus subtilis* Biosensor Strains for Antibiotic Discovery and MOA Determination

A panel of *B. subtilis* biosensor strains was constructed by Hutter et al. based on the expression profiles obtained after treatment of *B. subtilis* with a diverse set of antibacterial compounds as described in Sect. 5 [76, 78]. The biosensor strains were shown to be useful for predicting the MOA for antibacterial compounds, as well as

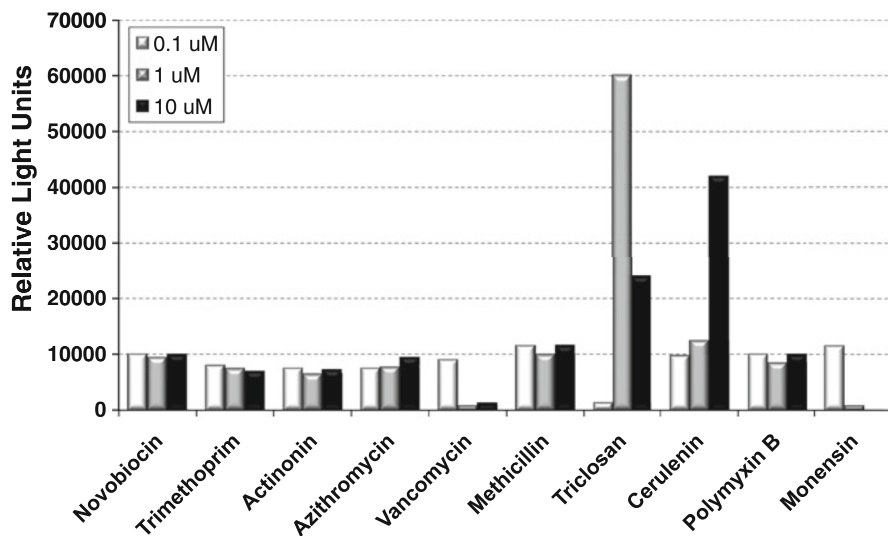

Fig. 28.5 *Bacillus subtilis* biosensor strain with a plasmid containing the *fabHB* promoter fused with the firefly luciferase (*luc*) gene was grown in the presence of antibiotics with known MOA. Induction of luminescence was dose-dependent and selective for fatty acid biosynthesis inhibitors triclosan and cerulenin (Adapted from Fischer et al. [70])

for pathway-specific antibacterial screening. In this study, promoters from several inducible genes were fused with the firefly luciferase reporter gene, and introduced into the *B. subtilis* chromosome. The resultant strains were then treated with 37 diverse antibacterial agents, and the response was analyzed and categorized according to the MOA of the compound versus the response of the biosensor strain. Twelve biosensor strains resulted as having a high signal and selective response to the antibiotic treatments.

Urban et al. continued to develop the *B. subtilis* biosensor approach by validating five strains for cell-based screening [77]. The authors proceeded to screen these strains against a diverse set of 14,000 natural products, many with inhibitory activity for *B. subtilis.* The successful application of the *fabHB* promoter reporter strain for screening described at the end of section 5 was a result of this effort (Fig. 28.5; [70]).

28.6.3 E. coli Biosensors for Detecting and Characterizing Antibacterial Compounds

Described below is a series of papers showing that fusions between the promoters of stress-response genes and reporter genes encoding β-galactosidase (*lacZ*) or

firefly luciferase (*luc*) are useful for detecting sublethal concentrations of known antibiotics.

Bianchi and Baneyx made a series of isogenic *E. coli* strains bearing single-copy gene fusions between the *lacZ* reporter gene, and the promoter regions of the major cold-shock protein CspA, the highly inducible cytoplasmic small heat-shock proteins IbpA and IbpB, and the P3 promoter of the *rpoH* gene which is transcribed by Eσ^E-bound RNA polymerase upon protein misfolding in the periplasm [74]. The *cspA*::*lacZ* fusion is induced by the so-called C-group translational inhibitors (e.g., chloramphenicol and tetracycline), which trigger the cold-shock response, and the *ibp*::*lacZ* fusion responded maximally to the H-group antibiotics targeting translation (e.g., streptomycin and neomycin), which activate the cytoplasmic heat-shock response and leave ribosomes with a vacant A site. Compounds that damage the outer membrane (e.g., polymyxin B) or interfere with peptidoglycan synthesis (e.g., carbenicillin) selectively activate the P3*rpoH* promoter.

As an example, in the *E. coli* biosensor strain containing the *cspA::lacZ* fusion, induction of β-galactosidase activity is time-dependent being maximally induced after 2–3 h exposure to chloramphenicol (Fig. 28.6a). Induction of β-galactosidase activity is also dose-dependent, with a maximum induction observed using 5 μg/mL chloramphenicol (Fig. 28.6b). Importantly, the induction of the *cspA::lacZ* fusion was specific for chloramphenicol and tetracycline (Fig. 28.6c).

Shapiro and Baneyx expanded on Bianchi's methods by showing that *E. coli* strains bearing a fusion between the SOS-inducible *sulA* promoter and *lacZ* are highly sensitive and selective for detection of antimicrobial compounds that interfere with DNA replication such as nalidixic acid and ofloxacin [79]. They further demonstrated that inactivation of TolC, the outer membrane channel of the AcrAB multidrug efflux system, allows for high signal-to-background detection of very low concentrations of model antibiotics, while addition of the outer membrane permeabilizer polymyxin B sulfate further enhances the sensitivity of the system to intermediate concentrations of hydrophobic antimicrobial compounds (Fig. 28.7). They also show that stress promoter-based detection of antimicrobial agents is scalable from a shake flask down to a microtiter plate format.

Shapiro and Baneyx continued to improve on the system [73]. They evaluated a red-shifted variant of *P. pyralis* luciferase (LucR1, [80]) for biosensor development using a single-copy translational fusion between the SOS-inducible *sulA* promoter and the *lucR1* gene that was inserted at the *malP* site of the *E. coli* chromosome.

They compared *cspA::lacZ* and *cspA::luc* biosensors integrated at the *att* λ site on the *E. coli* chromosome, and found that the luminescent sensor yielded high signals more rapidly than its chromogenic counterpart did (Fig. 28.8). More importantly, *cspA::luc* cells allowed high-confidence detection of very low amounts of chloramphenicol (1 μg/mL, approximately 15% of the MIC). Shapiro and Baneyx also described the construction of a bacterial biosensor, containing two distinct promoter–reporter fusions, suitable for the multiplex detection of translational inhibitors and DNA-damaging agents [73].

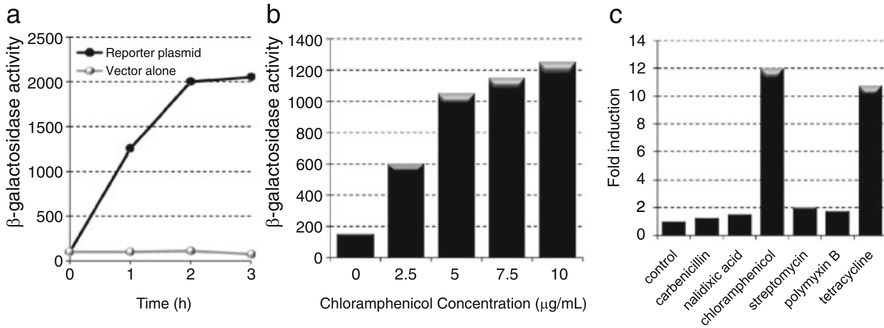

Fig. 28.6 *E. coli* biosensor strain containing the *cspA* promoter fused with β-galactosidase (*lacZ*) gene integrated at the λ *att* site: (**a**) was grown in the presence of 5 μg/mL chloramphenicol for 3 h, to evaluate the time required to achieve maximum signal. (**b**) was grown for 3 h at increasing concentrations of chloramphenicol to determine and evaluate the optimal concentration to achieve maximum signal. (**c**) was grown in the presence of antibiotics with known MOA. In this case, only the C-group antibiotics, chloramphenicol and tetracycline, induced a significant signal relative to the untreated control. Fold-induction is calculated as measured signal (β-galactosidase activity) normalized by the number of bacteria being assayed (Adapted from Bianchi and Baneyx [74])

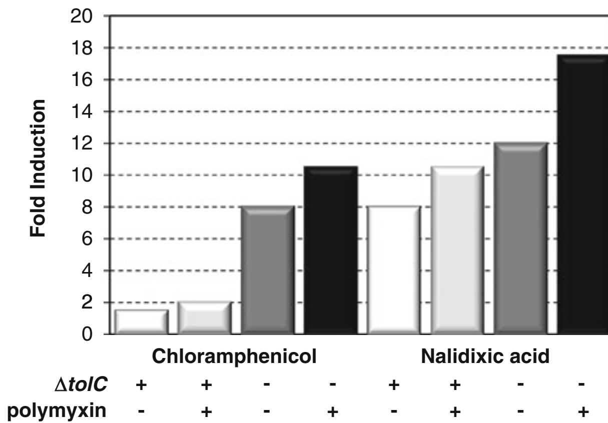

Fig. 28.7 Effect of *tolC* inactivation or addition of polymyxin on enhancing signal detection in *E. coli* biosensor strains. *E. coli* biosensor strains containing (+) or lacking (−) *tolC* were grown in the presence (+) or absence (−) of polymyxin. Strains containing the *cspA* promoter fused with *lacZ* integrated at the λ *att* site were treated with 1 μg/mL chloramphenicol (*left*), and strains containing the *sulA* promoter fused with *lacZ* integrated at the λ *att* site were treated with 5 μg/mL nalidixic acid (*right*). Inactivation of *tolC* and addition of polymyxin enhanced the biosensor signal (Redrawn from Shapiro and Baneyx [79])

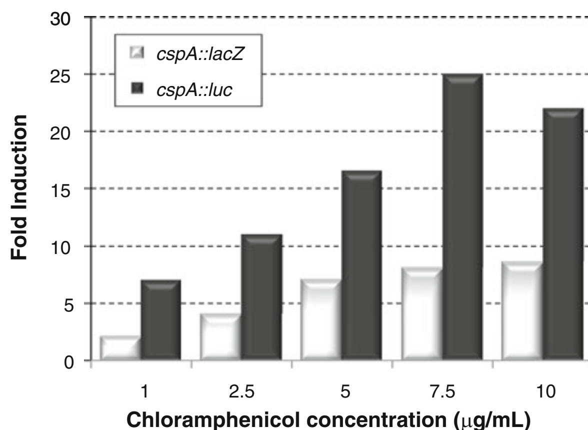

Fig. 28.8 Effect of reporter gene (*lacZ* vs. *luc*) on signal detection in *E. coli* biosensor strains in response to chloramphenicol. *E. coli* biosensor strains containing either *cspA::luc* or *cspA::lacZ* were grown in the presence of increasing concentrations of chloramphenicol to determine the effect of reporter gene on signal detection. In this case, the luciferase signal was significantly higher than the β-galactosidase signal (Redrawn from Shapiro and Baneyx [73])

28.7 Reverse Genetic Approaches for Identifying Targets for Antibiotics with Unknown Mode-of-Action

28.7.1 Target Identification by Mapping Antibiotic-Resistance

The section describes the use of genomic libraries to screen for targets by mapping resistance mutations. In a paper by Mills et al., they assessed the MOA for a novel series of benzimidazole derivatives that have potent and selective activity against *Helicobacter pylori* [81]. In this study, a λ phage-based genomic library from a resistant *H. pylori* strain was constructed. Selection for a resistance phenotype followed transformation of pooled λ clones into a sensitive *H. pylori* strain. A single 17-kilobase clone containing the entire *nuo* operon, encoding NADH:ubiquinone oxidoreductase, conferred resistance. DNA sequencing identified a single mutation in *nuoD* responsible for resistance. *nuoD* was established to be essential in *H. pylori* and therefore considered to be a good candidate target for the benzimidazoles. This particular approach is useful when screening libraries in naturally transformable pathogens such as *H. pylori* and *H. influenzae* where selection for resistance is possible through integration of the resistance mutation in the genome.

In a separate study, Brötz-Oesterhelt et al. constructed a plasmid-based genomic library from an acyldepsipeptide (ADEP)-resistant mutant. ADEPs are a new class of antibiotics with antibacterial activity versus Gram-positive bacteria and efflux-negative

Gram-negative bacteria [82]. Transformation of a susceptible efflux-negative strain of *E. coli* with the genomic library generated several daughter clones with high-level resistance to the ADEPs. DNA sequencing of the resulting clones identified ClpP, the catalytic core unit of the major bacterial protease, as the resistance determinant. ClpP is broadly conserved among eubacteria and binds ADEPs. Because the mechanism of action involves ClpP, typical MOA assays did not help to identify this target. Reverse genetics played an indispensible role in this determination.

In 2002, Belanger et al. published a novel approach to identify antibiotic targets in *S. pneumoniae* using PCR-based ordered genomic libraries [83]. The method relies on preparing an ordered genomic library composed of overlapping PCR amplicons generated under error-prone conditions to contain an increased frequency of random mutations. Several antibiotics of known MOA, including fusidic acid, rifampicin, and trimethoprim, established the validity of this method. Transformation with known amplicon pools generated resistant *S. pneumoniae* strains that ultimately led to identifying the correct target gene (e.g., *fusA*, fusidic acid; *rpoB*, rifampin; *dfr*, trimethoprim). The overall process for phenotypic screening is similar to the approach described above for lambda- and plasmid-based libraries of cloned DNA. The primary difference is that one library of PCR amplicons, containing random mutations, can be used multiple times for different antibiotics.

28.7.2 Target Identification Through Target Overexpression

Li et al. reported the use of "multicopy suppressors" to identify antibiotic targets for compounds of unknown mode-of-action [84]. The multicopy suppressor approach involves screening a plasmid-based genomic library for clones that suppress the growth inhibition phenotype of an antibiotic. In this case, the target is overexpressed, leading to a reduced susceptibility to the test compound. The suppressor phenotype is evaluated using a control strain, and pools of clones carrying a plasmid-based genomic library, which are exposed to increasing concentrations of antibiotic. The example used in the paper was trimethoprim. In this case, growth inhibition was observed at 0.078 μg/mL for the control strain, while suppressor clones increased the concentration required for growth inhibition to 0.64 μg/mL (eightfold relative to the control strain). In summary, three antibiotics of known mode-of-action generated clones containing the correct gene target. That is, trimethoprim-selected clones contained *folA*, fosfomycin-yielded clones containing *murA* and cycloserine-generated clones containing *ddl.* A small screening library (8,640 compounds) tested at a concentration of 50 μM against a hyperpermeable rough polysaccharide mutant of *E. coli* yielded 49 lead molecules with growth inhibitory activity. The majority of cloned suppressors for these lead molecules contained *acrB*, a component of a multidrug RND efflux pump in *E. coli.* It is important to note that all of the techniques described above involve phenotypic suppression of growth inhibition, and therefore require additional studies to confirm that resistance is mediated through the antibiotic drug target.

28.7.3 Multiapproach Method to MOA Determination

This last example illustrates the use of multiple techniques to identify the antibiotic target. This approach, exemplified by researchers at Pharmacia, is one of the most commonly used in antibacterial discovery [85]. In this instance, a chemical compound library was initially screened for antibacterial activity. The screen employed both *S. aureus* and *E. coli* strains, and a cutoff of 32 μg/mL was used for initial consideration. Additional criteria were used for compound selection, such as chemical tractability, serum binding, lack of fungal activity, purity, and inventory. Mechanism of action follow-up consisted of using radiolabeled precursors for the macromolecular processes of DNA, RNA, protein, cell wall, and lipid biosynthesis, and following the inhibition of these specific pathways by candidate compounds. Microarray analysis of mRNA responses were determined and compared with known antibiotic profiles. Resistant mutants (either ethyl methane sulfonate or spontaneous) against key compounds of interest were derived, and then tested against known classes of antibiotics. One molecule emerged, PNU-286607, a bacterial topoisomerase inhibitor, which was further characterized for inhibition of both purified DNA gyrase and topoisomerase IV. This exercise stands as an example of the broad application of modern molecular tools to compounds found by nontarget directed whole-cell screens.

28.8 Conclusion

This chapter focused on the use of bacterial strains to screen for antimicrobial activity. From simple growth inhibition assays employed in early programs, modern screening has evolved to increasingly sophisticated phenotypic cell-based screens. Many of these screens employ genetic technologies to modify the bacterial screening strains to enhance sensitivity and/or focus on specific targets or target pathways. An advantage of cell-based phenotypic screens over enzymatic inhibitor assays is the cellular context, which a requirement for includes not only intrinsic target potency but also factors in compound penetration and intracellular accumulation at inhibitory concentrations. Cell-based screens also play a role in determining the mechanism of action of newly identified inhibitors. Overall, the deployment of cleverly designed cell screens continues to play a major role in modern antibiotic screening and research.

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Chapter 29
Enzyme-Based Screens in HTS

David E. Ehmann and Stewart L. Fisher

29.1 Introduction

High throughput screening and the subsequent hit evaluation phase are critical components of target based drug discovery. Identification of suitable lead material remains a key milestone in any antibacterial strategy, and while target-based drug discovery has proven effective for a number of disease areas, the success rate of this approach has been challenged for antibacterial targets [23]. While there is always a component of luck in matching the right compound file with the chosen target, it is clear that careful and thoughtful assay design, execution, and hit evaluation can tilt the odds in favor of finding quality inhibitors that enable late stage drug discovery. In contrast, poorly designed assays and inadequate hit evaluation processes invariably result in the identification of questionable leads that require extensive resources and time to evaluate and usually have low probabilities for success. This chapter outlines the generic process from building a suitable assay for high throughput screening to the critical factors in selection of lead material for focused drug discovery prosecution.

29.2 Factors to Consider Before Screening

Before embarking on an HTS campaign, it is wise to review the strategic decisions that have consequences for the subsequent required laboratory work.

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29.2.1 Isozyme Selection

The selection of screening enzyme is one of the most important decisions made for HTS. If the goal of screening is to discover broad-spectrum antibacterial agents, then there will be dozens of species from which the screening target can be chosen. The ideal candidate will be one that can represent all relevant species in the sense that inhibitors from the screen will inhibit the target in other species. For novel targets, often the *E. coli* ortholog is selected because it carries the most evidence from the literature. In all cases, phylogenetic analysis of the target, preferably through structural genomics, should be performed in order to assess where the screening enzyme stands in relation to the other orthologs [4]. Furthermore, if prioritization among several targets is necessary, there are numerous ways by which to predict a target's "druggability," or the likelihood of finding small-molecule inhibitors [16].

29.2.2 Reagent Procurement

Depending on the scale of the screen, reagent procurement can be a limiting factor in the time it takes to complete a screen. To account for this potential delay, the prudent investigator will be aware of the size of the screening set and the approximate liquid volume required for various screening platforms. While it can be difficult at times to predict the precise quantities required for the screen prior to developing the actual assay conditions, often a rough estimate that includes ample overage is adequate since these reagents will be used for both the screening campaign and hit followup processes. All automated assay systems have dead volume, and the sum of this volume can be as high as 25% of the total required volume. With an accurate screen volume in hand, due time can then be allotted for reagent acquisition, be it protein purification, chemical ordering, or custom preparation of reagents.

29.2.3 Compound Library

The screening library is often not considered a variable in HTS development, especially if a dedicated screening facility is performing the screen. Nevertheless, before embarking on screen development one should know the typical screening compound concentration and amount of DMSO (or other solvent) added per well. In addition, it is desirable to know the physicochemical properties of the compounds comprising the screening library, because antibacterial compounds occupy a unique chemical space as compared to drugs from other disease therapy areas [21].

29.3 Assay Development for Screening

Developing an assay suitable for screening is a continuous process; however, it is useful to conceptualize these activities into four distinct phases: foundational work, assay window optimization, assay flexibility assessment, and final validation (see Fig. 29.1). The robustness of a given screen is a function of the assay performance – typically assessed in terms of sensitivity and the signal to noise ratio or assay window – and the flexibility of the assay to adapt to the demands of automation. Balancing these two elements, which often are in opposition, represents one of the key challenges in any assay development project. The compartmentalization into defined phases enables the isolation and evaluation of the individual components of the overall screening process and reveals the interdependencies between them. For example, the foundational work phase focuses on the detection system of the assay format whereas the assay window optimization phase evaluates the biochemistry of the target, as viewed through the detection system readout. Each element in the assay development format places constraints on the overall screen parameters, and these constraints propagate through the process to the final screening conditions. A robust screen has sufficient flexibility within the constraints imposed by each phase (see Fig. 29.2).

The sequential nature of this framework can and should be challenged on occasion, as it is prudent to prioritize the key risks and variables associated with a screen early in the development process, regardless of where these issues reside in the overall process. That said, the general experience to date has indicated that even when late stage risks (e.g., automation constraints) have been identified early in a program, a full understanding of the constraints imposed by the earlier phases

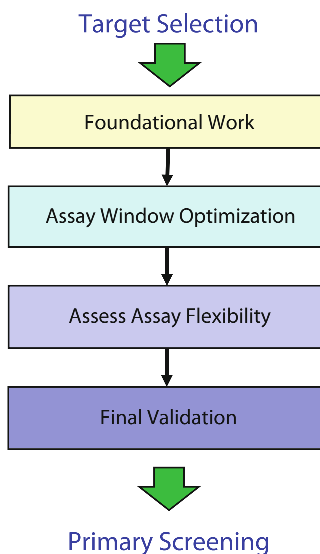

Fig. 29.1 Schematic outlining the four phases of assay development for high throughput screening

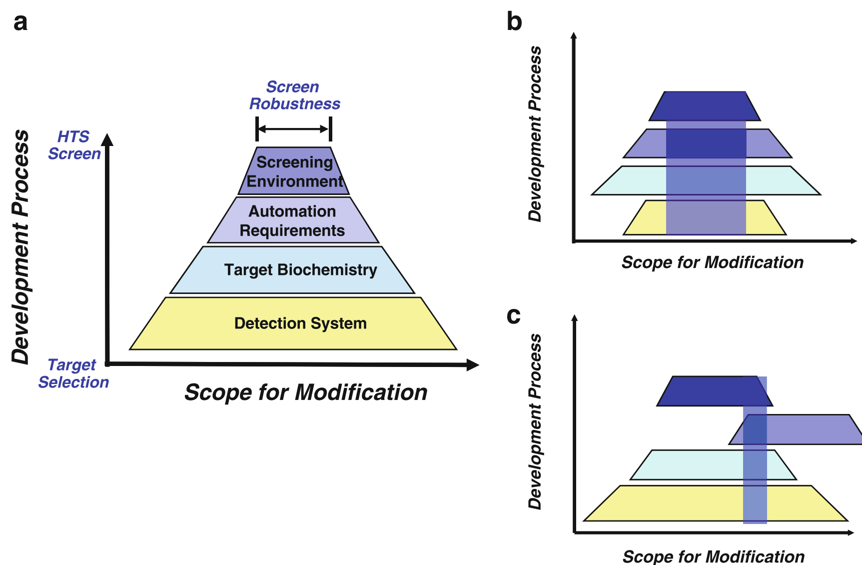

Fig. 29.2 The constraints pyramid for the assay development process. (**a**) each of the assay development phases places constraints on the scope for modification of the assay conditions and the overall robustness of the screen is assessed by the extent of overlap of the constrained conditions, (**b**) in this scenario, the constraints imposed by the detection system (e.g., pH sensitivity of coupling enzymes) limits the overall system, but the assay is sufficiently robust for primary screening. (**c**) in this scenario, the automation requirements of the assay (e.g., strict temperature control) are not aligned with the automation systems for screening; alternative equipment solutions should be investigated to improve the screen robustness

(e.g., foundational work) is required to put the key risks in context. In sum, since every component in the screening process (e.g., biochemistry of the target, automation system for screening, etc.) will have unique characteristics, and therefore different demands on the screening format; this categorization is best used as a guideline for understanding the interdependencies of the generic components of an in vitro assay format rather than a rigid structure for assay development progression.

29.3.1 Foundational Work: Selecting the Detection System

The initial phases of assay development for screening focus on the system used to detect the enzymatic reaction or binding event and the choice of conditions to begin evaluation of the target biochemistry. Key questions during this phase include the following:

- What is the range of product detection under the expected assay conditions?
- Is the detection system linear with the amount of product formed?
- What potential false positives and false negatives are anticipated with the detection system?
- What conditions should be used to begin the assay optimization?

Table 29.1 Examples of screening detection technology platforms

Technology	Practical sensitivity limit	Pros	Cons
Absorbance	~10 µM	Low cost Rapid High throughput	Prone to artifacts from compound absorbance; moderate sensitivity
Fluorescence[a]	~1 nM	Low cost Rapid High throughput	Prone to artifacts from compound absorbance; moderate sensitivity
Scintillation proximity assays	~0.5 nM	High sensitivity	High cost for reagents and waste disposal Prone to artifacts from compound absorbance
ELISA	~0.1 nM	High sensitivity Medium/high throughput	Requires extensive plate washing Wash steps may remove moderate potent inhibitors Prone to artifacts from compound interactions with detection system architecture (i.e., antibody capture)
AlphaScreen	0.1 pM	High sensitivity High throughput	Requires specialized detector High cost Prone to artifacts from compound absorbance and compound interactions with detection system architecture (i.e., antibody capture)[b]
Mass Spectrometry[c]	0.1 nM	Low throughput High sensitivity Direct	Requires rapid, robust chromatographic resolution of analytes

[a] [17]
[b] [33]
[c] [25]

Selection of an appropriate detection system for an assay represents one of the most critical factors in a successful screening campaign. In the most simplistic view, the detection system is the spectroscopic or analytical method used to assess target activity. Currently, there are a wide range of generic technology platforms that are amenable to medium and high throughput screening options, all of which have distinct advantages and practical limitations (see Table 29.1). Typically, emphasis is placed on the potential sensitivity of a given system, along with the technological innovations that underpin new assay technologies, and it is often assumed that these advances will translate into robust assays for screening. While there are certainly examples of successful screens using advanced and innovative approaches, the vast majority of screens are performed using spectroscopic methods (absorbance, fluorescence), due to the inherently low cost and convenience of these approaches. For example, the detection system for enzyme targets that utilize NAD(P)H cofactors is the direct absorbance or fluorescence measurement of NAD(P)H consumption over time. Examples of targets successfully screened in this manner include *E. coli* MurB [34], FabI [31], and Dxr [7].

These cases aside, it is relatively rare for a target reaction product to encode distinct spectroscopic signatures that are amenable to direct observation. This limitation is often overcome through the addition of auxiliary enzymes that couple the target reaction product to a downstream spectroscopic analyte (coupled enzyme systems). Ideal coupled enzyme assay systems utilize auxiliary enzymes that are low cost, have a high catalytic efficiency, and have high specificity for the target reaction product. Regardless of system, it is important to ensure that the concentration of the coupling enzyme activity is sufficient for rapid and complete conversion of target reaction product to the downstream spectroscopic analyte.

Direct product detection difficulties can also be overcome through the use of alternative substrates that encode spectroscopic signatures. This approach has been used effectively in the development of assays for proteases such as LepB [28] and protein kinase targets [24]. In these cases, chemically synthesized peptide substrates containing target-reaction sensitive chromophores are used to mimic the natural protein substrates. When used appropriately, these systems can provide robust assays with very high sensitivities; however, care must be taken to ensure that the inherent target biochemistry and enzymology are not compromised to ensure that the resulting leads from screening will inhibit the native state of the target.

As a general rule, the potential for false positive and negative results in the screening outcome rises is proportional to the complexity of the overall detection system. A false positive result is defined as a hit identified in the screen that is attributed to anything other than direct target inhibition; typically false positives arise as artifacts of the detection system (e.g., inhibition of coupling enzyme). In contrast, a false positive result represents the "opportunity cost" of an assay – in this case true inhibitors of the enzyme are not identified by the screen; these compounds are typically lost in the "noise" of the screen. Since no screen is perfect – all bona fide inhibitors identified with no false positives, it is important to identify and understand the potential weaknesses in the detection system. For example, insufficient coupling enzyme activity in an assay can result in a system where the coupling enzyme becomes the rate-limiting step in the overall reaction pathway. In effect, this condition results in a screen for the coupling enzyme rather than the target of interest thereby increasing the number of both false positives and negatives in the screening output. While this condition may be easily remedied (e.g., increasing coupler enzyme activity), it is critical to understand this potential early in the assay development process.

The best way to assess the limits of the detection system is to titrate the reaction product and measure the detection system response. The results of this experiment, termed the detection standard curve, will define the range of product that can be detected in the system and the shape of the response. It is important to perform these studies in conditions as close to the final assay conditions as possible, as additives to the reaction mixture can affect the range and shape of the detection system response. In cases where the detection system has a non-linear response with analyte concentration, the shape of the detection curve can have an impact on the hit distribution. Many systems are directly proportional to analyte concentration, and therefore a linear response is observed (see Fig. 29.3). However, non-linear responses – both

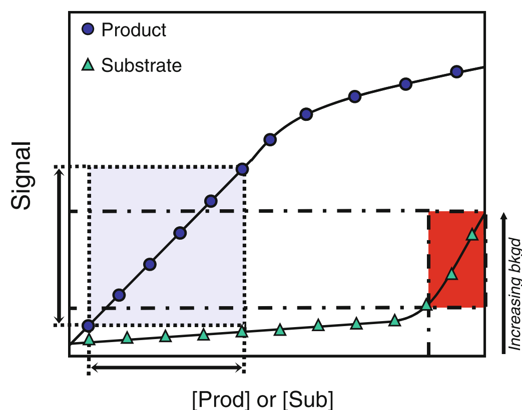

Fig. 29.3 Detection system standard curve. The detection system has a linear response at low to moderate product (*blue circles*) concentrations, but the system is non-linear at high concentrations. Background interference from substrate (*green triangles*) is minimal at low concentrations, but becomes significant at high concentrations. Ideal conditions for product detection are shown in the blue shaded area, but may be compromised at high substrate concentrations (*dashed lines*)

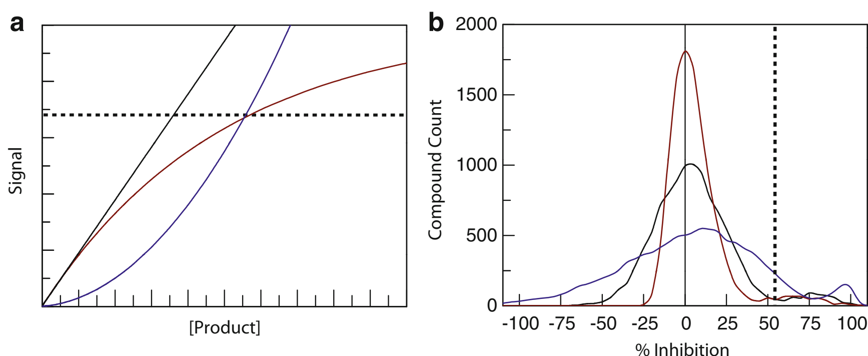

Fig. 29.4 Effects of uncorrected non-linear detection curves on the actives distribution from screening. (**a**) Linear (*black*), hyperbolic (*red*) and parabolic (*blue*) detection standard curves result in different active distributions from the HTS screen if the non-linearity is not accounted for in the analysis of the screening data. (**b**) The hyperbolic detection system results in a narrower distribution, with the potential impact of losing true actives in the noise of the screen, whereas the parabolic detection system results in a wider distribution, resulting in a higher rate of false positives

parabolic and hyperbolic – are possible and if these effects are not included in the analysis of the screening data the rate of false positives and negatives are increased (see Fig. 29.4). For example, if the system is assumed to be linear, but actually exhibits a hyperbolic relationship, a narrower inhibition distribution of actives will be observed, resulting in a higher false negative rate. By contrast, when a parabolic response is assumed to behave linearly, a wider distribution of response is observed thereby complicating the analysis of true actives from the false positives present in

Table 29.2 Systems for inorganic phosphate detection

Technology	Sensitivity	Advantages	Disadvantages
Malachite green	3–30 μM	Low cost Simple Long wavelength absorbance (650 nm)	Subject to interference from colored or charged compounds Discontinuous assay Short signal stability (<30 min)
Fluorescent Binding Protein[a, b]	0.05–5 μM	High specificity for phosphate High sensitivity Long signal stability	Expensive/difficult to prepare Non-linear detection curve
MESG/PNP coupled assay[c]	2–20 μM	Continuous assay High specificity for phosphate	Expensive Poor detection reagent stability Subject to interference from colored compounds
PNP/XOD/HRP-resorufin coupled assay[d]	0.05–10 μM	High sensitivity Long signal stability (>4 h)	Complex system Inactivated by reducing agents Moderate expense

MESG 2-amino-6-mercapto-7-methylpurine ribonucleoside, *PNP* purine nucleoside phosphorylase, *XOD* xanthine oxidase, *HRP* horseradish peroxidase
[a][22]
[b][3]
[c][32]
[d][29]

the noise. The core issue in these cases is the assumption of detection system linearity in the screening data analysis. While more labor intensive, these issues can be remediated through correction of the screening dataset for the non-linearity in the detection curve. Formulas for the dataset correction are often derived empirically through non-linear regressions polynomial curve fitting of the detection curve. Additionally, since substrate concentrations in enzyme reactions will often far exceed the concentrations of product formed, it is important to assess the effects of substrates over the range of concentrations from background interference effects (see Fig. 29.3). The net result of these product and substrate titration experiments will determine the overall limitations of the detection system. It is often the case that multiple technology platforms are suitable for a given target, therefore care must be exercised to understand the advantages and liabilities of each to select the most appropriate system. The detection of inorganic phosphate, the reaction product of many enzymes that utilize ATP, is a useful case study of multiple detection system options which include both direct measurement options using either colorimetric phosphomolybdate methods (e.g., Malachite green, molybdate blue) and fluorescein-modified bacterial phosphate binding protein [22] as well as coupled enzyme systems which that provide absorbance or fluorescence-based detection (see Table 29.2). In addition to the differences in detection limit sensitivities, each of these systems has unique advantages and liabilities. These characteristics make it difficult to identify the ideal generic detection system among these options; selection of the optimal system requires knowledge of the target biochemistry, the potential for substrate and product

interference and the spectroscopic options in the screening automation facility (e.g., absorbance vs. fluorescence).

Upon selection of the potential detection system(s) for further development, the focus of the assay development process can turn to the target of interest. To begin the assay optimization phase, initial conditions must be chosen and key parameters in this phase include the concentration of target enzyme, the overall reaction time and the initial substrate concentrations. Since all of these factors impact the amount of product produced by the system, some care must be exercised to ensure that the amount of product formed is within the detection system range. Often, it is possible to infer suitable starting conditions from literature reports; however, experience at the bench may be required to confirm the system is within range.

29.3.2 Optimizing the Assay: Evaluating Target Biochemistry

The majority of the targets of antibacterial screens are enzymes, and as a result, it is useful to review the basic principles of enzymology, since these concepts are often critical in the design of a successful screen. There are a number of excellent texts that cover these concepts in detail and scope [6, 11], but the goal of this section is to introduce the key elements as they relate to assay development and optimization.

First and foremost, enzymes are catalysts: they act as facilitators for the reaction to proceed by modulating the kinetic pathway of the reaction. In chemical terms, they do not alter the thermodynamics of a reaction, which is set by the relative potential energies of the starting materials and products, but provide a more favorable kinetic pathway for the reaction to proceed. As a result, enzymes affect the rate of a reaction, but do not impact the equilibrium state of the reaction progress. This distinction has a number of important, practical implications on assay optimization. First, the overall rate of product formation is dependent on the enzyme concentration. Often, these two parameters are directly proportional to one another through a linear relationship; however, there are exceptions to this rule. For example, some enzyme systems require dimerization or higher oligomerization states for full activity. In this case, rates measured from titrations of enzyme concentrations over the range where dimerization would be expected to show a supra-linear relationship. In contrast, titrations that reach the solubility limit of a target would exhibit depressed rates upon increasing target concentrations.

Establishing the enzyme: rate relationship has important implications for assay development, as it allows the investigator to modulate the desired reaction time according to the additional assay constraints in a programed manner. Often, issues arising from automation requirements or simple convenience will dictate the desired amount time for reaction completion and assay workup. It is a simple matter, once the enzyme concentration**:** rate relationship is understood, to adjust the enzyme concentration that delivers the appropriate amount product in the desired time frame.

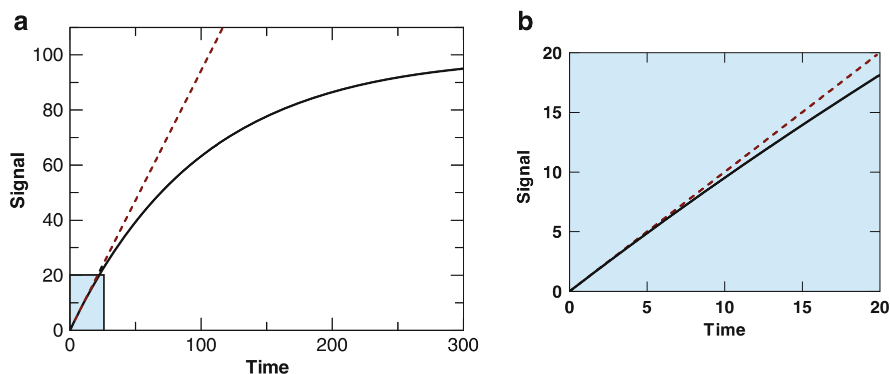

Fig. 29.5 (**a**) Typical steady state enzyme-catalyzed product progress curve. In well-behaved systems, significant deviations from the initial rate (*dashed red line*) occur from depletion of substrate or inhibition by product. (**b**) Under initial conditions (*blue shaded area*), the rate is assumed to be linear since the impacts of product formation and substrate depletion are minimized but they are never completely eliminated

A second, but equally important property of enzyme catalysis is derived from the relationship of substrates and enzyme to the rate of product formation. In technical terms, a plot of product formation over time is denoted a progress curve. Regardless of the enzyme and substrate concentrations used, a progress curve follows a rectangular hyperbolic (see Fig. 29.5). Descriptively, the initial portion of the curve appears linear, but over time the rate decreases eventually reaching an asymptote where the rate is effectively zero. Theoretically, this behavior is fully expected, since as substrate is turned over to product, the concentration of substrate available for enzyme encounters is depleted (substrate depletion) and the concentration of product available for rebinding to the enzyme increases (product inhibition).

When screening for inhibitors, it is important to understand the number and population of enzyme states formed under the assay conditions. Simply stated, target-specific inhibitors bind to distinct enzyme states, and the hit profile outcome will reflect the enzyme states present during the reaction conditions. For example, if the screening conditions are performed where significant substrate and product-bound forms of the enzyme exist, it is possible to identify inhibitors that bind to the free enzyme, substrate-bound, and product-bound forms of the target. Given this realization, one might consider running assays under conditions that cover the broadest array of enzyme states, since this would provide the largest set of actives for the target. In practice, however, it is often advisable to restrict the reaction progress covered in an assay to the initial linear phase for several reasons. First, if the progress curve follows a linear relationship, then the analysis of the extent of inhibition of the enzyme is greatly simplified. This situation is analogous to the effect of nonlinear detection curves on the hit distribution (see Sect 3.1); the inhibition profile becomes skewed if the hyperbolic formation of product formation is not appropriately accounted for in the screen data analysis. Second, it is often difficult to account

for the concentration of the product-bound forms of the enzyme, since the amount of product changes over the reaction time range. In the initial linear portion of the reaction progress curve, less than 10% of the substrate has been converted to product. As a result, the amount of substrate remains high and close to that added at the initiation of the reaction, and the amount of product formed is assumed to have a negligible effect. Under these conditions, termed initial velocity conditions, the number and population of the enzyme states can often be defined and modelled using mathematical equations, a key factor in understanding the mechanism of inhibition for confirmed hits.

Lastly, it is useful to include a note regarding the concentration of enzyme relative to the substrate concentrations. Typically, the enzyme concentration used in screening should be much lower than the chemical reactant concentrations (substrates and products). These conditions, the concentration of enzyme-bound substrate, are negligible relative to the total substrate concentration, therefore it is not necessary to account for this fraction when evaluating the amount of substrate in a reaction. In practical terms, these conditions are met when the enzyme concentration is >50-fold below the most limiting substrate concentration.

From an assay development perspective, it is important to ensure that the nonlinearity observed in reaction progress curves is attributed to the theoretical aspects noted above and not due to technical issues such as target instability or the detection system. In the former case, it is possible to observe rate depletion that is due to the enzyme degrading over time rather than substrate depletion or product inhibition. This factor is often relatively easy to evaluate – simply test the target activity as a function of preincubation time in the assay buffer. In the case of the detection system, it is best practice to confirm that the amount of product formed in the assay conditions remains within the appropriate range of the detection system.

In addition to the time dependency of product formation noted above, substrate interactions with enzymes are further governed by theoretical constraints. In an enzyme reaction, binding of substrate to the enzyme precedes catalytic turnover to products. Under the initial velocity conditions noted above, the enzyme concentration is maintained well below the substrate. However, the strength of the binding interactions and the catalytic power of the enzyme govern the rate of substrate conversion. As a result, titrations of substrate yield rates – determined under initial velocity conditions –diminish with increasing substrate concentration. In the ideal case, the initial velocity rates are related to substrate concentration, according to a rectangular hyperbola (see Fig. 29.6). The enzyme becomes *saturated* with substrate when the concentration of substrate exceeds the binding capacity of the enzyme concentration in solution and at full saturation; the enzyme turns substrate over to produce at the maximum possible rate. This maximal rate is often denoted V_{max} (maximum velocity). The concentration of substrate that corresponds to half the maximal rate is denoted the Michealis constant or K_M.

Inference is often made to the binding strength of a substrate based on the K_M value for an enzyme; strictly speaking, this is not often a direct measure of binding interactions, but a composite ratio of the factors governing substrate binding and turnover to product relative to substrate release back to solvent. In the rare cases

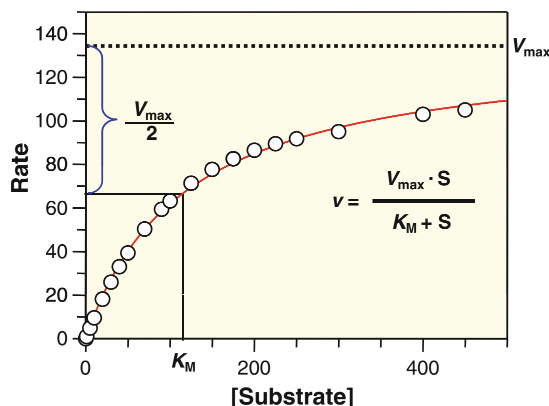

Fig. 29.6 Plot of reaction rate versus substrate concentration for an enzyme system exhibiting saturation kinetics

where turnover to product is very slow relative to the binding and release rates, the K_M value corresponds to the binding constant of the substrate. However, note that this is a rare case for two reasons: first, catalytic rates often approach the rate of substrate binding, and second this assumption breaks down when the enzyme utilizes multiple substrates that bind at varying rates. Single substrate enzymes are rare, and since individual substrates can impose changes upon the enzyme that either enhance or diminish the capacity for both additional substrates to bind and, ultimately, alter the rate of turnover to products it is often not appropriate to treat multi-substrate enzymes in a simplistic manner. Practically speaking, the K_M for one substrate is directly dependent upon the concentrations used for the additional substrates. It is beyond the scope of this review to discuss the kinetic details and implications of multiple substrate systems, but excellent texts are available on this subject for the interested reader [26]. Regardless of the number of substrates, both the K_M values and the maximum velocity are dependent on the conditions used in the assay. Changes in pH, salt concentration and detergents all affect the enzyme dynamics, interactions with the substrate and products and the catalytic power of the enzyme. As a result, the kinetic constants must be carefully monitored as assays are optimized or modified. Note that the K_M value is not dependent on the enzyme concentration, but the maximal velocity (V_{max}) is directly proportional to the enzyme concentration. Often, the maximum catalytic rate constant – denoted as the turnover number or k_{cat} – is calculated for an enzyme and is simply the ratio of V_{max} relative to the enzyme concentration under the specified conditions.

These kinetic values are critically important in understanding a well-designed assay because these values can be used to evaluate the population of enzyme forms in the reaction assay. Since, as noted above, inhibitors bind to distinct forms of the enzyme, it is possible to modulate the sensitivity of an assay to identify inhibitors that target a specific form of the enzyme. Generally speaking, there are three forms of inhibition that can occur relative to substrate for a given enzyme form: the inhibitor can bind to the same enzyme state and compete with the substrate for binding (competitive), inhibitor binding to the enzyme can require substrate binding (uncompetitive), or an

inhibitor can bind in the presence or absence of substrate binding (noncompetitive). In all cases, the concentrations of substrates, relative to the K_M values, will determine the extent of substrate bound forms in the reaction, and therefore modulate the ability for a compound to bind and inhibit the enzyme.

The process of optimizing the assay window is largely empirical, and often focuses on improvements in enzyme stability, enhancing activity and minimizing non-specific target enzyme binding to the assay plates. A largely overlooked, but often extremely important parameter concerns the selection of the appropriate assay vessel. Typical assays, particularly those for HTS efforts, utilize plastic microtiter plates. While it may seem that assay plates are all created equal, the number and type of plates available for assays is diverse, highly differentiated, and growing. An obvious, but critical factor in efficient assay optimization is an early commitment to the plate density format that will be used in the primary screening phase, which typically range from 96-well to 1,536-well format. While higher density formats have clear benefits in terms of reagent cost and volume and fewer overall plates per screening campaign, it is important to note that the challenges in reagent mixing are proportional to the format density due to the reduced volumes and increasingly columnar aspect of the wells in these plates. Achieving sufficient mixing to initiate or quench reactions can be a difficult empirical exercise; however, some general guidelines have emerged recently for the popular 384-well format [30].

Regardless of the plate density, the majority of plate vendors follow the Society of Biomolecular Screening plate mold standards that maintain the dimensions of the plate footprint to allow compatibility with various automation platforms; however, the size, shape, and coatings in the individual wells can vary widely across and within plate manufacturer offerings. These variations can dramatically impact the quality of an assay, since these factors affect the liquid meniscus and reagent mixing properties of the reaction mixture. Furthermore, the material used to prepare the plates should not be considered inert (many plastics can bind proteins or reagents), effectively reducing the concentration of these species in the reaction mixture. Some plastics can leach out substances that have inhibitory activity [20]. To make matters worse, it is not uncommon to find that adsorption of proteins to the plates is time dependent, resulting in a reduction in activity over time. To counter these effects and to ensure the best possible initial optimization position, it is best practice to profile the assay across a variety plate types early in the assay optimization phase. Once achieved, the best performing plate type can be selected based on assay performance, reagent stability, and cost.

