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Molecular Mechanisms of Antibiotic Resistance: The Need for Novel Antimicrobial Therapies

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Abstract

Despite the enormous success of antibiotics as chemotherapeutic agents, infectious diseases remain a leading cause of mortality worldwide. Bacteria coevolving with infectious microorganisms have been driven to develop protection against environmental bioactive chemicals, and to resist their own antibiotics and defense compounds. Such resistance in pathogenic microorganisms provides protection against chemotherapeutic intervention and can lead to infections that are notoriously difficult to manage. Here, we introduce briefly the molecular mechanisms of action for common antibiotic classes and the structural determinants of bacterial resistance to antibiotics. Bacterial resistance to antibiotics interfering with cell wall biosynthesis is discussed with examples from β-lactams and glycopeptides. The molecular determinants of bacterial tolerance to protein biosynthesis inhibitors are analyzed with examples from aminoglycosides, marcolides and tetracyclines. Fluoroquinolone tolerance is described in connection with DNA regulation in the presence of inhibitors. The action of β-lactam and glycopeptides antibiotics, which target cell wall biosynthesis, is evaded through target modifications and antibiotic deactivation. β-Lactamases can be deployed to deactivate β-lactam antibiotics, while penicillin-binding proteins with altered binding sites provide an example of modification of the antibiotic target. Alteration of peptidoglycan peptide termini helps protect cell wall synthesis from glycopeptides, while reduced cross-linking and thickened cell wall obstruct glycopeptides access at the cell wall periphery. Specific molecular changes within ribosomal structures can foil aminoglycoside, tetracycline or macrolide attack. Further protection can be achieved by positioning proteins to protect protein synthesizing machinery from the action of tetracyclines. Important resistance mechanisms protecting bacterial protein synthesis include enzymatic deactivation of aminoglycosides and efflux systems expelling incoming macrolides, aminoglycosides and tetracyclines. Renewed efforts to explore alternative strategies for infection management have brought to the top of scientific agenda defense peptides, lanthionine antibiotics, phage therapies and other antimicrobial techni-

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ques, which have not seen wide use against clinically significant pathogens and resistance to which remains uncommon.

1.1 Introduction

'It's time to close the book on infectious diseases, declare the war against pestilence won, and shift national resources to such chronic problems as cancer and heart disease.' These were the optimistic words of William H. Stewart, the United States Surgeon-General, in 1967. However, even during these years of antimicrobial optimism researchers still recognized that microbes would take the opportunity to reproduce about every 30 min as a fresh chance to mutate, migrate, adapt to new hosts and environments, and resist hostile agents that threatened them [1]. The honeymoon period of the 1950s and 1960s for antibiotic development was over, and even William H. Stewart had to admit later that his observation had been 'dead wrong' [2].

Despite the enormous success of antibiotics as chemotherapeutic agents, infectious diseases remain a leading cause of mortality worldwide. Antibiotics are compounds that are (literally) 'against life' – naturally produced by soil microorganisms and airborne molds in response to threats such as overcrowding [3]. Bacteria coevolving with these microorganisms have been driven to develop protection against environmental bioactive chemicals, and to resist their own antibiotics and defense compounds. Such resistance in pathogenic microorganisms provides protection against chemotherapeutic intervention and can lead to infections that are notoriously difficult to manage. The widespread use of antibiotics in recent history has placed significant selective pressure on bacteria in favor of less susceptible strains.

Prior to Fleming's discovery of penicillin in 1929, an antibacterial, pyocyanase, was isolated by Emmerich and Low in 1899 from *Pseudomonas pyocyanase*, and had been used therapeutically against infections caused by staphylococci. In the 1920s, Lieske, Gratia and Dath demonstrated that soil microorganisms like actinomycetes could, in most instances, produce antibacterial substances. Fleming was the first to recognize the potential of penicillin even though he never produced therapeutically usable amounts of penicillins. His discoveries opened the door for Chain, Florey and colleagues to obtain clinically usable amounts of penicillin and large-scale production commenced by 1940. Even at this early stage, the ability of some strains of bacteria to develop penicillin resistance had become evident. By 1946 around 15% of strains of *Staphylococcus aureus* isolated in London hospitals were resistant to penicillin G; 1947 saw this figure approach 40% and by 1948 it was 60% [4].