As noted earlier, individual targets will have specific requirements in terms of inorganic salts, pH, and detergents. These factors are typically determined empirically, based on optimal assay activity. It is important to utilize a fairly detailed approach, as the effects can be quite subtle and difficult to discern if sparse matrices are used exclusively as a means to identify optimal signal amplitude. It is often best to survey a wide range of salt concentrations, composition, and pH values initially and then perform more detailed analyses to identify optimal conditions. Detergents can be extremely useful as wetting agents for plates to minimize non-specific target binding and to maintain reagent stability and solubility over time. However, it is

important to consider the critical micellar concentration (CMC) of any detergents under consideration, since the formation of micelles can complicate the assay interpretation. This is due to the fact that micelles can act as depots of reagents, target, and inhibitors that may not reflect the concentration of the bulk reagent addition and it can become quite difficult to determine the actual concentrations that are affecting target activity. This is particularly important in the evaluation of any active compounds that are identified from HTS, since these will be assessed in dose response curves that are based on bulk addition concentrations. Conversely, small amounts of detergent have proven effective in minimizing the propensity to find active compounds that inhibit targets through non-specific interactions. These classes of compounds, often termed promiscuous inhibitors, are difficult to assess and are not suitable for lead optimization, since the target inhibition is usually associated with target unfolding mediated by compound aggregation.

In terms of overall risk mitigation of an assay, it is important to consider the effects of changes in the assay as well as the detection system. It is not uncommon to find that the optimal conditions for activity differ from the presumed cellular environment under standard physiological conditions. Under these situations, the power of the assay to predict activity in whole cells should be interrogated. Note, however, that these questions are frequently confounded, due to current limitations in understanding the microenvironments of the bacterial cell encountered by most targets and the realization that any in vitro assay system will never fully replicate the cellular environment. In the end, it is a matter of risk mitigation and a pragmatic approach in selecting conditions that represent the best compromise between assay performance and the anticipated cellular environment has proven to be the most successful to date. Further, as noted above, it is critical that the system remains within the limits of the detection system throughout the assay optimization phase. It is critical to assess the effects of changes to the reaction conditions on both the assay and the detection system to understand where the effects on the assay are manifested. In essence, optimization of the overall assay can occur in the target biochemistry, the detection system or both. Effects on the detection system are usually probed using a control plate where the expected amount of product formed under the assay conditions is added to the plate, but the substrate or the target enzyme is excluded. Deconvolution of the effects of additives on both the detection system and the target biochemistry provides clarity during the optimization phase and ensures that a robust screen is produced. This is particularly important in the special case where coupling enzymes are used in the detection system. Modifications to the assay conditions intended to improve the performance of the target enzyme may have antagonistic effects on the coupling enzymes, which can result in diminished coupling efficiency of the system. In this case, it is important to confirm that the coupling enzymes are maintained at excess coupling efficiency relative to the target enzyme to ensure that false positives attributed to inhibition of the coupling enzymes are minimized.

29.3.3 Assay Flexibility: Preparing for Automation

Once optimal assay conditions have been identified, it is important to evaluate the flexibility of the assay to ensure a smooth transition from the benchtop to automated platforms required for primary screening. The goal of this phase is both to assess fitness of the assay relative to the constraints imposed by the automation as well as provide potential contingencies for modifications when issues arise during the screening campaign. In general, the limitations of the automation will depend on the equipment used and the scale of the compound library. However, several common elements arise from current screening practices. First, most compound libraries are solubilized in dimethyl sulfoxide (DMSO). This solvent, while still polar relative to other organic solvents, is significantly more lipophilic than water (DMSO dielectric constant = 46.68, water dielectric constant = 78.5). Further, DMSO has the potential participating in reductive/oxidative reactions with assay reagents, including the target enzyme. As a result, it is common practice to assess tolerance of an assay as a function of DMSO concentration. Typical concentrations tested range from 0.1% to 10% v/v and any limits observed in the assay performance will constrain either the maximal concentration of compound screening concentration or force a pre-dilution of compound stocks in aqueous buffer.

Sensitivity to temperature fluctuations can also limit the assay flexibility. For convenience, assays are typically run at room temperature, but specific temperatures can be maintained if necessary, as most automation platforms have the capacity for maintaining temperature control during incubation periods. However, some fluctuation during plate movement and liquid additions will inevitably occur, and these effects become more pronounced as the temperature differential from room temperature increases. Regardless of the desired screening temperature, it is important to evaluate the temperature dependence of the assay, as temperature changes across and within plates can result in significant systematic error effects for those assays that exhibit extreme temperature sensitivity.

Typically, there is a drive to maximize the number of plates in a screening batch, since the time and reagent overage required to setup a screening run can be significant. Any reagents that require special handling or are unstable at room temperature compromise the batch size and will prolong the overall screening campaign. Since screens are often run during standard working hours, reagent stock stabilities exceeding 8 h are preferred and reagents with short stabilities (>2 h) present significant difficulties. Optimizing reagent stock stabilities is strictly empirical; however, we have noted that combining substrates with target enzymes in buffers can greatly enhance the stability of enzyme targets in a number of cases. Combining reagents, where possible, has the added advantage in that the overall number of reagent additions is minimized, reducing the overall complexity of the screen in terms of mixing and screening run setup time. For similar practical reasons, single reads are typically collected in primary screen, even when continuous assays are employed. The time required to read a plate can be significant, and it is not uncommon for data collection to be the rate limiting plate processing step. In these cases, a reaction quench

is often preferred over real time measurements of the reaction progression, since this allows flexibility in processing batches of plates: data collection can be done during off hours, on separate automation systems, or in overlapped with the next batch of plates in the campaign. The requirements of a suitable reaction quench are numerous, but instantaneous and complete reaction termination, extended signal stability with minimal interference with the detection system and compound collection are critical factors.

Finally, defined control conditions are an essential feature of any screen, since these are used in monitoring quality control of the screening runs and in the selection of active compounds from the screening run. The maximum signal condition is readily achieved through uninhibited reaction wells; however, selection of conditions that reflect the minimum boundary can be more difficult to identify. Often, it is not possible to set up conditions where substrates or target enzyme are removed due to the automation requirements for universal intra- and inter-plate processing and so a minimum condition is often selected empirically. Specific inhibitors of the target enzyme are preferred for setting the minimum signal condition, since these afford the most sensitive monitors of the assay system; however, reagents that affect only the detection system have been used when suitable alternatives can be identified. While not strictly required, it is useful to have a reference compound, typically a specific inhibitor of the system that can be used to confirm the activity range and performance of the assay. Often, the minimum signal control is obtained using a high concentration of the reference compound.

Upon completion of the stability assessments and the selection of the control conditions and DMSO tolerance range it is possible to draft the assay protocol and perform a full statistical analysis of the system. Assay performance is usually assessed using the assay window parameter Z′-factor [35]. In order to ensure an accurate assessment of this parameter, a sufficient number of replicates should be run where the controls are dispersed throughout the plate to minimize systematic errors due to edge or plate position effects [13, 19]. In addition, it is wise to confirm the relationship between enzyme concentration and reaction time under standard reaction conditions at this point, since this provides a measure of the flexibility of setting the reaction time to meet the automation requirements.

Often assay development is separated geographically and functionally from the screening center thereby requiring a protocol transfer step. In these cases, rapid confirmation of the assay performance and flexibility is essential for efficient screen prosecution. Typically, the experiments outlined in this section are repeated at both functional centers and upon satisfactory agreement between the two datasets, the primary screening can commence.

29.3.4 Primary Screening: Finding the Needles

The first step in setting up the primary screen involves the selection of the compound libraries and concentration for the screen. Compound concentration is a matter of

preference, but it should be noted that increasing the compound test concentration results in more potent activity theoretically allowing better segregation from the inactives, the potential for interference with the detection system, and non-specific binding effects is proportional to compound concentration. For pragmatic reasons, high throughput screens are typically performed with compounds set at 10 μM, which affords a reasonable balance between compound stock usage, reasonable inhibition potency measurements, and minimal detection system interference. In contrast, fragment based approaches, which utilize libraries of low-molecular weight and simple molecules suitable for rapid diversification – employ high concentrations (1 mM), since the inhibition of these molecules is expected to be significantly weaker than the standard HTS screening library. In these cases, the concentration of DMSO and the potential for interference with the detection system should be thoroughly assessed to minimize adverse effects on the assay performance. Finally, some organizations have moved to a multiplexed approach for screening, where the compound library is pooled into mixtures of compounds. This approach benefits from increased library screening coverage for a given plate batch size, but suffers from a more complicated hit deconvolution process and the potential for compound mixtures to exhibit antagonistic interference effects. In this case, active mixtures are segregated into single compound wells to identify the inhibiting compound(s) that resulted in the inhibition observed with the mixture.

Since the nature of screening is to find outliers within a large screening set, statistical sciences play a critical role in the prosecution of the primary screen. While most of the focus is on compounds that exhibit activity significantly different than the controls, the focus during the screening process is the identification of systematic errors resulting from deviations from the assay protocol or automation failures. The standard approach for monitoring these effects is to utilize control wells throughout the screen; however, the placement of the controls within the screen remains an ongoing debate [2]. Ideally, controls should be randomly positioned throughout the plates in screening batches, but this is not possible on the typical HTS plate preparation scale and the resulting demands on data processing would be extremely high.

An alternative approach is to use the compound wells of the screen as internal controls for the screen, using the assumption that most of the compounds (>98%) will be inactive and therefore return only random noise values. Using this approach, it is possible to use the full format of the plate for compound testing for most plates in the screen and plates containing only controls (Max, Min, Ref) can be prepared and tested periodically throughout the screening runs to ensure assay performance remains within specifications. This approach benefits from ease of implementation, maximal use of the plate format for compound testing and provides controls for all positions within the plate, assuming the control plates are prepared with this aim in mind. The limitation to this approach is that for screens that contain a high number of actives such as those anticipated in focussed or directed libraries the assumption that the bulk of the data will reside near the median value may break down. These arguments are relevant not only to monitoring the screen for performance but also

to the selection of actives. With control-based analysis, individual data points can be calculated based on the percent inhibition of the control range (Max-Min) and selection of actives can be obtained using a desired threshold of inhibition. Alternatively, the whole dataset can be processed as a function of a global median and active compounds can be selected as a function of the standard deviation from this value. The science of HTS informatics is an active and evolving science, and a number of reviews that deal with this subject in detail are available for the interested reader [10, 14, 18].

29.4 Follow-up of Hits

The phase after HTS is commonly called Hit Evaluation (HE), and its goal is to deliver series of compounds that are confirmed inhibitors of the target of interest. In Hit Evaluation the outcome of the HTS is first channeled through a cascade of biological assays that are designed to eliminate undesirable compounds. At some point in the progression, compounds are usually re-synthesized by a medicinal chemistry team. Additional biological tests are then performed to characterize the inhibitors, which in the end produce a lead series that is progressed to the next phase of drug discovery.

The HE process can be summarized as the progression from active to hit to lead. While the definition of these terms varies among organizations, typically an active is defined as inhibitory activity that resides in a compound from the screening collection. Often this inhibitory activity cannot be replicated, for reasons stated below. If the inhibitory activity can be confirmed and ascribed to a compound, then the compound becomes a hit. If the hit, when profiled in additional assays, continues to show promise, then it is termed a lead. Generation of leads is the ultimate goal of the Hit Evaluation process (see Fig. 29.7).

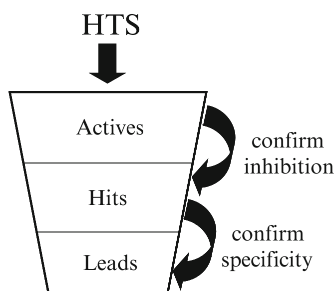

Fig. 29.7 Schematic of the hit evaluation process, from active to hit to lead

29.4.1 Artifact Assays

The simplest types of undesirable "active" compounds to identify and filter are false positives. Even though an "active" may have been tested in replicate and dose-response measured, other factors may have accounted for the apparent inhibition seen.

Every screening format is subject to interference with its particular readout. For spectrophotometric and fluorescence readouts, the most common problem is spectral overlap from the screening compound. In a typical assay, the screening compound is present at micromolar concentration whereas fluorophores and other readout technologies are often at nanomolar or picomolar concentrations. As a result, even minor absorbance or fluorescence of the screening compound can influence readout. This is especially a problem with near-UV and short-wavelength fluorescence readouts (<400 nm) as compared to luminescence and long-wavelength fluorescence. In one survey of a screening collection, 5% of the compounds screened at 5 μM were found to be as bright as 10 nM of a common 340 nm-absorbing fluorophore [27].

For enzyme assays, often a coupling reagent is used to provide a measurable signal. This reagent can be another enzyme in solution such as luciferase or a derivatized bead such as scintillant-impregnated beads. In these cases, inhibition of the coupler will manifest as apparent inhibition of the target.

For these simple false positive cases, a straightforward method to identify them is to test all of the "active" compounds in an assay that does not contain target enzyme but instead contains a fixed amount of the added enzymatic product. If the compound appears active in this assay, then it is interfering with the assay readout. These interfering compounds are often abandoned. The best way to determine if they are true inhibitors is to test them in a secondary assay.

29.4.2 Secondary Assays

The purpose of a secondary assay is to measure inhibition of "actives" in an assay that uses a different readout technology from the primary assay. For example, if a primary screen were run with phosphate detection via the Malachite Green dye and absorbance at 650 nm as the readout, a secondary assay could be phosphate detection via the purine nucleoside phosphorylase enzyme with an absorbance at 360 nm readout. Particularly for spectrophotometric and fluorescence assays, secondary assays should be designed to shift to a different coupling system and a different spectrophotometric readout. By doing this, secondary assays are powerful filters because they can screen out several types of false positive "actives" simultaneously. When designing a secondary assay, care should be taken to replicate enzyme and substrate conditions as in the primary assay otherwise IC_{50} values for the same compound in the two assays could vary significantly. For some targets, particularly

those that are not enzymes and require a binding readout, secondary assays become technically unfeasible, in which case compounds are progressed through Hit Evaluation at greater risk.

29.4.3 Compound QC

Although most of the assays in Hit Evaluation are biological, one important chemical assay is QC, or quality control of the compounds of interest. Most screening libraries are composed of individual compounds, to which is ascribed a single chemical structure. A QC test is simply a mass spectrometry measurement of the compound to verify that it matches the predicted mass from the assumed chemical structure.

In large screening collections, often the same compound exists in several locations and therefore the exact sample of compound used in the primary screen may not be the same sample used in subsequent testing. In this case, it is prudent to test the QC of the samples used in Hit Evaluation, especially if liquid stocks of compounds are being tested. Due to degradation during storage in DMSO, it is not uncommon for >10% of liquid samples to fail a QC test [8].

When a compound fails a QC, test a decision must be made whether or not to pursue the compound by identifying the species in the sample responsible for inhibition. As tempting as this may be, this is usually a difficult task. Often the inhibitory species is a trace contaminant, for example a heavy metal remnant from compound synthesis. Not only is chasing after trace contaminants a laborious procedure, but in the end, if the inhibitory species is identified, then it is likely to be an undesirable starting point for inhibitor design. For this reason, it is common practice to abandon "actives" that fail a QC test.

Artifact testing, secondary assays, and QC are three ways to eliminate false positive "actives" from further consideration. Compounds that survive these tests are "hits" and progress to additional tests to evaluate their potential.

29.4.4 Spectrum Assays

One of the largest challenges for antibacterial lead generation is finding broad-spectrum inhibitors. Drug candidates with the largest clinical utility will be those that cover the largest number of bacteria. To cover the largest number of bacteria, inhibitors of a target must maintain potent inhibition of the target across all pathogens, which is a difficult task due to the evolutionary diversity of bacteria. For this reason, the spectrum of activity of compounds in Hit Evaluation is often assessed early, in order to bin those that do not have a desirable spectrum.

The simplest way to assess spectrum is to assemble a panel of target enzymes chosen to represent key pathogens relevant to the chosen clinical indication and phylogenic diversity. For example, if a target were screened using the *S. aureus* version of the target, a useful spectrum panel for compounds that are targeted to lung (pneumonia) and skin infections could have the *H. influenzae, P. aeruginosa,*

S. pneumoniae, and *M. pneumoniae* target proteins. One would expect from a screen of an *E. coli* target that some inhibitors will hit only the *E. coli* enzyme, some will hit only the Gram-negative enzymes, and some will hit all enzymes. The goals of the screening campaign will define which bins of inhibitors are pursued (i.e., narrower spectrum or broader spectrum).

29.4.5 Promiscuous Inhibitors

A major breakthrough in the understanding of compound screening was the recognition of promiscuous inhibitors. Promiscuous inhibitors are compounds that exert inhibition via an aggregation phenomenon that is not specific to the screening target [5]. Typically this effect manifests as inhibition at low μM compound concentrations, which is unfortunately the same potency range used in HTS.

There are several methods to identify promiscuous inhibitors [12]; all of these methods rely on the fact that promiscuous inhibitors form aggregates, and these aggregates cause enzyme inhibition. While it is possible to employ a generic enzyme, such as β-lactamase, as a test for aggregators, promiscuous inhibition can be assay-dependent, and as a result, it is better to test for them in the actual screening assay. One simple way to do so is to run IC_{50} experiments at increasing enzyme concentrations. The IC_{50} of true inhibitors should not vary at increased enzyme concentration whereas for promiscuous inhibitors the IC_{50} will increase or often fail to inhibit at all at higher enzyme concentrations. Because of their non-specific nature, promiscuous inhibitors are usually abandoned once identified in hit-followup.

29.4.6 Covalent Inhibitors

Covalent inhibitors represent an attractive class of inhibitors, because of their tight, irreversible binding. Even though there are examples of antibacterial drugs that are covalent inhibitors such as β-lactams and fosfomycin, it is highly unusual to discover selective, covalent inhibitors. It is far more common to uncover non-selective covalent inhibitors due a reactive functionality of the compound. The negative safety ramifications of reactive compounds, combined with the high drug doses typically administered for antibacterials, present a high risk for progressing covalent inhibitors. For this reason, unless the compounds display exquisite selectivity covalent inhibitors are usually discarded in favor of less potent but reversible inhibitors.

29.4.7 Direct Binding Assays

In the process of characterizing hits from HTS, a valuable test to run is a direct binding measurement. If the HTS was a binding assay, then this step is unnecessary, but most antibacterial targets are enzymes that will be screened using an enzyme assay.

The results from direct binding measurement serve several purposes. First, it reinforces again the link between IC_{50} and target inhibition and provides confidence that the IC_{50} is not artifactual. If the binding method is quantitative, then it can link IC_{50} values, which are assay-dependent, to K_d values, which are assay-independent. The binding measurement can also define the enzyme state or states that are relevant for X-ray crystallography.

There are a large number of methods that can be employed to measure direct binding. A limiting factor for compounds from HTS is their weak potency, often low μM, which precludes many binding technologies. One method that has proven useful for measuring weak binding is protein NMR. This technology is sometimes employed to screen low molecular weight fragment compounds, but it is equally accessible to following up weak hits from HTS. Because bacterial target proteins are usually easy to express at high levels and soluble at >10 mg/mL concentration they are applicable to protein NMR methods, either ligand-observed or protein-observed [15].

29.4.8 X-Ray Crystallography/Molecular Modelling

Arguably the most valuable piece of data that can be provided for a hit series is a X-ray crystal structure with the target. Not only does the crystal structure again validate the series as specific binders but it provides instruction for medicinal chemists to optimize the series. Often selectivity and specificity observations from IC_{50} data can be explained by the structure and thereby provide confidence that the series has the potential to be enhanced to increase binding potency.

Another important use of a crystal structure is for modelling of binding to the target in other species. In the absence of antibacterial activity, it is difficult to assess whether a lead series has activity across all the bacteria needed for a clinical indication. With a X-ray structure in hand, one can model the binding mode of the series in multiple species and then estimate the likelihood of attaining the required cross-species activity.

29.4.9 Fragment-Based Methods

The topic of fragment-based lead generation is the title of a separate chapter in this book. Even though the primary screening methods are different from HTS, the HE process for fragments follows similar logic for HTS. In fact, efficiencies can be gained by screening both fragments and HTS collections at the same time and prosecuting the hits in parallel. Often scaffolds seen to hit in the fragment collection are exemplified as larger, more elaborated compounds in the HTS collection, allowing SAR conclusions to be drawn for the scaffold.

29.4.10 Enzymological Mode of Inhibition

For enzyme targets, the final piece of characterization to transform a hit series to a lead series is its enzymological mode of inhibition. This information is as critical as a direct binding measurement and X-ray crystallography to understanding how a compound achieves inhibition of its target.

As described above in Sect. 29.3.2, for any given target there are multiple modes of inhibition conceivable. In order to begin a mode of inhibition experiment, one must have knowledge about the kinetic mechanism of the enzyme. With this knowledge in hand, one can model the theoretical modes of inhibition. Often an inhibitor-bound crystal structure or obvious structural similarity to a substrate suggests the probable inhibition mode. To clarify this one can then gather a dataset where enzyme activity is measured at varying substrate and inhibitor concentrations. The likely inhibition modes can be modeled as rate equations and the empirical data fit to the models [9]. Statistical analysis distinguishes the fits and identifies the probable model. If direct binding measurements have not been performed yet, then they can reinforce the deduced mode of inhibition.

Identifying the correct mode of inhibition for an antibacterial lead has two major benefits: first, it allows derivation of the relationship between IC_{50} and K_i. An IC_{50} value can change as a function of substrate or product concentration whereas a K_i value does not. For convenience, IC_{50} values are usually reported to chemistry teams as a measure of binding potency and they are typically assumed to be of a similar magnitude to binding potency. This is true for uncompetitive inhibitors, as well as for compounds competitive with substrates for which the inhibition assay as been designed with substrate concentrations near K_M values. On the other extreme, there are inhibition modes such as product-competitive inhibitors for which IC_{50} values can be extremely misleading. One such example is the antibacterial agent triclosan, which inhibits an enzyme in fatty acid biosynthesis, FabI. FabI has two substrates and two products and is usually assayed in the forward direction. In such a format, the assay contains large concentrations of substrates relative to products. Triclosan binds specifically to the *product-bound* form of FabI, whose presence is biased against in the forward reaction IC_{50} setup. As a result, triclosan shows an IC_{50} of 2 μM against *E. coli* FabI in the forward reaction assay whereas its K_i value to the product-bound form of FabI is actually 38 pM [31]. In such cases, the forward reaction IC_{50} greatly underestimates binding potency and a different assay format should be found that will provide more accurate values.

The second major use of mode-of-inhibition data is to understand the relationship between IC_{50} and MIC. These two pieces of data comprise the cornerstone of target-based antibacterial drug discovery. IC_{50} measurements are used to gauge target binding potency and are assumed to translate to binding potency of the inhibitor inside the bacterium, at the native site for the target. But again, substrate and product concentrations inside the bacterial cell may be quite different from what is used in an IC_{50} assay, and these differences can lead to discrepancies between IC_{50} and expected MIC. One example is the thiazolyl-urea class of inhibitors of the phenylalanyl

tRNA synthetase enzyme [1]. These compounds are competitive with the substrate phenylalanine, and as a result, their binding potency is diminished by increasing phenylalanine concentration. The standard IC_{50} assay for these compounds uses a concentration of phenylalanine close to its K_M, 2 μM. Under these conditions, the compounds exhibit <100 nM IC_{50}s. Despite these low nM IC_{50}s the compounds show an MIC against *S. aureus* of >100 μg/mL. This lack of activity could be due to compound efflux, but in fact, it is caused by the high level of phenylalanine (>1 mM) present in standard MIC susceptibility media for *S. aureus.* When the concentration of phenylalanine is controlled for in the growth media, a direct relationship between phenylalanine concentration and MIC is seen. This example highlights the importance understanding target mode of inhibition and how it can affect MIC outcomes.

The Hit Evaluation process ends when the hit series has been characterized in enough detail to warrant further progression as a lead towards an antibacterial drug. To this end, the Hit Evaluation process is designed to demonstrate with high confidence that the lead series are specific inhibitors of the target and that the series has potential to optimized to increase antibacterial activity and improve pharmacokinetic parameters.

29.5 Conclusion

The target-based strategy of antibacterial discovery is predicated on the ability to screen targets to identify leads. Once the exclusive domain of large pharmaceutical companies, HTS, is now accessible to academic investigators, biotech companies, and smaller organizations. The above chapter is meant to introduce newcomers to HTS to the strategic decisions made in advance of screening and the tactical decisions made during prosecution of the screen results. With the capability of efficiently finding multiple lead series, HTS remains a powerful lead discovery tool for antibacterial agents.

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Chapter 30
Antibacterial Inhibitors of the Essential Cell Division Protein FtsZ

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30.1 Introduction

Antibacterials with new mechanisms of action are needed to treat the increasing number of life-threatening bacterial infections that are resistant to current therapies. Despite this urgent need, only three new classes of antibacterial compounds – oxazolidinones, cyclic lipopeptides, and pleuromutilins – have been approved for human clinical use since 1962 [19, 20]. In particular, the emergence and spread of drug-resistant Staphylococci, such as methicillin- and vancomycin-resistant *Staphylococcus aureus* (MRSA and VRSA) is of serious concern. Here we describe the motivation, discovery process, and characterization of a novel class of small synthetic antibacterials that have potent activity against Staphylococci, including drug-resistant clinical isolates.

The majority of clinically successful antibacterial compounds target just four essential cellular processes: protein, nucleic acid, folate, or cell-wall biosynthesis. The discovery of efficacious compounds against novel targets has proven surprisingly difficult [31]. Prolysis' founding scientist, Professor Jeff Errington, recognized that at least two barriers to Hit identification must be overcome to capitalize on the wealth of potential new targets identified by bacterial genomics. The first key barrier is sufficient knowledge about novel targets to enable effective target validation and to drive the generation of intelligent screening assays to find Hits. The second key barrier is entry into the bacterial cell. While it was proven to be relatively easy to identify *in vitro* inhibitors of novel target proteins, few Hit compounds from screening campaigns demonstrate antibacterial activity or have been modified leading to antibacterial activity. Professor Errington devised a novel antibiotic discovery process based on an in-depth knowledge of target biology and the use of

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genetically engineered bacterial cells to create a whole-cell screening platform that could identify inhibitors of essential target pathways such as bacterial cell division and DNA supercoiling [35]. This provided a different, advantageous starting point for optimization. Prolysis Ltd. was started to test drive the discovery process using these whole-cell screening assays, focusing on bacterial cell division, DNA supercoiling, chromosome segregation, DNA replication, and transcription.

Prolysis screened more than 100,000 synthetic compounds and natural products using the whole-cell screens. The results from these screening campaigns were mixed. Although the compound collection was selected to provide access to a diversity of compounds, few antibacterial compounds amenable to productive optimization were identified. One compound that was identified as a Hit in the cell division whole-cell assay screen was confirmed to be an inhibitor of the cell division protein FtsZ, demonstrating proof-of-principle for the whole-cell screening assay [35]. The Hit compound progressed into Lead optimization and more potent analogues have been synthesized and evaluated.

We considered that the whole-cell screening assay platform was effective but that the compound library lacked diversity and sufficient compounds with "antibacterial properties" to provide the progressable Hits we sought. It was clear that we either had to screen many more compounds, to identify compounds with physicochemical properties that increased the chance that they will have antibacterial properties, or to deploy an alternative approach to Hit finding.

Prolysis decided to focus on DNA supercoiling and cell division as key essential processes with the most potential for novel compound identification. Significantly changing the odds in high-throughput screening would have required increasing our library size ten-fold or more. This would have been costly and slow. The whole-cell screening assays, which could be run at 10,000 compounds per day, provide high-content, high-value results and are not readily amenable to ultra-high-throughput formats. Prolysis did explore the use of neural net technologies to improve library diversity through the identification of more antibacterial-like compounds in focused libraries of < 5,000 compounds. This did increase the Hit rate in screens but did not lead to optimization programs. We felt that although the whole-cell screens worked well, we lacked the right compounds to screen to identify productive hits. In order to create Prolysis' Lead programs, we turned to literature to identify alternative start points and deployed fragment-based and structure-informed approaches to develop novel chemical series into Hits and Leads. This review focuses on our experiences during the discovery of novel inhibitors of bacterial cell division.

30.2 Bacterial Cell Division and FtsZ

30.2.1 Background

The bacterial cell division machinery consists of a set of proteins that are recruited to the site of division where they assemble to form the divisome. Recruitment of these proteins to the site of division occurs in a specific order, with FtsZ at the top

of this hierarchy [8]. As such FtsZ is considered to be the most critical component of the division machinery [8]. FtsZ monomers undergo GTP-dependent polymerization to form protofilaments that aggregate into a macromolecular structure – termed the Z-ring – at the mid-cell. Other cell division proteins are then recruited to the Z-ring and a new septum is synthesized that enables the daughter cells to separate [8].

FtsZ is recognized as an attractive but as yet underexploited target for new antibacterial drug discovery target for a number of reasons [22, 39]. It is an essential protein for bacterial viability [1, 5, 33] and it is a highly conserved and potentially broad-spectrum antibacterial target. Although it has little primary sequence identity to mammalian β-tubulin, which has been successfully exploited for cancer therapy [7, 23, 27], it does have structural and functional homology, suggesting that FtsZ may also be amenable to inhibitor development. Finally, because cell division proteins are not targeted by any antibiotics in current clinical use, it is expected that there will not be any cross-resistance from existing drug-resistant bacterial populations.

The consequences of FtsZ inhibition in rod-shaped bacteria and in cocci, namely longitudinal filamentation and ballooning, respectively, have been described [1, 11, 24, 28, 33, 35]. This morphological effect of putative cell division inhibitors provides evidence that the mechanism of action of a compound is consistent with the inhibition of cell division but is not conclusive. For certain species of bacteria, cell morphology is dependent upon external factors and is responsive to changes in growth rate and other factors such as osmolarity. Compounds that alter the morphology of bacteria in this way have been described over many years. While some of these may directly interact with the cell division machinery, e.g., the beta-lactams inhibit septum formation leading to filamentation [13], many do not, e.g., nalidixic acid, a DNA supercoiling inhibitor [3] and mitomycin, a DNA synthesis inhibitor [37]. Several antibiotics, including protein synthesis inhibitors, disrupt septum formation and cell division [2] and the nitrofurans alter cell morphology [17]. Those searching for inhibitors of bacterial cell division, and inhibitors of FtsZ in particular, should therefore not rely on morphological evidence alone.

30.2.2 Cell Division Inhibitors

Several approaches have been taken to identify inhibitors of FtsZ. The use of in vitro assays to identify inhibitors of FtsZ self-polymerization or GTPase activity has been popular. The GTPase activity of FtsZ is dependent upon polymerization of FtsZ as the GTPase active site is formed at a dimer interface. Inhibitors of polymerization will therefore also be inhibitors of GTPase activity. It must be noted that GTPase and polymerization assays alone do not provide indisputable evidence that inhibitors target FtsZ. Polymerization is concentration dependent and requires a high concentration of protein *in vitro* which in turn means that high concentrations of inhibitor are required to be effective in assay systems. This means that the assays are relatively insensitive for screening and that they are predisposed to agents that nonspecifically diminish protein-protein interactions as well as nonspecific inhibitors

of GTPase activity. A compound demonstrating activity in the FtsZ GTPase or polymerization assay may be a specific inhibitor of FtsZ function or it might act indirectly through a nonspecific mechanism of action.

With our growing collective experience in compound screening, we have come to recognize that some classes of compounds regularly appear as screening hits across a wide variety of assays and assay formats. These classes are often referred to as promiscuous inhibitors [26]. One possible mechanism of action of the nonspecific promiscuous inhibitors is their propensity to self-aggregate forming micelles that adsorb target enzymes and thus interfere with their activity. It is possible that these types of inhibitor might result in false-positive readouts in FtsZ *in vitro* assays.

Berberine may be one example of a promiscuous inhibitor reported as being an inhibitor of *E. coli* FtsZ [6]. Berberine has been described as an antibacterial [14, 16], an antifungal [29], and an antiviral [10] compound. In addition to its potential as an anti-infective, berberine is also claimed to have anticancer [21] and antidiabetes [42, 43] activities. It is axiomatic that berberine cannot be a specific inhibitor of all of these processes. Close examination of the Domadia publication shows sharp, non-sigmoidal dose-response curves typical of nonspecific inhibitors and micelles in electron micrographs of berberine-treated FtsZ filaments, as predicted for nonspecific promiscuous inhibitors [26].

Researchers at Merck [40] used a fluorescence polymerization assay to screen a natural product library of more than 100,000 natural product extracts. From this high-throughput screen, they identified viriditoxin, and provided data to support its activity against FtsZ. Viriditoxin demonstrated broad-spectrum antibacterial activity, including against MRSA. Margalit *et al.* [25] screened more than 18,000 compounds in a high-throughput GTPase assay and identified a class of small-molecules (Zantrins) that perturb Z-ring assembly and have broad-spectrum antibacterial activity.

In a targeted approach, an analogue of GTP, 8-bromoguanosine 5’-triphosphate, was designed as a selective inhibitor of FtsZ [18]. 8-bromoguanosine 5’-triphosphate, which does not inhibit tubulin assembly, competitively inhibited FtsZ GTPase activity and polymerization. In another study, a collection of GTP analogues was synthesized by combinatorial chemistry and tested for activity against *Pseudomonas aeruginosa* FtsZ [30]. Derivatives that potently inhibited the GTPase of *P. aeruginosa* FtsZ were found. None inhibited the growth of *E. coli* but several did have MICs against *S. aureus*.

In an alternative approach, two studies used, as starting points, compounds with known inhibitory activity against mammalian β-tubulin. A set of alkoxycarbonylaminopyridines inhibited growth of susceptible and drug-resistant strains of *Mycobacterium tuberculosis* and inhibited the GTP hydrolysis and polymerization of *M. tuberculosis* FtsZ [41]. One of these, SRI-3072, did not inhibit the polymerization of bovine brain tubulin. Huang *et al.* [12] screened 120 taxanes and identified several that were active against *M. tuberculosis* with several having MICs of 2.5 μM, but no cytotoxicity observed at concentrations up to 80 μM.

FtsZ interacts with a number of other proteins *in vivo*. Researchers at Wyeth have exploited the interaction of FtsZ with one of these other proteins, called ZipA, in Gram-negative organisms and used structure-based drug design to create inhibitors of this interaction [15, 36, 38]. These inhibitors prompted the filamentation of *E. coli* cells *in vitro* but for the most part exhibited weak antibacterial activity.

30.3 Optimization of 3-Methoxybenzamide

We reviewed the literature and decided that *bona fide* FtsZ inhibitors should demonstrate antibacterial activity, morphological effects, genetic evidence, e.g., mutations within the FtsZ coding sequence that alter sensitivity to inhibitors, and GTPase inhibitory or stimulatory activity. From this perspective, the most compelling FtsZ inhibitor was 3-methoxybenzamide (3-MBA, [28], $C_8H_9NO_2$, relative molecular mass 151.16. Compound **1**; Fig. 30.1). Ohashi *et al*. provided morphological, antibacterial, and genetic evidences to support their hypothesis that 3-MBA targets FtsZ to inhibit cell division. Prolysis extended the validation of 3-MBA as an inhibitor of

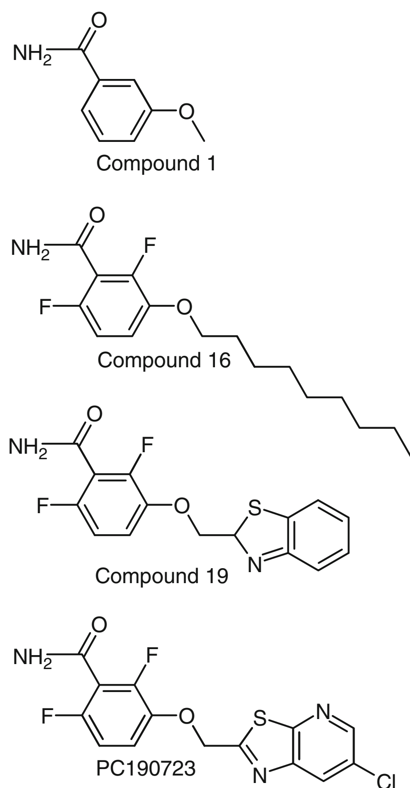


Fig. 30.1 Compound structures

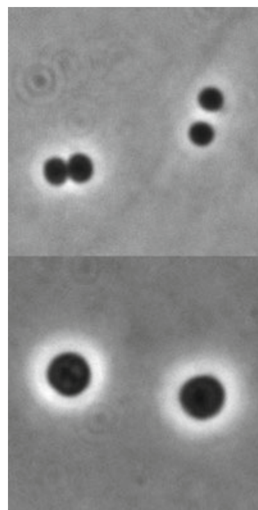


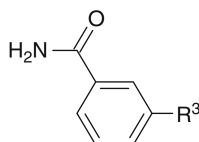
Fig. 30.2 Phenotype of *aureus* grown in the absence (*top*) and presence (*bottom*) of the cell division inhibitor **PC190723**

FtsZ and explored its use as a fragment-like starting point for FtsZ inhibitor design. 3-MBA may be the first FtsZ small molecule/ zuttietic inhibitor that is sufficiently robust and reproducible to be independently validated and confirmed.

3-MBA exhibits weak on-target activity (*Bacillus subtilis* minimum inhibitory concentration ((MIC) 2048 μg.ml^{-1}), but it is able to penetrate bacterial cells, which is often a barrier to novel antibacterial discovery. 3-MBA has also proven to be an effective starting point for, or has been a key feature in the discovery of, inhibitors in other therapeutic areas [9, 32, 34]. Our objective was to increase the potency of 3-MBA against whole bacterial cells while retaining the on-target cell division inhibitory activity. The biological activity of the compounds was characterized by measuring the MIC against *B. subtilis* and by morphometric analysis to determine on-target activity as expressed by filamentation of the bacilli due to continued short-term growth in the absence of cell division [35]. Selected compounds were also tested against *S. aureus* to determine both potency and on-target activity, expressed by cell enlargement ('ballooning') of the cocci in this species (Fig. 30.2) [11, 33]. In addition to the morphometric evaluation, the GTPase inhibitory activity of selected compounds and their activity against well-characterized bacterial strains with FtsZ point mutations that reduce sensitivity to inhibitors were measured. The target was confirmed genetically through the isolation of spontaneous mutants that conferred resistance to the compound and sequencing of their FtsZ genes. When resistant colonies could be isolated, mutations conferring amino acid substitutions in FtsZ were always identified.

The preliminary SAR exploration of compound **1** started with purchasable close analogues and extended to those that could be synthesized from commercial building blocks in one to four steps [4]. The amide and 3-ether substituents of compound **1** was critical for cell division inhibitory activity. Extension of the 3-alkyloxy substituent resulted in a substantial improvement in antibacterial activity and led to the

Table 30.1 *B. subtilis* and *S. aureus* MICs and cell division inhibitory activity for compounds 1-12



Compounds	R^3	MIC (μg.ml^{-1})		*B. subtilis* cell division inhibition[a] (μg.ml^{-1})
		B. subtilis	*S. aureus*	
1	Methyloxy	4000		500
2	Ethyloxy	2000		500
3	Propyloxy	500	256	375
4	Butyloxy	128	128	24
5	Pentyloxy	32	32	24
6	Hexyloxy	16	16	8
7	Heptyloxy	4	8	1.5
8	Octyloxy	1	4	0.37
9	Nonyloxy	0.5	2	0.18
10	Decyloxy	1	64	0.5
11	Undecyloxy	4	>256	1
12	Dodecyloxy	>256	>256	>128

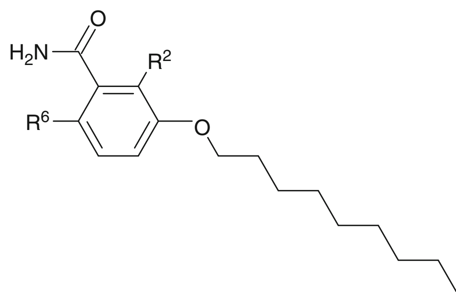
[a]Lowest concentration at which filamentation of *B. subtilis* is observed indicating on-target activity

identification of compound **9** with on-target activity and MICs of 0.5 and 2 μg/ml^{-1} against *B. subtilis* and *S. aureus*, respectively (Table 30.1). The SAR indicates that extension beyond the optimal nonyl alkyl chain results in a reduction of activity against *S. aureus*. Few substitutions of the benzamide ring were tolerated. R2 and R6 substitutions with small halogens were preferred leading to compound **16** (Fig. 30.1, Table 30.2), which demonstrated substantially higher (>10,000) potency and on-target cell division inhibitory activity in whole bacterial cells than compound **1**.

We believe these compounds are the most potent antibacterial agents targeting FtsZ and cell division described to date. However, the pharmaceutical properties of the series were more suited to the preparation of soaps than a high-value IV/oral therapy for the treatment of life-threatening staphylococcal infection. The next challenge was the replacement of the long alkyl substituent with more drug-like alternatives.

We created a ligand-docking model of the 3-MBA and alkyloxybenzamide analogue's interaction with FtsZ based on a 1.7 Å apo-crystal of *B. subtilis* FtsZ [11]. The highest and most consistent docking modes placed the ligands in a cleft formed by helix seven and the C-terminal domain, adjacent to the GTPase site (see Fig. 30.3). This cleft is analogous to the taxol-binding site in tubulin. In this model, the benzamide portion binds deeply into the cleft and the rest of the inhibitor binds into a hydrophobic channel. The benzamide-binding cleft, although conserved in Gram-positive species, is quite different in Gram-negative bacteria explaining the lack of sensitivity to benzamide derivatives observed in the Gram-negative bacteria.

Table 30.2 *B. subtilis* and *S. aureus* MICs and cell division inhibitory activity for halogenated 3-nonyloxybenzamide analogues 13-18



Compds	R^2	R^6	MIC (μg.ml^{-1})		Cell division inhibition[a] (μg.ml^{-1})	
			B. subtilis	*S. aureus*	*B. subtilis*	*S. aureus*
13	H	H	0.5	2	0.18	
14	F	H	0.125	0.5	0.125	0.5
15	H	F	1	8	1	2
16	F	F	0.125	0.5	0.125	0.25
17	F	Cl	0.5	1	0.25	0.5
18	Cl	F	0.5	2	0.5	1

[a]Lowest concentration at which filamentation of *B. subtilis* or ballooning of *S. aureus* is observed indicating on-target activity

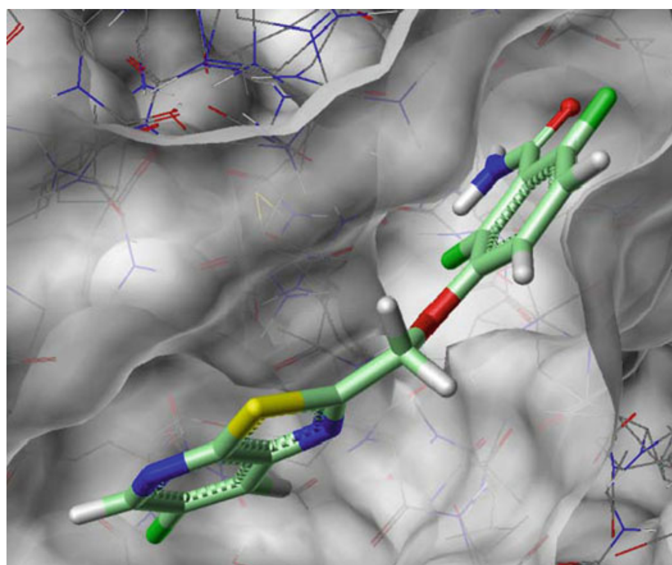


Fig. 30.3 The crystal structure of *B. subtilis* FtsZ putative inhibitor binding site and a model of the interaction between **PC190723** and FtsZ are shown

Furthermore, Bacilli and Staphylococci are unique in that they possess a more open cleft than other, benzamide-insensitive, species. In the apo-monomer of FtsZ from Bacilli and Staphylococci, this channel appears to be relatively open and solvent accessible. We used the model to explore what types of alkyl replacements might still bind into the cleft and used this information to prioritize the synthesis of compounds.

A library of hundreds of heterocyclic substituents linked via the methoxy group was synthesized and tested and resulted in the identification of more drug-like compounds with on-target antibacterial activity. One of the most potent of these substitutions was a benzothiazole (Compound **19**, Fig. 30.1). We expanded the series by the introduction of additional substitutions around the benzyl ring. Substitutions such as chloro, phenyl, or ethyloxy in the 5-position of the benzothiazole resulted in an 8- to 16-fold improvement in the on-target activity with MICs as low as 0.125 $\mu g/ml^{-1}$ against *S. aureus*. The antibacterial activity was specifically observed in all species and strains of Bacilli and Staphylococci tested, with the vast majority of other bacterial species being completely insensitive.

Although these 5-substituted benzothiazoles were more drug-like than the alkyl series, their protein binding, at >95% bound to plasma, was considered too high to progress. The addition of a second substituent onto the benzyl ring did not improve properties. Scaffold-hopping to the thiazolopyridine **PC190723** ([11], Figs. 30.1 and 30.3) not only slightly reduced the antibacterial activity, but also significantly reduced the plasma protein binding to <90%. Furthermore, the metabolic stability of the thiazolopyridine was improved compared to the 5-Cl-benzothiazole analogue resulting in an approximately 15-fold reduction in clearance following intravenous administration in the mouse.

The balance of antibacterial activity, plasma protein binding and metabolic stability of **PC190723,** resulted in the compound being tested in the murine septicemia model of staphylococcal infection [11]. A single subcutaneous or intravenous administration of **PC190723** at 30 mg/kg^{-1} resulted in a 100% survival of mice inoculated intraperitoneally with a potentially lethal dose of *S. aureus.* We believe that Haydon *et al*. [11] is the first publication of an FtsZ inhibitor that demonstrates *in vivo* efficacy.

It was important to verify that **PC190723** was an inhibitor of FtsZ [11]. Although **PC190723** evolved from 3-MBA, a well-characterized inhibitor of FtsZ, there were frequent checks at key stages during the evolution process to ensure that the analogues were still on-target. Several pieces of evidence demonstrate that **PC190723** directly interacts with, and inhibits, FtsZ function:

- Inhibition of purified *S. aureus* FtsZ GTPase activity with an IC_{50} of 55 ng/ml^{-1}
- Disruption of GFP-FtsZ localization from Z-rings to discrete foci in *B. subtilis*
- Induction of aseptate filamentation in *B. subtilis* and ballooning of *S. aureus*
- Identification of FtsZ point mutants conferring a degree of insensitivity to the compound
- Identification of FtsZ point mutants conferring compound dependency of growth

Taken together, the biochemical, cytological, and genetic evidence confirms that **PC190723** directly blocks FtsZ activity to prevent septum formation and cell division.

30.4 Future for Cell Division Inhibitors

Cell division has been recognized as an attractive target for novel antibacterial compounds for some time [22]. The program of work described here extends the validation of the target and describes the first cell division inhibitors that are active in animal models of infection. **PC190723** and related compounds are modeled to bind to an allosteric site adjacent to the active site and are specific inhibitors of Staphylococci and Bacilli that possess this site. The FtsZ proteins of other bacteria do not possess the same cleft and are therefore insensitive to the series of compounds. The anti-staphylococcal activity of **PC190723** is well suited to partner with molecular diagnostic tools in clinical practice to target the therapeutic and prophylactic challenges associated with drug-resistant staphylococcal infection and carriage. Furthermore, the specificity of the series means that antibiotic-induced colitis caused by ablation of commensal species will be minimized.

We consider that the series validates FtsZ as a target and should encourage the search for broader-spectrum inhibitors that bind to the GTPase site. Furthermore, we believe that this work suggests that inhibitors of the other essential septasome proteins would also be efficacious *in vivo*. This approach to the creation of a new class of antibacterials demonstrates the value of academic literature, fragment-based approaches, the use of a mixture of medicinal chemistry and structure-informed analogue design and the power of whole-cell screening and advanced bacterial cell biology.

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Chapter 31
Structure-Guided Discovery of New Antimicrobial Agents

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31.1 Background: The Protein Structure Initiatives

The global structural genomics initiative arose in the late 1990s, aiming to solve protein structures on a genomic-scale. These efforts have had a significant impact in enabling structure-guided antibacterial drug discovery, by solving the high-resolution structures of a large number of bacterial proteins.

In the US, the Protein Structure Initiative was established by the National Institutes of Health in 2000 as a 10-year project, broken into two 5-year project phases. While the National Institute of General Medical Sciences funded the majority of the project, two of the structural genomics centers were funded by the NIH National Institute of Allergy and Infectious Diseases, providing an infectious disease focus to the project. The pilot phase of the program (called PSI-1) provided $270MM between September 2000 – June 2005 for the organization and funding of nine structural genomics centers. The overarching goal of the structural genomics effort was to solve the structures of enough proteins to represent all of "protein space."

The great diversity of the eubacterial kingdom led to the desire to include bacterial proteins in the PSI-1 efforts. Compared with mammalian proteins, it proved much simpler and more efficient to clone and express proteins from many bacterial species. Thus, in the early phase of the structural genomics effort, the PSI-1 centers solved about 1,300 structures in the PSI-1 initiative (Fig. 31.1), and the vast majority of them were prokaryotic (Fig. 31.1; [49]). In addition to the general effort, the PSI-1 established the TB Structural Genomics Consortium (TBSGC), which solved the structures of many proteins from *Mycobacterium tuberculosis*, focusing on proteins that might have potential as targets for new antibiotic discovery [23, 37].

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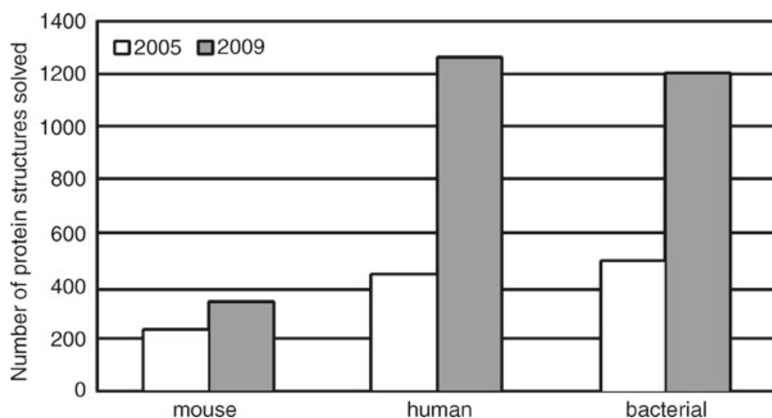

Fig. 31.1 Proteins solved in PSI efforts

Table 31.1 Status of PSI crystallization pipeline

Species	Solved structures in PDB[a] (2004)	Solved structures in PDB[a] (2005)	Solved structures in PDB[a] (2009)
Escherichia coli	70	86	279
Pseudomonas aeruginosa	9	36	107
Haemophilus influenzae	7	7	20
Staphylococcus aureus	5	14	51
Streptococcus pneumoniae	3	8	50
Enterococcus faecalis	4	11	68
Mycobacterium tuberculosis	70	70	75
Helicobacter pylori	2	3	18
Total	170	235	668

[a]Data from searches of the TargetDB database in June 2004 [48]; August 2005 [49]; and 25 May 2009. Search terms were "coli", "aeruginosa," "influenzae," "aureus,""*Streptococcus pneumoniae*," "faecalis," "tuberculosis," "pylori," as was performed in previous searches [49]

The Production Phase (PSI-2), was begun in 2005, and provides $325MM in funding for 14 centers to solve 3,000 structures over 5 years (until June 2010). Four of the centers have high throughput capabilities and aim to solve large numbers of structures, while other centers have specialized goals [39]. The PSI-2 efforts include active efforts to solve the structures of mammalian proteins, which has resulted in the more recent accumulation of solved structures from human and mouse proteins (see Table 31.1). Nonetheless, over the past 4 years (2005–2009), the number of structures of proteins from clinically important pathogens has grown from 235 [49] to 668 (see Table 31.1).

Certain specialized goals of the PSI-2 centers could have an impact on antibiotic discovery in the future. There are two specialized centers for structures of membrane proteins – one at UCSF and the other the New York Consortium on Membrane Protein Structure. These centers aim to determine structures of membrane proteins

from archaea, bacteria and humans, and to develop better methods for obtaining high-resolution membrane protein structures. The large-scale Midwest Center for Structural Genomics, has a PSI-2 goal of solving structures from pathogens, which should continue to add structures relevant for antibiotic discovery. In addition, the Integrated Center for Structure and Function Innovation at Los Alamos National Laboratory will include an effort to solve the structures of protein complexes, such as RNA polymerases, ribosomes, and other large protein and nucleoprotein complexes.

Today, in 2009, there are several databases that house information from the PSI initiatives. The longstanding Protein Data Bank (PDB, http://www.rcsb.org/pdb) was established in 1971, and it has become the worldwide archive for protein structure information. In addition, TargetDB (http://targetdb.pdb.org) was established in PSI-1 to manage the pipeline of structural genomics center's efforts, to allow coordination of effort, prevent target overlap, and provide status information of the effort, prior to deposition of the high resolution structures into the PDB. Funds were provided in PSI-2, to create KnowledgeBase, (http://kb.psi-structuralgenomics.org/KB/index.html), which serves as a community communication site, providing a portal to many types of structural biology information, in addition to summarizing the status of the PSI efforts. Suggestions for targets to be solved by the PSI centers can be submitted on this site. Furthermore, specialized resources such as targetTB have been established to synthesize a wide variety of information on potential targets of *M. tuberculosis* [45].

Despite the fantastic influx of new structures that can be used for antibiotic discovery, there are limits to what can be expected from the PSI efforts. Since the overarching goal is to map protein structure space, these consortia will be very unlikely to solve the structures of the second or third ortholog of a potential drug target. As shown by many individual drug discovery efforts, it is very valuable to have high-resolution structures for several key species when undertaking an antibiotic discovery effort.

31.2 Practical Issues in Using Structural Biology for Antibiotic Discovery

At the end of PSI-1, the estimated cost per solved structure was $138,000 [39], which would be approximately 6 months of effort in a commercial setting, using a fully burdened cost of $275 K/full time employee. (From the experience at Affinium (2001–2004), budgeting 6 months for solving the structure of a new protein is about right; some will be significantly easier, while a few will be much harder). To create a structure-guided discovery effort requires an interdisciplinary team – protein biochemists to provide high quality protein, structural biologists to solve protein structures, and obtain the structures of co-crystals with different molecules produced during lead finding and optimization, and computational chemists if *in silico* methods will be used to find chemical starting points, and to aid in the design of new molecules [6].

The amount of protein needed for crystallization can be relatively small using microfluidic systems such as TOPAZ from Fluidigm Corp., which require only tens of micrograms of proteins for assessing crystallization conditions in a sparse matrix screen. However, most structural genomics groups continue to use standard conditions, in which crystals are grown in microliter volumes as hanging or sitting drops in vapor diffusion wells and result in crystals that are tens of microns to low millimeter in size. To undertake a traditional sparse matrix screen by these methods typically requires tens of milligrams of protein, in order to have sufficient protein to identify initial crystallization conditions, then undertake the refinements that result in a well-diffracting crystal. Refinements of conditions are performed both to obtain larger crystals, to obtain crystals with better diffraction properties, and to create good freezing conditions for transport to, and use in, synchrotron radiation sources.

In antibiotic discovery, early lead optimization efforts generally also explore breadth of spectrum. This poses a challenge to structural biology efforts, since it is generally the case that only one or at best a few clinically relevant ortholog structures are available, while the necessary antibiotic spectrum may require potency in a dozen or more species. The selection of species for structural efforts can be made by selecting protein orthologs that have apparent differences in the pocket, based on primary sequence comparisons of key pathogens. Alternatively, an empirical selection may be based on the protein orthologs that crystallize most readily and yield well-behaved crystals. Such proteins will provide initial structural information most readily, and have the best chance to allow soaking of discovery ligands for co-crystal structures.

As shown by many individual drug discovery efforts, it is often valuable to have high-resolution structures for several key species when undertaking an antibiotic discovery effort. For example, a virtual screening effort to find inhibitors of methionyl-tRNA synthetase (MetRS) used the existing structure of *E. coli* MetRS. The inhibitors identified by this effort had activity against gram-negative MetRS, but not against gram-positive MetRS [25]. A subsequent virtual screen, using a *S. aureus* MetRS protein structure successfully identified novel inhibitors of *S. aureus* MetRS, but the majority of these were not inhibitors of *E. coli* MetRS [13]. Thus, in selecting a new target for drug discovery efforts, an investment in solving the structures of key pathogens will likely be desired, depending on the desired breadth of spectrum in the product profile.

Current methods are generally successful for the cloning of most bacterial genes in *E. coli*. Commercially available vectors (such as the pET expression vectors from Novagen) provide inducible expression and also facilitate the construction of N-terminal or C-terminal affinity tags (nearly universally hexa-histidine) for simple affinity-based purification. *E. coli* host strains for induction and expression of the protein are typically *E. coli* BL21(DE3), or derivatives, which has been satisfactory for expressing a wide range of bacterial proteins. Nearly all of the newly crystallized bacterial proteins from the PSI and other independent efforts are using variations of these methods [27, 37].

Not infrequently, the expressed protein will be produced as insoluble inclusion bodies after expression in *E. coli*. Aggregated protein doesn't crystallize, and

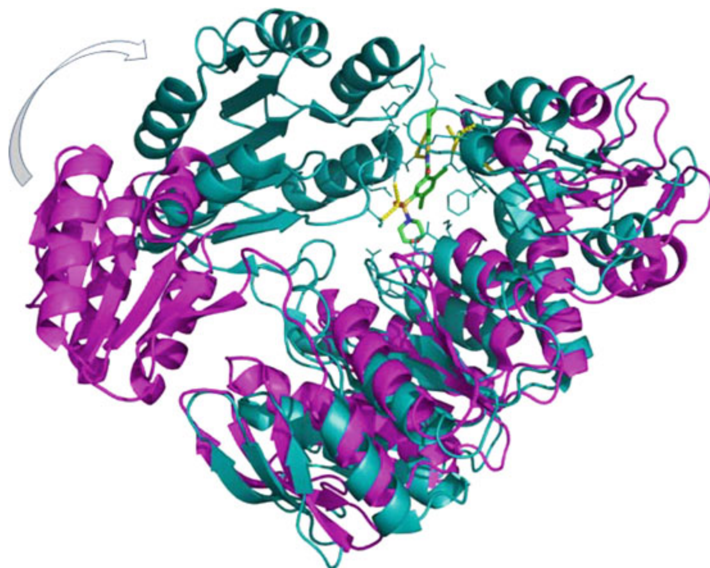

Fig. 31.2 The MurF protein undergoes significant motions upon binding of an inhibitory ligand. The *S. pneumoniae* MurF protein bound to a screening hit (2 AM1, [30]) was aligned with the *E. coli* apo-MurF structure (1GG4). While good alignment between the N-terminal domains is seen between the two proteins (*right-hand side*), corresponding amino acids in the C-terminal domains of the proteins are separated by nearly 30Å (*left-hand side*)

probably interferes with crystallization of the soluble protein in solution [20]. Thus, decisions are needed about whether to optimize protein expression to eliminate or minimize this aggregation or whether to undertake denaturation and refolding [31], or to separate the aggregated protein from non-aggregated and to scale up production and take low yield expression [19].

Once the protein is purified, and soluble protein is available, there are numerous "tricks" that have been anecdotally used by structural biologists over the years to coax a protein to crystallize into diffracting crystals. When the purified protein is well behaved, switching the crystallization screening conditions is often sufficient. There are now many commercially available sparse matrix screening plates, in addition to in-house sparse matrix screens that have been developed in academic laboratories [41].

The addition of non-hydrolyzable substrates or other ligands to the screening plates has been successful in coaxing many proteins into crystals [45], and is a useful and simple step. Even when protein function is not known, giving the protein something to bind has proven successful [58]. Occasionally, the presence of an inhibitor identified during screening can make a badly behaved protein crystallize more readily. Attempts to crystallize the *S. pneumoniae* MurF protein failed, until inhibitors were identified. Then, co-crystals of *S. pneumoniae* MurF with the inhibitors were readily obtained (Fig. 31.2; [30]).

If these efforts to obtain crystals continue to fail, then other simple changes in the protein construct sometimes will make a difference [19]. Thus, switching from N-terminal to C-terminal histidine tags, cleaving or not cleaving the tags, searching for better-behaved orthologs, or creating a protein fusion to a large soluble protein like maltose binding protein have all resulted in success in certain cases. Other radical changes that have worked in some cases, but are far more costly, such as mutagenesis, in which point mutations are introduced into the protein in an effort to make it better-behaved. In addition, sometimes N-terminal or C-terminal truncations or internal deletions will work, presumably by removing a flexible portion of the molecule. In any of these cases of changing the protein however, there will always be the possibility that the altered protein will have fundamentally different properties than the wild type protein. Orthologs often create more reasonable variety than lab-based changes of the protein [6].

Typically, high-resolution protein structures (<2.5Å) are necessary for structural information to provide a useful guide to lead optimization and *in silico* design of new molecules. However, even lower resolution structures can provide exceptionally valuable information for large targets such as the well-validated antibacterial targets of the ribosome [24, 54], and RNA polymerase [4, 36, 57]. The structures of these complexes, and especially the co-structures of known antibiotics with the structures, have provided key insights into the binding modes and mechanisms of action of antibiotics. Thus, despite relatively low resolutions, the structures of these complexes have contributed to the design of novel inhibitors against the targets [14, 15, 52, 59, 60].

31.3 Using Structural Information in Target Selection

There are numerous criteria that are used for selecting a new antibiotic target. When a novel target is being considered, the existence of a high-resolution structure from any ortholog can provide several key pieces of information that can be used to prioritize or de-prioritize the target.

First, the existence of the structure provides initial evidence that the protein can be coaxed into well-diffracting crystals. This is important, as some proteins are quite difficult, while others quite easy to crystallize. This difference was reflected in our experience at Affinium where we sometimes talked about “crystallophilic” proteins, which would crystallize under a wide variety of conditions and “crystallophobic” proteins, which did not crystallize. It was often true that for some proteins, nearly all orthologs would crystallize after reasonable effort in establishing crystallization conditions, while in other cases all orthologs behaved equally badly in crystallization. Fortunately, in many cases, one ortholog seemed to behave better than others.

Second, the existence of the structure can allow a preliminary examination of the pocket size and shape. While not foolproof, there are general ideas about what makes a “good” druggable pocket, and what will at least be a problematic pocket for small molecule discovery. Programs are available to aid the identification and measurement of the likely binding pocket in proteins with only apo-structures

[11, 28]. In addition, more recent efforts have resulted in exploration of algorithms that aim to predict the propensity of a protein pocket to bind small, drug-like ligands [22]. The calculated “druggability” index was reasonably well-correlated to experimental findings from both fragment-based affinity screening, and high throughput biochemical screens of drug-like diversity libraries. Overall, the assessment of 61 sites in 58 proteins suggested that nearly half of them should be relatively ”un-druggable“ [21]. The correlation of these predictions with experimental results suggests that such *in silico* druggability assessments will be a valuable tool to prioritize targets, especially since the algorithms can be performed using only a high-resolution protein structure. Refinements of the algorithm have attempted to account for dynamic motions in a protein, though so far, only a few examples have been reported [8].

The genetic diversity in the eubacterial kingdom is vast, and proteins in clinically relevant species can be quite distantly related. Sequence conservation in essential bacterial genes is higher than in non-essential genes, but it is still true that many essential genes are not well conserved between species [17]. Fortunately, the sequence conservation needed to accurately provide a homology model of a protein sequence to an existing structural model is only about 30%, and success with even lower percentages have been reported [26]. Thirty percent or more, allowing at minimum a homology model to assess problems of potency in certain species, relates nearly all orthologs of eubacterial proteins.

Finally, the existence of a structure allows the potential for both *in silico* and affinity-based screening methods to augment traditional experimental screening efforts. These methods should enhance lead finding and lead optimization potential, as described in the remaining sections of this article. Such extra potential should improve the priority of a target.

31.4 Using Structural Information to Find the Initial Chemical Matter

There are numerous screening methods and strategies to identify the initial chemical matter for a target, ranging from biochemical-based assays to cell-based assays. However, for antibacterial targets, experience has shown that traditional target-based screening methods are less successful than for targets in other therapeutic areas [43]. Thus, novel methods that can identify new or better chemical starting points for a target should be welcome drug discovery tools.

31.4.1 Experimental Methods Using Structure to Find the Initial Chemical Matter

Experimental fragment-based screening relies on smaller compounds – generally 150–250 molecular mass – and detection of physical interaction between the fragment and the protein target [5]. Initially performed by NMR-based detection of

bound compounds [51], the idea has been extended to detection of ligand binding by many other physical methods, including diffraction methods to assess the binding of fragments in protein crystals [38, 53], mass spectrometry [10], or even analytical centrifugation [42]. Fragment-based methods usually screen at high concentrations, and identify compounds that bind in the high micromolar-to-millimolar range, compared with more traditional screens that identify compounds with mid-to-low micromolar binding affinities. The concept of ligand efficiency – the binding affinity normalized by the size of the molecule – has been used to monitor the relative potency of molecules as the size of the molecule increases. In general, the potency of a molecule increases up to about 25 heavy atoms (ca. 300–350 molecular mass), and then plateaus [5].

Fragment screening can explore a larger fraction of chemical space than the screening of libraries of larger sized molecules. Theoretical calculations suggest that the size of the chemical universe below 160 molecular mass is only 14 million compounds [12], while the size of drug-like chemical universe is 10^{60} [7]. Thus, the screening of 10,000 fragments probes a much larger proportion of chemical space than when compounds of drug-like size are used.

The theoretical notion that fragment screening may be more comprehensive than traditional screening has been supported by the higher observed hit rates for fragment screens. A summary of recent Novartis screening efforts concluded that traditional target-based screening, using full sized ligands and IC50's less than 10 μM, had hit rates of 0.001–0.151%. In comparison, NMR-based fragment screening had hit rates of 3–30%, using fragments of 100–300 molecular mass and seeking compounds with millimolar affinity [50]. In addition, fragment screening has identified hits against targets in which biochemical screens of a large chemical diversity collection has failed or yielded few hits [21, 22]. Furthermore, the affinity-based screening methods, which serve as the foundation of fragment screening, provide the opportunity to identify inhibitors of targets of unknown function [27]. Thus, fragment-based methods substantially expand the range of the diversity and targets that can be explored, which may be exceptionally valuable to antibacterial discovery.

Fragment screening has been practiced successfully in many pharmaceutical organizations and therapeutic areas, including antibacterial discovery. Pfizer scientists recently described novel inhibitors of biotin carboxylase discovered by an interdisciplinary approach that used both virtual screening and fragment screening to identify chemical starting points [35]. In the fragment screening effort, a library of 5,200 fragments was screened in pools of ten compounds per well. Using a combined enzyme assay, and NMR-based approach, 142 hits were identified, six of which had IC50's less than 95 μM. Subsequent structure-guided efforts resulted in multiple optimized novel lead series with IC50's ranging between 7 and 330 nM.

Crystallographic methods for fragment screening have been highly effective. There are several criteria that simplify a crystallography-based fragment screening approach, including a crystal form with high symmetry (which typically decreases the diffraction data collection times), knowledge that ligands (such as a substrate or substrate analog) can diffuse into the crystal lattice, and a straightforward protein production and crystallization method to provide the necessary protein crystals.

Fragments that bind to protein crystals after soaking are identified by diffraction differences, which can be performed on the hundreds or thousands of fragments in a typical fragment library, most commonly screened in pools.

Fragment screening using an X-ray crystallographic approach resulted in potent inhibitors of a *S. aureus* enzyme from the folate biosynthetic pathway [47]. The *S. aureus* enzyme dihydropterin aldolase crystallized readily, and crystals diffracted to 1.6–2.0Å. A library of 10, 000 fragments was divided into structurally diverse pools of 100 compounds. Crystals were soaked with these fragment pools, diffraction data collected, and changes in the electron-density maps of the crystals were monitored. Initial hits were identified that had IC50 values of 28–80 μM, and the co-structure information obtained from the screening provided a sound pharmacophore model upon which to design larger molecules. From these efforts, several molecules with sub-micromolar potencies were identified, including one compound with an IC50 of 68 nM.

Importantly, fragment screening may provide a method to overcome the potential non-antibiotic bias of large chemical diversity libraries that have been questioned as one cause of the difficulty in identifying novel antibiotics [40].

31.4.2 In Silico Methods to Find the Initial Chemical Matter

In silico screening methods have been used successfully to screen large virtual libraries of drug-like compounds to identify novel chemical matter. The most challenging of the *in silico* screening efforts are fully de novo – starting with only an apo structure upon which to perform an *in silico* screen for chemicals that bind and inhibit the target. In some cases, it is not certain which pocket(s) can effectively serve to inhibit the enzymatic activity of the protein, yet even this uncertainty can be overcome in some cases. The catalytic domain of the *E. coli* DNA replication protein, DNA primase (DnaG), was used as a target for *in silico* screening of 500,000 compounds that had been prescreened for drug-like properties. Complicating this effort was a lack of understanding of which of three potential binding sites (identified by GRID software, ©Molecular Discovery [18]) would afford inhibition. All three sites were virtually screened using Glide (©Schrodinger, Inc. [46]) and subsequent visual inspection of the top *in silico* hits. Using this strategy, 2,500 virtual hits were examined and 68 diverse compounds were experimentally tested, resulting in four compounds with IC50's of 50 μM or less [1]. Though these initial hits lacked antibacterial activity, subsequent development of a pharmacophore model, and additional data mining and analog identification resulted in compounds with improved potency (3 μM) and antibacterial activity against efflux-deficient *E. coli* [1].

Most of the *in silico* screening methods account for ligand flexibility in a variety of ways, but leave the protein target as a rigid receptor. In some cases, a protein is known to undergo significant conformational changes upon binding inhibitors or substrate analogs, creating uncertainty about the best protein structure for *in silico* screening. Selecting the closed (inhibitor or substrate-bound) form of the MurD enzyme

(the D-Glu ligase, which adds the amino acid to UDP-MurNAc in peptidoglycan biosynthesis) resulted 50 top scoring compounds from the eHiTS program (©SymBiosys. [61], among which four showed IC50 values of 10–47 μM in experimental enzyme assays [56]. The *Helicobacter pylori* open form of dehydroquinate synthase was solved to 2.4Å, but in order to find inhibitors through virtual screening, a homology model of the closed form of the enzyme was based on an *Aspergillus nidulans* structure. This approach resulted in the testing of the top 100 virtual screening hits, among which two inhibitors were found with IC50's of 61 and 84 μM [29].

Pharmacophore models built from co-structures with known inhibitors are increasingly helping the virtual screening efforts. A recent virtual screen to identify novel inhibitors of *S. aureus* methionyl-tRNA synthetase (MetRS) used four available protein-inhibitor co-structures to build a pharmacophore model. The pharmacophore model was then used to identify novel inhibitors from an available diverse chemical library. Using this approach, 22 of 31 molecules identified from the virtual screen inhibited the enzyme at 100 μM; four of the molecules had IC50 values less than 10 μM [13].

Successful pharmacophore models can also be built from information in relatively distant orthologs of the desired target. In a search for inhibitors of *M. tuberculosis* chorismate mutase, there were no known bacterial inhibitors. Thus, a pharmacophore model was based on the *Saccharomyces cerevisiae* chorismate mutase, in which both substrate-bound, and inhibitor-bound structures were available. Using this model to filter a 3D compound database, 15,659 compounds were identified with the chemical and geometric requirements of the model. Of these, hits were identified with FlexX docking and scoring, combined with Sybyl scoring to yield 15 molecules for experimental testing, of which, four had micromolar inhibitory activity, with the best two characterized as having Ki of 5.7 μM and 17 μM [2].

Recently, successful reports of using *in silico* screening to identify fragment-sized molecules have appeared. The *in silico* screening of a library of 137,639 fragments against the AmpC beta-lactamase protein provided the ability to demonstrate that *in silico* fragment screening was very successful in identifying fragments with weak binding [55]. Of 48 top-ranked fragment *in silico* hits, 23 showed Ki values between 0.7 and 9.2 mM, consistent with the types of hits sought and found in experimental fragment-based screening. Experimental co-structures of eight of the fragment hits showed that four of these were bound in the mode predicted by the *in silico* efforts, while only two adopted very different poses [55]. The combination of *in silico* and experimental fragment-based approaches to finding new chemical matter may enhance our ability to find new antibiotic molecules against novel targets in the future.

Interesting approaches to identify dual inhibitors through structure-guided efforts, have taken advantage of the increased availability of the structures of interesting bacterial protein targets. Using the peptidoglycan biosynthetic ligase enzymes, MurD and MurE, dual inhibitors were sought and identified from a virtual screen [44]. Although this effort resulted in inhibitors with only modest enzyme activity (100 μM) and no detectable antibacterial activity, the approach is an interesting one that will likely be pursued in the future, to find molecules that through their dual mechanisms may resist resistance development.

31.5 Using Structural Information to Find and Optimize the Lead Series

The most important role of structural information is to prevent the medicinal chemist from working "blind" during lead optimization. The lead series identification phase of most therapeutic projects aims to improve the potency and selectivity of a series. The early stages of learning the SAR of a series by trial and error without structural information – making molecules and then testing their biological activities – is extremely inefficient. One of the most dramatic changes that have arisen from structure-guided discovery efforts is the willingness to work on compounds with weak binding. Experience has shown that structure-guided efforts can dramatically improve potency.

It is an important project goal to identify inhibitors with consistent binding modes, so that rational predictions can be made from the design and *in silico* modeling. In the initial characterization of inhibitors of the D-ala-D-ala adding enzyme (MurF), low micromolar inhibitors were identified, co-structures solved, and two inhibitors with nearly identical binding modes were found [53]. This helped in selecting these molecules for initial SAR exploration, and allowed an efficient progression to discover inhibitors with a 40-fold improvement in potency.

Understanding the binding interactions between one or more compounds and the atoms in the binding pocket of the target can improve lead optimization efforts, both by suggesting opportunities for increasing potency and by avoiding design ideas that are unlikely to be accommodated in the binding pocket. In a structure-guided search for dual inhibitors of Topoisomerase IV and DNA gyrase, a docking model of benzimidazole urea inhibitors identified several locations for potential modification of substituents that might yield improvements in potency. These efforts began with molecules showing low-mid micromolar potency against the two targets, and resulted in low-mid nanomolar inhibitors, with activity against both targets [9]. Structure-guided lead optimization takes much of the guesswork out of medicinal chemistry efforts to improve potency, allowing researchers to focus on incorporating desirable pharmacological characteristics into the molecules that are synthesized.

It is extremely valuable to have structural capabilities "early and often" during the early stages of exploring potential lead series, to assure that the design principles remain correct. Even when a high- resolution apo-structure exists and active sites are carefully identified, surprises can occur when the first co-structures are obtained, either because of significant conformational changes in the ligand-bound protein, or because inhibitors may not reside in the active site. A very careful examination of the N-acetylglucosamine-1-phosphate uridyltransferase (GlmU) apo-protein structure and binding sites [32] did not prevent surprise when the first co-crystal structure was obtained of an 18 μM GlmU inhibitor identified from a high throughput biochemical screen. The inhibitor was bound in a hydrophobic pocket adjacent to the substrate binding pocket [34], demonstrating an indirect, allosteric mechanism of inhibition.

Proteins can undergo a range of motions upon binding ligands. A protein can undergo drastic conformational change, such as seen in the bound and unbound

forms of the *S. pneumoniae* MurF protein. The *S. pneumoniae* MurF protein bound to a compound found through an affinity screen, has a "closed" conformation, with residues moving up to 30Å from their location in the "open" conformation seen in the apo structure from *E. coli* (Fig. 31.2; [30]). Protein motions can be more subtle, moving pocket residues only a few angstroms. Nonetheless, when the pocket is flexible, different ligands may create different pocket conformations, and hinder *in silico* design efforts. The co-structures of two different inhibitors bound to the LpxC protein suggested different binding modes, and prevented the successful modeling of the binding of the inhibitor CHIR-090 to the protein [16, 33].

The need for frequent structural information to guide design ideas is especially true when the chemical starting points are fragments, as the binding modes of fragments does not always predict the binding mode of larger molecules that incorporate those fragments [3]. A fragment-sized aminothiazole inhibitor of the β-ketoacyl ACP synthase enzyme (FabB) bound to a site distinct from the FabB natural product inhibitor, thiolactomycin (220 molecular mass), despite the strong shape similarity of the two inhibitors, and *in silico* screening results, which predicted that the two inhibitors would have identical binding modes [42].

The optimal situation for structure-guided lead optimization occurs when the ability to obtain co-structures can occur in a time frame that is similar to the chemistry design cycle time. When inhibitors can be soaked into well-behaved, highly diffracting crystals, the process of obtaining co-structure information can proceed nearly as quickly as obtaining biological assay data, and can keep pace with the speed of the discovery process. However, obtaining co-structures by soaking fails with some proteins and some inhibitors, for reasons not fully characterized. Conformational changes in the protein can diminish crystal-packing forces, resulting in highly fragile or cracked crystals. Sometimes the inhibitors are not sufficiently soluble to allow diffusion into the lattice in the aqueous protein crystal environment or sufficiently potent to bind to a high percentage of the binding sites in the crystal. And sometimes, it just is not clear why the soaks failed and co-crystallization worked, because the same protein conformation and crystal space group was obtained after co-crystallization [47].

Setting up new sparse matrix co-crystallization screens for a target protein with each new inhibitor considerably slows the ability to obtain co-structure information. Nonetheless, creative methods can overcome even these limitations, when a project team desires structural information to guide the lead finding and optimization processes. When it became clear that soaking would not provide co-structures of new inhibitors of the D-Ala-D-Ala adding enzyme (MurF), NMR screening methods were used to efficiently identify inhibitors that bound well to the MurF protein [53]. Heteronuclear Single Quantum Coherence (HSQC) experiments allowed rapid assessment of up to 50 compounds per day, and identified molecules that bound well to the target protein. This experiment was used as a gate to more labor intensive co-crystallization trials, and resulted in successful co-crystallization of 87% of compounds with good binding characteristics assessed by the HSQC spectra, and only 12% with compounds that had weak binding from HSQC spectra [53]. As a measure of efficiency and work required, starting with micromolar inhibitors of the

D-Ala-D-Ala adding enzyme (MurF), it required approximately 12 months, 300 molecules and 40 co-crystal structures to improve potency 40-fold [53].

Once a lead series has been identified with sufficient potency and selectivity, the project team often will switch focus to assessing and building in other drug-like properties in the molecule. In antibacterial drug discovery, an important property is often achieving antibacterial activity that matches the excellent biochemical potency of the molecules. Structural information can help significantly in these efforts again, by providing an *in silico* check of designs that might be aimed toward improving entry or minimizing efflux, or achieving any of the other necessary properties, such as solubility, stability or pharmacokinetics. Using structure to guide design efforts assures that the majority of molecules synthesized in this phase will retain biochemical potency in the effort to gain the necessary drug-like properties [9].

31.6 Conclusions

Structural biology has an important role in making the process of drug discovery more efficient. The explosion of new structures of antibiotic targets that are in the public domain provides a rich starting point for antibiotic discovery efforts in the future. The use of structural information – early and often – in the drug discovery process provides opportunities to expand the range of hits far beyond the molecules present in current diversity libraries. The use of fragment-based methods further extends the potential hit and lead series far beyond existing diversity molecules. Since several analyses point to the existing diversity collections as a barrier to new antibiotic discovery, structure based methods should be embraced strongly by the antibiotic discovery community.

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Chapter 32
NMR in Infection Research

Jun Hu and Gunther Kern

32.1 Introduction

In comparison to other research areas, antibacterial drug discovery provides many opportunities to employ nuclear magnetic resonance (NMR) spectroscopy in the process of drug discovery. First of all, there are a large number of validated targets that can be cloned and expressed and their biological function is well understood. Most targets can be over-expressed readily and purified at quantities needed for NMR. Meanwhile, structural information is frequently available for at least one representative of a given target. Furthermore, enzyme substrates and corresponding inhibitors are often available and characterized. In combination, these factors make NMR studies for bacterial targets both feasible and economical in industrial research.

The nuclei ^{1}H, ^{15}N, ^{13}C, ^{31}P and ^{19}F are well represented in proteins, and their ligands and typical drug like molecules are well observable by NMR spectroscopy. That explains why NMR is such a powerful biophysical tool that is widely applied in many branches of modern biology including infection research [3].

We intend to discuss practical aspects of applying NMR spectroscopy in exploring target/ligand interactions, rather than distract readers with complex NMR theory. For those who are interested in the theoretical background of biological NMR spectroscopy and NMR in drug discovery, there are already many excellent books and reviews in the literature focusing on these areas [1, 18, 30, 34]. In the next few paragraphs, we first introduce a modern set up of an automated NMR system, which is not commonly seen in most NMR labs. Then we touch upon sample conditions for NMR experiments. We will concentrate on two different NMR approaches that characterize target/ligand binding by observing the perturbation of either ligand signals or target signals. These methods are applied to a few bacterial targets to study enzymology and support hit evaluation.

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32.2 NMR Automation System

As illustrated in Fig. 32.1, the central part of the NMR spectrometer is the superconducting magnet, which generates extremely high field homogeneity in the center where the probe coil resides. In this highly homogeneous field, NMR active nuclear spins (such as ^{1}H or ^{13}C) are polarized and precess around the magnetic field axis at a frequency known as Larmor frequency [8, 19]. Different isotopes have different Larmor frequencies. For instance, in a 14 T Tesla (T) magnetic field, hydrogen spins precess at a Larmor frequency of 600 MHz, whereas for deuterium spins the frequency is only about 92 MHz. Instead of the field unit Tesla, proton Larmor frequency is often used to present the field of an NMR magnet. It turns out that the sensitivity of detecting magnetization of a certain nucleus is strongly dependent upon the external magnetic field. The higher the magnetic field the higher sensitivity

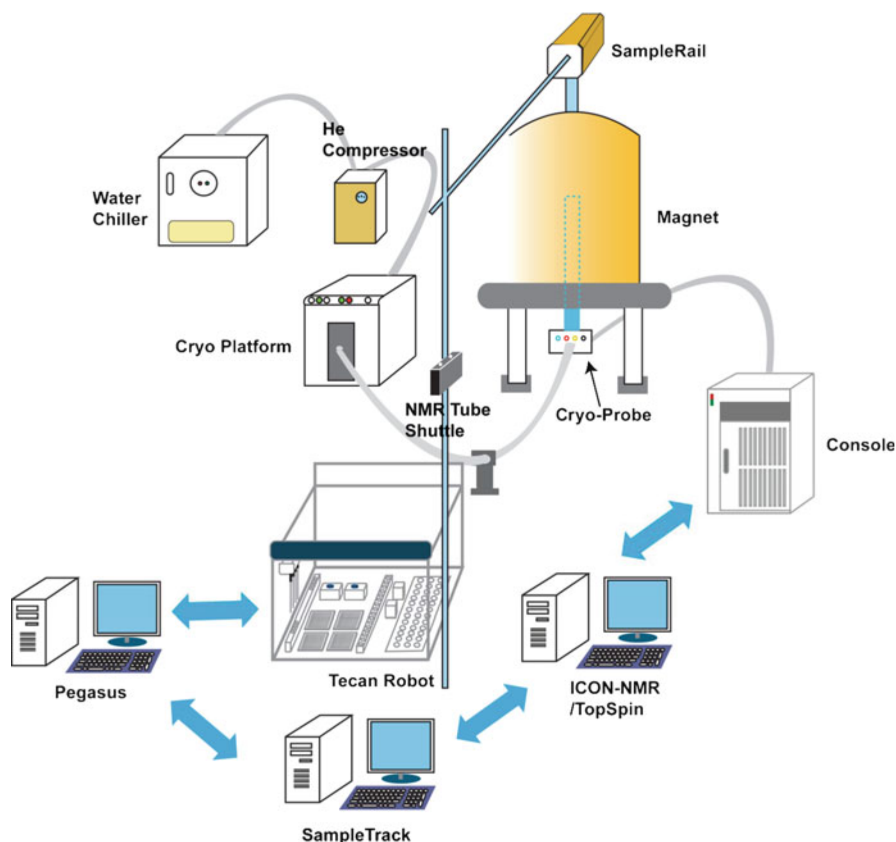


Fig. 32.1 A schematic illustration of a modern NMR setup with a cryogenic probe and automation system. This setup is based on the configuration of Bruker

and resolution. Therefore, the development of high field magnet is actively pursued [3]. As of 2009, the highest commercially available magnet, developed by Bruker, has field strength of 23.5 T, which is 1 GHz in terms of the proton Larmor frequency (http://www.bruker-biospin.com/avance1000.html). The probe is another important part of an NMR spectrometer. The probe imposes a tunable radio frequency on the sample and also measures the response. As mentioned above, it encloses the sample in the center of the magnet's field and is used to disturb the equilibrium of the observed sample nuclei with the magnetic field and also measures their response to this disturbance. The cryogenic probe is another newly developed technology taking advantage of the fact that the electronic noise level can be substantially reduced at very low temperature while being able to observe a sample at ambient temperatures. By cooling the probe preamplifier and coils using cold helium gas, the NMR sensitivity increases approximately fourfold due to noise reduction (details on the website http://www.bruker-biospin.com/cryoprobes.html).

Another unique feature shown in Fig. 32.1 is the automation system coupled to the NMR system. The automation system includes a Tecan pipetting robot and a SampleRail system that shuttles NMR samples between the Tecan robot and the Magnet. Communication between computers controlling different instruments becomes very important in order to perform automated experiments. In the Bruker automation setup shown here, a computer program (TopSpin) allows the NMR operator to talk to the NMR console and set up optimal parameters for NMR and automation. The console itself is connected to the probe and a computer. It controls the pulsing and gating of radio frequency pulses as well as data acquisition. ICON-NMR is the software that manages NMR data acquisition via TopSpin and sample preparation protocols via another program called SampleTrack. In SampleTrack, the operator chooses very specific experimental steps such as the order of adding compounds from different plate formats, the sequence of protein, compound or ligand addition, the amount of added material and the correspondent NMR pulse sequences. SampleTrack generates order files, based on the designed protocols, and it sends them to a program called Pegasus, which is located in another computer. The operator uses Pegasus to assign the location of NMR tubes, compound plates, protein sample and buffers. After all of the components are assigned by Pegasus, the program Gemini, which controls real sample pipetting and washing steps, is activated and commands the Tecan robot to execute the pipetting. The robot transfers the prepared NMR tube to the NMR tube shuttle and the shuttle transfers the tube into the NMR probe. Then ICON-NMR takes over and conducts the NMR experiments using previously set NMR parameters.

This automation system expands the NMR capacity in drug discovery. Compound binding, competition, and even library screening can be done efficiently and accurately without much labor and time involved but most importantly error free. As the sensitivity of NMR instruments keeps increasing, compound library screening may not anymore be limited by the time required to perform the NMR experiments but rather by the effort to perform thorough data interpretation. Although automated spectral processing is possible on modern NMR software, spectral interpretation still remains the rate limiting step in using NMR for drug discovery.

32.3 NMR Experiments

A typical bio-NMR experiment starts with an NMR tube containing protein with or without ligand in ~ 500 µl buffer. The NMR sample is placed in a strong constant magnetic field such that nuclear spins become polarized (aligned). A short burst of radio frequency electromagnetic radiation, transferred by the probe coils, rotates the spin magnetization away from the constant magnetic field axis. Because of the precessing motion of the spins, the rotating nuclei magnetization generates an electronic current in the NMR probe. This current signal is sampled and processed into an NMR spectrum. The spectrum of each nucleus is affected by its local environment, that is, the other atomic nuclei in its immediate vicinity. These shifts in spectrum provide important structural information.

NMR spectroscopy is a very versatile tool that can be applied to cover a broad range of biological aspects such as protein structure, protein/protein, or protein/ligand interactions, conformational exchange, thermodynamics, and protein dynamics. Here in this chapter we would like to focus on the NMR applications to characterize protein/ligand interactions, because this is one of the core issues in bacterial enzymology and drug discovery.

There are two ways of probing the protein/ligand interactions by NMR. One can examine signal changes of the ligand after protein addition called ligand-observed NMR, typically pursued by one dimensional (1D) NMR experiments. Alternatively, the protein response can be followed upon ligand binding which is termed protein-observed NMR. Typically two dimensional (2D) NMR experiments are needed to resolve the many signals observed from a protein. Before getting into the detailed discussion on the difference and advantages/disadvantages of these two methods, we first would like to talk about how to observe protein and ligand signals by NMR.

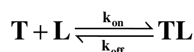
32.4 Analyzing Ligand-Target Interactions by NMR

32.4.1 Ligand-Observed NMR

Hydrogen is a ubiquitous element in all proteins, nucleic acids, and most biologically relevant chemicals, and ^{1}H (99.985% natural abundance) possesses the highest NMR sensitivity among all non-radioactive nuclei. It is simple to conduct one-dimensional ^{1}H NMR experiments on ligands because they normally possess a limited number of hydrogens and their ^{1}H frequencies are well separated in the NMR spectrum, by virtue of the individual ^{1}H local environment in the molecule. Here again, it is not our intention to explain the physical principles governing these NMR experiments. Instead, we focus on the interpretation of spectra.

Before getting into different ligand-observed NMR experiments, it is helpful to discuss an important concept governing almost all of the ligand-observed NMR

experiments and chemical exchange [3, 8, 19]. For a simple ligand (L)/target(T) equilibrium,



the ligand's free and bound states are under exchange. The exchange rate k_{ex} is defined as the following:

$$k_{ex} = k_{on} * [L_{free}] + k_{off}$$

For many natural substrates and hits from compound library screening, the binding affinity is normally between 10 μ and 1 mM which relates to fast exchange where $k_{ex} >> k_{on}$ (difference in resonance frequency between free and bound state). Assuming k_{on} is diffusion-limited (10^4–10^7 M^{-1} s^{-1}) and L >> T then k_{off} determines the exchange rate k_{ex}, which typically ranges from 10 to 1,000 s^{-1}. Consequently, many NMR properties of bound ligands, such as chemical shifts and relaxation rates, are averaged with that of the free ligand [9]. Under normal ligand-observed NMR condition with [Ligand]/[Target] > 10, the averaged signal still has a sharp line width similar to those observed for free ligands. In the case of slow exchange where $k_{ex} < \Delta\omega$ (where ω is the chemical shift difference between the frequencies of bound and free signal), signal reduction may not be obvious due to the excess amount of free ligands.

In the case of intermediate exchange where $k_{ex} \sim \Delta\omega$ NMR, signals can broaden to an extent that leads to apparent signal loss. In hit identification and hit evaluation, ligand affinities rarely are below 1 μM, and therefore k_{ex} is usually fast enough to allow efficient exchange between the bound and free state. In the rare case where the extent of observed inhibition (e.g., by using an enzyme assay) predicts tighter binding, NMR competition experiments or other biophysical methods can be used for efficient ligand binding characterization [18]. It is noteworthy that for many Nuclear Overhauser Effect (NOE) [8, 19]-based experiments described later on, slow and intermediate k_{ex} prohibits the observation of the NOE effect and therefore the experiments described below should be ideally used for fast exchanging ligands.

Binding of a ligand to a target perturbs the physical and chemical state of both the target and the ligand. When ligand/target interaction occurs, these changes can be manifested in chemical shifts, relaxation properties, and diffusion rates. Specifically designed NMR experiments can monitor those changes. For weak binders (K_d = ~ 10 μM – ~ 1 mM), the free and bound ligand molecules undergo fast exchange in the NMR time regime. Here again enzyme assays that can at least rank order-binding affinities by IC50 determination serve as useful tools to decide if the described 1D NMR techniques are appropriate for ligand binding confirmation. As a result, the population-weighted average of signals from both species are observed in the NMR spectrum instead of the individual signals from both bound and free species [18]. At a usual NMR sample condition, for instance, 100 μM ligand with 10 μM protein, it is very challenging to observe the chemical shift change, as the population of the free ligand is dominant. Therefore, normally chemical shifts of

ligands are not used to monitor ligand binding. However, relaxation rates can change dramatically when a small molecule binds to a macromolecule such as a protein and thus even a small population change becomes observable if the ligand is in sufficiently fast exchange between the bound and free form, since the ligand magnetization of the bound state is temporarily "memorized." The experiments described below all make use of relaxation and NOE effects that occur upon binding to the protein target, and thus are able to detect the binding of a small population of the total ligand to the protein target.