The discovery of the 6-aminopenicillanic (6-APA) basic nucleus of penicillin molecules in 1959 meant chemical synthesis could be employed to prepare virtually any penicillin structure. The 1960s and 1970s saw the introduction of semisynthetic penicillins, for example ampicillin and amoxicillin, which had good activity against both Gram-positive and Gram-negative bacteria, and methicillin and oxacillin, which were potent against penicillinase-producing *S. aureus*. However,

penicillins had their limitations. Most were only active against Gram-positive bacteria, some people developed life-threatening allergic reactions to them and resistance was developing rapidly. A solution came in 1945 from Giusepe Brotze who identified a mold growing near a sewage outfall as *Cephalosporium acremonium*, and showed it inhibited the growth of typhoid bacilli and other bacteria [3]. Thirteen years later Abraham and Newton elucidated the structure of the agent responsible. It contained a β-lactam ring fused to a six-membered ring containing a sulfur atom rather than the five-membered ring seen in penicillins. The Eli Lilly Company successfully removed the side-chain of cephalosporin C to produce a bare cephalosporin nucleus (7-aminocephalosporanic acid).

Strains of *S. aureus* and *Streptococcus pneumoniae* resistant to antibiotics can cause serious infections in hospitals and the community. Significant media attention in recent years has highlighted the problem and has coined the term 'superbugs' in reference, chiefly, to methicillin-resistant *S. aureus* (MRSA) and resistant enterococci. Although reported to a lesser extent, *Staphylococcus epidermidis* and *Staphylococcus saprophyticus* can colonize medical implants and cause serious antibiotic-resistant infections. Antibiotic-resistant commensal bacteria do not present a health problem in healthy individuals and only become problematic when they colonize atypical sites where they may cause persistent infections. In one example, *S. aureus* was a commensal in around 30% of the general population [4], while resistant hospital-acquired strains can lead to significant therapeutic challenges in immunocompromised patients.

In the 1960s, 10% of *S. aureus* strains produced penicillin-destroying enzymes (penicillinases); today the figure approaches 100%. Despite the introduction of methicillin in 1959 to tackle the increasing problem of penicillin resistance, it took only 3 years for MRSA to appear in 1961. Once resistance is genetically encoded it can spread rapidly within a population of bacterial species, or even to another bacterial species through transduction, transformation, conjugation or transposition.

Transduction involves phage replication that accidentally includes replication of resistance-encoding bacterial DNA. When newly replicated phages infect another bacterium the resistance genes can recombine into the DNA of the new host. Transformation involves recombination with the bacterial chromosome of new resistance genes present on absorbed fragments of exogenous bacterial DNA. Conjugation allows plasmid DNA conferring resistance to be transferred via the sex pilus to another bacterium and is a major mechanism of gene transfer in enterococci. Transposons confer resistance to antibiotics by transferring genes between different plasmids or from plasmids to chromosomes.

Social awareness of antibiotic-resistant hospital acquired infections, most notably with *Staphylococcus* and *Clostridium*, is at all times high. Each year in England and Wales about 5000 lives are lost to these infections, and the annual costs to the National Health Service amount to £1 billion. With resistance to antibiotics rapidly spreading, progress in search of novel chemotherapeutic agents is slow. The United kingdom has one of the highest levels of incidence of MRSA in Europe [5]. In 1993 there were 216 deaths where *Staphylococcus* infection was the final underlying cause of death. This figure rose to 546 deaths in 1998 [6]. Sophisticated resistant gene transfer

mechanisms are partly responsible; however, it is our usage of antibiotics that places evolutionary pressure on bacteria and selects those conferring resistance. This is exacerbated by prescribing at subtherapeutic levels and poor patient compliance, and declining antibiotic research and development has not helped. Hospital antibiotic usage demands attention because the large numbers of immunocompromised patients in hospitals creates a suitable environment for the development of antibiotic-resistant bacteria. This environment is primed further by the fact that only around 30% of all hospital antibiotics are used for definitive therapy, where susceptibility patterns for the infection-associated pathogen are known [7].