32.4.2 Relaxation-Edited 1D NMR

NMR, similar to other spectroscopic techniques, shares a common physical property called relaxation [3, 8, 19]. NMR relaxation describes a phenomenon that a disturbed spin magnetic state returns to the thermal equilibrium state over a period of time. Although there are many factors that affect the rate of spin relaxation, molecular motion usually plays a dominant role in how fast the spin magnetization relaxes (i.e., the relaxation rate). In a 1D NMR spectrum, NMR relaxation governs the line widths of resonances (peaks) after the acquired NMR signals are processed by the Fourier transformation. Simply speaking, for big molecules such as proteins the molecular motion is slow and NMR signal line widths for them are broad, whereas for enzyme substrates and small organic compounds that are small and tumble very fast in solution, line widths are sharp.

$T_{1\rho}$ and T_2 relaxation, so called spin-lattice relaxation in the rotating frame and spin-spin relaxation, are both very sensitive to molecular motion [23]. Ligand binding to a protein translates into shortened $T_{1\rho}$ and T_2 relaxation properties because of a reduced tumbling of the complex relative to the free ligand [12]. A $T_{1\rho}$ or T_2 filter is usually applied in the pulse sequence in order to manifest this effect. As illustrated in Fig. 32.2a, an obvious signal reduction indicates compound binding, while for compounds that do not bind to the target or the binding is beyond NMR detection, no change of signal intensities will be observed. In some favorable cases, signal reduction from ligands in complex with large proteins can be observed directly without any filter in a simple 1D ^{1}H spectrum.

32.4.3 STD and Water LOGSY 1D NMR:

Another widely applied ligand-observed NMR technology takes advantage of the nuclear Overhauser effect (NOE) [3, 8, 19] to detect the physical contact between the bound ligand and the target. Essentially, NOE originates from the dipole-dipole interaction between nuclear spins through space. This interaction is also a function of distances between nuclei that are not covalently bound to each other. Commonly seen in the literature are transferred NOE (TRNOE) [9], saturation transfer difference

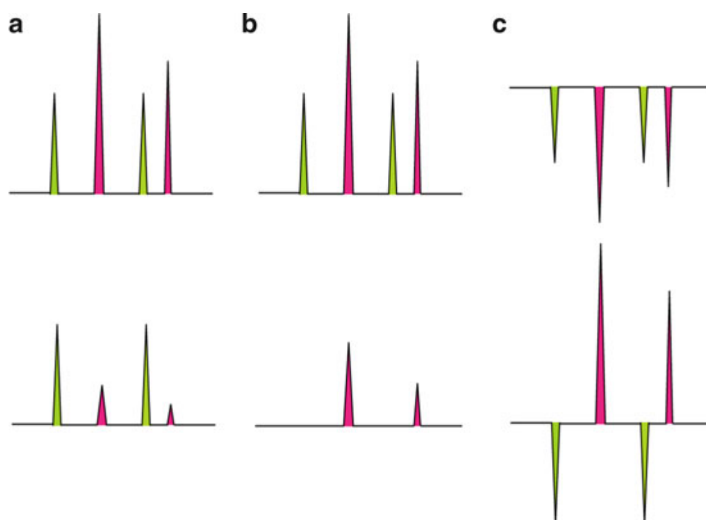


Fig. 32.2 Schematic illustration of three typical 1D ^{1}H NMR experiments applied in detecting ligand binding. They are (**a**) relaxation edited NMR such as $T_{1\rho}$ or T_2 experiments, (**b**) STD and (**c**) WaterLOGSY. Assuming there are two compounds as a mixture in aqueous buffer, peaks in green represent signals from the compound that does not bind to the target, while red peaks are from real binders. For **a** and **c**, the *top* and *bottom* spectra illustrate possible outcomes in the absence and presence of the target, respectively. For **b**, the *bottom* is the difference spectrum between the on and off target irradiated target

(STD) spectroscopy [21] and the Water-Ligand Observed via Gradient SpectroscopY (WaterLOGSY) [5, 6]. In essence, fast exchange between free and bound states is the prerequisite for those methods to be effective, as the NOE effect of the bound state must be observed when the ligand is in the free state. Also the rate of exchange between the free and bound states must be much faster than the proton relaxation rate in the bound state. Normally weak binders satisfy this condition. Thus, NOEs from ligands in the bound states will be transferred to free ligands and observed in a common two-dimensional ^{1}H–^{1}H NOE spectroscopy experiment. The transferred NOEs are readily distinguished from those of free ligands, as their phases are opposite. The transferred NOEs not only confirm the binding but also can be used to determine the conformation of the bound ligand if the NOE data are handled carefully [4, 10, 25]).

STD: Upon ligand binding to a target, the change of polarization of one partner will affect the polarization of the other by the so-called "cross-relaxation" effect. This is the fundamental principle underlying a popular 1D ligand-observed NMR method called saturation transfer difference (STD) spectroscopy [21]. In the STD experiments, the target is continuously irradiated by selective radio frequency pulses for 1 –2 s in order to saturate the magnetization of the whole target. This is accomplished by a phenomenon called spin diffusion, which occurs mostly via dipole-dipole interactions between proximal spins. The selection of the frequency is usually in the

aliphatic region to avoid irradiation of the ligand of interest. Because of the proximity of the target to the ligand, this saturation is then transferred through space to the bound ligand while the selective pulses do not affect the free ligand. In order to observe this transferred effect, a difference spectroscopy has to be involved, namely, the data are recorded with target magnetization saturated (on-resonance irradiation e.g., in the aliphatic region) and target magnetization untouched (off-resonance irradiation e.g., at –15 ppm where neither protein nor ligand is affected.). The subtraction of these two data is the STD spectrum demonstrating the physical proximity of ligand to the target (Fig. 32.2b).

WaterLOGSY: The water-ligand-observed-via-gradient-spectroscopy (Water-LOGSY) is another 1D NMR technique utilizing similar principles [6]. But, instead of target magnetization, it is the bulk water magnetization that is selectively perturbed. For bound ligands, the cross-relaxation rate of dipole-dipole interaction between water and ligand is negative, while it is positive for free ligands in bulk water. The sign difference of cross-relaxation rate translates into 180° phase difference in the WaterLOGSY NMR spectrum. WaterLOGSY takes advantage of this NMR property to differentiate free and bound ligands (Fig. 32.2c).

Among the ligand-based NMR experiments discussed above, $T_{1\rho}$ and T_2 edited experiments are the most sensitive ones to detect ligand binding. WaterLOGSY, although less sensitive than the relaxation-edited experiments, is more reliable and robust to monitor ligand binding [16]. Thus, in practice, these two experiments are conducted together to minimize false negatives and to avoid discarding weak but valuable hits. STD is less sensitive than WaterLOGSY. Moreover, highly deuterated buffer is required for optimal sensitivity. This prohibits its application in screening compound libraries because the automation system almost exclusively uses H_2O to wash the pipetting system.

On the other hand, STD provides more valuable information contents than other 1D methods. One of the most common applications for STD is group epitope mapping [22]. STD can differentiate between the region of a ligand that is in proximity of protein residues and areas that are solvent exposed. Here the binding region of a ligand can be identified, so called epitope mapping. Hajduk and co-workers came up with a special STD method using *S*tructural information, *O*verhauser effects and *S*elective labeling called *SOS* – STD [13]. They use targets that are perdeuterated but contain specific amino acid residues that are selectively protonated. This method combines X-ray crystal structure information and STD positive and negative data to search the ligand-binding pocket. From STD signal intensities as well as distance restraints, they successfully determined the protein/ligand complex structures which are consistent with the ones determined by X-ray crystallography. SOS-NMR has a great potential in areas where both X-ray crystallography and traditional NMR methods are challenging to generate complex structures. Furthermore, this technique does not require protein resonance assignments, which indicates that many large size proteins can be assessed by this method.

TRNOE is the least sensitive method compared to the others. Nevertheless, it is still a powerful means to determine structures of bound ligands, which can be combined with SOS-NMR data in the protein/ligand structural determination.

32.5 Protein-Observed NMR

In previous sections, we introduced the concept that ligand binding to proteins can be monitored by observing ligand signals. Here we will discuss NMR methods that detect protein signal changes upon ligand binding. Since protein hydrogen signals show severe overlap in the one-dimensional ^{1}H spectrum and the ^{1}H signal line width in proteins is significantly broadened due to their fast T_2 relaxation, a better signal separation is necessary. Two-dimensional NMR uses a second dimension obtained from another nucleus in the biomolecule to provide a resolution that allows better isolation of the many proton signals in a protein. Heavily used experiments in 2D protein NMR are the ^{1}H-^{15}N heteronuclear single quantum spectroscopy (HSQC) or the HSQC. This pulse sequence has been further optimized to detect signals in larger proteins and is called TROSY. ^{1}H-^{13}C heteronuclear multiple quantum spectroscopy (HMQC) [3] is another heavily used 2D experiment.

The ^{1}H-^{15}N HSQC/TROSY experiment focuses on protein backbone amide protons and links the chemical shift of backbone amide protons to the backbone nitrogen that they are attached to. The ^{1}H-^{13}C HMQC links the chemical shift of protons to the chemical shift of their covalently bonded ^{13}C. In drug discovery, this technique is mainly used to monitor protein side chains, especially methyl groups from methionines, leucines, isoleucines, valines, and alanines. These two types of 2D NMR experiments offer the "finger print" of the target protein, and these resonances can sense changes in their chemical environment and are thus very sensitive to changes such as pH, temperature, salt concentration, or ligand binding. It is also worth mentioning that normally ^{1}H-^{13}C HMQC is more sensitive than ^{1}H-^{15}N HSQC/TROSY primarily because of the higher gyromagnetic ratio of ^{13}C and the presence of three protons in methyl groups.

Since the natural abundance of ^{15}N and ^{13}C is extremely low, 2D HSQC and HMQC NMR methods almost exclusively require isotope (^{15}N and/or ^{13}C) enrichment for the protein investigated. For large proteins (> 60 kDa), ^{2}H enrichment for non-exchangeable protons is needed to obtain high quality TROSY spectra [24]. In essence, this trick slows down the loss of magnetization via neighboring protons since magnetization transfer via deuterium is much weaker. Isotope labeled proteins can be obtained by expression in minimal medium supplemented with ^{15}N-ammonium salt and ^{13}C-glucose [2]. More rigorous conditions are required for deuterated proteins. For optimal labeling, ~ 99.8% D_2O and deuterated glucose must be used to prepare growth medium but rarely >85% deuterium labeling is needed to obtain good quality TROSY spectra even for big proteins. Only if selective labeling is the goal, is a high deuteration of the protein desirable. To avoid ^{1}H contamination, normally all medium additives are pre-lyophilized and followed by dissolution in D_2O. Usually cell growth in D_2O is dramatically reduced and there are commercially available isotope-enriched growth media to boost the cell growth.

Besides the uniform deuterium labeling, the technology on selective labeling has been advancing recently as more and more NMR researchers become interested in

challenging proteins such as high molecular weight (> 100 kDa) proteins [29] and membrane proteins [28]. Particularly, selectively labeling of $^{13}C^{1}H_3$ groups in leucine, isoleucine, valine and alanine residues while keeping the rest of the protein deuterated, marks a milestone in bio-NMR [31, 32].

In order to correlate each cross peak in a 2D spectrum to a defined location in the protein, an assignment needs to be done. This assignment effort relates each cross peak in a 2D NMR spectrum to a specific atom of a given target protein. If structural data for this target are available, the effect of a ligand on a specific cross peak can be immediately localized in the target structure. Since ligand binding will affect the environment of many atoms in a protein, the binding event is reflected by a chemical shift of the cross peak for the atoms in proximity. These shifts can then be mapped onto the structure, as shown in Fig. 32.10. This will provide a coarse resolution picture of the site of binding for a ligand. In addition, this mapping will also show if conformational changes occur at sites in the protein that are distant from the binding pocket. This can be very useful information on the right choice of existing crystal structures. For example, if the chemical shift patterns show large conformational changes upon ligand binding, rather the ligand bound structure than the apo structure of a target should be used to develop structure activity relationships (SAR); however, one should keep in mind that cross peak assignment requires extensive and sometimes also expensive labeling. While current software now allows rapid assignment for proteins < 35 kDa, this task becomes exponentially more challenging for proteins that exceed this limit. This is due to overlap of signals in the 2D and 3D NMR experiments required for assignments and the loss of signal intensity due to decreased tumbling rates in bigger proteins. Assignment should therefore only be pursued if the information on binding cannot be acquired by other methods and is absolutely needed for advancing ligands into drug-like molecules. One should also be aware of the fact that for large proteins the effort to obtain an assignment for a high enough number of cross peaks is not always warranted. This is especially true for proteins that are mostly helical since the chemical shifts crowd in a narrow range.

To get around the need for assignments, we use the following strategy. The chemical shift patterns of known ligands for a target are characterized and compared to novel hits. Hits that behave in a similar way, will produce similar chemical shift patterns. In the case where a crystal structure of the protein exists and the binding site is known, the number of residues involved in direct binding can be estimated and compared to the observed shifts. This information can be used to predict a static or dynamic behavior of the target upon ligand binding. In a static case, a similar number of peaks, as predicted from the structure, will shift while in a dynamic case many more peak shifts are observable. If questions around a specific residue in the active site arise, then the site directed mutation of the suspected amino acid of interest could be pursued to identify this amino acid and to probe for a specific interaction.

32.6 Screening by NMR in Antibacterial Drug Discovery

Most targets in antibacterial drug discovery allow development of enzyme assays. These can be used to test for inhibition of the target enzyme during catalysis and are thus probing all relevant conformations of a target of interest. Fortunately, a good enzymologist can develop assays that are sophisticated enough to test for inhibition of activity at compound concentrations up to 200 μM, which will allow efficient screening of molecular fragments and weak binders. This deprioritizes NMR as a tool for primary hit identification in antibacterial drug discovery. NMR is rather used to rapidly characterize hits that result from these screens. NMR serves as an excellent tool to exclude false positive hits such as nonspecific binders or ligands that bind to a non desirable pocket on a given target since 1D and 2D NMR methods can quickly characterize the site of binding for a compound of interest by competition (1D NMR) or chemical shift patterns (2D NMR). This strategy allows for faster screening of libraries and focuses NMR use on hit deconvolution and characterization. Thus targets can be screened more rapidly with fewer resources than by using strictly NMR based screening technologies.

Only in cases where adequate assays cannot be established or where a certain form of the enzyme is targeted in a screen, NMR can become an efficient screening method to identify novel fragment hits. In this case 1D NMR screening using $T_{1\rho}$, STD or WaterLOGSY pulse sequences becomes the primary screening approach. Here no isotope labeling is required and the amount of protein needed is tenfold less than for a 2D NMR screen. Since NMR screening uses mixtures of 5–10 compounds in a single binding experiment, 1D NMR does not need deconvolution of the mixture to identify the binding fragments since mixtures are assembled in a way that each compound has at least one unique signal in the spectrum. 1D NMR is ideally geared to identify weak and medium tight binders (mM to ~ 10 μM). However, in contrast to 2D NMR, non-specific binders cannot immediately be separated from specific hits by 1D NMR; therefore, appropriate 1D NMR based control experiments, such as competition experiments, should be performed to uniquely identify specific hits.

32.7 Examples for NMR Applications in Infectious Research

Understanding the enzymatic mechanism of bacterial targets is crucial for designing antibacterial inhibitors at both initial and later stages of drug discovery. A thorough knowledge of the enzymatic reaction mechanism not only guides the development of biochemical assays for compound library screening, but also drives the optimization of inhibitors if the mode of inhibition is known. In this respect, NMR spectroscopy is a very powerful technique that can be combined with other biochemical and biophysical tools to study the order of substrate addition, substrate binding constants and mode of inhibition. In the following sections, we will provides examples that are applied in infectious research.

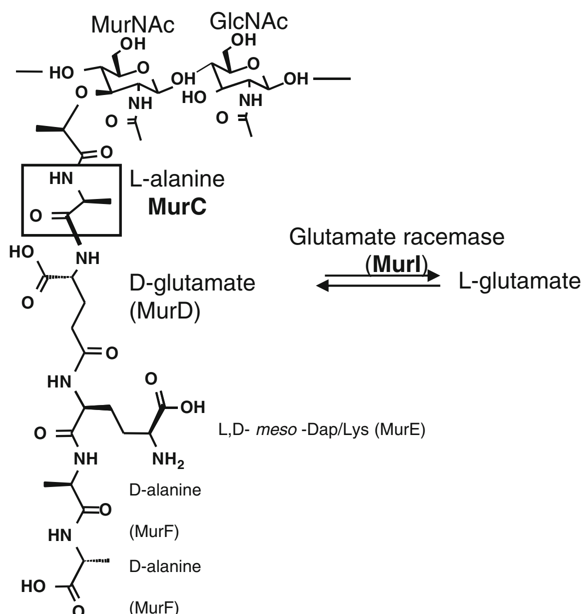


Fig. 32.3 Pentapeptide assembly. The L-alanine added by the enzyme MurC is boxed in the pentapeptide structure. The D/L glutamate racemization reaction by MurI is indicated right to the pentapeptide structure

32.7.1 1D NMR Applications

32.7.1.1 Peptidoglycan Synthesis Pathway

One focus of interest in antibacterial research is the bacterial cell wall [14], which is assembled by the *Mur* enzyme family. Here two enzymes from the *Mur* enzyme family, MurC and MurI (see Fig. 32.3 Pentapaptide assembly), are used as examples to elucidate how 1D and 2D NMR are applied to study the order of substrate binding, the right enzymatic form for screening and ligand binding characterization, respectively.

32.7.1.2 MurC

The three-substrate reaction (Fig. 32.4) catalyzed by the MurC enzyme provides an appropriate system for exemplifying the power of NMR to study the enzyme function, substrate binding and inhibition mechanism.

For *E.coli* MurC, the well-documented binding mechanism [7], which also best fits the kinetic data, is the Ordered-Ter-Ter mechanism, namely for the three MurC substrates, ATP, UNAM and alanine, ATP binds to the enzyme first, then UNAM and lastly alanine. Here we applied 1D ^{1}H WaterLOGSY to investigate the substrate binding at different conditions.

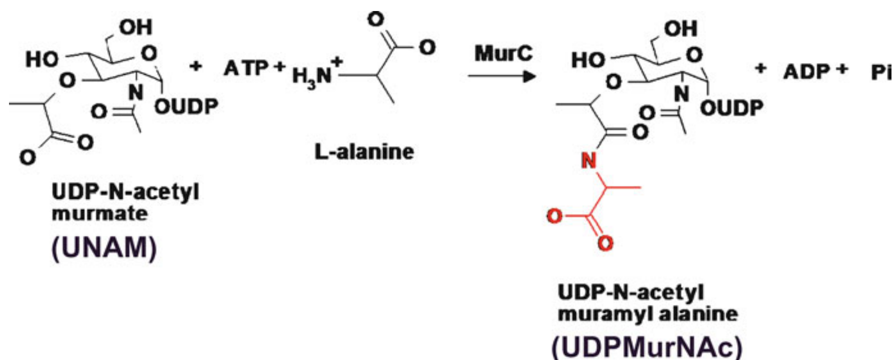


Fig. 32.4 The MurC enzyme reaction

Although fitting steady-state kinetic data with different kinetic models can shed light on the binding mechanism, these results do not always differentiate kinetically related models and ambiguity may persist since different models satisfy the fitting criteria equally well. Only elaborate studies with inhibitors can discriminate between related mechanistic models. This requires data collection using a multidimensional matrix where inhibitors and ligands are varied against each other with relatively high accuracy and often the development of the correct mathematical models for data fitting. In this case, NMR can provide quick unambiguous answers by detecting the substrate binding individually and concurrently and thus complement the fitting results of steady-state data.

32.7.1.3 ATP Binding

In the *E. coli* MurC ordered-Ter-Ter mechanism, ATP is the first substrate to bind. In Fig. 32.5a, free ATP shows negative WaterLOGSY signals as expected. Positive WaterLOGSY singals of ATP after the addition of *E.coli* MurC demonstrate the substrate binding. Therefore, ATP can bind to the enzyme without any substrate. Moreover, the signals for ATP do not alter in the presence of UNAM or alanine (data not shown), which suggests it to be the first substrate binding to the protein.

32.7.1.4 UNAM Binding

Similar WaterLOGSY experiments were conducted to examine UNAM binding. Interestingly, UNAM alone demonstrates binding affinity toward *E.coli* MurC (Fig. 32.6). However, the UNAM WaterLOGSY signals are significantly enhanced after the addition of ATP (Fig. 32.6c), indicating that optimum UNAM binding requires the ATP bound form of the enzyme. If the substrate addition were random, then the UNAM binding would not be affected by the addition of other substrates.

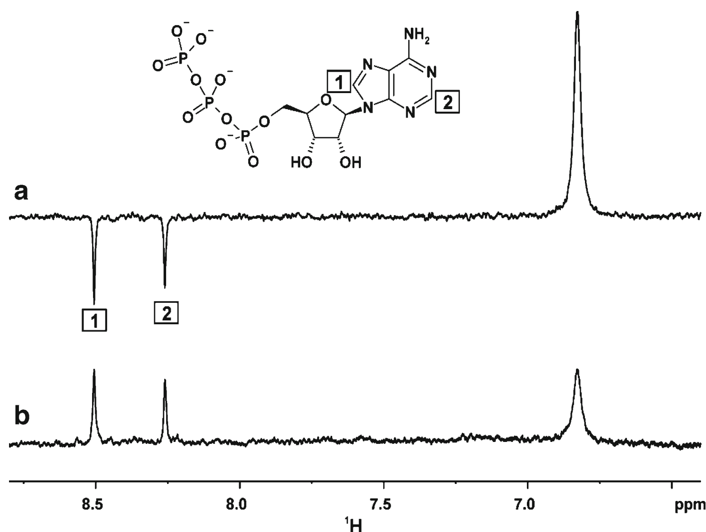


Fig. 32.5 ATP Binding to MurC: 1D ^{1}H WaterLOGSY spectra of 200 µM ATP in the absence (**a**) and presence (**b**) of 20 µM *E. coli* MurC. Only signals at low field are shown here. The assignments of the aromatic proton signals are labeled in the chemical structure of ATP. As expected, free ATP shows negative signals at 8.26 and 8.51 ppm in spectrum (**a**). The broad and positive signal at 6.83 ppm is from the chemical exchangeable NH_2 protons. ATP binding to *Eco* MurC is apparent since the signals at 8.25 and 8.51 ppm become positive in the WaterLOGSY spectrum (**b**)

Thus, the NMR observation strongly supports the Ordered-Ter-Ter mechanism, which proposes that UNAM is the second substrate after ATP.

Interestingly Fisher and co-workers studied the mode of inhibition of a transition-state inhibitor of *E. coli* MurC [11]. Although the inhibitor exhibits nanomolar binding affinity in the presence of ATP, this inhibitor binds to the enzyme without ATP with a $K_d = 2.30$ µM. Since the transition-state inhibitor shares large similarity with UNAM, in this regard moderate UNAM binding to *E.coli* MurC without ATP is not surprising.

32.7.1.5 Alanine Binding

Based on the Ordered Ter Ter mechanism and enzyme kinetic analysis, alanine is the last substrate to bind to the enzyme; however, the addition of alanine leads to rapid product formation interfering with direct measurement of binding for alanine. To avoid the formation of the product UNAM-ALA product, the use of a substrate mimetic can be pursued to prevent the reaction. In the case of MurC an ATP mimic, AMP-PCP can be tried to examine alanine binding; however, this attempt was unsuccessful due to very weak binding affinity of AMP-PCP. Therefore

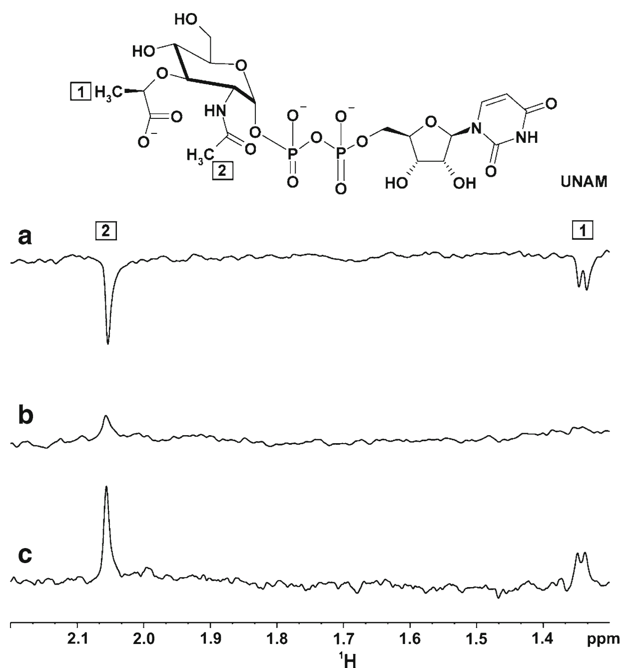


Fig. 32.6 UNAM binding to MurC: Comparison of 1D ^{1}H WaterLOGSY spectra of 200 μM UNAM at different conditions. Only signals of methyl groups are presented here for clarity. Free UNAM gives rise to negative WaterLOGSY signals in (**a**). With 20 μM MurC, signals are partially inverted (**b**), indicating that the substrate UNAM binds to MurC. However, upon addition of 200 μM ATP the positive signal observed in spectrum **b** is significantly enhanced (**c**), suggesting an ordered binding mechanism where ATP binds first. The assignments of each signal corresponding to the UNAM chemical structure are labeled on the NMR spectra

after addition of alanine in presence of the other two substrates the reaction proceeds and only the binding of product can be directly monitored in the case of MurC. Alanine being the third substrate to bind can be verified indirectly by testing binding to the apo or ATP bound form of MurC. In a strictly ordered system where Ala binds last, no binding should be observed as is demonstrated in lanes A and B of Fig. 32.7.

Studying the steady-state kinetics is one of the commonly applied approaches to determine the mechanism of substrate addition for multi substrate enzymes; however, fitting kinetic data does not always provide an unambiguous answer especially when kinetic data satisfy more than one mechanistic model within the error of the data. Here, we demonstrate that NMR spectroscopy is a very convenient tool to quickly differentiate between models that satisfy fitting algorithms. Coupled with kinetic data, a few simple NMR experiments can provide clear and straightforward answers supporting a distinct substrate addition mechanism.

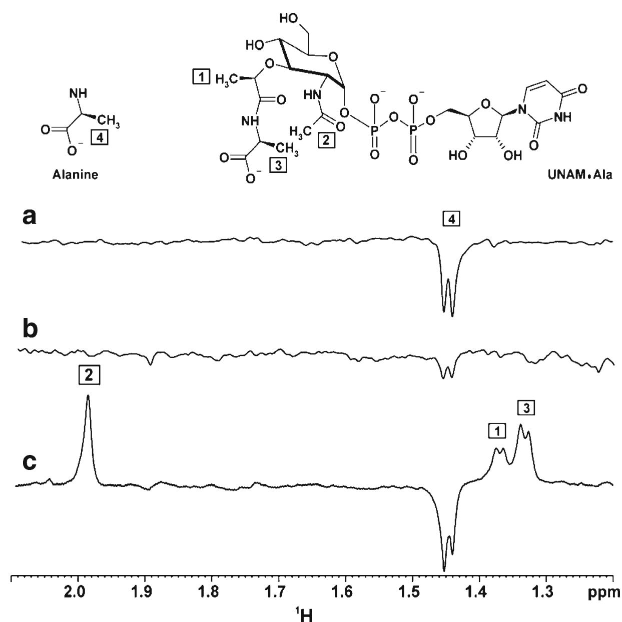


Fig. 32.7 Alanine binding to MurC: The 1D [1]H WaterLOGSY spectra for studying alanine binding. Only the signals from methyl protons are presented. Methyl proton signals from 200 μM alanine in solution are negative in the WaterLOGSY spectrum (**a**). Even in the presence of 200 μM ATP and 20 μM *E.coli* MurC, methyl proton signals from 60 μM alanine have a reduced signal intensity but are still negative in the WaterLOGSY spectrum (**b**), indicating that alanine binds very weakly to the enzyme. The addition of 500 μM alanine, 200 μM ATP and 20 μM *E. coli* MurC to 200 μM UNAM converts UNAM to UNAM-ALA which also binds to the enzyme (**c**)

32.7.1.6 Hit Evaluation of MurC Compounds

Besides its use for compound library screening and because of its low false positive hit rate and strong capacity to detect weak binders, NMR also serves as an excellent tool to evaluate hits from high-throughput screening or other screening methods. 1D NMR can typically detect the binding affinity for K_d values from 1μM to 1 mM [26], which is normally the affinity range for screening hits. The first NMR experiment for hit evaluation will confirm simple hit binding to the enzyme. It appears to be a straightforward experiment to do. Thorough study of the target enzymology and cautious design of the NMR experiment are required because the apo enzyme is not always necessarily the best acceptor for hit compounds. A good example here again is the transition-state inhibitor for *E.coli* MurC mentioned previously. The inhibitor can bind to *E.coli* MurC with K_d of 2.30 μM. However, in the presence of ATP, the binding affinity is boosted 20-fold to 102 nM. Clearly, the binding of this inhibitor requires ATP binding. Therefore, the compound binding by NMR against all possible enzymatic states should be examined to give a complete picture of

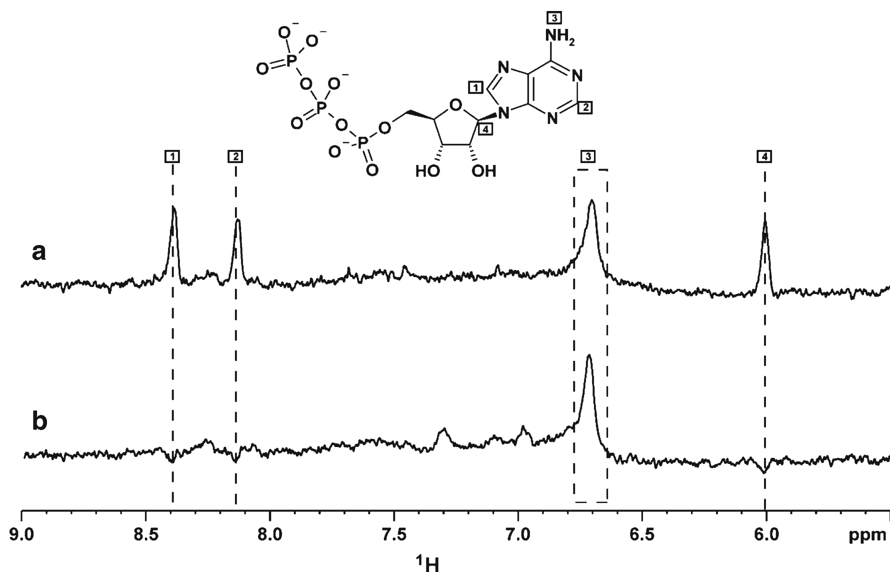


Fig. 32.8 Hit evaluation of an *E.coli* MurC compound by 1D NMR competition experiments. (**a**) 1D WaterLOGSY spectrum of 200 μM ATP in the presence 20 μM Eco MurC. Positivie signals indicate that ATP binds to the enzyme. (**b**) In the second step, 200 μM compound was added to the NMR sample shown in spectrum A. Because of the compound's tight affinity (IC50 < 0.39 μM), ATP is almost completely displaced from the binding site and its signals therefore become negative in the WaterLOGSY spectrum. Also note that the $-NH_2$ (annotated as the Signal 3) always gives rise to positive WaterLOGSY signals due to its chemical exchange with water

compound binding. Simply testing binding to the apo enzyme can potentially miss valuable hits or lead to misinterpretation during hit evaluation.

If NMR confirms compound binding, then the question arises where these compounds bind. Although investigating the shift pattern in 2D NMR spectra can provide the binding site information, MurC is too large for protein-observed NMR. Instead, competition between compound and substrate or compound with a known binding site by 1D NMR can indicate the compound binding site; however, caution should be administered since competition observed by 1D NMR does not necessarily prove that the compound binds at the competitor binding site because allosteric inhibitors can also affect substrate binding. X-ray crystallography or more sophisticated NMR experiments such as SOS-STD NMR [13, 15] can provide binding site information. The spectra shown below (Fig. 32.8) demonstrate a typical 1D NMR competition experiment for *Eco* MurC hits. The technique is well suited in early hit evaluation since it is optimal for the affinity range typically observed for HTS or HCS screens.

The above-described 1D NMR techniques used to analyze binding do not work well for tight binders since the off rate is too slow to allow transfer of magnetization to the free form of the ligand. In theory line shapes of a bound ligand should change but this would require protein concentrations that are equal to the ligand

concentration. The experiments referred to in this chapter use an excess of ligand over protein thus limiting straightforward detection of binders in the < μM affinity range. Ideally hit evaluation by 1D NMR should be combined with some information on a ligand's or substrate's potency such as K_m, $K_{d,}$ or IC_{50} typically obtained from enzyme assays. This would allow selection of very potent hits for other binding confirmation techniques such as isothermal caloremitry (ITC). For *E.coli* MurC, the K_ds of ATP, and UNAM are 145 μM and 117 μM, respectively and therefore can be used in 1D NMR competition experiments to detect specific binding of unknown ligands. Even potent ligands could be detected in this case, since one would monitor release of the weak substrate form MurC upon addition of a potent ligand that competes for the substrate binding site. The spectra shown below (Fig. 32.8) demonstrate a typical 1D NMR competition experiment for *E.coli* MurC hits. Here the signals from the substrate ATP were monitored since the compound affinity is high (IC50 < 0.39 μM). In this case, competition experiments with weak and known compounds or substrates, as shown in Fig. 32.8, are very suitable for hit evaluation.

32.7.1.7 Enzyme Kinetics and Compound Inhibition of MurC

Although it is usually not the best method to study enzyme kinetics, NMR can monitor the change of substrate and product as a function of time. Zawadzke et al. observed the reaction catalyzed by *E.coli* MurC by the decrease of the substrate signals and the gradual enhancement of signals from the product UNAM-Ala [35]. A hit identified after high throughput screening was characterized by comparing the product signal with and without the inhibitor after 20 min of the MurC reaction. The inhibition of MurC by the compound was directly confirmed by changes in the product and substrate signals. Combined with powerful robotics and rapid data acquisition, this could be a useful approach for a medium throughput screen where development of a normal plate based enzyme assay is prohibitive.

32.7.2 2D NMR Applications

The following examples show how 2D NMR can address the following questions:

1. Is a given protein in exchange between different confirmations and what is the effect of ligands on this dynamic behavior?
2. What is required to efficiently bind a novel ligand?
3. Where does a novel ligand bind? (very efficient if a target structure is available)
4. Is a protein present in the right form or state?
5. What conformational state of a protein do the ligands bind to?

Fragment library screening is often pursued to identify chemical starting points for inhibitors that bind specifically to the enzyme. Those fragment hits can be further developed using structural information. When enzyme assays are used, the protein of interest naturally cycles through its different conformations and only the correct

choice of substrate concentrations or the direction of the reaction become important (see chapter 29 on Enzyme-Based Screening). In contrast, NMR binding screens often test binding to a specific form of the enzyme. Whenever an enzyme undergoes substantial conformational change or exists in multiple forms such as described above for MurC, choosing the right enzyme form for screening is essential in order to obtain a reasonable hit rate. We will use MurI as an example to explain how screening a substrate-bound form can successfully deliver hits.

32.7.2.1 The Glutamate Racemase MurI

Glutamate racemase is an enzyme essential to the bacterial cell wall biosynthesis pathway [27]. In a search for selective inhibitors, we identified a series of inhibitors specifically targeting *Helicobacter pylori* glutamate racemase. We used 2D-TROSY NMR to characterize ligand binding. When recording a TROSY ^{15}N–^{1}H spectrum for apo MurI, the spectrum lacked resolution. The indole amide region in the spectrum (Fig. 32.9, top left, Arrow) showed four instead of the two expected peaks (two tryptophans in the protein). This indicated that the protein is dynamic at the used conditions reasoning the loss of many cross peaks due to exchange broadening. Indeed, adding the substrate glutamate, produced the spectrum colored black in Fig. 32.9, top right. The good resolution and presence of >90% of the expected cross peaks indicated that the substrate was stabilizing a defined conformation of the MurI protein.

This 2D NMR method was then used to quickly identify conditions where the inhibitor produced a chemical shift (red colored spectrum, top right) for some of the peaks. It was found that glutamate was required for inhibitor binding which is consistent with an allosteric binding mode. One can clearly see that the tryptophan indole signals experience a strong shift upon compound addition indicating a substantial change in the chemical environment of the tryptophan residues (Fig. 32.9, right side). Indeed the crystal structure later showed that a tryptophan is rotated out of position to allow inhibitor binding. This 2D NMR method proved very useful to quickly identify conditions where a structure of the inhibitor containing MurI protein could be obtained. We went on to assign the backbone amide signals for *H. pylori* MurI. This allowed us to quickly map the chemical shifts onto the liganded MurI structure (Fig. 32.9, bottom), which served as a quick tool to assess the binding mode of other inhibitors from this screen without the need for additional crystallography. There the observed shifts upon ligand addition were mapped onto the MurI crystal structure to define the inhibitor binding location (Fig. 32.9, bottom).

32.7.2.2 DNA Ligase

DNA ligase joins the DNA fragments (Okazaki pieces) occurring on the lagging strand during DNA replication and participate in DNA repair reactions. It is an essential enzyme present in all bacteria and therefore another attractive target for

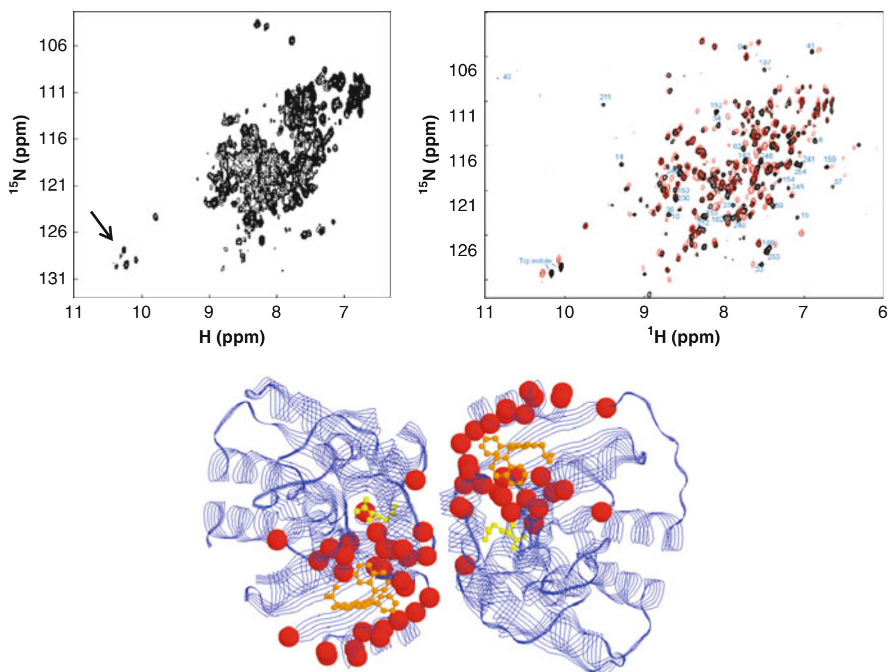


Fig. 32.9 (*Top left*) ^{15}N^{2}H TROSY 2D NMR spectrum of *H. pylori* glutamate racemase in the absence of glutamate. The *arrow* indicates the signals observed in the region typical for the amide proton present in the Trp side chain. (*Top right*; From [20]) ^{15}N^{2}H TROSY 2D NMR spectrum of *H. pylori* glutamate racemase in the presence of glutamate without (*black cross peaks*) and in presence (*red cross peaks*) of the pyrimidinedinone inhibitor (structure see figure insert). (*Bottom*) Crystal structure of *H. pylori* glutamate racemase in the presence of glutamate (*yellow*) and the pyrimidinedinone inhibitor (*orange*). *Red spheres* indicate chemical shifts observed in the 2D ^{15}N^{2}H TROSY NMR spectrum of *H. pylori* glutamate racemase with bound glutamate upon adition of the pyrimidinone inhibitor

drug discovery [33]. The enzyme uses NAD+(Fig. 32.10, top) to adenylate itself at the ε-NH_2 group of a lysine residue within a conserved KXDG motive. NMN (Fig. 32.10, top) is then released from the protein and the adenylated form of the enzyme is present as shown in Fig. 32.10 below. In a second step the adenosine is transferred to the 5′-phosphate in a DNA nick to energize subsequent strand ligation. 2D-NMR is a highly useful tool to differentiate between the adenylated and apo form of the enzyme. The shift patterns created by binding to the adenylation site (Fig. 32.10) can be used to identify binders to this site. Since the shifts for binders to the NMN binding pocket are distinct from those observed for the adenylation site (data not shown), binding to these sites can be differentiated. 2D NMR can test compounds with an unknown mode of inhibition for binding and analysis of the chemical shifts created upon their addition allows discrimination between binding to the adenylation site or the NAD binding pocket. DNA ligase is an example to show where 2D NMR can be used to rapidly identify the binding location of unknown inhibitors by simple shift pattern analysis without the need for assignment of the peaks.

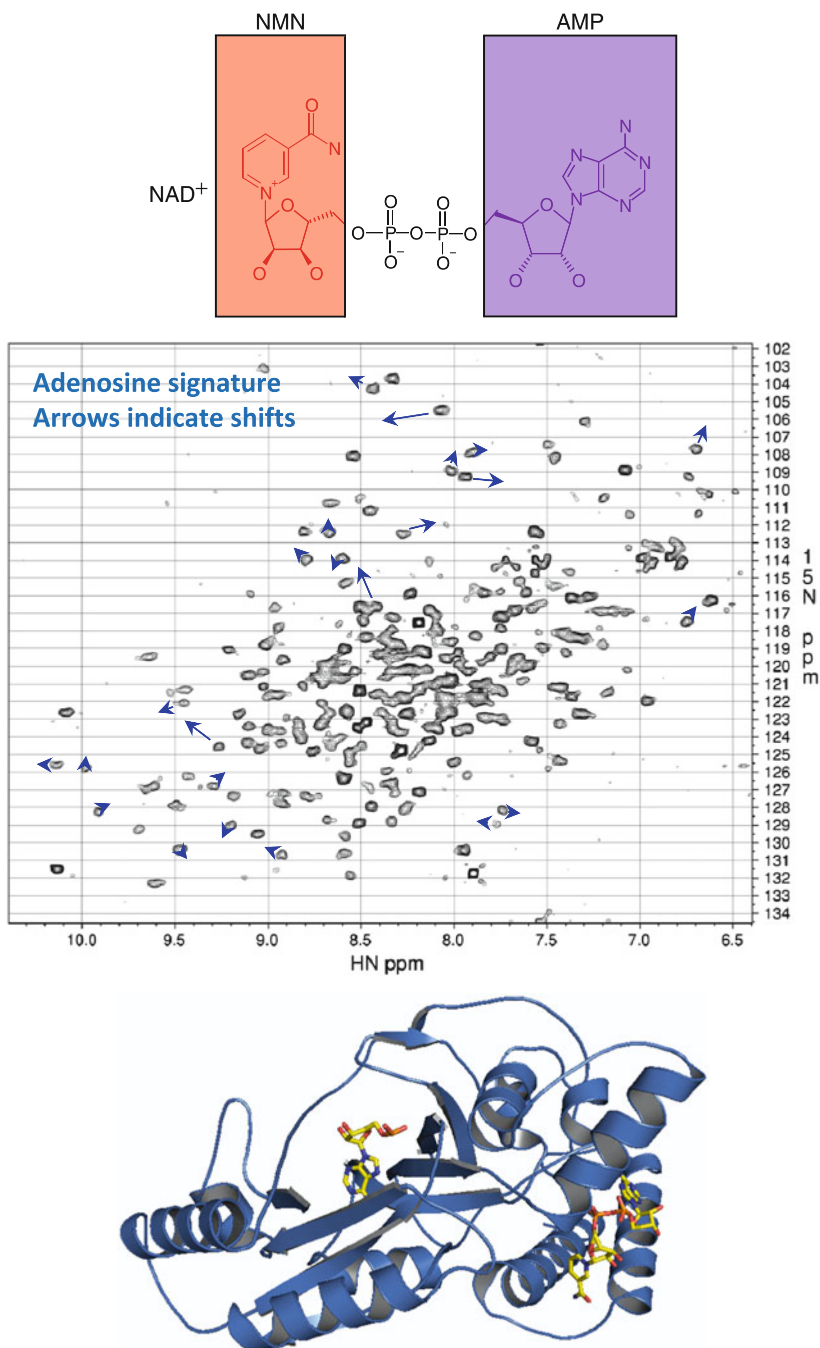


Fig. 32.10 (*Top*) Chemical structure of NAD. The NMN and adenosine portion are shaded in *red* and *blue*. (*Middle*) 2D $^{15}N^{2}H$ TROSY NMR spectrum of *H. influenzae* DNA Ligase in its apo form. *Arrows* show shifts upon adenlyation of DNA Ligase. (Spectra were recorded by the AstraZeneca NMR group in Alderley Park, United Kindom). (*Bottom*) Crystal structure of *H. influenzae* DNA Ligase in its adenylated form with NAD bound to the NMN binding pocket [17]

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Chapter 33
A Review of Animal Models Used for Antibiotic Evaluation

Andrea Marra

33.1 Introduction

One of the foremost challenges of drug discovery in any therapeutic area is solidifying the correlation between *in vitro* activity and clinical efficacy. Intermediate between those two points is the validation that affecting a particular target *in vivo* will lead to a therapeutic benefit. In antibacterial drug discovery, there is an implicit advantage from the start, in that the targets are bacteria, and it is relatively straightforward to ascertain *in vitro* whether a compound has the desired effect (i.e., bacterial cell killing) and to understand the mechanism by which that occurs. The downstream criteria, whether a compound reaches the site of infection and attains levels necessary to affect bacterial viability, can be evaluated in animal models of infection. That is, once it is clear that a test compound is able to kill bacteria, and it is established that it can achieve appropriate concentrations in infection sites, it is possible to extrapolate that the desired clinical effect can be expected. In this way, animal models of infection can be a highly valuable and predictive bridge between *in vitro* drug discovery and early clinical evaluation.