Here, we introduce briefly the molecular mechanisms of antibiotic action, and discuss the molecular and structural determinants of bacterial resistance to antibiotics. Bacterial resistance to antibiotics interfering with cell wall biosynthesis is discussed with examples from β-lactams and glycopeptides. The molecular determinants of bacterial tolerance to protein biosynthesis inhibitors are analyzed with examples from aminoglycosides, marcolides and tetracyclines. Finally, fluoroquinolone tolerance is described in connection with DNA regulation in the presence of inhibitors.

1.2 Molecular Mechanisms of Resistance

Understanding the molecular mechanisms underlying antibiotic resistance requires an understanding of bacterial structure and function. Structural and metabolic differences between bacterial and mammalian cells make it possible to selectively kill bacteria with antibiotics. For example, the presence of a cell wall in bacterial, but not mammalian, cells can be exploited by antibiotics. Gram-negative bacteria have an inner cell wall made up of a thin layer of peptidoglycan and an outer wall referred to as the outer membrane owing to its resemblance to the cytoplasmic membrane. The periplasm lies between the outer cell wall and the inner cell wall. The Gram-positive cell has no outer membrane and therefore no periplasm. Instead, the cell wall is made up of a thick layer of peptidoglycan and accessory polymers. Mechanical strength in the cell wall is crucial as it must withstand osmotic pressures in excess of 5–15 atm without rupturing [8].

β-Lactam and glycopeptide antibiotics target cell wall biosynthesis, to which there are three stages [8]. First, UDP-*N*-acetylglucosamine (GlcNAc) is converted to UDP-*N*-acetylmuramyl-pentapeptide in the cytoplasm [9]. UDP-GlcNAc is made by linking glucose-1-phosphate (from glucose or glucosamine) to UDP (from pyrimidine biosynthesis starting with glutamate). MurC, D and E sequentially add L-Ala-D-γ-Gln and Lys (Gram-positives) or meso-diaminopimelate (DAP) (Gram-negatives) in ATP-dependent amide-forming steps to create UDP-muramyl-tripeptide. MurF adds a preformed D-Ala-D-Ala in the fourth amide-forming step to create the UDP-muramyl-L-Ala-γ-D-Gln-L-Lys-D-Ala-D-Ala pentapeptide. Transpeptidase catalyzed cross-linking between the third amino acid (L-Lys in Gram-positives, meso-DAP in Gram-negatives) and the penultimate amino acid (D-Ala) of adjacent peptidoglycan chains, with loss of the terminal D-Ala, gives the cell wall rigidity.

The second stage involves transferring muramyl-pentapeptide from UDP to a C55 isoprenol-P carrier. The MraY-catalyzed movement of the muramyl-pentapeptide to the membrane interface makes lipid I. The peptidoglycan chain consists of alternating *N*-acetylmuramic acid and GlcNAc, linked via β1–4 links in a reaction catalyzed by MurG. This generates the disaccharide-pentapeptide attached to bactoprenol-PP, which is lipid II. Lipid II flips the disaccharide-pentapeptide across the plasma membrane to its outer face. Here, transglycosylation involves transglycosylases catalyzing binding of the disaccharide pentapeptide to the 4-OH group of GlcNAc of the existing peptidoglycan strand.