Unlike other therapeutic areas, researchers in Antibacterial Drug Discovery have the advantage of being able to isolate the target (i.e., the bacterial pathogen) and manipulate it outside the context of the host (*in vitro*) in order to (1) understand the mechanism of action of a test compound, (2) identify the concentration necessary for bacterial eradication and (3) be able to predict the events that occur when a compound acts on a pathogen (such as growth inhibition, resistance development

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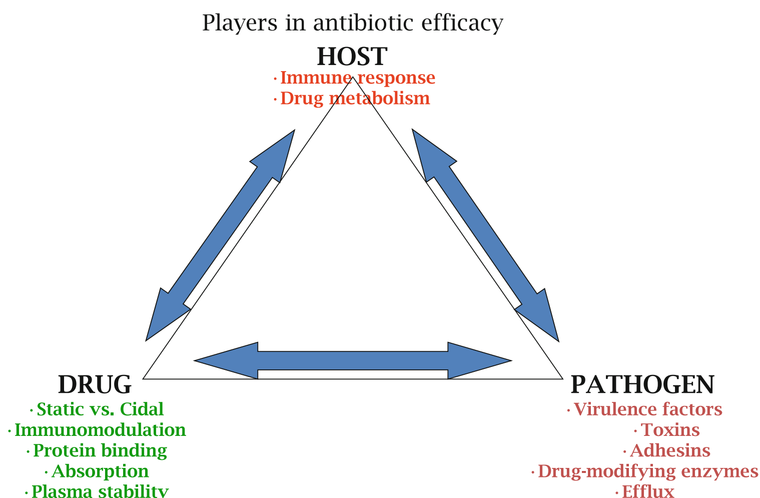

Fig. 33.1 The complexities involved in host–pathogen–antibiotic interactions

or cidality). Animal models are most useful for confirming *in vitro* findings with the added complexity of the host environment, for helping to learn what the host does to the drug, and for what effects the drug may have on the host. The ideal situation is one where host changes to the drug are such that the drug is still efficacious against the organism, at concentrations that have minimal, if any, adverse effects on the host. It is also possible to develop infection models that closely mimic human disease, because the infecting organisms can be recent clinical isolates, and the routes and sites of infection are the same in the model and in humans. This chapter will focus on considerations for model development in rodents and the benefits and drawbacks of different classes of infection models.

But of course rodents are not people, and where as they can provide some confidence in the efficacy of a novel agent, there is not a direct correlation to the human situation. The value of testing a compound in a whole animal is without parallel – the presence of the immune system, the trafficking of the drug through the host, the effects the host has on the drug, the effects the bacteria have on the host (Fig. 33.1) – none of these can as yet be modeled *in vitro* and the dynamics and complexities of them all interacting concomitantly enables a better understanding of what a potential drug can do. Of particular value, *in vivo* models provide the opportunity for frequent sampling of host tissues for antibacterial drug concentrations and/or colony-forming unit (cfu) determinations – data which is not possible to obtain in humans [1]. With bacterial infections, perhaps the most easily accessed and most commonly sampled site in patients is the middle ear or blood – whereby bacterial eradication can be correlated with dose and/or drug concentrations [2]; other sites require a much more invasive procedure to obtain samples. There are many challenges to animal infection models; however, not all human pathogens are virulent in rodent or other animal

species, and therefore large inoculum sizes or host immune system suppression is often required. A major issue is the pharmacokinetic differences between humans and animals [1, 3]. These issues will be discussed in more detail below.

33.2 Advantages of Animal Models

Even beyond the ability to study the infectious process and the effects of therapy within the complex system of the whole animal, *in vivo* infection models allow investigators to ask questions that are not possible in any other system, be it tissue culture or human infection. Although there are some basic understandings that can be gained by the use of tissue culture cells interacting with pathogens, for example, adherence to or invasion of mammalian cells, however, the results of these studies merely suggest that such interactions are possible *in vivo*, but not that the interactions necessarily occur. Isolated cells outside the context of other cell types, biological fluids, and host responses represent the simplest possible system; it is unclear how the mammalian cell or pathogen gene expression differs from that in the whole animal, where so many disparate interactions take place simultaneously. Therefore, infection models can serve to validate the findings of these other systems, but they also can provide information that extends far beyond. They have the added advantage of being able to provide a system for investigation and sampling not possible in human patients.

More specifically, animal infection models enable one to do the following:

- Monitor bacterial levels *in vivo* during/after therapy (including rebound upon cessation of therapy)
- Determine virulence of genetically manipulated or spontaneous mutants (including lethality, dissemination, tissue damage, replication at host sites)
- Follow the kinetics of infection at different host sites and understand how the host response limits bacterial dissemination
- Study infection in hosts carrying mutations in genes of interest, to understand how these genes impact the progress of infection
- Monitor *in vivo*-generated resistance with sub-efficacious dosing
- Compare multiple doses and different dosing regimens for efficacy
- Correlate drug dosing regimen with bacterial burden reduction
- Identify bacterial reduction required for eradication (+/− immune-competence)
- Examine relationship between MIC and tissue levels of drugs and bacterial reduction and understand how resistant mutants are affected by treatment
- Use the specific information gained to form a prediction of the efficacy in humans

The key is being able to use this information to predict how pathogens and drugs will behave in patients upon therapy with the ultimate goal being able to treat optimally, to eradicate the infection, and to prevent emergence of resistance [1]. These kinds of studies are particularly important for treating tissue-based infections in which plasma levels of drugs may not be a good predictor of efficacy.

33.3 Choice of Organism and Host

A collection of recent clinical isolates is a valuable resource for testing novel compounds *in vitro* as well as *in vivo*. The advantageous characteristics of such isolates include the following: range of MICs of marketed agents of interest; different levels of virulence; genetically representative of infection-causing pathogens (in terms of pathogenicity islands or toxin expression, etc.). When establishing a new infection model, it is ideal to match organisms with the model that best simulates the human infection. One drawback of recent clinical isolates is that they are "primed" or adapted for infecting human hosts; it is often difficult to demonstrate infectivity in a murine or other animal host. Some tricks can be employed to increase pathogenicity in mice such as limited passage in animals via intraperitoneal, intranasal, intravenous routes of infection that are followed by harvesting of organisms after 2–24 h from the peritoneal cavity, lungs, or blood, respectively [4]. Although it is not entirely clear how this *in vivo* passaging of bacteria alters the organisms, it can lead to a selection for clones that are the most fit for infecting that host site due, for example to differential gene expression. It is also possible to grow some organisms under conditions that aim to mimic the host environment such as reduced iron media, in the presence of carbon dioxide, or in blood or urine. These activities often have the ability to better potentiate bacteria for rodent infections by "acclimating" them to this host, again, likely involving bacterial gene induction.

The inverse approach to boosting infection potential by tinkering with the bacteria, is to set up infections in different mouse strains or by treating mice with immunosuppressive agents. Due to their small size, mice are generally the preferred species used in infection models for drug discovery because their size allows for less bulk chemical synthesis for testing, lower cost, and less vivarium space. The choice of mouse strain can be a crucial one for the establishment of murine models of infection, as it has long been known that certain strains are more susceptible or resistant to infection by different pathogens (Fig. 33.2 – a thorough analysis can be found in [5]). Susceptibility to infection in humans is also controlled by genetics, though the correlation between mice and humans in this regard is ill-defined [6]. For Gram-negative bacterial pathogens, several genetic loci have been identified that limit infection in some manner. Some of the earliest reports focused on the *Lps* gene, as it had been recognized that closely-related inbred mouse strains, C3H/HeN and C3H/HeJ respond very differently to infection by *Escherichia coli* or *Salmonella typhimurium* [7, 8]. This resistance or susceptibility can be measured by determining the number of organisms required for a lethal infection or LD by following the growth of the organisms in the liver and spleen following systemic challenge or by monitoring the time to death of the animals [7, 9]. It was generally found that mouse strains for which organisms had the highest LD_{50}s also, when monitoring time to death, the mice had the most prolonged infections [8].

The means by which the host can limit infection has been characterized as well via the inflammatory response. In this case, it has been demonstrated that C57BL/6 mice were more susceptible to pulmonary infection by *Pseudomonas aeruginosa* compared with BALB/c mice [9]. This susceptibility was manifested in higher

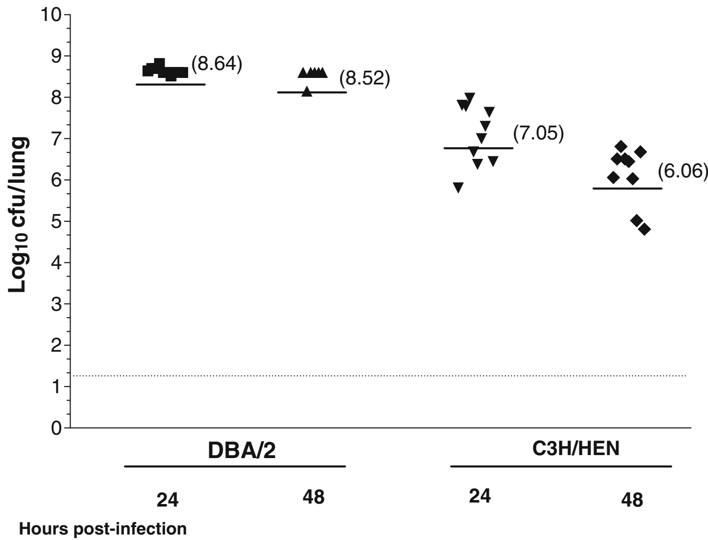

Fig. 33.2 Bacterial titers in lungs of DBA/s and C3H/HeN mice infected intra-nasally with *Acinetobacter calcoaceticus*. Mice were infected via intranasal instillation with *A. calcoaceticus* at the inoculum level shown in 40 µl volume. Mice were euthanized, the lungs were harvested at 24 and 48 h, and lung homogenates plated for bacterial enumeration. Each point on the graph indicates lung counts for a single mouse. Horizontal dotted line indicates limit of detection. Numbers in parentheses indicate geometric means

bacterial counts in the lungs, increased clinical signs, and greater mortality, and it was associated with strong neutrophil recruitment and massive tissue damage in the lungs. The inappropriate response is the inability to control the infection whereby the death of the host is an eventuality. A similar story was spun out of studies of *Streptococcus pneumoniae* murine pulmonary infection [10]. When a panel of inbred mouse strains was examined for susceptibility to *S. pneumoniae* lung infection, BALB/c mice were found to be resistant whereas CBA/Ca and SJL mice were susceptible. The resistance exhibited by BALB/c mice was due to their ability to limit bacterial growth in the lungs, prevent dissemination to the blood, early and increased recruitment of neutrophils to the lungs – none of which were seen in CBA/Ca mice. CBA/Ca mice carry a mutation in the xid gene, and thus cannot raise antibodies to cell wall and capsule components of pneumococci. It is not clear from these studies whether *xid* is the only defect in the CBA/Ca mice with respect to pneumococcal infection.

Genetically, such responses have been deciphered, and the genes identified point to a mechanism to limit infection. The aforementioned *Lps* gene, which is mutated in C3H/HeJ mice and as such renders lymphocytes and macrophages unable to recognize the lipid A moiety of LPS, has been identified as *Tlr4*, one of the Toll-like receptors that is important in innate immune system response to infection [5]. The lack of LPS recognition results in reduced macrophage activation and a consequent

decrease in oxidative burst and phagocytosis such that these mice are supremely susceptible to Gram-negative infection. Another gene, Nramp1, also known as *Ity*, is able to control replication of intracellular organisms; again, Nramp1 mutants are far more susceptible to *S. typhimurium* infection than mice carrying the wild-type gene [5].

Immunosuppressive agents such as cyclophosphamide are often used in murine studies to deplete the number of neutrophils in the circulation, and infections are initiated at time of minimal neutrophil numbers. Such treatment allows a temporary advantage to the bacteria, enabling them to initiate infection in the absence of a robust assault from the immune system. The literature variously reports different cyclophosphamide doses and schedules: 200 mg/kg injected i.p. once at 4 days prior to challenge [11], i.p. injection of 300 mg/kg once at 3 days before challenge [12], 150 mg/kg injected i.p. at 4, 2 and 0 days before infection [13], or two i.p. injections of 150 and 100 mg/kg at 4 and 1 day prior to challenge. The optimal dose and schedule will depend on the severity and length of the infection and should be determined empirically as the models are being established. Investigators are encouraged to monitor neutrophils in blood following administration of the different doses of cyclophosphamide to determine optimum dosing schedule for their model.

33.4 Mouse Protection Tests

33.4.1 A Word About Ethics

It bears mention that any studies involving living animals must be scientifically justified and carefully designed so as to maximize the information gained and the clarity and certainty of the results while also minimizing the numbers used. Of utmost importance is that these studies are planned to minimize any pain and/or suffering experienced by the test subjects. Anticipated pain should be appropriately managed or justified by the expected gain in knowledge and understanding of the disease and/or treatment. In order to ensure that the results obtained are sound and to reduce the need for repeat studies, relevant control groups must be included; these can help relate the data to those obtained in previous studies and inconsistencies or variability noted. For most animal infection studies in which therapy is administered, there should be both positive and negative control drugs (to demonstrate efficacy and failure, respectively) and untreated control groups should be also included. These groups will provide information to confirm that the test organism and inoculum size are appropriate. A careful consideration of the ethical issues is provided in Zak and O'Reilly, 1993 [3].

It is also important that all researchers understand that they bear full responsibility for the ethical treatment of test subjects. Experimental designs should adhere to the three R's: replace, reduce, and refine. To fulfill these objectives, investigators need to consider replacement of animal models with other models, either invertebrate or non-*in vivo* models (such as cell culture) or computer simulations. Reducing the number of animals used can be accomplished by performing pilot studies to

perfect techniques and skills and to determine variability; this can help ensure that control groups are appropriate and aid in maximizing the amount of information that can be obtained from each study or animal. Models can be refined by perfecting or minimizing use of techniques that cause pain or distress to the animals and by including the use of analgesics and more humane endpoints whenever possible. Researchers are encouraged to consult the IACUC Guidebook and the attending veterinarian.

There is an obvious conflict between the need to evaluate the efficacy of novel antibacterial drugs and using animal infection models to do so. It is hoped that the ethical issues will help push development of better models that can more closely predict effects in humans. There are still no set guidelines available to help direct these studies or to improve consistency across laboratories and so a review of the literature often can present confusing data.

33.4.2 The Mouse Protection Test

The mouse protection test (MPT) has been used since the 1930s [14] to evaluate the protective effect of a substance (in the earliest experiments, an antipneumococcus serum) against a lethal infection in mice. The most common MPT used is initiated via intra-peritoneal challenge (acute septicemia). Even from the earliest days of this test, investigators observed a heterogeneous response among animals within a group whereby some mice receiving the same bacterial inoculum and the same dose of anti-pneumococcal serum survived and others died. The same conclusions from these first studies are true today: (1) that all mice do not respond identically to infection and/or therapy and (2) group size (n) in such tests must smooth out this variability by being large enough to be able to identify clear trends. Also evident was that the timing of therapy relative to challenge greatly influenced whether the treatment was efficacious. For this reason, antibiotic administration typically begins 30–60 min post-challenge and may include a second dose 4–5 h post-challenge.

The acute septicemia model is known as a basic screening model [15, 16], as it allows a rough determination of antibiotic efficacy, safety, and tolerability. An intraperitoneal injection of bacteria (usually on the order of 10–1,000 times the lethal dose, or LD) is used to initiate this infection; within a few hours, bacteria will have spread from the peritoneal cavity to all organs to reach ~10^7 colony-forming units (cfu) per gram of tissue or per milliliter of blood. Depending on the organism and inoculum size, lethalities can occur within 24 h. Given that antibiotics are administered soon after challenge, treatment may be considered to be prophylactic rather than therapeutic. Many human Gram-negative and Gram-positive pathogens can be used in this model, and in some cases, even as few as <10 organisms can cause a lethal infection. Adjuvants such as hog gastric mucin or Brewer's yeast (up to 3% of either; it is necessary to titrate these to optimize infection obtained) are often used; these will allow a lower inoculum. Lethalities are monitored for 5 days. An example of the results observed in such a study is shown in Table 33.1.

Table 33.1 Example of an LD study in CF-1mice with three *K. pneumoniae* strains to determine inoculum for MPT

Bacterial Strain	# mice infected	# cfu/mouse	Number dead or euthanized Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	# Survivors end of study
A	10	1.15E+09	10	0	0	0	0	0	0
	10	1.15E+08	10	0	0	0	0	0	0
	10	1.15E+07	10	0	0	0	0	0	0
	10	1.15E+06	10	0	0	0	0	0	0
	10	1.15E+05	10	0	0	0	0	0	0
	10	1.15E+04	9	1	0	0	0	0	0
	10	1.15E+03	1	4	2	2	1	0	0
	10	1.15E+02	0	2	1	0	1	1	5
			$LD_{50} = \sim 1.15 \times 10^2$						
B	10	2.50E+08	7	3	0	0	0	0	0
	10	2.50E+07	5	5	0	0	0	0	0
	10	2.50E+06	4	3	0	0	0	0	3
	10	2.50E+05	0	0	0	0	0	0	10
	10	2.50E+04	0	0	0	0	0	0	10
	10	2.50E+03	0	0	0	0	0	0	10
	10	2.50E+02	0	0	0	0	0	0	10
	10	2.50E+01	0	0	0	0	0	0	10
			$LD_{50} = \sim 10^5 - 10^6$						
C	10	4.50E+08	10	0	0	0	0	0	0
	10	4.50E+07	9	1	0	0	0	0	0
	10	4.50E+06	7	1	2	0	0	0	0
	10	4.50E+05	1	1	0	0	0	0	8
	10	4.50E+04	1	0	0	0	0	0	9
	10	4.50E+03	0	2	0	0	0	0	8
	10	4.50E+02	0	0	0	0	0	0	10
	10	4.50E+01	0	0	0	0	0	0	10
			$LD_{50} = \sim 10^5 - 10^6$						

The other mouse protection test commonly used is the mouse respiratory tract infection model or RTI. This infection is initiated via an intranasal instillation of bacteria applied to the nares of anesthetized mice. Mice are held in an upright position with the thumb covering the mouth to prevent aspiration; a small volume of the bacterial suspension applied to the nares is then readily breathed in. Depending on the organism, the bacteria remain localized to the lungs for 1 or 2 days; however, usually once a critical bacterial threshold number is reached the infection can often disseminate to the blood and other organs. Therapy can begin the day of or following challenge and continue for several days, and lethalities are monitored for up to 10 days.

Some parameters must be established prior to using either model to evaluate antibacterial activity of test agents (Fig. 33.2). To choose the pathogen, a collection of recent clinical isolates can be screened for growth in a target organ or for lethality,

by infecting groups of mice with dilutions of organisms and monitoring cfu counts or deaths over time. (Table 33.1 describes the results of a typical study).

Once a range of inocula is identified, further studies can be used to fine-tune the inoculum number and to obtain an LD such that a reproducible infection can be established. A time course of the infection is useful to determine the time points of peak bacterial level; this is necessary The kinetics of growth during infection help to decide at what time point therapy should be initiated, if not completed, before the animals succumb to the infection. The kinetics of growth during infection help to decide at what time point therapy should be initiated. Inoculum size can have a major impact on the efficacy of an antibiotic, as drugs are sensitive to even a one- or two-log increase or decrease in cfu. Other factors that can influence the outcome of this test are antibiotic pharmacokinetics and the strain, age, and sex of the mice [15]. Detailed protocols for these models can be found also in [17].

33.5 Bacterial Reduction Models

33.5.1 Tissue-Based Infection Models with a Focus on Non-Surgical Models That Are Initiated Via a Simple Procedure

Infections that remain fairly localized can yield a great deal of information by allowing the evaluation of drug concentrations and bacterial burdens at a specific site or tissue either at a fixed time point or over a period of time during and following therapy. Such tissue-burden models differ from the MPTs described above by utilizing an endpoint of bacterial reduction instead of lethality. The advantages presented by these models relate to the information gained by the ability to monitor bacterial cfu over time in an infected animal or tissue in conjunction with antibacterial drug levels. In addition, these models allow optimization of dosing level and regimen to optimize anti-bacterial effect, particularly for organisms with elevated MICs [18, 19]. This section will focus on localized tissue-based infection models that are initiated via a simple injection, as opposed to a more elaborate, time and resource-consuming surgical procedure. A number of models will be considered individually below, but the approaches toward developing the models are similar for each (Fig. 33.3). The reader is referred to reference [16] for more complete details on these and many other models.

- Identify bacterial strain to use. This is accomplished by performing ID (Infectious Dose) studies – inoculum titrations whereby groups of mice are infected with different dilutions of bacteria and following the progress of the infection over time (Table 33.1 and Fig. 33.2)
- Identify appropriate inoculum level to use. The number of viable bacterial counts used to infect can only be determined after overnight growth on plates containing serially diluted organisms; the obvious difficulty is that one does not know until

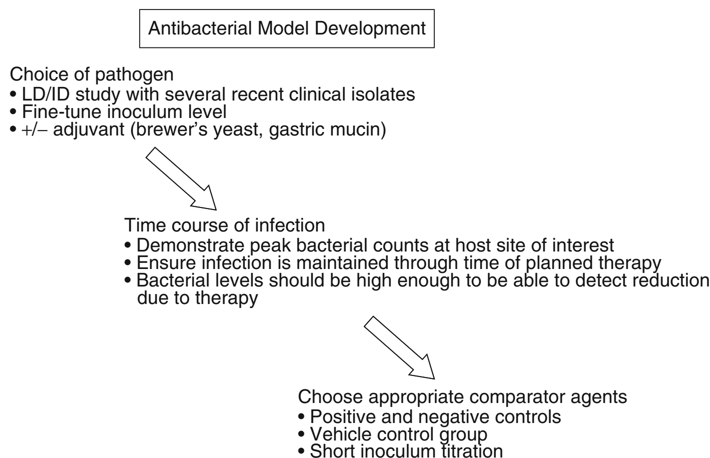

Fig. 33.3 Considerations in animal model development

the colonies are enumerated (usually the next day) what *actual* number of bacteria was used to infect the animals. For this reason, it is essential to have a standard method whereby the absorbance of a bacterial suspension is measured in a spectrophotometer and compared to a standard curve, or stock vials containing the appropriate number of bacteria are prepared and stored frozen at −80°C

These first two points may also be applied to setting up the mouse protection test:

- Identify the appropriate time point to harvest samples. For the initial studies, one should set up the infection in enough animals to be able to euthanize animals and sample tissues at specific time points (for example, every 24 h) to follow bacterial counts. If bacterial counts decrease with time in the tissue of interest, one might try passaging the organism (as described above), establishing the infection in immune-suppressed animals, or infecting a different host strain. If bacterial counts increase over time, it is important to know how quickly they reach a plateau and how long that level is maintained. The ideal situation is one where the bacterial numbers remain at high levels for the duration of therapy (without declining or causing the animal to succumb to the infection); in that case, the difference in bacterial counts between treated and untreated groups will be most apparent
- Identify a dosing regimen that can eradicate or significantly reduce the bacterial burden using an appropriate comparator agent in the same class. It is important demonstrate that the infection can be controlled by a known agent before testing a novel one. The number and timing of doses should be defined and adhered to for efficacy studies so that data can be compared over time. The ability to distinguish subtle differences in bacterial reductions (as opposed to the black-and-white results of the MPT) can aid greatly in setting appropriate doses for therapy.

This was clearly demonstrated for the antibiotic azithromycin, as a single dose delivered superior efficacy in terms of survival and bacterial burden reduction in three different infection models in animals, compared to administration of the same total dose over 2 or 3 days [20]. These results were corroborated in patients whereby single dose azithromycin was more effective than the same total dose delivered over 3 days, the traditional course of therapy [21].

Respiratory Tract Infections: Murine respiratory tract infection (RTI) models are commonly used to help understand whether a novel antibacterial agent can penetrate the lungs and achieve levels adequate for bacterial eradication. In addition, this model also enables one to study the kinetics of lung infection, to understand the tissue tropism that occurs, and to determine the location of the bacteria. In this model, bacteria are introduced into the lungs via intranasal or intra-tracheal instillation. The former is performed by placing a drop (typically 30–50 μl) of an appropriate bacterial inoculum onto the nares of anesthetized mice; the thumb gently holds the mouth closed while the mice breathe the inoculum in through the nose. Intra-tracheal inoculation requires a surgical procedure to visualize the trachea; inoculation using this method is via an injection through the trachea and delivers the bacteria deeper into the lungs than the intranasal method. A more detailed protocol for intra-tracheal inoculation can be found in ref. [16]. Either method distributes the bacteria to both lobes of the lungs. Depending on the organism, bacteria can disseminate from the lungs into the blood, typically once a critical threshold number of bacteria is reached; this is the sequelae with *S. pneumoniae*, and once this occurs, animals will rapidly succumb to the infection. For *S. pneumoniae*, treatment can begin the day following infection, as deaths in the untreated control groups begin sometime before the second day. In the case of other organisms, for example, *P. aeruginosa*, the massive inflammation that occurs is enough to kill the mice due to tissue damage and fluid accumulation in the lungs. Prior treatment with cyclophosphamide can aid in limiting this inflammatory response (unpublished observation), and therapy should be initiated early enough after infection to be able to protect the mice –within several hours appears most efficacious. Efficacy is determined in these models by euthanizing animals at predetermined time points, harvesting and homogenizing lungs in 1–2 ml of PBS, and diluting samples. Aliquots are plated for determination of bacterial load; in this way, differences in bacterial load between treated and untreated groups, or wild-type and mutant organisms can be easily detected. Starting inoculum will also affect outcome (Fig. 33.4).

Murine kidney abscess (also called descending urinary tract infection or hematogenous pyelonephritis): Some organisms will preferentially colonize a particular organ following systemic infection, as is the case with *S. aureus*; when this pathogen is injected into mice via the intravenous route, bacteria rapidly leave the blood and establish infection in the kidneys [22]. Upon necropsy, infected kidneys are seen to be larger and paler in color than naïve kidneys, and foci of infection are easily observed. Depending on the strain, mice can remain alive for 5–7 days, and bacterial counts in the kidneys can attain 10^7–10^8 cfu/tissue within a 2–3 day period. In addition, bacteria may also be found in the bladder, after having disseminated

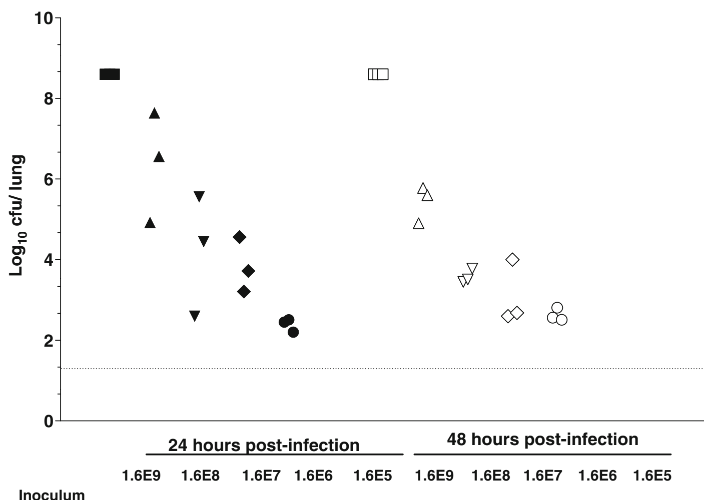

Fig. 33.4 Bacterial titers in lungs of C3H/HeN mice infected intra-nasally with *Klebsiella pneumoniae*. Mice were infected via intranasal instillation with *K. pneumoniae* in a 40 μl volume at the inoculum level shown. Mice were euthanized, lungs were harvested at 24 and 48 h, and lung homogenates plated for bacterial enumeration. Each point on the graph indicates lung counts for a single mouse. Horizontal dotted line indicates limit of detection

there from the kidneys. At a predetermined time point following infection, mice are euthanized and both kidneys removed aseptically (and weighed as necessary). Kidneys are placed in 1 ml of sterile buffer and homogenized; aliquots are diluted in buffer and plated for bacterial enumeration [23, 24].

Murine ascending urinary tract infection (UTI): An alternate method for establishing a urinary tract infection in mice involves a simple injection via the urethra into the bladder. This procedure is typically done with female mice, as the clinical infection occurs predominantly in women. In order to ensure that bacterial ascension from the bladder to the kidneys occurs during the course of the infection, and is not due to vesicoureteral reflux during the inoculation process, the volume of bacterial inoculum should be minimal, on the order of 10–40 μl [16]. Some bacteria will naturally ascend to the kidney whereas others, in this model may not; this must be determined empirically by investigators. The bacterial inoculum is prepared (typically a concentrated suspension so as to deliver ~10^8 cfu/mouse) and loaded into a 1 cc syringe with a 30 G needle fitted with polyethylene tubing, 0.61 mm outer diameter ×~2.5 cm long [25]. To better ensure direct bladder inoculation, slightly longer tubing may beused; shorter sections of tubing will deliver bacteria intraurethrally, and bacteria may then migrate to the bladder [16]. For this procedure, the mouse is anesthetized and placed on its back; the papilla is grasped on one side carefully with tissue forceps and pulled slightly away from the abdomen (for photographs of the procedure, see ref. [16]). The tubing is inserted ~1.0 cm into the urethral opening and the bacterial inoculum is slowly injected. It is useful to gently

express urine from the bladder prior to infection. The tubing is withdrawn and the mice are allowed to recover from the anesthesia, and at predetermined time points, mice are euthanized and bladders and kidneys are harvested for weighing, homogenization, and bacterial enumeration.

For some bacteria, it has been demonstrated that a glucose diuresis regimen enhances bacterial colonization of the bladder and kidneys [26–28]. This colonization is achieved by removing all but 1 g of food per mouse per day from the food hoppers and replacing the water with water containing 5% glucose. The mice are kept on this regimen for up to 1 week prior to infection; no overall decrease in weight or activity is observed in these animals over this time. After the infection, the animals are continued on the 5% glucose regimen through the end of the study.

Thigh abscess Infection: A commonly used soft-tissue infection model is the murine thigh abscess model. This model is initiated by a direct intramuscular injection of bacteria into the thigh muscle. The resulting abscess remains fairly localized throughout the course of the infection; some organisms can cause the skin around the thigh to become necrotic after several days, at which point the study must be terminated. This model has been used with a broad variety of Gram-positive and Gram-negative bacterial pathogens, including *S. aureus*, *S. pneumoniae*, *M. tuberculosis*, *P. aeruginosa*, *K. pneumoniae*, and *E. coli* [16]; due to its technical facility, its reproducibility and applicability to both *in vivo* efficacy and pharmacokinetic/pharmacodynamic evaluation. The importance of this model rests in its ability to demonstrate whether a compound is able to penetrate tissues at high enough levels to clear an infection. Depending on the organism and/or goals of the study, mice may or may not be immunosuppressed with cyclophosphamide; immunosuppression focuses the efficacy squarely on the antibiotic tested without assistance from the immune system. The typical study using this model lasts 24 h from the time of challenge; one or two doses of drug may be given in that time period to evaluate efficacy (usually within the first 8 h of infection), or a dose fractionation paradigm may be used whereby the same total dose of compound is distributed over one to eight doses within the 24 h period [29]. At predetermined time points, the mice are euthanized and thigh tissue is harvested, homogenized, and titrated for bacterial enumeration.

Since the infection remains localized, the mice may be infected in both thighs either with the same or different pathogens, allowing duplicate sampling and reducing the numbers of mice used. Additionally, infecting with two different organisms allows the investigator to study two pathogens within the same set of animals, resulting in a well-controlled study. Bacteria can attain 10^7–10^8 cfu/thigh in this model, allowing a large window to differentiate efficacious compounds from non-efficacious ones.

Otitis Media: Middle ear infections, known as otitis media, are common childhood infections that are caused by the spread of bacteria from the naso-pharynx, via the Eustachian tube, to the middle ear cavity. The resulting inflammation and infection leads to fluid accumulation and painful distension of the ear drum. The gerbil model of otitis media has been used to reproducibly model the disease as it is seen in humans.

Gerbils (also chinchillas) are appropriate surrogates, as their bullae, or middle ear cavities, are proportionately larger compared to those of other rodents, which more closely resembles the case in children. The infection in gerbils is typically established with *Streptococcus pneumoniae* or *Haemophilus influenzae* via a direct intrabullar injection under anesthesia; more detail can be found in [16]. Samples of middle ear fluid are taken after euthanization of the animals by injecting 100 μl of PBS or other diluent through the eardrum into the middle ear cavity using a 1 cc tuberculin syringe; the fluid is flushed two to three times and withdrawn for diluting and plating. *H. influenzae* infection remains localized in the middle ear, but the *S. pneumoniae* infection can disseminate and spread to the cerebral spinal fluid, causing meningitis (which also occurs in humans, but rarely). Bacteria can reach densities of 10^7–10^8 cfu/ml of middle ear fluid, and animals may eventually succumb to the infection. Accurate quantitation of antibiotic concentrations in MEF can be challenging, due to the small and variable volume of fluid contained therein. For such studies, an internal standard can be used [30].

33.6 Rat Granuloma Pouch Model

There is an inherent variability in the models described above, which is typically due to the outbred strain of mouse used and to the requirement to serially sacrifice animals at specific time points for tissue or blood sampling and cfu enumeration and/or drug quantitation. Since the same animals are not able to be followed over time, arger group sizes are used to reduce observable variability. The rat granuloma pouch model, however, offers the distinct advantage of enabling the investigator to serially sample the same animal at several time points over the course of a study; therefore, an anomalous response or time point can be easily discerned, and fewer animals can be used in a study. As will be described below, this model also gives researchers many additional options and capabilities that belie its rather simple procedural method.

The rat granuloma pouch model has been used since the 1970s, and the procedure has remained fairly consistent over the years. Rats (200–300 g) are given a subcutaneous injection of ~20 ml of air into the loose connective tissue between the shoulders. This is followed by injection of an irritant, which will initiate a localized inflammatory response in the air pocket just formed. The irritant used is generally croton oil, 0.5–1% in an inert carrier (for example, olive oil or com oil). One milliliter of the irritant mixture is sufficient to establish the granulomas. The rats are maintained for 7–10 days at which time a bacterial inoculum is injected into the pouch (it may be necessary to remove some air and/or exudate at this point if the pressure is too great). The number of bacterial cells, and the need for an adjuvant such as Brewer's yeast must be determined for each strain examined. Depending on the organism, the pouches can remain intact for 5–30 days, and small volumes can be withdrawn from the pouch even twice per day. Many bacterial pathogens have

been used in this model, including *Pseudomonas aeruginosa* [31, 32], *Staphylococcus aureus* [32–35], *Escherichia coli* [32, 35, 36], and *Klebsiella pneumoniae* [37].

One limitation of other bacterial burden models has been that bacterial counts reach a maximum level on the order of 10^7–10^8 cfu/g of tissue or milliliter of blood, and that level is either maintained for only a few days or the animal dies from the infection due to dissemination. Since it is often of interest to examine rates of resistance frequencies *in vivo*, it is desirable to attain bacterial levels higher than those mentioned; indeed, only resistance frequencies higher than one in 10^8 would in theory be detectable. The rat granuloma pouch model offers another advantage here in that bacterial numbers can rapidly reach 10^8 cfu/ml of exudate and remain at that level for up to a week or more, so that simply administering subefficacious levels of therapy should be predicted to result in the proper conditions under which resistant mutants can be isolated.

Since this infection remains localized, exudate samples also contain host factors and secreted bacterial products; it is possible to utilize bacterial mutants to study whether certain virulence factors trigger a host response, or indeed, what their effects are, as described in an early report that compared *P. aeruginosa* strains that produced different levels of secreted proteases [31]. Conversely, this system has been used to determine the effects of sub-inhibitory doses of antibiotics on α-toxin expression by *S. aureus in vivo* [34] as well as how the host responds by cleaving α-toxin with an elastase produced by neutrophils. This model has also been used to examine characteristics of bacteria that are important for survival in the host. Strains of *E. coli* with different levels of sensitivity to serum had correspondingly different abilities to survive and grow in this model [36]; but there was also an inoculum dependence seen in that higher inocula could in some strains overcome the serum sensitivity, indicating that there is a threshold number of organisms above which the host cannot control.

Different agents and therapeutic regimens for the same agent can easily be compared in this model and pharmacokinetic – pharmacodynamic relationships examined. Exudate samples can be analyzed for both bacterial numbers and drug concentrations, and the pharmacokinetics of a new agent can be monitored to determine drug levels needed for bacterial eradication [32, 33], or to determine how long or whether bacterial rebound occurs once therapy is stopped [32]. The first report of this model suggested that there was a parallel between serum drug levels and exudate drug levels for some drugs [35], but others have reported more of an inverse correlation: as serum levels decline, exudate levels increase with time [33] at any rate, the decrease in cfu achieved by a drug correlates with the drug concentration in the pouch [35]. The relationship between a drug's MIC for a given organism and the concentration achieved by the drug in the pouch is, as expected, the major determining factor in its ability to clear the bacteria. This was demonstrated using four strains of *K. pneumoniae* with different MICs to ciprofloxacin [37]. The investigators used a single dose of ciprofloxacin to treat an acute infection (dose given at 3 h post-challenge) or a chronic infection (dose given at 3 days post-challenge). The best cfu reductions were seen in the acute infection against actively-growing bacteria

with lower MICs to ciprofloxacin. Infections caused by organisms with higher MICs were more difficult to eradicate, most likely due to the inability of the drug to achieve the required concentration (determined to be three times the MIC) even with higher doses. These types of studies also help elucidate under which conditions bacteria *in vivo* are most susceptible to antibiotic effects.

The rat granuloma pouch model offers another advantage over other tissue burden models: the ability to serially sample the same animals reduces both compound bulk requirements and also the numbers of animals that are used. Since it is likely that the data obtained from this model are more consistent, it is possible that smaller group sizes would be possible as well.

33.7 Importance of Control Groups

Whether inbred or outbred strains are used, models of infection can demonstrate significant variability, depending on the model, the organism, and the host. It is often assumed that parameters such as LD_{50}s and PD_{50}s are absolute; that these values are consistent through multiple studies. This is unfortunately not true, as any practitioner in the field will attest to a formerly reliable pathogen suddenly "losing" virulence such that the inoculum size must be increased. It is important that the investigator control as many variables as possible (Table 33.2), for example, using the same age/weight of animals from the same vendors, and staying within the same timeframe of arrival. The stresses of travel can cause increased variability in the animals' response to infection, so it is a good idea to allow a period of acclimation, generally 2–7 days, before any procedure is undertaken. The preparation of the bacterial inoculum should also be standardized as much as possible. Different organisms are optimally grown on plates or in broth overnight, with or without 5% CO_2, and with or without aeration. A careful preliminary study to determine which growth conditions are most conducive to bacterial infection should be undertaken, and that method should be strictly adhered to. One simple way to standardize inocula is to prepare a stock culture that can be frozen at −80°C; the stock is then simply thawed and diluted in preparation for a study. Several times per year it is wise to check on the viability of these stocks to ensure that the number of CFU remains

Table 33.2 Control groups for in vivo infection studies

- Positive and negative treatment control drugs
- Uninfected group
- Infected, untreated group
- Vehicle control group
- Inoculum titration – tenfold higher and lower
- Inoculum test for contamination
- Vehicle test for sterility

consistent over time. This method may not be easily applied to all pathogens, but can facilitate study set-up where possible.

No matter the method of inoculum preparation, at some point either before or very soon after the infection procedure has been performed an aliquot of the inoculum must be diluted and plated in order to verify the actual bacterial counts contained therein. This step also serves as a check for the scientist to determine the homogeneity of the inoculum, to see whether it is the correct, desired organism or whether it is contaminated with another pathogenic or non-pathogenic organism. Plating of this titration on discriminating media such as Tryptic Soy agar containing sheep blood (to detect hemolysis), MacConkey agar (to select for enteric bacteria) or antibiotic-containing media to maintain selection of a recombinant organism, can aid in determination of the presence of contaminants in the infecting bacterial suspension. If the inoculum preparation is not pure, or the cfu count is lower (or higher) than desired, the infection study will be compromised, so all of the animals unfortunately must be euthanized, as it is not until the day after the infection has been established that the inoculum check can be interpreted. That is one of the more frustrating realities of bacterial challenge models.

In designing a study, it important to consider what controls are needed; if the goal of the study is to evaluate the efficacy of a novel test compound, the question is not how well does this agent perform, but how well does it perform relative to a known agent. If the novel drug is a member of an existing class, the comparator should also be in that class. If not, then the researcher may choose an agent with known efficacy against that organism in that model. A negative comparator agent should be included in the study as well, as presumably the new compound bears some feature such as improved pharmacokinetics or potency against a resistant organism, and it is important for the novel agent to demonstrate superior in vivo activity.

One control group that may seem unnecessary but can be very valuable is a group "infected" with the vehicle only. That is, the broth or PBS or other diluent in which the bacterial inoculum was prepared. An unexpected result in the study could signal some contamination of the inoculum preparation; if the animals injected with the vehicle alone succumb (which they should not), it is likely that there was an issue with the vehicle. For this reason, it is also valuable to plate an aliquot of the vehicle as a sterility check.

Perhaps most important for efficacy studies, is the uninfected control group, as these animals will receive the infection but no treatment. No matter whether the model is a protection test or a tissue burden model, all of the animals receiving treatment must be compared to an untreated control set of animals. For protection tests, this group can be monitored for the number of lethalities, the time over which these occur, and then compared to animals on therapy. For tissue burden models, the infected, untreated control group offers the amount of bacterial replication that has occurred during the study (ideally higher than what was initially inoculated) and a means of determining the cfu decrease that has occurred during therapy.

33.8 Alternate Models

33.8.1 Surrogate Hosts for Infection Studies

Many investigators have attempted to simulate host-pathogen interactions *in vitro*, for example by using cell lines, or modeling the interplay using surrogate infection models such as *Caenorhabditis elegans* or silkworms [38–42]. The literature contains many reports of the discovery of bacterial virulence factors that have been identified via such assays; the studies are typically extended to include evaluation in mouse infection models. Given that there is not a simple one-to-one correlation between factors identified in surrogate or *in vitro*, these models are generally of limited value for antibacterial drug discovery.

Some enthusiasm has been generated for the use of invertebrate or plant species as surrogate infection models for which to study bacterial pathogenesis. This line of research involves some genetically-tractable invertebrates such as the nematode *Caenorhabditis elegans* [43], the fruit fly *Drosophila melanogaster* [44, 45], the moth *Galleria mellonella* [46], the silkworm *Bombyx mori* [47], the plant *Arabidopsis thaliana* [48], and more recently, the vertebrate zebrafish *Danio rerio* [49]. In a surprising demonstration of the host range and spectrum of disease caused by *Pseudomonas aeruginosa*, it has been reported that this pathogen can infect mice in a burn model, can kill *C. elegans* when used as a food source, and can cause lesions in an *A. thaliana* leaf infiltration model. Subsequent experiments demonstrated that a subset of *P. aeruginosa* virulence factors is required for pathogenesis in all three models [48]. Other Gram-negative pathogens that have been shown to have lethal effects when fed to *C. elegans* include *Salmonella typhimurium*, *Serratia marcecens*, and *Burkholderia pseudomallei* [43, 50]. More recently it has been shown that *C. elegans* is also susceptible to killing by Gram-positive bacterial pathogens, including *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Enterococcus faecalis*, implicating specific proteins as being important for this pathogenesis [42, 51]. Lethality in the *C. elegans* model appears to be organism- and medium-dependent [52]. Despite this, it is likely that a major utility of the worm model would be a greater understanding of highly conserved and universal virulence factors, as mutant bacteria can be rapidly screened.

A major drawback to applying these invertebrate models to drug discovery is the inability to assess antibiotic efficacy in a meaningful way. Along those lines, it has been reported that when silkworm larvae (*Bombyx mori*) are injected with *S. aureus, P. aeruginosa*, or *Vibrio cholerae* the larvae die within 2 days [47]. This observed killing is inoculum-dependent, involves bacterial replication, and can be treated effectively with antibiotics. Indeed, it has been shown that infections in this model with sensitive *S. aureus* strains can be cured by a variety of antibiotics whereas drugs such as ampicillin and oxacillin failed against methicillin-resistant *S. aureus*. Issues of pharmacokinetics cannot be addressed using these surrogate models, but their possible use to rapidly screen large numbers of compounds in small quantities may prove fruitful.

Given that the host plays a role in the infection process as well, these models present an opportunity to genetically identify factors important for this interaction. Humans mount two distinct responses to invading organisms, the innate and the adaptive immune responses [53]. The former is rapid, non-specific and is primarily triggered by bacterial lipopolysaccharides; whereas, the latter, the adaptive immune response is more delayed, inducible, and far more specific. The insect models discussed above share common features with the innate immune system of humans, specifically the Toll family of receptors, which activate a cascade of intracellular signaling in response to certain pathogen-elicited molecules [54]. It is possible that insect and other surrogate models could elevate our understanding of this response.

33.9 Resistance

A major driver behind the need to discover novel antibacterial drugs is the ability of bacteria to develop resistance to an agent. Depending on the drug's target and the organism, this can occur easily and without any adverse impact on the pathogen's ability to cause disease [55]. Rapidly bactericidal drugs are more likely to prevent the emergence of resistant organisms, provided they achieve the necessary concentrations *in vivo* [56]. For example, if the rate of resistance to an antibacterial agent is 10^{-7} and a tissue site contains $>10^8$ organisms, then it is possible that resistance will arise during treatment. For this reason, it is important to dose antibiotics at a level that will not only eradicate the organisms, but will also prevent the emergence of a resistant population [57, 58]. This drug level and the mutant prevention concentration (MPC) relates to the MIC of the least susceptible organism in the population; this can be difficult to achieve *in vivo*, and it must be balanced by the potential for toxicity [57]. The requirement for such high doses for antibacterial drugs sets this therapeutic area apart from most others.

With the goal of bacterial eradication, it is important to consider the infection site where the bacteria reside as the primary target for optimizing drug concentrations. For tissue-based infections, plasma concentrations of drugs can be misleading, as different classes of drugs penetrate tissues to significantly different degrees [2, 59]. The ability to determine tissue concentrations of drugs over time and to correlate this with bacterial burden reduction and dosing regimen is the unique remit of animal infection models. Such information is difficult to obtain in patients without an invasive procedure, so such correlations must often be extrapolated from a clinical improvement. A positive response from the patient, known as clinical cure, is differentiated from the more tangible bacteriological cure that is more readily determined in *in vivo* models [2]. But here too, antibacterial drug concentrations can be misleading, as the methods by which these determinations are made involve homogenizing whole tissues such that intracellular and extracellular concentrations, or drug levels in different compartments within tissues, cannot be distinguished [1]. For pneumococcal pneumonia, drug concentrations measured from whole lung homogenates do not account for drug levels in the epithelial lining fluid, which are

all-important for this infection [59]. This is key because in order to eradicate bacteria and prevent resistant mutant emergence, the only drug concentration that matters is that to which the organism is exposed.