Aminoglycoside, tetracycline and macrolide antibiotics target protein synthesis. The 70S bacterial ribosome consists of a 50S and a 30S subunit. The 50S subunit contains one molecule of 55S RNA and one molecule of 23S RNA plus 32 proteins. The 30S subunit contains one molecule of 16S RNA and 21 proteins. Bacterial mRNA binds to the 30S ribosomal subunit, attracting *N*-formylmethionyl-tRNA (fMet-tRNA) to its AUG initiator codon and forming the 30S initiation complex. The 70S initiation complex is completed by adding the 50S subunit. fMet-tRNA binds to the peptidyl donor (P)-site, which is adjacent to the aminoacyl acceptor (A)-site. Vacant at first, the A-site is where aminoacyl-tRNA bearing the appropriate anticodon and its specific amino acid will bind to. Affinity of aminoacyl-tRNA for the A-site is low and elongation factor (EF)-Tu, which hydrolyzes GTP, is required to increase affinity of the 70S complex for aminoacyl-tRNA. Aminoacyl-tRNA becomes bound to the A-site in the form of a ternary complex: EF-Tu·aminoacyl-tRNA·GTP. Codon–anticodon recognition at the A-site is associated with hydrolysis of GTP and results in the release of EF-Tu·GDP. Following this release is formation of a peptide bond catalyzed by peptidyltransferase which is located in the 50S subunit. Peptide bond formation involves the transfer of *N*-formylmethionine (or peptidyl residue in subsequent chain elongation cycles) from tRNA in the P-site to aminoacyl-tRNA in the A-site, where it is joined to the new amino acid. Deacylated tRNA is lost to the exit (E)-site, the newly formed peptidyl tRNA will move from the A-site to the P-site, and the mRNA and ribosome move to incorporate the next codon into the A-site. The peptidyltransferase-catalyzed transfer of the *N*-formylmethionine to the new amino acid is called translocation and is repeated for subsequent tRNA complexes. However, the maximum rate of translocation is dependent on EF-G, which hydrolyzes GTP to promote translocation.

Bacterial DNA is replicated in a stepwise manner along the DNA circle, progressing along a continuously advancing point, the replication fork [9]. Replication involves separation of the double-stranded DNA and alignment of nucleotides with their complementary bases on each single strand. However, separation of strands wound in a circular helix generates loops called positive supercoiled twists in the single strands. If this was unresolved, positive superhelicity would increase until the rising torsional strain prevented any further unwinding of the DNA at the replication fork [10]. DNA gyrase and topoisomerase IV work in a coordinated fashion to restore the proper conformational structure of DNA and safeguard against the occurrence of high-level replication-induced helical stress.

Discovering new antibiotics requires a comprehensive understanding of the molecular mechanisms and structural determinants of resistance, and how new

drugs may be affected by existing mechanisms and ones which we can anticipate emerging [11]. Destroying or inactivating the antibiotic, pumping out the antibiotic and modifying the antibiotic target are important mechanisms conferring bacterial resistance, and will be reviewed in the context of specific antibiotics.

1.3 β-Lactams

Penicillins, cephalosporins and other β-lactam antibiotics contain a four-membered amide ring and target cross-linking in the final stage of cell wall biosynthesis (transpeptidation). The four-membered ring is structurally similar to the terminal acyl-D-Ala-D-Ala unit of the nascent peptidoglycan (Figure 1.1). Both contain the CO–N bond known to be cleaved by penicillin-binding proteins (PBPs) found in the cell membranes of bacteria. There are usually four or more PBPs with transglycosylation and transpeptidation activity. They are named numerically relative to their molecular size (PBP1 is the heaviest). High-molecular-mass PBPs have an essential role in peptidoglycan synthesis, whereas low-molecular-mass PBPs have only a minor role. Normally, PBPs utilize the D-Ala-D-Ala moiety of the pentapeptide as a substrate, forming an acyl-enzyme intermediate with release of the terminal D-Ala during transpeptidation. β-Lactam antibiotics act as pseudosubstrates and subvert this process by acylating the active sites of the PBPs [12]. Transpeptidation activity is essentially inhibited because deacylation of the penicilloylated PBPs is so slow.