As opposed to the static nature of many *in vitro* tests of potency (e.g., the MIC), the concentration of a drug in the animal, and within tissues and compartments, fluctuates over time [1, 2]. The dynamic nature of drug exposure to bacteria in the whole animal contributes to resistance emergence, because as the drug concentrations drop below the MIC, any surviving bacteria can continue multiplying. Optimization of dosing can limit the outgrowth of organisms (as does the immune system), and here animal models can also be used to evaluate different doses and regimens for maximum efficacy and resistance suppression. As more clinical data are collected, it is hoped that a stronger correlation between preclinical species and humans can be established.

33.10 Use of Non-Invasive Imaging to Monitor Infection

The last decade or so has seen the development of technology that aims to generate real-time, serial information on infection and therapy in a small set of animals, typically mice. The technology relies on construction of a mouse-pathogenic bacterial strain carrying a stable gene encoding a bioluminescent reporter [60]. Bioluminescence occurs naturally in several species and genes, for at least three so-called luciferases have been cloned to use as tools for gene expression and infection imaging studies [61]. Since some wavelengths of light are able to pass through tissue, bioluminescent bacteria can be detected in a living animal during infection.

Much effort has been put forth to demonstrate that the bioluminescence expressed by the bacteria correlates with the number of viable, metabolically active bacteria present both *in vitro* and *in vivo*. Rocchetta, et al., [62] performed a set of careful studies aimed at correlating bacterial counts with luminescent signal *in vitro* and *in vivo*. The *in vitro* results, those of MIC and MBC determinations as well as growth curves, correlated very well with the traditional methods. Depending on the time point and antibiotic treatment, there was somewhat more of a discrepancy between cfu counts and bioluminescence for the *in vivo* results. The *in vivo* correlation, while good, seems to depend on the organism, treatment, and model being employed. Perhaps improvements in echnology can help overcome these differences thereby lending more confidence to this approach.

This system has several advantages over traditional methods of performing *in vivo* infection models for evaluating antibiotic efficacy and studying infection: it can be utilized with clinical strains of bacteria as long as they are genetically manipulable; it can save the investigators' time by abrogating the need to harvest and prepare tissue samples for bacterial quantitation; it allows for serial real-time monitoring of the same animals, with the potential to reduce variability of data points; since the whole animal is imaged, bacterial dissemination to other, even unanticipated tissues, can be detected; it enables one to compare the virulence of different

bacterial mutants; and the effectiveness of therapy can be followed over time and in a smaller set of mice, thereby reducing compound bulk requirements [61, 63]. Of course, the main advantage is the anticipated reduction in the numbers of mice needed for *in vivo* efficacy studies. This technology has been applied to a broad range of bacterial pathogens, including *S. aureus*, *P. aeruginosa*, *S. typhimurium*, *S. pneumoniae*, *S. pyogenes*, and *Listeria monocytogenes* [60–64]. It has been demonstrated in the more routinely used pneumonia and thigh abscess models [62, 64] and also in an *in vivo* biofilm model [65].

There are definite caveats to the broad application of this technology in its present state. To ensure bacterial counts are attributed to the proper organ, it is wise to image tissues from animals following necropsy, particularly in cases where the initial infection was not tissue-directed [61]. Another limitation regards the requirement for oxygen for some bioluminescent reporters to the extent that bacteria in hypoxic or anaerobic microenvironments may not be accurately imaged [63]. Additionally, if a study involves treatment with an antibiotic that inhibits bacterial protein synthesis, the luminescent signal, which depends on protein synthesis, will be blocked, likely affecting quantification. The limit of detection, depending on the organism and infected tissue, may also reduce the utility of this approach [62]. As this technology improves, however, it should be possible to apply it more routinely, which can increase confidence by generating more consistent data, and reduce the numbers of animals used overall.

33.11 Caveats to Animal Models

Given the difficulties inherent in evaluating microbiological efficacy in patients, animal models represent a rich source of information regarding the progression of infection, the tissue distribution of drug, the ability of a drug to promote survival, or bacterial eradication within a tissue, all of which give more detail as to how and whether a drug is working, than simply whether the patient "feels better." With these significant benefits, it is easy to think that animal infection models can supply all the answers an investigator requires in order to make predictions about how that drug will behave in humans. That is obviously not the case, since there are so few new antibacterial agents in clinical development or on the market [66]. Foremost among the reasons for termination of clinical development for an antibacterial compound is human PK; in addition, due to the high doses needed in this area to both eradicate organisms and to prevent development of resistance, safety is also a major concern.

Why should this be? Animal models allow us to determine a great number of details regarding infection and treatment, so how could so much information be misleading? Obviously, rodents share only a few traits with humans, and among those from which the two species diverge, metabolism and immune response account for the major differences leading to drug failure in clinical trials. The pharmacokinetic differences between rodents and humans make it very difficult to translate animal model data to predictions for humans; the rapid clearance exhibited by

rodents is a major issue [67]. Both the innate and adaptive immune system activation and response to infection differ between mice and humans; the complexities of these systems mean that there is no feasible strategy to minimize them [68]. Perhaps the more reliable information that can be gleaned from animal models of infection with regard to translation to humans is the tissue distribution of a drug and the concentration of the drug that is required for bacterial eradication; the limitation here is the differences in protein binding (which limits the amount of drug available to the bacteria) between the species.

Likewise, in many cases, pathogens have evolved to prefer one host over another so that bacterial pathogens of humans are not as capable of infecting other (i.e., rodent) hosts. For this reason, it is sometimes necessary to passage organisms through a mouse, or immunosuppress the mice prior to challenge [1]. These methods skew the advantage more in favor of the bacteria thereby allowing the infection to establish.

Finally, the differences between people and the comorbidities exhibited by patients with severe infections, cannot be modeled in animals. The patient's age, genetics, immune status, other pharmacologies, and other diseases all contribute to a drug's pharmacokinetics and thus a patient's outcome. Without animal models we would have no information about a drug, except that is it capable of killing bacteria in a test tube; so as long as the limitations of these models are clear, we should be able to use them to our advantage to derive the correct information and make the most informed predictions about potential drug candidates.

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Chapter 34
In Vivo Pharmacodynamic Modeling for Drug Discovery

Jared L. Crandon and David P. Nicolau

34.1 Introduction

The use of animal infections models to assess antimicrobial efficacy is hardly a novel science. This practice dates back many decades, and, as might be expected, an exorbitant number of infection models in many different species have been employed over that time. Some of the more popular infection models include the following: thigh infection, lung infection, meningitis, sepsis, and urinary tract infection. Entire textbooks have been devoted to describing these models methodologically, and to do so again is not within the scope of this chapter. The purpose of this chapter, however, is to discuss some of the important concepts involved in developing any one of these models of infection to create meaningful data that can then be used to better characterize antimicrobials pharmacodynamically and ultimately to understand how these findings can translate to the clinic.

34.2 Model Development

Development of a reliable animal infection model or adoption of a well-published model within a new laboratory can be a rather daunting task. If one considers the number of steps required to start with a healthy animal and end with a reliable

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endpoint, then it is no wonder these studies are performed in a relatively small number of laboratories. As an example of this, consider the number of steps and potential for errors encountered in one of our commonly used neutropenic murine models of infection, the Staphylococcal lung infection model. The first step is selection of an appropriate mouse strain to be infected. In this instance, not just any mouse will do. During initial attempts at the model, we found that the outbreed ICR mice we most commonly use would not establish as reliable an infection as the inbreed Balb/c strain (Nicolau and Crandon, unpublished data 2007). Once the appropriate animals have been obtained, they must next be rendered neutropenic. For this, mice are weighed, a proper weight based dose calculated, and in the case of cyclophosphamide, each mouse must receive an intraperitoneal injection of the drug. This procedure must be repeated again 3 days later to fully induce neutropenia [1]. Once immunosuppression is established, mice must then be inoculated. First and foremost in this process is the selection of the actual isolate that will be studied. In our experience, a certain percentage of isolates are clearly not as pathogenic as others and do not establish as predictable an infection as would be required for research studies. As such, before moving forward with a pharmacodynamic study in a large number of animals, it is prudent and our current practice is to conduct sufficient growth control experiments for each prospective isolate. The physical process of inoculation is a technical skill garnered through rather extensive training, especially for lung infection. Some groups have had success with direct intra-tracheal [2] or intranasal [3] inoculation and others, like us, instill the inoculum into the buccal cavity and block the nares of the mouse to induce aspiration [4, 5]. The goal of this procedure is to assure that each animal is receiving approximately the same amount of the bacterial inoculum and thus the same general number of bacterial colonies. For this reason the inoculum itself can also play a vital role in the reproducibility of infection models. For many isolates, small changes in the bacterial density of the inoculum can result in either inadequate infection or acute morbidity, not to mention the potential effects inoculum can have on therapeutic outcomes as discussed below. For this reason it is also important to consider the time required to inoculate the lot of animals needed for a given project and to consider how long a prepared inoculum remains confidently at a given bacterial density. After inoculation, a predefined amount of time must pass before antibiotic treatment is initiated; this is an important concept and will be discussed later. For antimicrobial administration, animals must again be weighed and an appropriate dose calculated; then the dose must be administered through one of the various routes (i.e., oral, subcutaneous, intraperitoneal, intravenous). Dosing must then continue for the duration of the study. The number of doses varies greatly by study, but each dose must be reliably administered to all animals, which creates a high potential for error in a less seasoned laboratory.

The next potential area of error involves the harvesting and processing of the sample, in this case the lungs. For this each animal must first be euthanized and the lungs removed under sterile conditions. This is a multi-step procedure that introduces the potential for contamination with each step. Once the lungs are harvested

and placed in sterile water, they must be completely homogenized; again under sterile conditions. Of note, lack of complete homogenization could falsely decrease the recovered bacterial density, which is another easily overlooked source for error. Next, this homogenate must be sterilely filtered to rid of any tissue particles that may hinder the accurate dilution of the sample. Serial dilution is the next step in the processing of a sample and could also greatly impact the results of a study, if it is done incorrectly or without proper quality control measures. Next, diluted samples must be plated on an appropriate media either by hand or through use of a machine (i.e., spiral plater) to allow for bacterial enumeration, while controlling for potential antibiotic carryover. The last step in turning a tissue sample into a useable endpoint and another potential source of error is in counting the number of colonies found on each of the agar plates to determine the bacterial density within the lungs of each mouse. While the preceding description illustrated the steps involved in conducting a pharmacodynamic study, a similar multi-tiered description of pharmacokinetic analyses could also be created. Aside from the ability to procedurally establish a reliable infection in animals, there are also a number of other concepts that must be considered to produce meaningful data and critically evaluate it.

34.3 General Concepts

34.3.1 Inoculum Effect

As an *in vitro* phenomenon relating to susceptibility testing, the inoculum effect is defined as the observation that the minimum inhibitory concentration (MIC) of a given antimicrobial increases when a higher than standard inoculum is used. Not all agents fall victim to *in vitro* inoculum effects, but it has been most commonly reported among the β-lactam class [6]. It is unclear how these effects translate *in vivo* or their relevance within the clinic. Data generated in animal models by our group and others showed no significant effects on exposure-response targets of cefepime when extended-spectrum *B*-lactamase (ESBL) producing Enterobacteriaceae were tested as standard versus high inoculums [7, 8], despite these effects being present *in vitro* [9, 10]. Yet, an *in vivo* analysis of the fluoroquinolone marbofloxacin against *Escherichia coli* showed increased AUC/MIC exposures were required for high inoculum relative to standard [11], an observation similarly noted *in vitro* for other members of the fluoroquinolone class [12]. Of note, it was observed during the *in vivo* analysis that the use of a high inoculum resulted in the selection of resistant mutants, which was likely responsible for the findings [11]. In light of these conflicting data, it seems prudent to note the bacterial density of the inoculum when comparing the results of similar *in vivo* analyses and further highlights the importance of consistent inoculum preparation when conducting these types of studies.

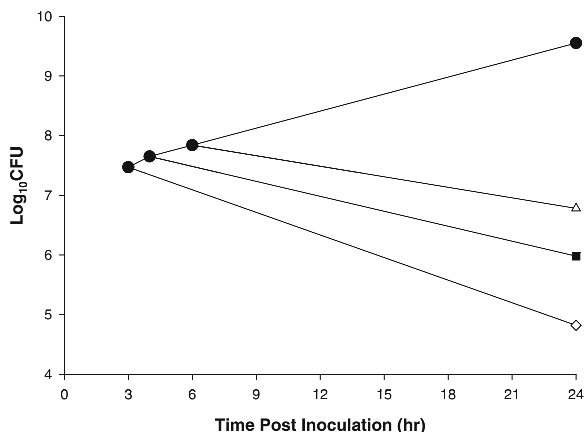

Fig. 34.1 Comparative efficacy of tigecycline 25 mg/kg given to *A. baumannii* lung infected mice 3 h (*open diamond*), 4 h (*closed square*), or 6 h (*open triangle*) after inoculation as compared with untreated control mice (*closed circles*) (15)

34.3.2 Timing of Antimicrobial Therapy

Another important concept to consider when comparing the results of similar analyses or developing an infection model is the timing of the initiation of antimicrobial therapy relative to inoculation. Namely, after animals are inoculated, a certain amount of time must pass to allow the establishment of the infection before antimicrobial therapy is initiated. As a general rule, the incubation time is inversely proportional to the efficacy of the antimicrobial agent. Depending on the study endpoint, as the time to initiation of therapy is decreased, a non-treatment related decrease in overall mortality [13] or bacterial burden [14] could result. During the development of our *Acinetobacter baumannii* murine pneumonia model, we noted this effect in mice given 25 mg/kg of tigecycline starting 3, 4, or 6 h after inoculation [15] (Fig. 34.1).

34.3.3 Evaluable Endpoints

One advantage of *in vivo* analyses in comparison with *in vitro* studies is the multitude of potential endpoints available to evaluate drug therapy. If the chosen infection results in mortality, then the proportion of animals surviving after a given time can be plotted against exposure or dose to develop an exposure/dose-response relationship. Given the ethical issues associated with mortality studies, many investigators have resorted to evaluating signs and symptoms associated with morbidity as a marker for mortality to reduce suffering in the animals. This can be troublesome as it requires extensive monitoring and may introduce subjectivity into the study if animals that may have survived for many more hours were sacrificed secondary to perceived morbidity. It should also be noted, that mortality observed in an animal model is a very complex process, with bacterial burden being just one of the many

factors involved (i.e., organ dysfunction, host factors, physical stress, etc.) again complicating the findings of such analyses.

Probably the most commonly evaluated endpoint *in vivo* is bacterial burden within tissue or fluid at a given time point. The use of this endpoint eliminates subjectivity and allows for detection of subtle changes in bacterial efficacy across a dosing range. Aside from the primary production of a dose-response curve, this method also allows analysis of other measures such as determination of whether a drugs activity is likely static or cidal, assessment of the emergence of resistance, or determination of a required exposure for some magnitude of effect (i.e., stasis, 1 log decrease, etc). Further, assessment of bacterial burden over time instead of a single time point (i.e., 24 h) affords the ability to construct time-kill curves similar to those often developed *in vitro*. These types of analyses also allow the evaluation of the potential post antibiotic effect. However, unlike *in vitro* studies where a single apparatus can be sampled over time, the attainment of a sample *in vivo* usually occurs though terminal sampling and thus requires a large number of animals.

Another interesting endpoint that has gained popularity in recent years is assessment of the immunomodulatory effects of antimicrobial agents. For these studies, concentrations of cytokines and/or chemokines can be analyzed in various fluids or tissues to ascertain potential host modifying effects that may exist exclusive of antibacterial activity [16, 17]. Often, these measures can be evaluated in addition to studies of bacterial burden to add an extra dimension to efficacy studies.

34.3.4 Immunocompetent Versus Immunocompromised Models

As might be expected, the development and implementation of an immunocompromised animal model is far easier to execute than an immunocompetent model. And unquestionably, the immunocompetent model results in a greater degree of variability than the neutropenic model. However, studies incorporating the innate immunity of the host may better approximate infections within humans, the majority of whom posses a competent immune system. This observation was highlighted in a study performed by our group in which tigecycline pharmacodynamics were evaluated against *E. coli* and *Klebsiella pneumoniae* in both the neutropenic and immunocompetent thigh infection models [18]. When comparing our results to that of a pharmacodynamic study in humans, we found that while the pharmacodynamic targets found in neutropenic mice over predicted human derived *f*AUC/MIC targets, results in competent mice were in line with human findings [19]. While the best correlate to humans is a debatable topic, it is reasonable to suggest that the immunocompetent animal model represents the best-case scenario for a given antimicrobial while the neutropenic model represents the worst-case scenario (pure interaction between bug and drug); knowledge of both ends of the spectrum are advisable. As a further advantage, the ability to induce infection in an immunocompetent animal also allows the examination of other aspects of the host-infection relationship that may be suppressed along with the neutrophils in a neutropenic model.

34.4 In Vivo Pharmacokinetics

One of the primary objectives of *in vivo* pharmacodynamic modeling is to relate some measurement of exposure to one of the evaluable endpoints mentioned above. The ability to accurately accomplish this task relies heavily on the quality of the pharmacokinetic analyses conducted for a given antimicrobial agent within the test species. Further, as more and more developmental compounds rely on data gathered during preclinical studies to tailor clinical studies, it cannot be overemphasized how important accurate pharmacokinetic analyses are in drug development. In addition to the considerations provided below, one must also take into account the potential for *in vivo* drug-drug interactions (i.e., cyclophosphamide or uranyl nitrate) with the target drug profile especially if variations in the model (immunocompetent vs. compromised) and/or dose of the interacting substances will be altered in the same experimental series.

34.4.1 Animal Selection

The approach we take and recommend to others when selecting animals for pharmacokinetic analyses is to conduct all studies in animals infected exactly as those used during efficacy portions of the study, preferably in the hands of the same investigator. The last point may seem excessive, but we have had occasions where previously generated pharmacokinetic data gathered from other groups varied greatly from those gathered within our laboratory with only slight variations in handling procedures (Nicolau and Crandon, personal communication, 2011). As mentioned, it is important that all pharmacokinetic studies are conducted in infected animals of the same strain, preferably with the actual infection to be studied (i.e., thigh, lung). The importance of this is highlighted in a study we conducted comparing the pulmonary penetration of tigecycline in mice infected with either the thigh or lung infection models [20]. As shown in Fig. 34.2, we found that although serum concentrations were similar between the two infection models, mice infected in the pulmonary compartment had much greater epithelial lining fluid concentrations than those with infections confined to the thigh. As such, if the pulmonary pharmacokinetics of lung-uninfected mice were used to establish exposure-response relationships within the lungs of infected mice, a gross under estimation would result.

34.4.2 Sampling

In the purest sense, a pharmacokinetic study is simply the analysis of drug concentrations in a fluid or tissue over time. While seemingly simple, selection of appropriate sampling times and procedures are imperative to developing robust

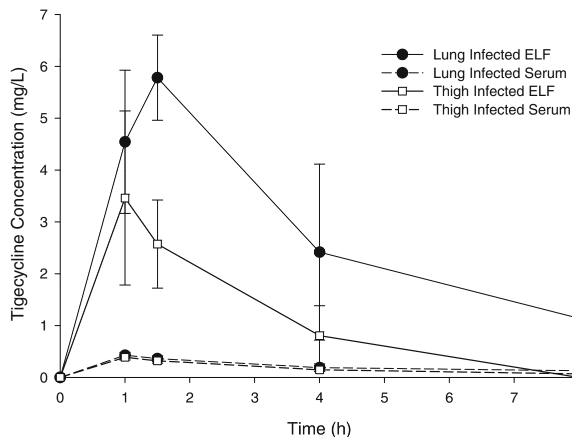

Fig. 34.2 Comparative ELF penetration of tigecycline 25 mg/kg given to lung infected and thigh infected mice [20]

pharmacokinetic models. In larger animals, such as rats and rabbits, the ability to sample a single animal over time using an indwelling intravenous or intra-arterial catheter makes development of an individual pharmacokinetic profile very similar to those used in humans. Another advantage of conducting pharmacokinetic studies via this method is the ability to collect both pharmacokinetic and pharmacodynamic data from the same animal. Unfortunately, while pharmacokinetic data are easily attainable, robust pharmacodynamic data sets in these models can be very costly and are thus often limited to only a few observations. For mice studies, pharmacokinetic blood sampling is a terminal procedure with the most prolific procedure being cardiac puncture in euthanized animals. For this reason, groups of mice must be sampled at each time-point and a composite pharmacokinetic curve must be constructed. There is no steadfast rule as to the number of mice required per group. But, given the variability noted in these analyses, we have adopted six mice per time-point. A similar but alternative approach would be to construct a population pharmacokinetic model using data from many single data points.

While the retro-orbital sampling technique has also been advocated as a mechanism to reduce the utilization of animals in pharmacokinetic studies, its limitation on the number of samples over the dosing interval and the low volume of resultant biological matrix recovered reduce the viability of this approach.

When designing a pharmacokinetic study, one important consideration is the selection of sampling times. To fully characterize the pharmacokinetics, it is typically recommended to sample for at least a couple of half-lives, if possible. Given that many animal studies are evaluating novel compounds, these data are usually unavailable to guide sampling time selection. Thankfully, as a general rule, the half-life of most drugs in small animals is very short and rarely requires longer than 24 h sampling. Some have purposed the used of d-optimality criteria to minimized the number of sampling times [21]. However, given the rapid pharmacokinetics noted in small animals, there is not much room for vast reductions in sampling times and thus these methods are rarely employed.

34.4.3 Drug Concentration Determination

The analysis of drug concentrations within the blood, fluid, or tissue of an animal can be determined by a number of different methods, ranging from high performance liquid chromatography (HPLC) to microbiological assays. Secondary to its sensitivity, accuracy, speed, and increasingly widespread availability, most laboratories have adopted HPLC as a primary means of concentration determination. When small sample volumes and/or very low concentrations are present, mass spectrometry is often very useful. When developing assays for any method, it is important to prepare all standards within the appropriate biological matrix (i.e., plasma, serum, tissue homogenate). When minimal amounts of the physical matrix are available, development of a cross matrix in a similar media is often used. In addition, careful consider should be given to the possibility of drug loss due to freeze-thaw procedures and non-specific binding to storage containers.

34.4.4 Pharmacokinetic Modeling

The modeling of concentration-time profiles can be accomplished through a number of accepted methods. With the luxury of a robust data set, use of compartmental modeling methods is preferred; this approach is used exclusively in our laboratory. In instances where only the terminal portion of the concentration-time profile is available, non-compartmental methods offer a viable modeling solution, although these models severely hinder the ability to further simulate data. The value of these simulations cannot be understated, as they allow calculation of the ratio between a given pharmacodynamic parameter and MIC and the ability to humanize a dose. We, and others, have adopted WinNonlin as our program of choice for pharmacokinetic modeling; however, there are a number of other programs that would work equally as well (i.e., ADAPT II, Kinetica). Further, as mentioned above, programs designed specifically for population pharmacokinetic analyses (i.e., NONMEM, NPAG) are also of use in certain situations.

Once the pharmacokinetic data are fully described and pharmacokinetic parameter estimates are determined, it is then possible to simulate concentration-time profiles for various regimens not undertaken in pharmacokinetic studies, including multi-dose regimens (i.e., twice daily). Further, these data can also be used to develop human simulated dosing regimens for pharmacodynamic studies, an approach that will be described in detail below.

34.4.5 Protein Binding

Another important issue to consider relative to the conduct and interpretation of pharmacokinetic studies is the percentage of drug bound to proteins *in vivo*. It is generally recognized that the protein bound fraction of an antimicrobial is

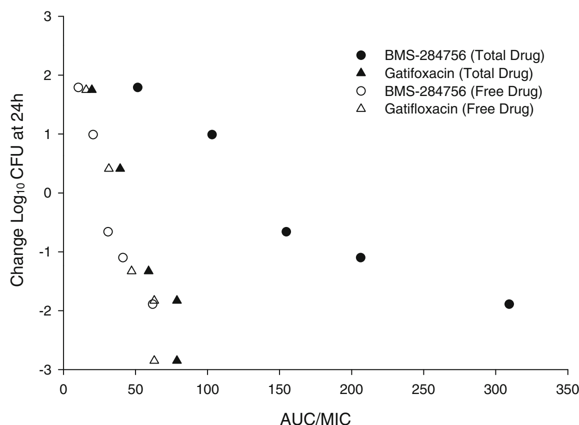

Fig. 34.3 Exposure response curves of BMS-284756 (80% protein bound) and Gatifloxacin (20% protein bound) against a single isolate of *S. pneumoniae* in a neutropenic murine pneumonia model

microbiologically inactive and thus should be accounted for during pharmacokinetic interpretation. While considerable data are available describing this phenomenon *in vitro*, far fewer studies have been conducted *in vivo* [22]. A study conducted by Merriken *et al.* demonstrated the importance of protein binding *in vivo* [23]. In that study, they compared the mortality rate of mice infected with *S. aureus* via the sepsis model given different analogs of penicillin exhibiting different degrees of protein binding (36–98%). They found that while the total drug pharmacokinetics and *in vitro* MICs were similar between analogs, there were large differences in the dose required for 50% survival, correlating with percent protein bound. We noted similar results using the *Streptococcus pneumoniae* neutropenic thigh infection model to evaluate the efficacy of two fluoroquinolones, gatifloxacin (20% protein bound), and BMS-284756 (80% protein bound) [24, 25]. As shown in Fig. 34.3, it was the free drug exposure-response relationship that predicted antibacterial killing for both agents.

Clearly, the importance of protein binding as it relates to antibacterial efficacy cannot be ignored; similarly, there are methodological considerations in conducting protein-binding experiments that also need careful attention. A number of different methods can be used to determine the protein binding of antimicrobials. The most common of these are equilibrium dialysis, ultrafiltration, and microdialysis. The premise behind each method is that protein bound drug, secondary to its increased molecular weight, will not penetrate a given semi-permeable membrane while unbound drug easily passes through the membrane. Concentrations are then measured on both sides of this membrane and the free fraction can be easily determined. There are positives and negatives to each method, and to comment on each would be rather cumbersome for this chapter. Instead, we refer you to a review article by Beer et al. for a rather thorough discussion of the pros and cons of each [22]. Regardless of the chosen method, it is imperative that non-specific binding is accounted for when performing protein binding experiments. Non-specific binding refers to the extent at which the antimicrobial binds to the semi-permeable membrane or apparatus in the absence of protein; not adjusting for non-specific binding when present could result

Table 34.1 Comparative protein binding values for various antimicrobials in mice and man [5, 26–28]

Antimicrobial	Mice (%)	Humans (%)
Ceftobiprole	79	22
Clindamycin	82	77
Doripenem	25	9
Ertapenem	95	85–95
Vancomycin	30	50

in a gross over prediction of protein binding for a given compound. When conducting *ex-vivo* studies, it is also important to consider the source of the serum that will be used for protein binding experiments. It is our practice to use only fresh blood collected from the species of interest immediately prior to the conduct of these experiments. While this practice is more costly and laborsome, we have found that use of purchased frozen serum yielded discordant results relative to freshly collected serum (Nicolau and Crandon, unpublished data 2009). Further, given the inherent complexity of the interaction between antimicrobials and protein, it is in the researcher's best interest to eliminate as many variables as possible. Another important variable to consider when accounting for protein binding *in vivo* is the potential variation in the degree of protein binding between animal species, and in some instances, strains within a given species. This becomes increasing more important when human equivalent exposures are simulated in animals as considerable variation exists for many antimicrobials between animals and humans [5, 26–28] (Table 34.1).

Most antimicrobials exhibit linear protein binding, referring to the observation that regardless of the test concentration, the degree of protein binding is equivalent over a targeted concentration range; however, there are a select number of agents in which the percentage of protein binding depends on the concentration. The later scenario is referred to as non-linear or concentration-dependent protein binding. For most agents exhibiting non-linear binding, the percentage of free drug increases as concentrations increases (i.e., macrolides, fluoroquinolones) [29, 30]. However, for other agents such as the glycylcycline tigecycline, free drug concentrations decrease as concentrations increase [31]. In order to rule out the possibility of concentration-dependent binding, it is important to conduct protein-binding studies across a wide range of concentrations. When *in vivo* pharmacokinetics have been described prior to the conduct of protein binding experiments, it is preferable to include concentrations within the purposed concentration-time profile of likely doses, always including the maximal concentrations (C_{max}). For drugs exhibiting linear protein binding, calculating the free drug concentration profile is straightforward; the entire dosing intervals for all test doses are corrected by a fixed percentage. However, the correct method to account for concentration-dependent binding is more complicated and controversial. Two general correcting methods have been utilized. The first and most widely accepted method is to correct the entire concentration-time profile by the percentage of protein binding noted at the C_{max} for the given exposure. The second potential method consists of correcting each observed concentration within a profile by the actual percentage of protein bound drug determined at that concentration [29]. We recently examined both methods of correcting concentration-dependent

protein binding in a study of tigecycline in the neutropenic thigh infection model [32]. Given the inverse relationship noted above for the protein binding of tigecycline, the later correction method resulted in a false extension in the half-life and an ultimate doubling of the calculated free area under the concentration time cure (*f*AUC) exposure when compared with the C_{max} method. Based on these results, further supported by the pharmacodynamic findings, we concluded that calculation of the free fraction of tigecycline is best accomplished by correcting the entire dosing interval by the bound percentage noted at the C_{max} [32].

Additionally, it has also been noted that some compounds such as daptomycin may have reversible protein binding (i.e., due to a low binding affinity for the protein), and thus its free drug profile may not be wholly predictive of its *in vivo* efficacy. When concerns regarding discordance between the apparent *in vivo* free drug exposure and efficacy arise, it may be prudent to fully characterize the binding affinity of the compound to its target protein.

34.4.6 Human Simulated Dosing Regimens

As previously noted, once the pharmacokinetic parameters have been determined, there are limitless possibilities to potential dosing simulations. An area of simulation that has become increasingly popular is the creation of human simulated dosing regimens. The purpose of these simulations is to create a dosing regimen in a given animal that simulates the exposure profile noted in man given a specific dose of the antimicrobial. Given the ethical issues surrounding conduct of randomized controlled trials in man, the ability to humanize doses in animals can prove invaluable during both the developmental and post-marketing stages of drug discovery. The potential utility of these studies are endless, some common examples include the following: to support the efficacy of a dose predicted using Monte Carlo simulation [27, 33], to provide evidence to suggest a susceptibility breakpoint [34, 35], to garner efficacy data to support the use of a given antimicrobial against emergent pathogens [26, 36], or to compare the efficacy of multiple agents in a head to head study [28, 37].

The humanizing of a dose in animals can be a rather daunting task, due to the marked differences between the pharmacokinetics in animal species and humans. These differences are increasingly more evident in smaller animals such as mice. Many of the differences noted between the pharmacokinetics of animals and humans can be described by allometric relationships; that is, the relationship between species dependent physiological variations and body weight [38]. As it relates to pharmacokinetics, the most prominent difference between small and large animals is that small animals eliminate drug much faster than larger animals (i.e., humans).

To overcome differences in pharmacokinetics between animals and man, one must either alter the way drug enters or exits the system. For entry, this could be as simple as giving more doses. As levels fall below a desired threshold, the agent of interest is repeatedly dosed to a target concentration throughout the dosing interval.

The actual number of doses can vary, but could easily be required upwards of every hour for the study duration. The use of electronic infusion pumps represents a viable option for slowing entry in all but the smallest animals, especially with the ability to purchase pre-catheterized animals. Using these pumps, one can either change the infusion rate of a fixed drug concentration over time or infuse the drug solution into the animal at a constant rate and change the drug concentration of the infusate with a pump controlled drug-free solution.

To affect the way in which drug exits the system, one must alter either metabolism or excretion. For drugs that are largely excreted unchanged in the urine, induction of renal impairment is a commonly employed option. Most groups, ourselves included, have had reliable results using a single dose of uranyl nitrate 5 mg/kg given 3 days prior to the initiation of infection [34]. Of note, the use of uranyl nitrate is only advised for short-term studies (i.e., 24 h), as the renal toxic effects are only temporary. For antimicrobials that are metabolized by the cytochrome P450 enzyme system, it is possible to give an inhibitor of these enzymes prior to the initiation of therapy. One such example is 1-aminobenzotriazole which is a suicide inhibitor of both hepatic and pulmonary P450 enzymes [39].

As mentioned above, it is important to account for the variations in protein binding between species when designing the human exposure to simulate within an animal. For many agents, it is not imperative that the exact free concentration-time profile is simulated. Instead, the simulation of the pharmacodynamic parameter of interest (see discussion below) as it relates to MIC is a viable substitute, and in many cases, a much easier profile to simulate. To highlight this observation,, we conducted a study comparing two simulation regimens of ertapenem 1 g every 24 h in a neutropenic mouse thigh infection model, both achieving the same percentage of the dosing interval free drug concentration exceed the MIC (*f*T > MIC) across a range of MICs[40]. The first was a simplified regimen of 50 mg/kg every 6 h while the second was a complex regimen of nine doses of varying concentrations. Against a collection of *E. coli* and *K. pneumoniae* isolates with MICs of 0.032–16 mg/L, both regimens produced similar fT > MIC over the 24 h study period and thus similar reductions in colony forming units (CFU) at the end of therapy. While this study evaluated *f*T > MIC as the exposure-response target of interest, similar observations would be expected for the remaining targets, certainly *f*AUC/MIC.

Unfortunately for some agents, the use of complex dosing regimens is required to simulate the human pharmacodynamic profile for the target organisms over the range of clinically anticipated MICs. This was the case in a recent analysis we conducted of doripenem 2 g every 8 h (3 h infusion) where simulated *f*T > MIC for isolates with MICs of 16 and 32 mg/L were 52.5% and 0%, respectively[33]. In that study, 8 subcutaneous doses were required to simulate each 8-h human simulated dosing interval, assuring the peak concentration never exceeded 32 mg/L (Fig. 34.4).

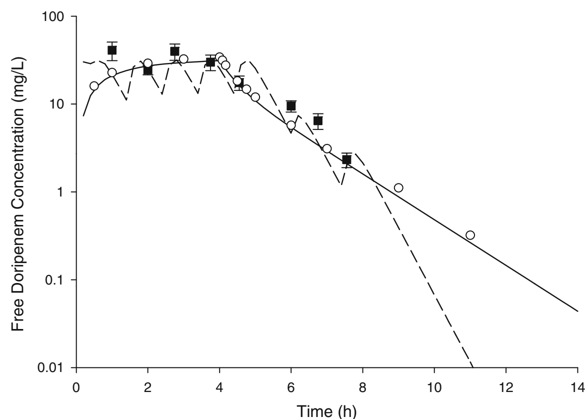

Fig. 34.4 Concentration-time profiles of human simulated doripenem 2 g every 8 h (4 h infusion) in mice (*closed squares*, *dotted line*) and man (*open circles*, *solid line*). Mice were given doses of 22, 9, 18, 18, 18, 18, 3, and 1.5 mg/kg at 0, 0.5, 1.5, 2.5, 3.5, 4.5, 6, and 7.5 h [30]

34.5 Pharmacodynamics

Pharmacodynamics is defined as the relationship between some measure of drug exposure and drug effect at its target of interest. Specifically for antibacterial agents, this is the relationship between drug concentrations and antimicrobial effects. The actual study of these effects *in vivo* can range from relatively simple to very complex analyses. The simpler approach refers to studies evaluating some marker for drug exposure (i.e., dose) while the more complex and modern approach incorporates the entire concentration-time course. The following is a discussion of the general concepts of pharmacodynamics and their study within *in vivo* models.

34.5.1 Pharmacodynamic Theory

In the treatment of a bacterial infection in patients, there are three variables that together predict the overall outcome: the host, the pathogen, and the antimicrobial agent. Of these three, the antimicrobial agent is the only modifiable factor [41]. Our ability as clinicians to modify this variable consists not only of drug selection, but also of the chosen dosing regimen. The importance of proper drug and dose selection during the initiation of care has been highlighted in a number of publications. Each noted that appropriate initial therapy resulted in decreased mortality when compared with inappropriate initial therapy [42–45]. The Clinical and Laboratory Standards Institute in the USA and the European Committee on Antimicrobial Susceptibility Testing in Europe have derived susceptibility breakpoints for each antimicrobial agent to aid the clinician in proper drug selection based on some measure of *in vitro* drug potency (i.e., MIC, Kirby Bauer disk diffusion). For some agents, however, these susceptibility breakpoints may not correlate with the actual ability of an agent to achieve adequate concentration *in vivo* and ultimately positive outcomes [46–49].

However, a complete understanding of a drug's pharmacodynamics may help develop methods to overcome these discrepancies.

The primary premise behind modern antimicrobial pharmacodynamic theory is that the shape of the concentration-time curve has a direct correlation with the antibacterial effect of an antimicrobial. The three matrixes that characterize the shape of the concentration time curve are the following: the percentage of the dosing interval that drug concentrations remain above a given threshold, AUC, and C_{max}. The microbiological effect of all antibacterial agents is determined by one or more of these parameters when combined with some measure of in vitro potency. While some have proposed other measures, the MIC typically serves as the relational comparator of *in vivo* potency. Accordingly, each of the matrixes above become: T>MIC, the ratio of the AUC to the MIC (AUC/MIC), and the ratio of the C_{max} to the MIC (C_{max} /MIC).

β-lactam antibiotics are the prototypical example of drugs that garner their antimicrobial effects by T>MIC. The activity of these agents is completely independent of concentration once levels are above the MIC; namely, increasing the concentrations many times above the MIC of the infecting organism does not result in enhanced killing. This has to do with the mechanism of action of these agents, the acylation of penicillin binding proteins [50]. Within each pathogen, there are a limited number of binding sites available. Once each of these has been saturated, addition of more drug would not gain an increase in activity. It is important to also point out, that it is not necessary that the concentrations of the *β*-lactams remain above the MIC for the entire dosing interval. The actual percentage of time required for maximal bactericidal activity varies by class with the carbapenems requiring the least (40%), followed by the penicillins (50%), and then cephalosporins (60%) [51]. In an attempt to optimize the pharmacodynamics of many *β*-lactams, the use of prolonged or continuous infusions has been used. These dosing strategies result in an increase in *f*T>MIC using similar or lesser total daily doses when compared with standard infusions [51].

The second exposure-response relationship is that of C_{max} /MIC. For agents that display this pattern (i.e., aminoglycosides, fluoroquinolones, daptomycin, and metronidazole), higher concentrations kill organisms at a faster rate and more extensively than lower concentrations. These agents typically exhibit an extensive post antibiotic effect (PAE), that is, previously drug-exposed bacteria take more time to regrow by 1 $\log_{10}$CFU/mL than do non-exposed bacteria. This ensures persistent killing even when concentrations fall below the MIC. Accordingly, the goal of dosing these agents is to maximize the peak concentrations [52]. Of note, secondary to the co-linearity between C_{max} and AUC, agents exhibiting concentration-dependent killing may also display a relationship with the AUC/MIC ratio.

There are, however, another group of compounds for which the AUC/MIC ratio is the linked pharmacodynamic parameter but they are not concentration dependent. Unlike the *β*-lactams, these agents exhibit extensive PAE and are typically either bacteriastatic or poorly bactericidal in their activity. Examples of these agents include: glycopeptides, glycylcyclines, macrolides, tetracyclines, and oxazolidinones. As expected, dosing regimens for these agents should be designed to maximize the total exposure.

34.5.2 *In Vivo Pharmacodynamics*

34.5.2.1 Dose-Response Studies

The simplest study of pharmacodynamics in an animal model is the development of a dose-response curve. After choosing one of the potential study endpoints previously discussed (i.e., change in bacterial density, mortality, etc.), one would give an antimicrobial at various doses and evaluate the relationship between dose and the chosen endpoint. Construction of the dose-response curve would then consist of the mg/kg dose on the x-axis and outcome on the y-axis. After fitting the E_{max} model [53] to this plot, one could then determine the dose required for maximal efficacy (E_{max}), the dose required for 50% of maximal efficacy (ED_{50}), or in the case of bacterial density studies, the dose required to achieve bacterial stasis [54]. In light of the previous discussions surrounding *in vivo* pharmacokinetics, penetration, protein binding, etc., caution should be exercised when comparing stasis or ED_{50} values between antimicrobials without those supportive data. However, when the pharmacokinetics are adequately described, these data can be used to provide a more complete understanding of the bug-drug interaction for a given antimicrobial agent.

34.5.2.2 Exposure-Response Studies

As discussed above, the efficacy of each antimicrobial agent is determined by the shape and time course of the concentration-time curve. The purpose of these studies is to link each matrix with a measure of efficacy across the exposure profile. To do this, one incorporates the data gathered from pharmacokinetic, *in vitro* potency, protein binding and dose-ranging pharmacodynamic studies. The first step is to simulate the free concentration-time profile for each of the regimens used during the dose-ranging studies. Linking the exposure of each regimen with the *in vitro* potency of the agent (i.e., MIC) allows the ability to calculate a value for each pharmacodynamic index (i.e., *f*T>MIC, *f*AUC/MIC, fC_{max}/MIC) for all doses analyzed in the dose-ranging studies. Finally, exposure response curves can be constructed just as was done for dose-response studies except that each pharmacodynamic index replaces dose on the x-axis. Comparing the correlation coefficient of each of these curves can then help define the exposure-response relationship most highly correlated with efficacy.

Once the most pertinent pharmacodynamic index has been identified, one can use the exposure-response curve for that index to identify specific pharmacodynamic targets. It is not clear exactly what the best reference point to define theses targets is, but some common examples included the following: the exposure required to achieve 50% (EI_{50}), 80% (EI_{80}), or 90% (EI_{90}) of maximal efficacy, or for bacterial density studies, the exposure required for stasis or some drop in CFU (i.e., 1 log or 2 logs). To create a more robust dataset, often-individual data generated from isolates over a range of MICs are combined to create a composite exposure-response curve from which targets can be identified. With the help of Monte-Carlo simulation,

the identified exposure-response target coupled with the Phase I pharmacokinetic data in humans can provide a systematic approach to dose selection for novel compounds [55].

When interpreting the results of exposure-response studies there are a few things that must be considered: the first is to emphasize exactly how much impact the MIC of the infecting organism has on the calculated pharmacodynamic parameters. Given the dilutional nature of MIC testing, a onefold increase or decrease in the reported MIC can have drastic effects on the reported pharmacodynamic target. For this reason, it has been our practice to conduct all MIC testing as a minimum of three independent tests via the broth microdilution method [56], reporting the modal MIC value. Next, it is important to note that the identified targets may not be the same for all organisms. As an example when evaluating the efficacy of third and forth generation cephalosporins against *S. aureus*, *S. pneumoniae*, and gram-negative bacilli neutropenic thigh infections, Craig and colleagues found that stasis derived T > MIC targets for *S. pneumoniae* and gram-negatives (37–38%) were greater than required for *S. aureus* (24%) [57]. Similarly, when comparing the *f*AUC/MIC targets derived for tigecycline, we found that in the neutropenic lung infection models of *S. aureus* and *A. baumannii*, EI_{80} exposures required for *S. aureus* infections were far less than those for *A. baumannii* (3.04 vs. 17.2, respectively) [4, 15]. Lastly, a vast majority of studies correlate efficacy to exposures in the serum, while for most, the infection is established in a different compartment. This may not be worrisome for drugs that exhibit similar site penetrations between animals and man, but could prove problematic if penetration is different. An exposure-response based study designed to support clinical dosing of ceftobiprole for the treatment of pneumonia serves as an excellent example [58]. In that study, median penetration into the epithelial lining fluid (ELF) of lung infected mice and healthy human volunteers was found to be 69% and 15.3%, respectively. While these differences were accounted for in the analyses of that study, if they had not been, serum derived T > MIC targets in mice would have underestimated the exposure likely required in humans.

34.5.2.3 Dose Fractionation Studies

As discussed above, there is a tremendous amount of interdependence among the various pharmacodynamic indices. Pharmacodynamic analyses utilizing dose fractionation strategies are an excellent approach to help differentiate between these indices. The design of these studies is relatively simple: a single mg/kg/day regimen is given as a single, twice daily, and four times daily dose. By design, each of the doses has the same *f*AUC while the fC_{max} is decreased and the *f*T > MIC increased as doses are given more frequently. These studies should always be conducted in a parallel, and it is best if at least two total daily dose regimens are evaluated. When selecting the dose(s) to assess, it is best to identify regimens near the ED_{50}. If the selected total daily dose resulted in near maximal or near minimal efficacy for each of the divided regimens, it would be difficult to delineate between parameters.

Of note, the conduct of dose fractionation studies can be quite difficult for agents that exhibit concentration-dependent protein binding as an equivalent total daily dose will likely not equate to comparable *f*AUCs for divided doses. If dose fractionation analyses are to be attempted for these agents, one must, through mathematical simulation, assure each dose has an equivalent *f*AUC while varying the *f*T>MIC and fC_{max} before undertaking these studies *in vivo*.

34.6 Summary

The study of antimicrobial pharmacodynamics, while not a new science, has blossomed over the last two decades. Much of the data contributing to our current understanding of pharmacodynamic theory has been gathered from research conducted within animal models of infection. It stands to reason then that these models will continue to play a vital role in the study of pharmacokinetics and pharmacodynamics for the foreseeable future. Given the importance of these models, coupled with their inherent complexity, it is imperative that we as investigators strive to understand the nuances involved in the study of antimicrobials in animals to ensure data generated during these analyses are of utmost scientific rigor, as are the interpretations of their results. By doing so, we formulate a data set that is invaluable to a drug discovery program from early in pre-clinical development to well into the post marketing stages.

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Chapter 35
Applications of Pharmacokinetic/Pharmacodynamic Models for the Development of Antimicrobial Agents

April Barbour and Hartmut Derendorf

35.1 Introduction to the Characterization of Antimicrobial Pharmacokinetics/Pharmacodynamics

35.1.1 Model-Based Drug Development

The learn/confirm paradigm for drug development, as discussed by Sheiner [29], has provided a relatively new but more efficient method for development of an investigational pharmaceutical. This model-based approach suggests the focus of drug development should be an understanding of the science rather than using empirical evidence to make decisions. While sequential drug development may be slightly more time consuming early in the process, numerous examples in literature have been cited in which model-based drug development has been used to make critical decisions that include lead compound selection, trial design, and dose selection [7, 21]. All of these examples have ultimately increased efficiency by saving time, money, and resources. The FDA has also stated its support of model-based drug development [34] and approved gabapentin for post-herpetic neuralgia, based partly on efficacy evidence provided by PK/PD modeling [21].

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35.1.2 Antimicrobial Drug Development

The techniques mentioned above can also be applied to the development of antimicrobial agents and post-approval for the evaluation and possible adjustment of recommended dosing regimens. Unique to antimicrobials, the pharmacodynamics can be extensively and accurately characterized using *in vitro* models and *in vivo* animal models with good correlation to the effect in infected patients [1] as the site of action is the same in all systems – the bacteria. In order to predict efficacy and select the appropriate dosing regimen, antimicrobial PK/PD has been traditionally related to one of three indices; the time the free concentration remains above the minimum inhibitory concentration (MIC), the maximum free concentration to MIC ratio, and the area under free 24 h concentration time curve to MIC ratio [9]. Dose fraction studies are typically performed to select the most appropriate index and magnitude of the index for each agent [2, 3, 10, 35]. Additionally, Monte Carlo simulations are performed to find the most appropriate dosing regimen or evaluate a current regimen based on the pharmacokinetic variability and pharmacodynamic variability, i.e. inter/intrapatient variability and the MIC distribution [13, 17, 18, 24]. Although these techniques have greatly improved antimicrobial development over empiric methods, there is an opportunity and means to improve the processes currently used.

35.1.3 Streamlining Antimicrobial Drug Development

Traditionally, plasma samples are used as the pharmacokinetic input in PK/PD models. It has now been widely accepted that free concentrations should be used in these models, as only free drug is active [23, 26, 27]. Also, it is important to consider the free concentration at the site of action. Although plasma may be the site of action, i.e. bacteremia, this is often not the case. For example, in regard to complicated skin and skin structure infections, it is more meaningful to determine the concentration within the interstitial space fluid of subcutaneous soft tissues, e.g. skeletal muscle or adipose tissue. This can be accomplished by using the microdialysis sampling technique, which has been proven suitable for the measurement of free ISF antimicrobial concentrations in virtually any tissue in both healthy volunteers and patients [5, 14, 31, 32].

As previously mentioned, the pharmacodynamic parameter most often used with antimicrobials is the MIC. However, there are many limitations if the MIC is used as the only pharmacodynamic parameter. Mainly, the MIC does not characterize the antimicrobial activity over time but is a static, one-point in time measurement. It is possible for an agent to display different kill-kinetics but result in the same bacterial load after a given incubation period (Fig. 35.1). The MIC also does not indicate the degree of pharmacological effect, e.g. a 1-log kill or a 2-log kill over 24 h. The MIC has a twofold variability and is somewhat subjective, as it is based on visual detection of bacterial growth. Finally, this parameter does not measure the presence or

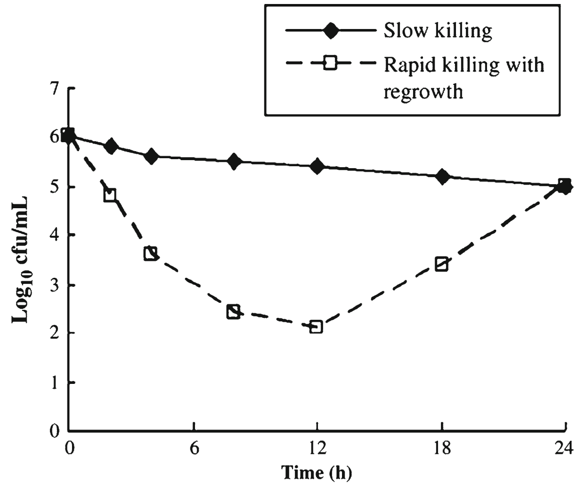

Fig. 35.1 Two different pharmacodynamic profiles which result in the same bacterial concentration at 24 hours are displayed

concentration of persistent/resistant bacteria that may be present but may not have grown to a detectable limit by the unaided eye.

Time-kill curves are an experimental technique to characterize the pharmacodynamic activity of an antimicrobial agent and circumvent many of the limitations of using the MIC as the pharmacodynamic parameter. These experiments measure the antimicrobial activity over time, determine the extent of antimicrobial activity, and may detect persistent/resistant populations. The concentrations used in these experiments may be static or dynamic, i.e. changing to simulate the half-life. Dynamic experiments also have the additional benefit of capturing the antimicrobial activity after concentrations drop below the MIC (i.e., post-antibiotic effects). Due to the numerous advantages of this technique over the MIC, time-kill curve experiments have gained in popularity. The mathematical models that have been developed and applied to these experiments for pharmacodynamic characterization are compared below. Additionally, the applications of these experiments and models are mentioned.

35.2 Pharmacodynamic Models and Applications

35.2.1 One Population Models

The primary goal of time-kill curves is to characterize the pharmacodynamic activity of an antimicrobial agent, often with the intention of using this information to optimize the dosing regimen. These experiments are performed by exposing bacteria to a range of concentrations, generally as multiples of the MIC. The results are then typically fitted to an E_{max} model, $E_{max} = \frac{\varepsilon * C}{C + EC_{50}}$, to characterize the activity

Table 35.1 Definition of each symbol used throughout the chapter

Parameter	Definition
ε	Maximum kill rate constant
EC_{50}	Concentration needed to produce half-the maximum effect
C	Concentration of antimicrobial
H	Hill factor
N	Number of bacteria
λ	Growth rate constant
N_{max}	Maximum number of bacteria
dg	Delay in growth
dk	Delay in kill
t	Time
N_S	Number of susceptible bacteria
N_R	Number of persistent/resistant bacteria
k_{SR}	Transfer rate constant of bacteria into the persistent/resistant stage
k_{RS}	Transfer rate constant of bacteria into the susceptible stage
B_{max}	Maximum number of viable bacteria in compartment 1
k_{death}	Natural bacterial death rate constant
VG_{max}	Maximum growth velocity
MIC	Minimum inhibitory concentration
N_m	Number of bacteria at which replication is half maximum
SIT_m	Median effect value of C/MIC (value at which the drug effect is half maximal)
δ	Maximum fraction increase of k_{death}
C_r	Concentration which induces adaptive resistance
IC_{50}	Concentration of the adaptive resistance at which ε_1 is half maximal
z	Delay factor
k_e	Elimination rate constant
tl_{ag}	Initial lag time for adaptive resistance
k_{ecr}	Rate constant for the decrease in the concentration which induces adaptive resistance
C_0	Initial antibiotic concentration
β	Maximum adaptation factor
Φ	Adaptation factor

and calculate the pharmacodynamic parameters as a function of the change in the number of bacteria over time (Eq. 35.1) [19, 22]. This model can be modified to account for a delay in growth and/or kill and saturation in the number of bacteria in the *in vitro* system (Eq. 35.2) [33]. The symbol definitions are provided in Table 35.1.

$$\frac{dN}{dt} = \left(\lambda - \frac{\varepsilon * C^H}{C^H + EC_{50}^H}\right) * N \tag{35.1}$$

$$\frac{dN}{dt} = \left(\lambda * \left(1 - \frac{N}{N_{max}}\right) * (1 - e^{-dg*t}) - \frac{\varepsilon * C^H}{C^H + EC_{50}^H} * (1 - e^{-dk*t})\right) * N \tag{35.2}$$

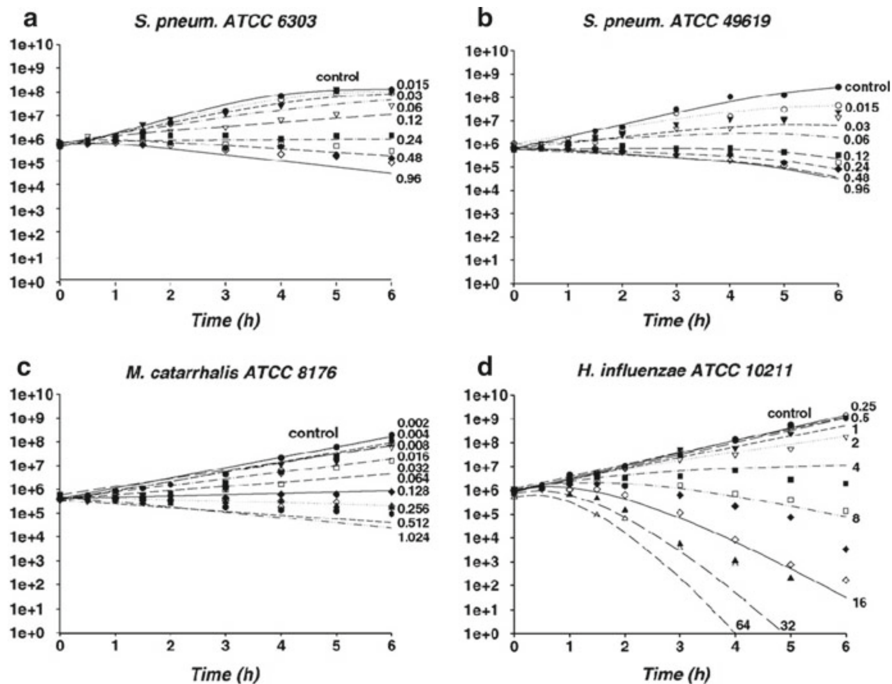

Fig. 35.2 Model fit of four bacterial strains with various constant antibacterial concentrations (μg/ml) (bacterial concentration represented as log10 CFU/mL): (**a**) azithromycin against *S. pneumoniae* ATCC6303; (**b**) azithromycin against *S. pneumoniae* ATCC 49619; (**c**) azithromycin against *M. catarrhalis* ATCC 8176; (**d**) azithromycin against *H. influenzae* ATCC 10211. A monophasic activity profile is displayed over 6 h [27] (Need Copyright)

From Equation 35.2, and displayed in Figure 35.2, a monophasic kill pattern is described as the bacteria are all assumed to have the same susceptibility. However, if these experiments are carried out over a sufficiently long time period, a biphasic antibacterial effect is usually observed (Fig. 35.3). Therefore, models that account for differences in susceptibility have gained much popularity as they better characterize the pharmacodynamics.

35.2.2 Two-Population Models with Persistent Bacteria

Many pharmacodynamic models have been developed which describe the bacterial population as two distinct sub-populations, one which is susceptible to drug and one which is not. In these models, the susceptible population is growing while the resistant/persistent population may be dividing or in a state of hibernation. The presence of a persistent/resistant population has been experimentally validated [4, 12, 16, 30], but it can be difficult to discern whether resistance or persistence is the rationale

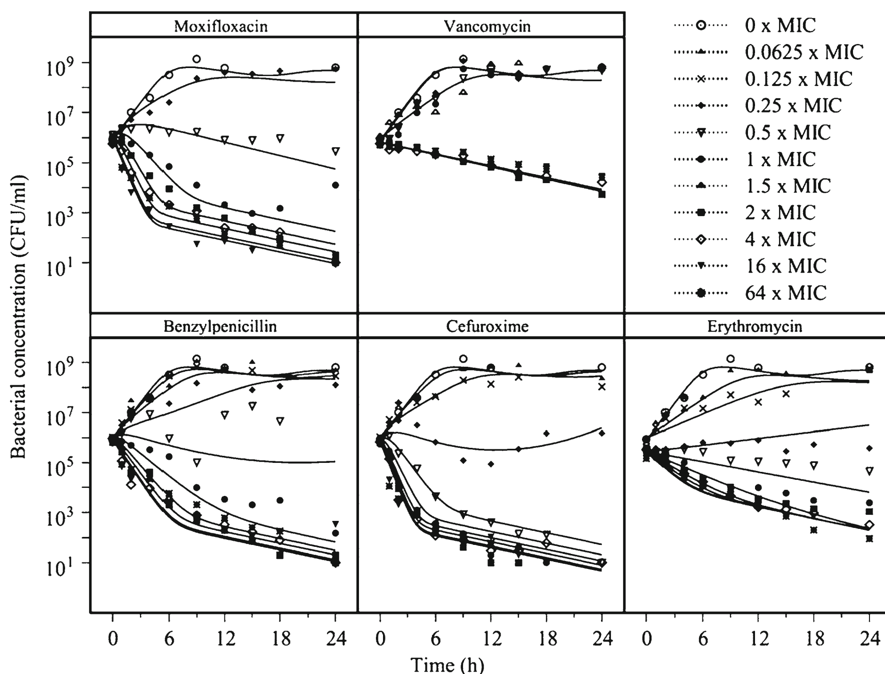

Fig. 35.3 Observed (points) and model predicted (lines) time-kill curves for *S. pyogenes* after exposure to several antibiotics and a range of antibiotics concentrations. Four of the five activity profiles are biphasic

behind the biphasic profile of antimicrobial activity, as it could be a combination of both. The difference between the two populations is that resistant bacteria have a genetic mechanism of resistance and grow on agar supplemented with antibiotics, while persistent bacteria remain sensitive to the antibiotic when they resume normal growth. It has been suggested that if a large inoculum is used then the biphasic profile is more likely to be attributed to resistance, as the resistance mutation rates are often in the 10^{-7}–10^{-8} [30]. If a smaller inoculum is used, e.g. the standard of ~10^6 CFU/mL, then the presence of persisters may be more likely than a genetically-acquired resistance mechanism [25]. Many authors have applied the persistence theory to model the kill kinetics of antimicrobials from time-kill experiments.

A persister model was developed during a comparison of the *in vitro* bactericidal kinetics of S-4661, a new carbapenem, to meropenem, imipenem, cefpirome, and ceftazidime using several bacterial strains which included *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* [36]. This model places the bacteria in one of two states, susceptible and dividing or persistent (Eqs. 35.3 and 35.4) [36].

$$\frac{dN_S}{dt} = \lambda N_S (B_{max} - N_S) - \frac{\varepsilon * C}{EC_{50} + C} N_S - k_{SR} N_S + k_{RS} N_R \quad (35.3)$$

$$\frac{dN_R}{dt} = k_{SR} N_S - k_{RS} N_R \quad (35.4)$$

A similar model was developed by using an additional term to account for the natural death rate of both the persistent and susceptible bacteria (Eqs. 35.5–35.8) [25]. This model also takes into account the transition of susceptible cells to persisters, where this transition, k_{sr}, is described by a proportionality constant times the total bacterial concentration in the system. The purpose of this experiment was to identify a robust model that could be used for several drug/bacteria combinations with the promise of eventually aiding in dose optimization of new antimicrobials. This model was able to fit the kill-kinetics of several antimicrobials from different classes against *Streptococcus pyogenes*, including the penicillin benzylpenicillin, the cephalosporin cefuroxime, the macrolide erythromycin, the fluoroquinolone moxifloxacin, and the glycopeptide vancomycin. In this paper, several models were applied to the data that placed the antimicrobial effect on the growth rate (Eq. 35.5) as an additive effect on the natural death rate (Eq. 35.6) or as a proportional effect on the natural death rate (Eq. 35.7). The equation for the persister population was the same during the comparison of the models (Eq. 35.8). It should be noted that k_{rs} was fixed to zero and a mixture model was used so the initial inoculum was either in logarithmic growth or a mixture of logarithmically growing bacteria and persisters. If the starting inoculum was mixed with both persisters and susceptible bacteria, then it was estimated that 5% of the population were persisters.

$$\frac{dN_S}{dt} = \lambda * (1 - Drug) * N_S - k_{death} * N_S - k_{SR} * N_S + k_{RS} * N_R \quad (35.5)$$

$$\frac{dN_S}{dt} = \lambda * N_S - \left((k)_{death} + Drug\right) * N_S - k_{SR} * N_S + k_{RS} * N_R \quad (35.6)$$

$$\frac{dN_S}{dt} = \lambda * N_S - k_{death} * (1 + Drug) * N_S - k_{SR} * N_S + k_{RS} * N_R \quad (35.7)$$

$$\frac{dN_R}{dt} = -k_{death} * N_R + k_{SR} * N_S - k_{RS} * N_R \quad (35.8)$$

In these equations, the term Drug is the E_{max} model with a Hill factor. Equations 35.5–35.7 presented above for the antimicrobial effect fit the data equally well.

35.2.3 Two-Population Models with Resistant Bacteria

As previously mentioned, it is also possible to have two distinct populations within a bacterial culture, one susceptible to drug and one resistant. These populations may in fact display different profiles for growth and kill, presented below as a net effect model (Eqs. 35.9 and 35.10) [6]. This model provided a better fit than a model which had similar growth and death rate constants or one which allowed for adaptation and the appearance of resistance. The purpose of model development was to characterize the antimicrobial effect of ciprofloxacin *in vitro* on both susceptible and resistant populations after exposure to concentrations equivalent to those observed *in vivo* after various ciprofloxacin dosing regimens.

$$\frac{dN_S}{dt} = \left(\lambda_S * \left(1 - \frac{(S+R)}{N_{\max}}\right) - \frac{\varepsilon_S * C}{EC_{50S} + C}\right) * N_S \tag{35.9}$$

$$\frac{dN_R}{dt} = \left(\lambda_R * \left(1 - \frac{(S+R)}{N_{\max}}\right) - \frac{\varepsilon_R * C}{EC_{50R} + C}\right) * N_R \tag{35.10}$$

In a subsequent study, two-populations models were compared in order to examine which and how the parameters are affected by the antibiotic [8]. The models compared included the net effect model (Eqs. 35.9 and 35.10), the growth inhibition model (Eqs. 35.11 and 35.12), the death stimulation model (Eqs. 35.13 and 35.14), and the MIC based model (Eqs. 35.15 and 35.16) [20]. It was found that based on goodness-of-fit, bias of observed vs. predicted, precision of the estimates, and predictive performance that the net effect model and growth inhibition model were the superior models.

$$\frac{dN_S}{dt} = \left(\lambda_S * \left(1 - \frac{(S+R)}{N_{\max}}\right) * \left(1 - \frac{\varepsilon_S * C}{EC_{50S} + C}\right) - k_{sdeath}\right) * N_S \tag{35.11}$$

$$\frac{dN_R}{dt} = \left(\lambda_R * \left(1 - \frac{(S+R)}{N_{\max}}\right) * \left(1 - \frac{\varepsilon_R * C}{EC_{50R} + C}\right) - k_{rdeath}\right) * N_R \tag{35.12}$$

$$\frac{dN_S}{dt} = \left(\lambda_S * \left(1 - \frac{(S+R)}{N_{\max}}\right) - k_{sdeath}\left(1 + \frac{\varepsilon_s * C}{EC_{50S} + C}\right)\right) * N_S \tag{35.13}$$

$$\frac{dN_R}{dt} = \left(\lambda_R * \left(1 - \frac{(S+R)}{N_{\max}}\right) - k_{Rdeath}\left(1 + \frac{\varepsilon_R * C}{EC_{50R} + C}\right)\right) * N_R \tag{35.14}$$

$$\frac{dN_S}{dt}=\left(\frac{VG_{max}}{N_m+N_S+N_R}-k_{death}\left(1+\frac{\delta*\left(\frac{C}{MIC}\right)^H}{SIT_{mS}^H+\left(\frac{C}{MIC}\right)^H}\right)\right)*N_S \tag{35.15}$$

$$\frac{dN_R}{dt}=\left(\frac{VG_{max}}{N_m+N_S+N_R}-k_{death}\left(1+\frac{\delta*\left(\frac{C}{MIC}\right)^H}{SIT_{mR}^H+\left(\frac{C}{MIC}\right)^H}\right)\right)*N_R \tag{35.16}$$

Theoretically, it is possible to have several populations of bacteria within a given inoculum. In the above MIC model, originally proposed by Meagher et al., three different bacterial populations were assumed, i.e. three different values for SIT_m (the concentration needed to produce the median effect for C/MIC [20]). However, the pharmacodynamics were adequately described with only two values, where SITm is either susceptible (SIT_{mS}) or intermediately susceptible (SIT_{mR}), with all other parameters remaining the same between the different populations (Eq. 35.17) [20]. Additionally, in this model growth was a function of the bacterial load in the system and not a constant. This model was originally proposed to characterize the pharmacodynamics of ciprofloxacin and model the effects of different ciprofloxacin dosing regimens, an extended release and an immediate release.

$$\frac{dN}{dt}=\frac{VG_{max}*N}{N_m+N}-\left(1+\frac{\delta*\left(\frac{C}{MIC}\right)^H}{SIT_m^H+\left(\frac{C}{MIC}\right)^H}\right)*(k_{death}*N) \tag{35.17}$$

35.2.4 Adaptation Models

Thus far, models have been discussed that assume two distinct populations. It is also possible to model a bacterial population that undergoes adaptive resistance in the presence of an antimicrobial agent. For example, to explain the biphasic kill profile a model was developed which assumed that adaptive resistance changed the rate of bacterial kill from an initial rapid kill rate, ε_1, to a slower permanent kill rate, ε_2. This model was also developed for comparison of the pharmacodynamics of two different dosing regimens of ciprofloxacin, a twice daily immediate release and an once daily extended release, by simulating *in vivo* concentrations in an *in vitro* model, i.e. concentrations changing according to the half-life (Eqs. 35.18 and 35.19) [28].

$$\frac{dN}{dt} = \left(\lambda - \frac{\left(\varepsilon_1 \left(1 - \frac{C_r}{IC_{50} + C_r} \right) + \varepsilon_2 \right) * C}{EC_{50} + C} \right) * N * (1 - e^{-z*t}) \tag{35.18}$$

$$C_r = C_0 * \left(e^{-k_e*(t - t_{lag})} - e^{-k_{ecr}*(t - t_{lag})} \right) \tag{35.19}$$

In the above model, the adaptation was placed on the kill rate constant. However, it is also possible to say that the adaptation is due to a change in the susceptibility and not the rate of kill, i.e. a change in the EC_{50} (Eqs. 35.20 and 35.21) [30]. This model was developed to characterize the pharmacodynamics of meropenem against *Pseudomonas aeruginosa*. Like many other models, it is robust and has the potential to be applied to other drug/bacteria combinations [30]. To ensure the presence of resistant population(s) at the start of the study, a large initial inoculum, 10^8 CFU/mL, was used.

$$\frac{dN}{dt} = \left(\lambda * \left(1 - \frac{N}{N_{\max}} \right) - \left(\frac{\varepsilon * C^H}{C^{H+} (\alpha * C_{50k})^H} \right) \right) \tag{35.20}$$

$$\alpha = 1 + \beta * (1 - e^{-C*t*\Phi}) \tag{35.21}$$

Similar to the approaches used during model development in two-population models, the possibility of adaptation due to a change in the growth rate was also examined (Eq. 35.22) [22]. This model was developed to compare the pharmacodynamics of two different dosing approaches with ceftazidime, continuous infusion and intermittent infusion.

$$\frac{dN}{dt} = \left(\lambda * \left(1 - \frac{N}{N\max} \right) * (1 - e^{-\Phi*t}) - \frac{\varepsilon * C^H}{{EC_{50}}^H + C^H} \right) * N \tag{35.22}$$

35.3 Summary

In vitro experiments to determine the antimicrobial activity of a particular agent often correlate well with the *in vivo* situation, partly due to the site of action in both cases being bacteria. Kill-curve experiments are particularly useful as they provide a detailed pharmacodynamic profile, a major advantage over the static MIC approach. For example, two different dosing regimens may produce the same AUC/MIC ratio, but different profiles of activity (Fig. 35.4). Several examples have been given in the

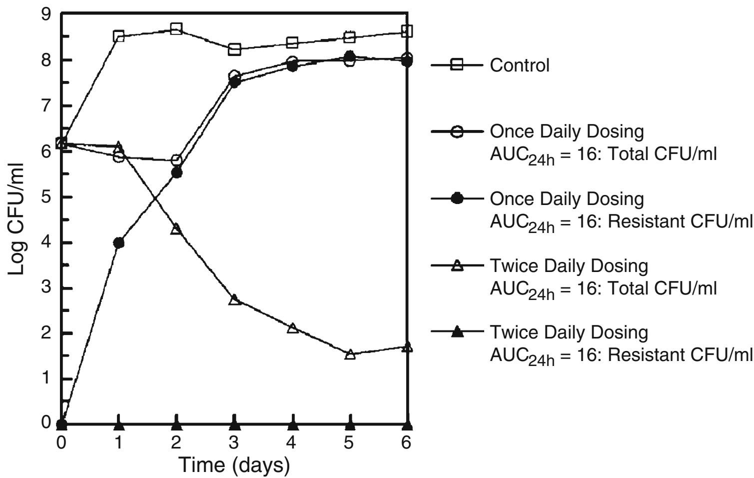

Fig. 35.4 The effect of dosing regimen is displayed as two equivalent daily exposures (AUC24/MIC=256) were administered as either two equivalent doses every 12 hours or a single daily dose. Twice daily dosing prevented resistance development and reduced bacterial load while once daily dosing permitted resistance development and bacterial load increased

literature using kill-curves to evaluate and/or select the optimal dosing regimen. A few examples include comparing continuous and intermittent infusions of ceftazidime [22], a comparison of an extended release and immediate release formulation of ciprofloxacin [20, 28], dose selection of levofloxacin against *Bacillus anthracis* [12], and optimizing the dosing regimen to prevent resistance development [6, 15]. While most examples use *in vivo* plasma data combined with time-kill curves, it seems more meaningful to explore the PK/PD relationship based on antibiotic concentrations at the site of infection, e.g. subcutaneous adipose tissue for complicated skin and skin structure infections, to make a dosing recommendation [11].

Many semi-mechanistic models have been proposed to model the pharmacodynamic activity of antimicrobial agents from time-kill experiments. As can be seen from a comparison between the models, they share many similarities and are not entirely unique. More research is needed to fully understand the mechanisms that can lead to a biphasic pharmacodynamic profile. Currently, the models presented within this chapter all seem justified and serve the purpose of their development.

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Part VIII
Antibiotic Drug Development

Chapter 36
Antibiotic Drug Development: Moving Forward into the Clinic

Jane E. Ambler and Greg G. Stone

36.1 Introduction

The cornerstone of all antibacterial drug discovery and development organizations is the microbiology and microbiological profiling from early hit analyses in drug discovery to post-launch in the marketplace. In the early stages of drug discovery, many inhibitors of bacterial targets can be identified, but if that activity does not translate into bacterial killing and pharmacological properties, the molecule will not advance to phase III clinical studies. An understanding of microbiology is essential in lead optimization where the activity against the targeted pathogens is to be optimized along with additional pharmacological traits such as *in vitro* and *in vivo* safety and efficacy. In early drug development, an agent needs to clearly demonstrate safety and efficacy in preclinical models and, thereby, explore the spectrum of potential clinical use and predict safety and efficacy in humans. In phase II and phase III clinical trials, microbiology is essential for the demonstration of pathogen eradication and correlation with clinical outcome for the establishment of clinical breakpoints for the differentiation of susceptible and resistant bacterial populations. Finally, microbiology remains an essential component of life cycle management mandatory regulatory agency periodic safety updates, antimicrobial surveillance programs for monitoring emergence of resistance, and support for supplementary indications.

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36.2 Antibacterial Profiling at the Preclinical Stage

36.2.1 Susceptibility Testing

Preclinical profiling of potentially new antibacterial agents can be assessed in numerous ways. It can be as simple as a crude “dip-disk” technique [3, 40] or more complicated using an agar or a broth dilution method. The dip-disk method involves the use of sterile 6–8-mm paper disks that are dipped into a liquid vial of a known concentration of an agent and then placed onto a freshly inoculated lawn of a bacterial strain on a standard agar plate. This is then incubated overnight under the appropriate growth conditions, and the presence of a zone of inhibition around the disk is determined, which would indicate antibacterial activity. This is a crude non-standardized method that does not provide a quantitative level of antibacterial activity but shows evidence of, or lack of, activity. It is rarely used today (except for screening plant or other biological extracts) with many more refined methods available.

More methods involve the use of doubling dilutions of the antibacterial agent in either agar or broth. The agar dilution method involves the incorporation of the antibacterial agent into molten agar over a wide range of doubling dilutions. The broth dilution method either in macro (~1–10 mL) or micro (100 μL) volumes is another method of susceptibility testing. In either case, the minimum inhibitory concentration (MIC) is determined as the lowest concentration that inhibits the culture from growing determined by eye or by more automated methods, such as microtiter plate readers. It is beyond the scope of this chapter to delve into all the nuances and differences in the methods which have been reviewed thoroughly elsewhere [44]. Basically, the agar dilution method has the advantage over the broth methods in that multiple strains and species can be evaluated on the same agar plate containing the antibacterial agent. The disadvantage is that it is a much more labor-intensive method than broth-based methods for drug discovery. The broth-based (particularly the micro format) method has the greatest advantage for drug discovery purposes since it is relatively miniaturized and is amenable to automation using liquid-handling workstations much more easily than other dilution methods.

Any growth medium and conditions that support the growth of the organisms to be tested could theoretically be used to test the inhibitory effects of the agent in question because the agent in question has not gone through a validation or standardization process. However, susceptibility testing in the clinical laboratories in the United States and other parts of the world use the most widely accepted methods and current guidelines developed and published by the Clinical Laboratory Standards Institute (CLSI). Resources are not readily available in a drug discovery setting to evaluate molecules under the wide range of conditions; therefore, the best place to start is the basic method developed by CLSI that is defined for most antibiotics on the market today. In preclinical microbiological evaluations of novel compounds, the marketed comparator agents are (or should be) tested according to the appropriate CLSI-approved methods as well. This gives the discovery team the best perspective on the activity profile in relation to the established comparator agents.

Ultimately, this may not be the appropriate method that will be used in clinical laboratories for the agent in question but is a good starting point from which methodological issues may be addressed.

In screening novel compounds in the lead-optimization phase, microbiological screening should continue to be performed according to the basic CLSI standards for inoculum cell density, medium, growth conditions, and length of incubation for the respective organisms [8, 9]. This is in the absence of other information on the organism/medium/mode-of-action. The structure-activity relationships (SAR) are thus optimized under these conditions and little or no manipulation of the method is needed during the development phase of the agent. Studies that are altered from the standardized testing conditions (e.g., medium effects, inoculum effect, CO_2 effect, etc.) will undoubtedly need to be performed and presented to the regulatory agencies to demonstrate how these different conditions affect the susceptibility testing methodology (see below). CLSI has defined exceptions for some agents such as tigecycline, which requires the medium to be less than 12 h old [6], and daptomycin, which requires additional calcium chloride to be used for susceptibility testing [14]. These exceptions are specific to these compounds and were found to be necessary in order to help discern the resistant population from the susceptible population. The impact of the testing methodology could not have been determined without prior knowledge of the inhibitory effects of the agent.

Irrespective of the method employed to evaluate compounds in drug discovery, the compound and potential target organisms are screened or evaluated in MIC tests for SAR. This is especially important during the lead-optimization phase when potential lead candidates for development must be identified. This is the stage at which compounds are being differentiated from each other not only from a microbiological perspective but also from other existing antibiotic classes in which the potential label indications are being sought. The microbiological profile of any development candidate may suggest potential for treating specific indications as well as for positioning of the agent in the marketplace. This is true not only for novel agents that are unrelated to any other class on the market but also for modification or improvements of compounds in existing classes. Both approaches have the same ultimate goal of identifying an agent for the unmet medical need of combating resistance to existing agents.

36.2.2 MIC Population-Based Studies

During lead initiation and the transition into lead optimization, it is necessary to establish the spectrum of activity of an agent through several different evaluations against a broad range of bacterial species. The initial microbiological profile dictates the direction the discovery team will take in optimization and the potential for intended indications that a pharmaceutical company, otherwise known as a "sponsor," wishes to seek when interacting with the regulatory agencies. These

evaluations (or screens) are usually performed against an initial primary screen, which frequently consists of a panel of individual strains representing the key Gram-positive and Gram-negative species important for the indications being targeted. This usually leads onto secondary evaluations that comprise populations of targeted pathogens. During the lead-optimization phase of drug discovery for an antibacterial, it is necessary to evaluate larger sets of organisms in order to gather population-based summary data (i.e., MIC range [minimum and maximum], MIC_{50} and MIC_{90} values for populations of specific pathogens obtained from clinical sources). This allows for a better characterization of the *in vitro* activity of a given compound against multiple strains of the same species. It can also help in the differentiation between compounds being selected for nomination for further development. The number of strains to include in these types of studies is often debated, as few as ten isolates of the same species can be used to gain some understanding of the population distribution in addition to determination of the MIC_{50} and MIC_{90} values. In the earlier stages of lead optimization, large panels of clinical strains may not need to be tested, but as the project progresses toward nomination, evaluation of atleast 100 recent clinical isolates from diverse sources should be considered to demonstrate activity. Inclusion of strains with known resistant to established agents to assess cross-resistance to comparator agents should be considered. This allows for a comprehensive comparative evaluation of antibacterial spectrum against other antibacterial classes.

36.2.3 Bactericidal-Based Studies

36.2.3.1 Minimum Bactericidal Concentration

The MIC is a valuable piece of information in drug discovery, but it has limitations in that the MIC test is a static test that only measures the inhibitory activity of a given agent at a given time. There are measures of activity beyond the MIC test to better understand the bacterium–drug interaction. These assays investigate the bactericidal or bacteriostatic effects of the agent. One of these tests is the minimum bactericidal concentration (MBC) test, is relatively straightforward to perform. This is usually performed from the MIC test plates whereby the broth remaining in the MIC test wells at the MIC and above the MIC is plated onto an appropriate medium. The MBC is determined by counting the colonies and calculating a 99.9% reduction in CFU/mL from the starting inoculum after overnight incubation. The concentration of drug above the MIC that results in this 99.9% reduction is the MBC. Like the MIC test, it is amenable to automation and can be performed routinely. However, it does have limitations in the interpretation of the results. It is also a static test in that the end result is a concentration that kills 99.9% of the starting inoculum which is determined at the end of the MIC test (after ~20 h of incubation). Both the MIC test and the MBC test are discrete endpoint assays that do not provide any data on the time-course of killing during the incubation period. Also, there is some

debate as to whether the MBC test from the broth microdilution MIC test is reliable and reproducible [19, 36]. It can be adopted in drug discovery programs to gain some perspective of the relative reduction of bacterial survival at drug concentrations above the MIC, but results should be interpreted with some caution.

36.2.3.2 Time-Kill Studies

How rapidly or slowly a bacterial culture is killed by an agent over time is a valuable piece of information in drug discovery. Studies on killing kinetics investigate the concentration of compound, often expressed as a multiple of the MIC at a single fixed endpoint required to kill 99.9% of a growing culture of bacteria in a time-dependent fashion (usually 24 h). This assay yields a more dynamic evaluation of the bactericidal nature of an agent compared with the MBC. The viability of the bacterial population (CFU/mL) is determined at several time points rather than at a single time point as is the case for the MBC assay. Fluoroquinolones typically demonstrate rapid bactericidal activity (99.9% reduction in 4–6 h) whereas macrolides and oxazolidinones are bacteriostatic and do not demonstrate a 99.9% reduction in 24 h. These aforementioned compounds are typically concentration-independent. Aminoglycosides, on the other hand, demonstrate a concentration-dependent killing. These assays have traditionally been applied to understanding and putting greater context around deciding dosages and the parameters that are the driver of efficacy (T>MIC, AUC/MIC, Cmax/MIC). Recently, *in vitro* dynamic models such as hollow fiber systems are being incorporated into drug discovery programs to help elucidate drivers of efficacy in addition to the time-kill studies.

36.2.4 Resistance Selection Studies

36.2.4.1 Spontaneous Selection

Resistance to an antibiotic is a key question that all drug discovery programs must address. What makes a bacterium resistant to an antibiotic can be defined differently depending on the particular situation. In the clinic, resistance is defined as the categorization of the MIC against a given pathogen isolate that is above the clinically defined breakpoint for that compound. In the laboratory, resistance is sometimes defined as any gain of fitness by an organism after exposure to an agent allowing it to survive. The theory of these resistance-selection studies relies on the naturally occurring random mutations in a growing population of bacteria. By exposing a large inoculum (approximately 10^{10} CFU) of an organism to an agent at multiples above the MIC (2×, 4×, 8×), there may be a subpopulation existing in that inoculum that can survive and grow. Experiments are often carried out in the laboratory utilizing this technique to help elucidate the mode-of-action of the agent (other parameters are important, see below). Typically, the frequency of resistance is calculated by count-

ing the number of colonies growing on the 4x MIC plate after 48 h of incubation and dividing it by the actual starting number of CFU that was initially inoculated onto the plate. That starting inoculum can be determined by simply making doubling dilutions of the starting inoculum and plating on drug-free plates simultaneously to plating on the drug-containing plates. Plates with countable colonies (typically between 30 and 300) after incubation are counted and the CFU/mL is determined. After 48 h of incubation, colonies from the drug-containing plates are inoculated to another drug-containing plate containing the same concentration of drug as that from which the colony was originally isolated. This is to ensure a single clone is isolated and to inhibit any susceptible bacteria that may have survived on the drug-containing plate. These colonies are further passed onto drug-free plates for at least three passages to ensure stability of the isolate resistance. The degree to which the isolates have become resistant can be assessed by MIC determination. If the target is known, the coding region of DNA of the suspected target can be sequenced to investigate any amino acid changes or ribosomal RNA changes if the agent targets the ribosome. Any changes can then be followed up and cloned back into a naïve wild-type strain to understand whether this change was indeed responsible for the resistance.

For drug discovery, the frequency and degree of loss of activity against the surviving colonies are important parameters to understand. Theoretically, the MIC against the mutant isolates should be at least fourfold above the MIC (4x MIC) against the parent strain if taken from the 4x MIC plates. There is no hard and fast rule on what is acceptable for a single-step increase in MIC, but a general rule of thumb is that an eightfold increase in the MIC between the parent and mutant strains in a single-step is a warning flag; a ~32-fold increase would certainly be a cause for alarm. There are examples of agents on the market that have a high frequency of spontaneous resistance (e.g., rifampin) and very low frequency of spontaneous resistance (e.g., linezolid); however, most marketed agents fall between these two extremes. Often, the chances of developing spontaneous resistance are greater when targets are encoded by a single gene. In the case of rifampin, the target is RNA polymerase, which is encoded by a single gene; the frequency of resistance is high ($\sim 10^{-6}$ range) because any one of several amino acids can mutate, resulting in elevated MICs. For linezolid, the spontaneous resistance is extremely low ($<10^{-10}$), particularly in *Staphylococcus aureus*. The target for linezolid is the ribosome and *S. aureus* has 6 copies of the gene encoding ribosomal RNA, which linezolid is known to interact with. It is generally regarded that ~50% of the genes encoding ribosomal RNA need to be mutated to observe clinical resistance and these mutations are acquired sequentially on serial passage exposure to the agent.

36.2.5 Luria-Delbrück Fluctuation Test

The frequency of resistance determination does have limitations. It only indicates the phenotype selected in the population of the growing culture. The Luria and Delbrück fluctuation test [29] is an extension of the frequency of resistance

measurements which determines the rate of spontaneous mutants per generation that arise from the growing culture prior to exposure of the selecting agent. Luria and Delbrück found that mutations are randomly generated during the growth phase of the bacteria and are not directed by the selecting agent. It is a more accurate way of determining the frequency of occurrence of each mutation that can confer resistance. In a frequency of resistance study, a mutation that arises early in the growth cycle of the experiment prior to exposure of the agent can multiply (a clonal-expansion effect) and the rate at which the mutation arose may be exaggerated. The fluctuation test is conducted in a way which eliminates this from the experiment. By growing many replicates of small inoculum cultures that are statistically below the possibility of having a preexisting resistant mutant in the population prior to exposure of the agent, the determination of the rate at which resistance mutations arise can be calculated. Since Luria and Delbrück's pioneering work, several other authors [2, 11, 20, 23, 24, 53] have described more refined mathematical models for estimating mutation frequencies.

36.2.5.1 Serial Passage Studies

Additional resistance-selection studies should be performed by serial passage. These studies investigate how an organism responds to the repeated daily exposures to an agent either at the MIC or just below (1/4, 1/2 x MIC). Upon repeated exposure, the MIC may be observed to increase over time. The assay does not necessarily reflect the situation in the clinical setting, but is a useful tool to help understand the bacterium–drug relationship at sublethal concentrations upon repeated exposure. These are slightly different than spontaneous resistance studies where a subpopulation of resistant (>4 x MIC) colonies are selected within a preexisting large population of bacteria. In the serial passage studies, it is quite possible to select for incremental or small changes in the MIC upon repeated exposure. The changes in the organism that confer the incremental increase in the MIC may or may not be related to the mode-of-inhibition of the agent. The number of serial passages that result in a significant increase in MIC is usually characteristic of a given antibiotic class.

These studies are usually carried out by first performing the standard MIC test by broth microdilution and then subculturing the liquid at the highest concentration where growth occurs (½ x MIC) after measuring the MIC. This is then used as the inoculum for performing the MIC test again. The experiment is repeated for a number of passages until higher-level MICs have been achieved. It is important to investigate if resistance to other agents is induced by these passage studies as well to assess potential cross-resistance, so testing comparative agents is essential.

Other methods have been developed to investigate resistance development by serial passage such as an agar dilution method. This is performed exactly as the broth method but instead the assay is performed on agar plates containing fixed concentrations of the agent [42]. Gradient plates have been employed as well [21, 41] where the leading edge of a standard inoculum is streaked across an agar plate

containing a concentration gradient of the selecting agent. An adaptation of this method is employed by spiraling drug out onto the plate with a spiral plater [7, 54], resulting again in a changing concentration of drug across the plate surface.

36.2.6 Addressing Heteroresistance

The term "heteroresistance" can best be described as resistance of a small subpopulation of bacteria within a total population of bacteria from a clinical specimen or bacterial culture. It was known for some years with methicillin resistance and first described for vancomycin in staphylococci in 1997 by Hiramatsu [18], and is becoming an increasing clinical problem in *S. aureus*, particularly with methicillin or vancomycin. Heteroresistance has also been reported with other bacterial species and other agents [32, 35]. It is not easily detected in clinical laboratories where susceptibility testing is usually performed by either disk and/or MIC test. A heteroresistant subpopulation is hidden within the normal distribution of MIC values and usually falls within the susceptible category. This leads to the proliferation of the resistant subpopulation and the emergence of a resistant strain, and thus resulting in a clinical problem. In recent years, heteroresistance in *S. aureus* to vancomycin (hVISA phenotype) prompted a review of the clinical breakpoints by both the Food and Drug Agency (FDA) and the CLSI in the USA [43]. Clinical evidence that heteroresistance in *S. aureus* to vancomycin is linked to clinical failures is rare and somewhat anecdotal; however, various investigators have reported heteroresistant isolates to clinical failures [28, 30, 39].

Heteroresistance poses a unique issue for new agents in future drug discovery and development programs. How does one address heteroresistance in the preclinical setting for completely novel agents unrelated to any class that already exists for clinical utility? It is much easier to address for new agents of an existing antibiotic class to assess in the laboratory when a recognized heteroresistant population of strains already exists. Carry-over of heteroresistance to the new agent from the existing class can be determined by testing known heteroresistant strains. However, demonstration of heteroresistance in a novel agent is difficult. This is primarily because a novel agent has had no human exposure to allow the selection of a heteroresistant subpopulation. The antibacterial activity of the novel agent can be tested by way of a population analysis.

The population analysis profile (PAP) is a test that can indicate whether more than one population exists within a bacterial culture. Several different methods are described in the literature [27, 32, 33, 52] and can be used to demonstrate heteroresistance (or lack thereof). Deciding on strains to assess for the presence of heteroresistance necessitates examining the population distribution of MICs from a rather large set of strains (~100), and selecting strains that are on the upper, middle, and lower ends of the distribution. The tighter the distribution of MICs the less likely that heteroresistant strains exist in the population; however, a broad distribution of MICs that does not necessarily show more than one mode may indicate a heteroresistant

population. The theory is that strains at the low end of the distribution would be true wild type and more than likely would not have a heteroresistant subpopulation. Strains at the upper end of the distribution may be quasi-resistant (or intermediate) in the sense that a subpopulation of a heteroresistant strain has now become the homogeneous population (similar to what is seen with hVISA and VISA). Strains in the middle of the distribution may increase the chances of detecting heteroresistance, if heteroresistance does indeed exist. Particularly, if there is a "shoulder" in the distribution where there may be more strains that test on the upper end of the distribution versus the lower end of the distribution. The middle part of the distribution may be where heteroresistance exists. Performing a PAP on representative strains of this population may be able to detect a heteroresistant subpopulation.

36.2.7 Combination Approaches

Combination antibiotic therapy is used to provide a broader spectrum of coverage, in particular in seriously ill patients. Aside from the idea of an inhibitor of a resistance mechanism to protect the antibiotic such as a β-lactam/β-lactamase inhibitor combination, the concept of combining two or more agents is multifactorial in an empirical situation. It increases the spectrum of coverage when agents that primarily target Gram-positive bacteria are combined with an agent that targets Gram-negative bacteria. It also allows for the coverage of a specific pathogen that may be resistant to one of the classes prior to pathogen identification and the availability of susceptibility test results needed to select appropriate antimicrobial therapy. It also can provide double coverage of the offending pathogen that may develop resistance during therapy to one of the classes being used and the second agent will still be able to exert an effect on the pathogen. Still, other potential benefits are the possibility of a synergistic effect between the two agents that may improve the outcome of the treatment and the potential to reduce dosages where dose-related toxicity is known to occur. The drawback to combining two or more agents may be an antagonistic effect either for pharmacological (drug metabolism, absorption, etc.) or microbiological reasons. Central dogma for microbiological antagonism is the combination of a bacteriostatic agent, such as protein synthesis inhibitor, with a bactericidal agent, such as a β-lactam antibiotic. This is because the bacteriostatic agent slows the growth of the organism which in turn slows the bactericidal effect of the β-lactam since β-lactams require growing cells to exert their effect [26, 50, 51].

In drug discovery, combination studies are performed to evaluate if a synergistic effect (or lack of an antagonistic effect) occurs between two agents. In some situations, discussions are centered on combining two inhibitors in the same biochemical pathway that may result in a synergistic effect as seen with trimethoprim and the sulfonamides. In later stages of preclinical programs, combination assessments are required to understand if there is potential for an antagonistic effect between agents (see below). These studies help determine if combination therapy either during clinical trials or in clinical use is appropriate.

The simplest method to assess drug synergy or antagonism is the diffusion method where two 6-mm paper disks, one impregnated with drug A, the other with drug B, are placed on an inoculated agar plate at a set distance from each other. This method is interpreted by the shape of the zones around the disk [31]. It is a qualitative method that does have some limitations. The checkerboard method in a broth microtiter plate format is most often employed for investigating combinations in an *in vitro* microbiological evaluation [16, 31], but other methods involving agar dilution have been developed [31] The broth microdilution checkerboard method is amenable to automation and is relatively simple to perform. It involves the doubling dilution of one agent in one direction on the microtiter plate and the doubling dilution of the second agent in the perpendicular direction. The end result is a combination of different concentrations of the two agents in each well of the microtiter plate (a checkerboard pattern). Interpretation of the result is by the pattern of MICs after incubation. A fractional inhibitory concentration index (FIC) is calculated from the multiple MICs in the different combinations in relation to the MIC of the agents alone as described by Eliopoulos and Moellering [31]. It is generally regarded that a FIC of <0.5 is considered synergistic, 0.5 to 4 is considered additive/indifferent, and >4 is considered antagonistic. In these experiments, it is usually clear when there is an antagonistic or synergistic effect. However, many antibiotic combinations demonstrate an additive/indifferent effect in the test. Like the MIC and MBC tests, it is a static test that is read at a single time point (usually after 18–20 h of incubation). A more labor-intensive method that investigates the combination between two agents is the kill kinetic method involving a matrix of a few concentrations of the two agents. In this method, synergism is considered when there is a ≥100-fold increase at the 24 h time point in the kill rate in combination over the most active agent and antagonism is considered when there is ≥100-fold decrease at the 24 h time point in kill rate when compared to the most active agent. There are many assumptions to these interpretations, and they are outlined in more detail by Eliopoulos and Moellering [31].

36.2.8 Documentation of Preclinical Data

At some point during the drug discovery process, all data associated with a given agent that is being nominated for testing in humans need to be collated and presented to regulatory agencies, most commonly the Food and Drug Agency (FDA) for trials involving US subjects and/or the European Medicines Agency (EMA). Lead-optimization phase is the best time for a team to put into place a documentation system that has complete reports demonstrating *in vitro* and *in vivo* efficacy and safety in various species. Individual study reports should describe the experimental protocol in detail, results, and conclusions. The batch (or lot) number(s) of the investigational compound(s) including comparator agents is (are) to be reported, along with dates, laboratory notebook pages, etc. The source of bacterial species used in experiments should also be detailed, and include the year and country of isolation of clinical isolates tested. These reports become reference source material for all further submissions to the regulatory authorities, including the final submission.

36.3 Microbiological Considerations during Early Clinical Development (Phase I and Phase II)

The United States FDA has published Draft Guidance for Industry (2009) that focuses on the specific microbiology data required for the development of an antibacterial agent [47]. In addition, this guidance provides advice to sponsors (i.e., pharmaceutical company or other entity submitting the required data package for review) regarding the data analysis and presentation needed to support the clinical development of a new antibiotic. This initial process is known as an "investigational new drug application" (IND). This Draft Guidance for Industry also describes the data analysis and presentation needed to support a submission for approval and marketing authorization, a document known as a "new drug application" (NDA). The content and format for presentation of data in the microbiology subsection of US product labeling are also outlined in the Draft Guidance.

The microbiology section of the IND contains all the necessary information to provide the regulatory agencies with a broad understanding of the antibacterial activity of the agent prior to the initiation of Phase I human volunteer studies. It is highly recommended that a sponsor engages the FDA early in pre-IND discussions, to introduce the product, ask questions as required, and advise the FDA of their intention to submit an IND. These face-to-face meetings provide the sponsor an opportunity to exchange information on the intended use of the agent, the early microbiological profile, susceptibility testing methods, pharmacokinetics, and design of the Phase I studies, and discuss further development plans. The IND should be considered an evolving document that is continually updated throughout the development program. By the time a sponsor reaches Phase II/III of development, a more complete *in vitro* and *in vivo* microbiological data package should be demonstrated to the regulatory authorities. In addition to the IND, much of the same data are documented in an Investigator's Brochure that is provided to physicians recruiting patients into clinical trials. Although this section of the chapter separates early clinical development from late clinical development, these activities are undoubtedly continuous and oftentimes simultaneous.

There are five major points to consider for the microbiology section of an IND that the FDA guidelines highlight for early clinical development. For additional details, consult the Draft Guidance for Industry [47]:

A. Demonstration of *in vitro* and *in vivo* activity against targeted pathogens
B. *In vitro* culture conditions that may impact the assessment of antimicrobial activity to establish quality control parameters
C. Impact of human body fluids and secretions on *in vitro* activity (serum, lung surfactant, etc.)
D. Provision of information on the mechanism of action or inhibition (MOA/MOI), cidality, potential for resistance development, and cross-resistance to other antimicrobials
E. Determination of interactions with other antimicrobial agents and potential interactions with other drugs

36.3.1 Demonstration of In Vitro and In Vivo Activity Against Target Pathogens

The initial IND application filing should include demonstration of the in vitro spectrum of activity, to indicate target pathogens and identify the possible indications to be studied in the clinic. Multiple strains (at least 50 would be recommended) of the key pathogens for the indications being sought should be included. In addition, the *in vivo* effects against the same pathogen(s) should be studied in relevant animal models of infection to support the justification of testing humans. For instance, if a sponsor seeks to achieve the approval to test an agent in humans for the treatment of acute bacterial skin and skin-structure infections against *S. aureus,* both in vitro and appropriate animal models such as the mouse thigh infection model should be demonstrated. From an *in vitro* standpoint, it would certainly add value to demonstrate the activity of the agent against the most recent clinical isolates that can be obtained and tested, and efforts should be made to include relevant antibiotic-resistant isolates.

36.3.2 Factors That May Influence Susceptibility Test Results

As outlined earlier in this chapter, there are numerous methods for evaluating the antibacterial effects of any given agent in the laboratory. Any growth medium and conditions that supports the growth of the organisms could be used to test the inhibitory effects of the agent in question. Factors affecting susceptibility testing have been reviewed by Amsterdam [1]. The key factors are medium, inoculum, pH, temperature, atmospheric conditions, and cations, and these are discussed below. Other factors such as surfactants and supplements such as hemoglobin can be investigated as well to understand the impact of these on susceptibility testing. Factors affecting susceptibility test results are to be reported to the agency in early development (preferably early Phase I) so that the agency has a good understanding, prior to the initiation of pivotal Phase II/III trials.

36.3.2.1 Media Type

It has been recognized for years that different media can influence the results and reproducibility of tests. Over the years, cation-adjusted Mueller-Hinton Broth (CA-MHB) has become the most widely accepted and is the recommended choice of medium for performing broth microdilution susceptibility testing in the United States and other countries that use CLSI methodology. Magnesium and calcium cations are adjusted to 10–12.5 mg/L and 20–25 mg/L, respectively, in MHB and are in the physiological range in human serum (discussed below). The corresponding agar dilution–based methods utilize Mueller-Hinton Agar (MHA) without adjustment of

the cations. CA-MHB/MHA has been shown to be reproducible and has become the reference media by CLSI. The more fastidious organisms usually require some sort of nutritional supplement or environmental condition to support their growth. For instance, streptococci require the addition of 2.5–5% lysed horse blood (LHB) to the broth medium and 5% defibrinated sheep blood for agar dilution testing.

It is recommended that comparative studies of medium type be performed, particularly broth versus agar dilution to establish equivalency between the two methods (see below). However, during Phase I, different media effects that can affect the MIC test should be evaluated using protocols described in the CLSI M23 document [9]. It should be noted that some countries use different media and/or methods, such as those endorsed by the British Society for Antimicrobial Chemotherapy (BSAC), as used in much of the UK and Ireland, and it is necessary to evaluate the agent under those conditions in order to establish a method in that country.

36.3.2.2 Inoculum Effect

With all antibacterial agents, it is considered that starting with a larger inoculum is always more difficult to inhibit than starting with a smaller inoculum. Therefore, the standard density of inoculum used in susceptibility testing is critical, particularly when broth microdilution tests are carried out. The standard inoculum as defined by CLSI is a final concentration of ~5×10^5 CFU/mL for broth microdilution and 10^4 CFU/spot for agar dilution tests. This is usually achieved by an adjustment of an exponential-phase bacterial culture to 0.5 McFarland units. If it is assumed that a homogenous progeny of cells develops in a growing culture of bacteria, the probability of a mutant or variant is greater with a larger inoculum relative to a smaller inoculum. Sanders et al. [38] indicates that mutant subpopulations are detected better at inocula greater than 10^5 CFU/mL, hence one of the reasons for the starting inoculum for the MIC test. In the MIC test, an inoculum effect is considered positive when there is ≥ four-fold increase in the MIC when the inoculum is increased by 0.5 McFarland units.

It is not clear if there are any clinical implications with the inoculum effect, but it is necessary to evaluate its potential impact on the standardization of the susceptibility testing methods for any given agent through the development process.

36.3.2.3 Cation Concentration

The concentration of cations has long been known to affect the results of the MIC test, particularly for the aminoglycosides when testing against *Pseudomonas aeruginosa* [4, 5, 22]. Early evaluations of MICs in Mueller-Hinton broth (MHB) were shown to be quite variable when using different lots and different manufacturer's media due to the variations in magnesium and calcium content. To gain consistency, Reller et al. [37] suggested that MHB be supplemented with 25 mg/L magnesium and 50 mg/L calcium. This helped with early lots of some manufacturers' media

where the cation content was minimal, but later lots of MHB had higher and variable cation contents and supplementing with 25 mg/L magnesium and 50 mg/L calcium might have been excessive. As a result, Barry et al. [5] suggested a lower adjustment of 10–12.5 mg/L magnesium and 20-25 mg/L calcium would more appropriate. The MICs of netilmicin when tested under these conditions against *P. aeruginosa* correlated well with clinical outcome [4]. Today, contemporary media from the various manufacturers have contents of 10–12.5 mg/L magnesium and 20–25 mg/L calcium and the total content is specified on each bottle and lot of medium that is made. As mentioned above, the corresponding Mueller-Hinton agar (MHA) is unadjusted, and achieving consistent cation content is a little more complicated owing primarily to the variable amounts of cations in the agar component itself [22]. Therefore, CLSI adopted a reference lot of MHA from the work of Pollock et al. [34] as the standard to which other lots of MHA are compared when tested under quality control conditions by manufacturer.

Even with the standardization of the cation content in contemporary susceptibility testing, it is necessary to assess the impact of varying cation content in MHB on newer agents. Ultimately, the current content may not be the most appropriate for reproducible MIC tests for the newer agent. An example is daptomycin, which has been demonstrated to have a calcium-dependent mechanism of action [25] and supplementation of 50 mg/L in MHB is necessary for stable MIC determination [15].

36.3.2.4 pH Effect

The buffering capacity of CA-MHB is such that there is a minimal drift in the pH of the medium during incubation in ambient air. It is recommended by CLSI that the pH for susceptibility testing be in the range of 7.2–7.4, which is in the same range as human plasma; however, there are reports that describe testing at a pH that may be more representative of different body compartments (such as simulated urine, which would have a lower pH), but the clinical relevance is not clear. Nonetheless, it is expected that studies investigating the effect that pH has on the activity be performed and presented to regulatory authorities. If there is an effect observed, it can usually be attributed to relative strength of any ionizable groups on the drug molecule. Agents with non-ionizable groups or agents that are weak acids do not tend to show variations in MICs when the pH of the medium is reduced. On the other hand, there is a profound effect on the MIC for agents such as aminoglycosides and macrolides when the medium pH is raised due to the relatively high pKa of the ionizable groups on these agents.

36.3.2.5 Incubation Conditions

As previously stated, minimal drift in the media pH is observed when plates are incubated in ambient air; however, when plates are incubated in CO_2, a medium can become more acidic. Agar dilution MIC testing for organisms such as *Haemophilus influenzae* and *Streptococcus pneumoniae* is performed in the presence of 5% CO_2

due to the fastidious environmental requirements of these species. Susceptibility tests with and without 5% CO_2 should be performed and the results presented to regulatory agencies. Incubation in an anaerobic atmosphere can also influence the interpretation of the MIC results. It is recommended that agents being developed be tested against aerobes in an anaerobic atmosphere.

The length of incubation (hours) can have an impact on the interpretation of the MIC values. The recommended MIC test is usually read between 16 to 20 h for non-fastidious organisms and 20 to 24 h for fastidious organisms. There are few exceptions, specific organisms and drugs, such as vancomycin and *S. aureus*, where a full 24 h of incubation is needed to determine reduced susceptibility to vancomycin in a test strain. It is recommended initially to vary the incubation period for development agents from 16 to 24 h so that a recommendation on the appropriate incubation period prior to reading the MIC can be evaluated.

36.3.3 Development of Susceptibility Test Methods

During Phase I studies, validated MIC and disk methods for susceptibility testing are to be established for aerobic, fastidious, and anaerobic bacteria. Recently, such studies are commonly outsourced to contract organizations that specialize in microbiological testing. Use of a recognized reference method developed by a recognized antimicrobial susceptibility testing organization, such as the US-based CLSI or the European Committee of Antimicrobial Susceptibility Testing (EUCAST), is highly recommended. The most commonly employed reference methods for global antibacterial development programs are those of the CLSI, as described in the CLSI M23 document [9]. However, on occasion, a modified standard method might be more appropriate (e.g., the addition of any substance). If modification to the standard method is proposed by a sponsor, such as the addition of a medium supplement (e.g. Ca^{2+}), then justification for the modification is required, and the effect on susceptibility results is to be evaluated. In such instances, regulatory authorities recommend that a sponsor performs quality control (QC) testing during drug development in order to establish acceptable QC ranges for antimicrobial susceptibility testing (AST) to ensure confidence in the test performance, and the generation of precise, accurate, and reproducible susceptibility data. Scientific advice from regulatory agencies should be sought prior to implementing any modification to a reference method. Studies investigating the influence of growth medium, inoculum density, and incubation conditions will need to be repeated if a modified reference method is to be established.

36.3.4 Preliminary Quality Control Parameters for In Vitro Susceptibility Testing

Preliminary QC testing should include procedural variations that are known to influence antimicrobial susceptibility test (AST) performance for other structurally related approved antimicrobial agents (e.g., inoculum, pH, temperature, atmospheric

conditions, cations, etc.). Preliminary QC parameters should be elucidated in Phase I, prior to additional susceptibility testing to ensure the generation of accurate and reproducible results. Quality Control studies should be performed with all appropriate CLSI reference methods to establish equivalency of methods (e.g., agar dilution and broth microdilution). Ideally, testing is to be performed with at least 500 contemporary clinical isolates representing all clinically relevant species to the indications sought. Isolates should be gathered from various geographical locations to represent strains commonly isolated and subjected to susceptibility testing by the routine clinical microbiology laboratory. The organisms to be studied should also include resistant phenotypes or genotypes (if known) in addition to wild-type susceptible strains with no known mechanisms of resistance, isolated from patients with clinical infections resident in the US and ex-US (CLSI defined Tier 1 Preliminary QC Study) for US applications.

The focus of further testing is the establishment of the acceptable ranges with a given method (agar dilution, or broth microdilution) by a single laboratory, between multiple laboratories and between reagent lot results (CLSI defined Tier 2 QC study). To examine inter-laboratory variation, data from at least seven laboratories (at distinct institutions) are to be analyzed. Ten replicates of each QC strain are to be tested on three different test medium lots, frozen panels for MIC evaluations and at least two lots for disk diffusion studies (sourced from two different manufacturers, if available) over 3 days. Individually prepared inoculum suspensions are to be used for each replicate strain tested. Established QC strains recommended by CLSI should be obtained from a reputable strain collection recognized by regulatory authorities such as the American Type Culture Collection (ATCC®) or National Type Culture Collection (NTCC®). For detailed procedural guidance for preliminary QC testing and establishing acceptable QC ranges, consultation of CLSI document M23-A3 is recommended [9].

The FDA Draft Guidance recommends that preliminary QC ranges and supporting data are submitted to the reviewing microbiologist for approval prior to the initiation of Phase II clinical trials. Following FDA review, expected ranges established with Tier 2 QC studies are to be presented to the CLSI AST Subcommittee, together with proposed abbreviation(s) (maximum of three letters) to be utilized by the manufacturers of antimicrobial susceptibility testing devices, for publication in CLSI M2 documents. In addition, requirements for the preparation of antimicrobial stock solutions, such as diluents and/or special supplements, should be presented together with any limitations on the standard method, plus special instructions for reading reference tests. Following publication in the CLSI M2 documents, preliminary QC ranges may be reported in the investigational agent's Investigators Brochure, as once they are established, QC data are to be continually monitored for variability throughout the entire microbiology development program.

Currently, there is no specific requirement to present QC data to European regulatory agency (EMA) or the European Committee of Antimicrobial Susceptibility Testing (EUCAST) prior to the submission of a Marketing Authorisation Application (MAA), the European equivalent of an NDA. It should be noted that EUCAST recommended QC strains are similar to those recommended by CLSI, except for *Haemophilus influenzae* and *S. aureus* testing. The EUCAST Web site

is updated frequently and should be consulted for guidance regarding QC strains and QC testing. For additional QC testing with EUCAST QC strains, consultation with EUCAST is encouraged.

36.3.5 Provision of Information on the MOA/MOI, Cidality, Potential for Resistance Development, and Cross-Resistance to Other Antimicrobials

By the lead-optimization stage of drug discovery, some aspects on the mode-of-action (MOA) and mode-of-inhibition (MOI) should be understood. At least the biosynthetic pathway (DNA synthesis, protein synthesis, etc.) affected by the agent should have been elucidated. For new compounds within existing antibiotic classes, there is usually a wealth of information available in the literature suggesting the MOA of the molecules. In lead optimization, it becomes a matter of demonstrating improvements and differentiation over the existing molecules in that class. For new molecular entities with novel MOA, limited information is available. When the MOA/MOI is not clearly understood and the factors that impact the MOA/MOI are even less understood for novel classes, the microbiological activity in preclinical settings can be misleading. Supplements or alterations to the susceptibility test medium may be necessary to exert an effect, as is the case with daptomycin where a saturating amount of calcium chloride is added to the susceptibility test medium [15]. This would not be known without some knowledge of the MOA/MOI. It may not be feasible to demonstrate a complete picture for the MOA/MOI to the regulatory authorities in early development, but some information relating to the pathway that is being inhibited should be demonstrated. As further knowledge is gained on the inhibition of the target and the pathway being inhibited, better microbiological studies can be designed for assessing the impact of susceptibility testing methods. These should be provided to the regulatory authorities during the development process to demonstrate a clearer picture of the MOA.

36.3.6 Other Miscellaneous Studies

Individual studies may examine the effects of the novel agent on target bacteria and/or interactions with the host. These phenomena include, but are not limited to, post-antibiotic effect (PAE), post-antibiotic leukocyte effect (PALE), sub-MIC effects, MICs in the presence of human serum, effects on endotoxin release, effects on the normal flora, and interactions with the host immune system. Although the clinical significance of these phenomena is unknown, it would be useful to provide this as part of the overall understanding of the potential activity of the antimicrobial in the human body. This is not an exhaustive list of the various studies that can be investigated, but does address some that have been investigated in the past with approved agents.

36.4 Microbiological Considerations during Late Clinical Development (Phase II and Phase III)

36.4.1 Confirmation and Re-assessment of Quality Control Ranges

Microbiology testing QC data are to be continually monitored at both the central and local laboratory test sites of Phase II and III clinical trials, together with the central laboratory performing the *in vitro* antimicrobial surveillance program. These data are to be assessed for variability and presented in the final submission documentation known as the "Common Technical Document" (CTD). Failure to provide this information may invalidate the susceptibility test data (FDA 2009 Draft guidance). These additional QC data will confirm the appropriateness of the preliminary QC ranges established, and may indicate the need to tighten the range, or for a complete reassessment.

Quality Control data from the clinical trials should also be presented to CLSI to confirm the appropriateness of the preliminary QC range previously established (Tier 2 study). Occasionally QC ranges may require reassessment. Detailed guidance regarding the content and format of final QC parameters for dilution techniques (MIC tests) and diffusion techniques (measurement of zone diameters) in US labeling can be found in FDA Draft Guidance to Industry [47]. It should be noted there is no requirement to present QC ranges in European product labeling, known as the "Summary of Product Characteristics" (SmPC).

36.5 Development of Antimicrobial Susceptibility Testing Devices

In the routine healthcare setting, clinical microbiology laboratories use a variety of antimicrobial susceptibility test devices. Those processing high numbers of specimens daily commonly use automated or semiautomated devices to report the susceptibility results. However, the most common device in use worldwide is the paper disk impregnated with drug. For a newly approved antimicrobial agent to be tested in the routine clinical laboratory, the sponsor will need to have the agent incorporated into the various susceptibility testing devices. At least one method (disk, MIC, etc.) must be developed and approved for susceptibility testing. Discussions with the regulatory authorities early in development of the agent should occur on methodological issues particularly if there are circumstances that may prevent the development of one of the susceptibility testing methods because it does not accurately predict susceptible versus resistant strains. In addition, contact with AST device manufacturers must occur relatively early in drug development if preliminary QC ranges are to be available for Phase II clinical studies (requires the development of two disk batches

from different manufacturers and MIC microdilution panels from a single manufacturer). Device manufacturer development studies for incorporation of the new agent into additional AST devices can be performed at a later date, however not later than Phase III, if they are to be available to the routine laboratory after product approval. There are three main types of AST device in use: disk diffusion, gradient diffusion, and instrument-based automated and semiautomated devices.

36.5.1 Disk Diffusion Test

The disk diffusion test is a simple test that is easy to conduct and inexpensive to perform. It relies on the diffusion of the test agent into the agar surrounding an impregnated 6-mm paper disk. These are placed onto the agar after the plate has been inoculated with the organism. Following incubation, a zone diameter (mm) is read and interpretive criteria applied to define the susceptibility of the test organism (susceptible, intermediate, or resistant) to the agent tested. The disk diffusion test is however subject to many variables that can affect the diffusion of the agent into the agar. These variables have previously been described by Turnidge and Bell [44].

In order to develop a disk test, a validated dilution test (broth dilution or agar dilution) needs to be established in order to correlate MIC results with zone diameters from the disk diffusion test. Initial studies investigate the quantity of drug (mg) to be impregnated to the disk to provide reasonable zone diameters (mm) that predict susceptible, and possibly intermediate and resistant categories. To establish tentative interpretive criteria for disk diffusion testing for use in Phase III trials, the zone diameters of a population of strains are correlated to broth or agar dilution MIC values.

In addition the effect of disk storage conditions (up to 52 weeks) on test results should also be investigated, by both the disk manufacturer and the sponsor. These data are to be presented in the CTD.

36.5.2 Gradient Diffusion Test

This device is a variation of the disk diffusion test, which is relatively simple to perform. It consists of a plastic strip impregnated with a gradient of the test agent, and unlike the disk test provides an MIC value. Results are read at the point where the growth of the bacterium intersects the strip where the concentration of drug is indicated on the strip. The MIC result obtained by this method should be comparable to an MIC obtained by either the broth dilution or agar dilution method. Once again, in order to develop this test, and validate the method a validated dilution method (broth dilution or agar dilution) needs to be established to allow comparison of MIC data.

36.5.3 Automated Susceptibility Testing Devices

There are several manufacturers of instrument-based automated susceptibility testing devices available. The advantage that many of these devices offer is the ability to determine pathogen identification in addition to the susceptibility profile (antibiogram). There are also disadvantages to some of the systems, in particular the rapid tests available on some systems. The history of these instrument-based systems has been reviewed by Felmingham and Brown [13].

Contact with the manufacturers of these instrument-based devices is warranted before Phase III in order to allow sufficient time for development studies to be conducted for the agent to be incorporated on the device so that the consumables (cards, cassettes, trays, etc.) are ready and available by marketing approval. This may take as long as three to five years.

36.5.4 Pharmacokinetic and Pharmacodynamic Considerations

Pharmacokinetics (PK) describes the relationship between the administered dose and the observed levels in serum, tissues, and other bodily fluids over time. Pharmacodynamics (PD) is concerned with the magnitude of the observed pharmacological effect, bacterial inhibition, (exposure) over a given period of time. The advancements in PK/PD analysis over the past decade have been considerable, such that these investigations are central to antibacterial development programs today, and are recommended by both the FDA and EMA. Despite the current rate of these advancements, there is no standardized procedure for the PK/PD evaluation of antibiotics. The EMA recommends that the evaluation of PK/PD relationships be performed in collaboration with experts in the field who are at the forefront of developing and improving the techniques used for these analyses. The overall PK/PD assessment should be sufficient to establish reasonable confidence in the efficacy of the antibacterial agent. PK/PD analyses should cover the highest MIC values of the target pathogens observed in surveillance programs.

36.5.5 In Vitro Models

A variety of *in vitro* kinetic models (one-compartment, multiple compartments) have been developed to simulate specific conditions (reviewed by Gloede et al. [17]). It is a usual practice to simulate free drug serum concentrations; however, a key advantage over *in vivo* studies is the flexible nature of these models, which permits almost any concentration versus time profile to be produced allowing human PK to be easily simulated and drug activity to be assessed against a range of target pathogens. The PK/PD parameters can be investigated to establish the best correlation with drug efficacy (Time above MIC [T > MIC], AUC/MIC ratio; Peak Concentration/MIC ratio [C_{max}/MIC] – see Crandon et al., Chapter 34). The main limitation of these

models is that they model infection in an immunocompromised host (since there is no cell-mediated immune response in an *in vitro* model), and thus do not always reflect the intended patient population, where the immune system usually plays a key role in the overall clinical and microbiological response.

In addition, these models evaluate the emergence of resistance, time-kill data, persistent effects (post-antibiotic effect, post-antibiotic sub-MIC effect, post-antibiotic leukocyte enhancement), identification of PK/PD indices, and support optimal dose selection.

36.5.6 In Vivo Models

Numerous animal models have been developed that closely imitate the characteristics of a human infection, with clearly defined infection endpoints (death/moribund condition) comparable to humans. The β-lactams and fluoroquinolones are the most studied antibacterials to determine the impact of drug concentration on killing kinetics for specific infections. Modeling of the relationship between drug concentration and efficacy allows determination of the PK/PD parameter that best correlates with outcome. The main disadvantage of animal models is the marked difference in PK, e.g., metabolism, biliary, or renal transport between animal species (particularly small animals used in PK studies) and humans. These differences are most marked for β-lactams. Consideration of these PK differences is pivotal to both study design and data interpretation. The dosage can be adjusted (by modifying drug input or drug elimination) to simulate human PK.

Of the animal models used to study PK/PD relationships, the most frequently employed in drug development is the neutropenic murine thigh model. Other animal models include the acute pneumonia model (performed in mice, rats, hamsters, and rabbits); osteomyelitis (rat and rabbit), endocarditis (rabbit), meningitis (rat, guinea pig, cat, rabbit, dog, goat, and monkey), pyelonephritis (mice and rats); and septicemia model (mouse and rat). In early development, efficacy should be investigated in a variety of these models against the most commonly implicated pathogens relevant to the indication(s) sought, e.g., for community-acquired pneumonia programs, efficacy should be demonstrated against *Streptococcus pneumoniae*, *H. influenzae*, *S. aureus*, and *Moraxella catarrhalis* susceptible strains in addition to challenging resistance phenotypes or specific bacterial serotypes. In addition, strains with elevated MICs to the study drug should also be studied in animal infection models, to provide evidence in support of proposed susceptibility breakpoints. In addition, these models may be utilized to measure the post-antibiotic effect *in vivo*.

36.5.7 Characterizing the PK/PD Relationship

Analysis of the data derived from *in vitro* and *in vivo* non-clinical studies (such as hollow fiber models and the murine thigh infection model) can be useful in

determining the PK/PD index best associated with the activity of a new antibacterial agent as well as the magnitude of the PK/PD index necessary to achieve the desired endpoint. Ideally, animal infection studies should be conducted in both neutropenic and immunocompetent mice to evaluate the impact of an intact immune system on efficacy. All target pathogens relevant to the indication(s) sought should be tested in the appropriate animal model(s).

The MIC distributions for wild-type populations of the target pathogens sought for labeling should be taken into account so that the PK/PD analyses cover the highest MICs considered to be treatable with well-tolerated dose regimens.

The results of non-clinical studies should be considered in the context of PK data derived from Phase I studies to help identify appropriate dosing regimen for Phase II and III studies. Whenever possible, it is recommended that the PK/PD analyses used for dose regimen selection be based on PK data obtained from infected patients rather than from healthy volunteers. If not, the initial analyses should be repeated using patient PK data when these become available to reassess the validity of the initial conclusions.

It is recommended that the PK/PD relationship is further explored during Phase II and III clinical studies in each indication studied and using the *in vitro* susceptibility data of the clinical trial isolates, patient PK data, and clinical and microbiological outcomes. These investigations might constitute sub-studies within larger clinical studies.

36.5.8 Clinical Trials

Both the FDA and EMA have developed draft guidance documents that provide general considerations for conducting clinical trials and the evaluation of microbiological data. The employment of a central laboratory for microbiological testing, including susceptibility testing, is highly recommended. If commercially prepared susceptibility testing microtiter panels (either frozen or freeze-dried) are to be employed to determine MIC results for Phase III clinical isolates, a validation study demonstrating the comparability of MIC data is required.

In addition, FDA Draft Guidance for Industry for specific indications has been developed; e.g., community-acquired bacterial pneumonia [46], nosocomial pneumonia [49], uncomplicated and complicated skin and skin-structure infections [48], and complicated urinary tract infection and pyelonephritis [45]. The FDA Web site (http://www.fda.gov/) should be consulted for the most updated draft guidance. The Community-acquired Bacterial Pneumonia Draft Guidance [46] provides detailed guidance regarding clinical trial design, implementation, monitoring, and collection of relevant data for analysis, and appropriate types and numbers of analyses.

Before initiating a Phase III clinical trial program, it is highly recommended that scientific advice be sought from regulatory agencies in both North America (FDA) and Europe (EMA) regarding the proposed clinical trial protocol design and to review the current non-clinical and clinical data that are available. These should include preliminary QC ranges and provisional interpretive criteria for susceptibility testing.

36.6 Provisional Interpretive Criteria for Susceptibility Testing

Provisional interpretive criteria are based on the limited data available following the completion of Phase II trials. These include PK data reported for human volunteers from the Phase I studies and from Phase II efficacy data and limited patient population PK data; the PK data of any microbiologically active metabolite should also be considered and the data presented in the same format as for the parent molecule.

Comparative MIC distribution data for at least 500 isolates relevant to the target pathogens for the indication(s) to be sought, as well as zone diameter data, are required and should be analyzed as frequency distributions for each target species. For the establishment of preliminary breakpoints, it is recommended that potentially resistant populations are excluded from the susceptible category when they exist. Further details can be found in FDA draft guidance [47] and CLSI M23 documents [9].

36.6.1 Antimicrobial Surveillance

During the microbiological and clinical development program, sufficient antimicrobial surveillance data to further characterize *in vitro* activity against recent clinical isolates from various countries and regions worldwide is to be collected for assessing the epidemiology of resistance. The species selected for susceptibility testing should be clinically relevant to the indications sought. The EMA provides specific guidance relating to the conduct of antimicrobial surveillance studies. While definitive numbers of organisms to be tested are not suggested, general considerations are provided: The number of organisms to be tested will be dependent upon the agent's spectrum of activity. In addition, consideration is given to whether the agent under study belongs to an existing antibiotic class for which data on the prevalence of acquired resistance and the potential for cross-resistance are well established versus a new antibiotic class. Recent European draft guidance [10] further defines recent isolates as those obtained within approximately 5 years prior to submission of an MAA, while the FDA specifies for the investigation of a new molecular entity that isolates used to generate data in a span of no more than 3 years from the date of the NDA submission. Marketing authorization applications (MAAs) require the isolates to include a representative sample from within the European Union (EU). For NDA (US) submissions; a representative sample of isolates from the US is required and the testing of isolates from other regions is encouraged.

The number of isolates tested for a given species and their source requires justification in the MAA. The frequency with which various pathogens cause infections within the indications sought should be used to select the proportion of the total number of organisms to be tested for a given species. For commonly encountered species, it should be possible to test several hundred isolates of a given species, and include representative numbers of organisms that demonstrate common resistance phenotypes (individual and multiple antibiotic classes). If the study agent belongs to

a known antibiotic class, then adequate data should be obtained in order to document the degree of cross-resistance within the class that can be expected. For rare or less frequently encountered pathogens, it is preferred that at least ten organisms of each species be tested.

While the presentation of MIC summary data (number of isolates tested, MIC range, MIC_{50} and MIC_{90}) is sufficient for a spectrum of activity studies, the preferred presentation format of surveillance data is MIC distributions for each species sought for labeling. When appropriate, MIC distributions by phenotype (with and without specific resistance mechanisms of particular interest) for a given species should be presented (e.g., *S. aureus* [all strains], *S. aureus* [methicillin-susceptible], *S. aureus* [methicillin-resistant]). The selection process should also take into account information that may be already available on the prevalence of bacteria that possess various types of mechanisms of resistance and the known or unknown potential for these mechanisms to affect the activity of the antibacterial agent under evaluation.

Examples of surveillance programs undertaken for the approval of a new antibiotic can be found in the literature and CHMP European Public Assessment Reports (EPARs). For doripenem, a broad-spectrum carbapenem approved in Europe, approximately 10,000 recent worldwide clinical isolates were tested to characterize the *in vitro* activity. Isolates included aerobic and anaerobic Gram-negative and Gram-positive species (CHMP Assessment Report for Doribax, EMA) [12].

36.6.2 Establishing Final Interpretive Criteria for Susceptibility Testing

In the US final interpretive criteria for susceptibility, testing should be proposed by the sponsor in the NDA criteria for all the bacteria listed in the INDICATIONS AND USAGE section of the label. The FDA will establish breakpoints at the time of product approval, and these are included in the label. Sponsors may also present proposed interpretive criteria to CLSI for possible publication in CLSI reference documents that are used by routine clinical microbiology laboratories worldwide.

It should be noted these independent processes may produce discrepant results resulting in the establishment of different breakpoints by the FDA and CLSI. Every effort should be made to resolve such discrepancies. When the CLSI subcommittee approves a breakpoint that is different from the established FDA breakpoint, the sponsor is encouraged to submit the data package that led to the approval of the CLSI breakpoint to the regulatory agency and/or request additional assessment or reassessment by the CLSI subcommittee.

In the European Union, a standard operating procedure (SOP/H/3043) regarding the establishment of antimicrobial interpretive criteria for susceptibility testing exists between the EMA and EUCAST as part of the centralized procedure for the assessment and approval of new drugs. This SOP outlines the conditions under which the sponsor, the rapporteur, and co-rapporteur appointed by the Committee

for Medicinal Products for Human Use (CHMP), the EMA, and EUCAST will work together in confidence. Further information regarding the procedure, including the SOP, can be downloaded from the EUCAST Web site (http://www.eucast.org/information_for_industry). Sponsors should decide early in the development program whether they will participate in an agreement that will allow interpretive criteria (breakpoints) to be evaluated and agreed by EUCAST since this decision has potential implications for the *in vitro* susceptibility testing program. If the sponsor opts out of the arrangement, then the breakpoints will be established by CHMP. In either case, the final decision regarding breakpoints will be made by the CHMP at the time of approval. Additional breakpoints maybe sought at a later date with the submission of supplemental indications or may be changed if current breakpoints are deemed no longer optimal.

Data from the MAA will be shared with EUCAST, who will review all data relevant to establishing breakpoints before a final opinion regarding approval is reached by CHMP. Subject to the agreement of the CHMP, EUCAST breakpoints will be included in section 5.1 of the SmPC). The review process by EUCAST will adhere to an agreed timetable drawn up by the aforementioned bodies for each centralized procedure. The EUCAST Steering Committee plays a key role in the process, and the committee meets regularly, with the dates of upcoming meetings being posted on the EUCAST Web site. A plethora of valuable information to assist industry with the process can now be found on the EUCAST Web site (http://www.eucast.org/). In brief, sponsors are encouraged to make an initial presentation to EUCAST prior to initiation of the centralized procedure. The scientific secretary will provide the names of the current Steering Committee members needed for the preparation of confidentiality agreements.

A preliminary presentation to the Steering Committee should ideally contain an outline of the microbiological activity of the drug, the clinical indication(s) sought and the organisms sought for labeling, and MIC distributions of target pathogens. In addition, planned administration formulations, dosing regimens, and PK data obtained from healthy subjects and from patients should be presented, together with relevant *in vivo* infection models that support proof of concept, available analyses of the possible relationship between dose, and clinical and microbiological outcomes by MIC if available.

In addition, EUCAST requests that sponsors assemble all the relevant data for establishing breakpoints in a document format known as the "Company Rationale Document." A template document can be downloaded from the EUCAST Web site. This document lists data relevant to dosing, indications, target organisms, MIC distributions for target organisms, existing breakpoints (from other AST committees or regulatory authorities) if available, PK/PD data, and clinical response by MIC, and will serve as one of many sources of information used by EUCAST to establish clinical breakpoints. This document should be submitted as early as possible, preferably prior to filing the MAA or at the latest when filing with EMA. The existence of a Company Rationale Document will help highlight differences between the Sponsor and EUCAST with respect to data and their interpretation. The Company Rationale Document can be amended or updated by the Sponsor at

any time during the process, whereby all text modifications must be highlighted and a copy sent to EUCAST and the Rapporteur. EUCAST Rationale Documents for approved agents with established EUCAST breakpoints can also be viewed at the EUCAST Web site.

The establishment of clinical breakpoints is based on the following key features of the antimicrobial agent or the pathogen: (1) clinical and microbiological outcome, (2) PK and PD of the antimicrobial agent (*in vitro*, *in vivo*, and in humans), and (3) MIC distribution of the target pathogen(s).

The following analyses should be considered for the establishment of breakpoints:

- Analyses of the clinical trial data to establish the correlation of clinical response and microbiological eradication (presumed eradication) by individual indication sought, by pathogen (all species sought for labeling) with susceptibility test result (MIC, zone diameter), phenotype(s), and virulence factors (as appropriate).
- Analysis examining the relation between PK/PD parameters and efficacy: to include, but not to be limited to, discussion of the primary PK/PD index, the index magnitude predictive of efficacy derived from appropriate *in vivo* efficacy infection models, peak serum or plasma levels, MIC ratio, and area-under-the-curve serum drug concentration (AUC): MIC ratio. Data on the post-antibiotic effect in all species relevant to the indications sought and impact of increasing drug concentrations on bacterial killing.
- The MIC distributions of all the isolates from the clinical trials and surveillance are to be compared. Isolates showing important resistance mechanisms such as methicillin-resistant *S. aureus* (MRSA) and coagulase-negative staphylococci (methicillin-susceptible and -resistant strains) should be included in the evaluation of anti-staphylococcal agents.

36.6.3 Microbiological Considerations for Labeling

36.6.3.1 Food and Drug Agency Considerations

The FDA Draft Guidance for Industry, Microbiological Data for Systemic Antibacterial Drug Products [47] recommends the content and format for presentation of microbiological data and provides an example format for the microbiology subsection of the label.

36.6.3.2 European Medicines Agency Considerations

Recommendations for the content and presentation format of the microbiology data in section 5.1 Pharmacodynamics of the Summary of Product Characteristics (SmPC) can be found in the EMA draft guidance [10].

36.6.3.3 The Common Technical Document: Location of Microbiology Information

North American and European regulatory agencies currently (since January 2010) recommend that submissions be made using the electronic common technical document (eCTD) format. Specific guidelines describe the eCTD process (Providing Regulatory Submissions in Electronic Format – Human Pharmaceutical Product Applications and Related Submissions Using the eCTD Specifications, 2008).

General guidance regarding the location of microbiology written and tabular summaries, non-clinical and clinical study reports is provided in the FDA draft guidance [47]. More detailed guidance regarding all the European CTD modules can be found in Volume 2B Notice to Applicants Medicinal Products for Human Use (http://ec.europa.eu/health/files/eudralex/vol-2/b/update_200805/ctd_05-2008_en.pdf).

36.7 Post-approval Studies of Resistance

36.7.1 United States

After approval, additional susceptibility testing data including QC data to monitor the performance of MIC tests will become available. It is important that the sponsor continually monitors these data to ensure that the interpretive criteria listed in the product labeling reflect the most current information. The susceptibility of certain species to antibacterial products may change over time. Information relevant to changes may include additional data on susceptibility and response to therapy and/or new mechanisms of resistance that result in decreased susceptibility to a particular agent. Such changes in susceptibility may translate into a lack of efficacy and/or safety concerns when outdated susceptibility criteria are applied, which may lead to treatment failure.

Procedures for updating labeling with regard to susceptibility testing can be found in the FDA guidance document "Updating Labeling for Susceptibility Test Information in Systemic Antibacterial Drug Products and Antimicrobial Susceptibility Testing Devices."

36.7.2 Europe

After approval of an antibiotic in the EU, the sponsor should continue (for approximately three to five years) to monitor the susceptibility of the new drug against key pathogens relevant to the approved indications in order to assess the emergence of resistance. These surveillance studies may need to be prolonged if resistance issues arise during the initial three to five year surveillance period. These studies should be mentioned in the Risk Management Plan and specific commitments should be listed in the Letter of Undertaking.

Information regarding the emergence of antibiotic resistance or changes in susceptibility should be promptly reported via the Periodic Safety Update Report (PSURs). The marketing authorization holder (MAH) is expected to provide a critical evaluation of the benefit/risk balance of the product in the light of new or changing post-authorization information. This should include whether further investigations are required for the assessment or whether changes should be made to the marketing authorization (e.g., to the product information, the SmPC).

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Part IX
The Economics and Incentives of Antibiotic Drug Discovery

Chapter 37
Stimulating Antibacterial Research and Development: Sense and Sensibility?

Steven J. Projan

37.1 Getting Back to First Principles

When HIV/AIDS became epidemic in the early 1980s, aggressive funding of academic research (and a flexible regulatory regime) led to the discovery, development and approval of over 20 novel therapeutic agents. The CDC reported that in 2007 the deaths due to MRSA in the U.S. exceeded HIV/AIDS. Indeed deaths in the United States due to bacterial infections exceed HIV/AIDS by more than an order of magnitude; however, this is clearly not reflected in our current academic funding priorities. Before we can expect to see robust industrial pipelines, we will need to take the same academic funding approach to multi-drug resistant bacterial infections in this decade that we did (and frankly still do) for HIV in the 1980s. Ah but where are the monies for this research going to come from? Well one could adjust our current funding priorities (sort of like stealing from Tony to pay Stuart) or one could impose the equivalent of a carbon tax on the burning of fossil fuels (i.e., a "resistance tax" on generic antibiotics (for all purposes, including animal husbandry)). It is now well known to all that the emergence and dissemination of resistant strains is proportional to the amount of antibiotic use, so while it is imperative that patients receive antibiotics it is also clear that such use contributes to the emergence and dissemination of resistant strains that may negatively affect the next patient receiving these drugs. Therefore, such a tax is eminently fair. But then, assuming we get the appropriate levels of funding, we are still left with the problem of insufficient numbers of industrial scientists who are capable of translating the academic knowledge into the next generation of antibacterial drugs. So how do we incentivize the biopharmaceutical industry to once again invest in infectious disease drug discovery and development?

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37.2 Incentives? Yes, But What is Going to Work?

The IDSA has previously called for "modified wild-card" exclusivity where, if a company actually received regulatory approval for a novel antibiotic addressing a key unmet need, then the company could receive an extended period of market exclusivity for another anti-infective drug in their portfolio. Such a change would require new legislation and would, no doubt, be proclaimed a "give away to the drug companies" or "corporate welfare," and given our current demagoguery-filled climate of political discourse such legislation has not and will probably never see the light of day. Even if it does see the light of day there is something illogical in extending the market for another drug when really it is the novel therapy that should receive the reward. Something quite interesting happened this year (2010) when the recent healthcare insurance reform bill became law: novel biologic drugs will now have 12 years of exclusivity in all therapeutic areas. Indeed, the anticipation of such a change has already resulted in increased activity to discover and develop novel biologic drugs by large and small companies alike. It takes only a small amount of reflection to propose that novel antibacterial drugs that gain regulatory approval for designated unmet medical needs should be afforded the same 12 years of exclusivity. Given that patents now run for 20 years from the initial application, this may sound like a disincentive; however, take the case of tigecycline, which was discovered in 1993, and the U.S. patent issued in 1996, but it was not approved until 2005 (2006 in the E.U.), and the drug comes off patent in the U.S. in 2013. Twelve years of exclusivity would provide an additional 4 or 5 years at the back end of the exclusivity period (when drugs are typically most profitable) and would be a billion to multi-billion incentive for a drug, even if it has a below average market. Yes, this does subject the patient to higher prices for perhaps an additional 4 or 5 years before a generic version may become available, but for the most part, in the crowded and confused antibacterial market, the patient is still getting a bargain. Indeed, it is the view here that antibiotics are not only among the most effective of all drugs (even in the face of resistance), but they are probably the most under-priced in medicine. It is not unusual for a state-of-the-art therapy for cancer, which extends a patient's life for 3 months, to cost upwards of $40,000.00 whereas a course of even the most expensive of antibiotics that extends a patient's life for decades almost always costs less than $1000.00.

37.3 Eliminate the Disincentives

One of the great disincentives for pursuing novel antibacterial drugs has been the ability of companies large and small to navigate the very choppy (and often mine-infested) regulatory waters. There currently appears to be a significant divergence between the attitudes and positions of the EMEA in Europe and the US FDA; the inconsistencies of approach as to what is required for regulatory approval essentially

makes it less and less likely that a set of clinical trials can be designed which can be used to gain regulatory approval in both venues. This may well double the cost of development. In today's current climate, the industry is being pushed to perform larger and larger studies with more restrictive inclusion criteria, unrealistic or inconsistent endpoints (and the hunt for novel biomarkers), and less and less relevance to clinical practice. Ironically, we in the industry have been repeatedly lectured that the one, well validated, biomarker, the elimination of the pathogenic bacterium, is not acceptable for approval purposes. Many of us have also been subjected to lectures about the importance of large patient safety databases as part of regulatory submissions, but the largest safety studies pursued for a novel antibiotic was for telithromycin, which ultimately received marketing approval only to be confined to the dust bin because of alleged safety issues. Indeed, the safety issues identified for telithromycin that derailed its commercial (and therefore medical) success as an antibiotic were not (and could not) be discovered by even the largest antibiotic clinical trial in history. The irony is, as well demonstrated in an analysis by Shlaes and Moellering [4], telithromycin has a safety profile that places it about in the middle of many currently used antibiotics, and it is certainly safer than penicillin (which in today's regulatory environment would not get approved; but that is probably true of over a dozen currently used antibiotics). What would make the most sense in all regulatory regimes is provisional approval based on solid phase II data and then to go directly to phase IV and see how the drug works and how safe it is in real patients and in numbers that can reveal whether there are actual safety issues.

While it is hard to remain optimistic given the public, private, and governmental failures in this critical area, it is clear that the current approach is failing us as a society in very real and global terms. When the public finally wakes up to this fact as the worldwide plague of bacterial resistance hits home more and more often, and at the cost of tens of thousands of lives, then and only then will the tide turn. Despite all the barriers we currently confront, there has been much scientific progress made; this will eventually result in improved strategies to reduce the burden of infectious disease, and I think we all realize that failure is not an option.

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