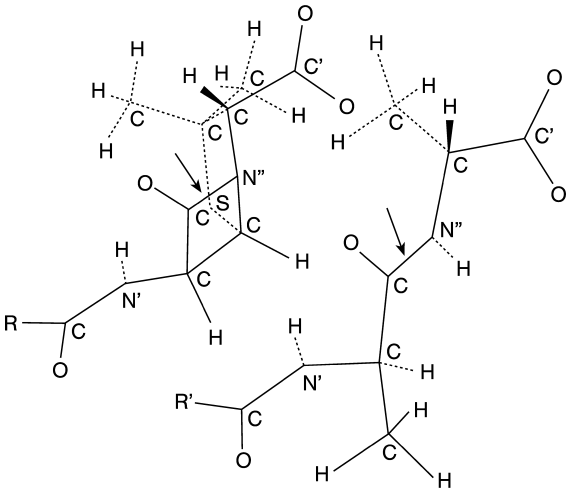

Figure 1.1 Dreiding stereomodels of penicillin (upper left) and of the acyl-D-Ala-D-Ala end of the nascent peptidoglycan (lower right). Arrows indicate CO–N bond common to both structures and the heavy lines indicate the portion of the penicillin molecule believed to resemble the peptide backbone of acyl-D-Ala-D-Ala [10].

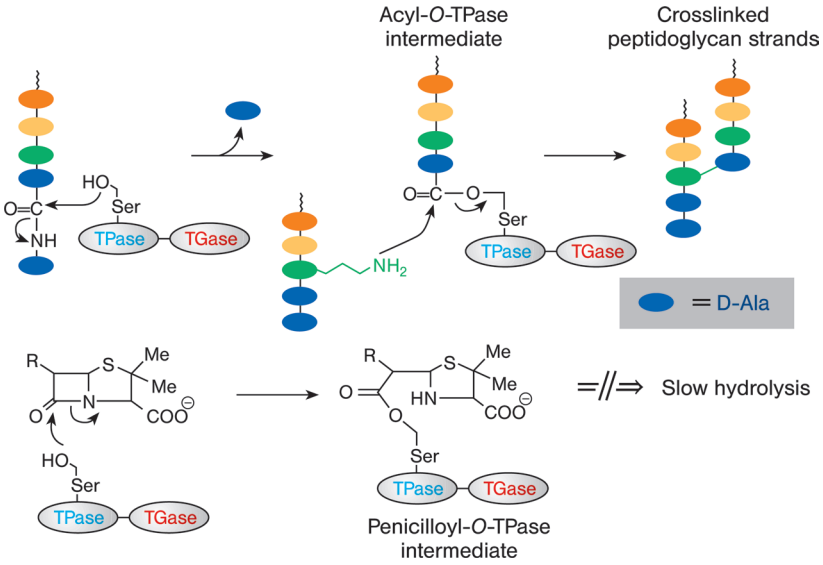

Figure 1.2 Inhibition of transpeptidase activity by penicillins [12].

Fifty-five percent all antibiotics are β-lactams [13], so resistance in this class is clinically important. β-Lactam hydrolysis by β-lactamases was observed by Abraham and Chain even before penicillin was introduced into the clinical setting. There are four classes of β-lactamases – classes A, C and D, which utilize an active-site serine, and class C, which requires divalent metal cations (Zn^{2+}) for catalysis [17]. The four-membered, strained lactam ring is the chemically activated functionality in the drugs which acylates and irreversibly modifies the cell wall cross-linking PBPs (Figure 1.2).

β-Lactamases (penicillinases) open the β-lactam ring by hydrolyzing the CO–N bond that is normally involved in the acylation of PBPs. The resultant ring-opened structure (penicilloic acid) has no activity as a pseudosubstrate of PBPs. Deactivation occurs in the periplasm, so that penicillin is deactivated before it reaches its cell wall target (Figure 1.3). Gram-negative bacteria produce β-lactamases within their cytoplasm and then secrete them into their periplasm, whereas Gram-positive bacteria secrete β-lactamases into the external environment. β-Lactamases confer high levels of resistance as a single β-lactamase molecule can hydrolyze 10^3 penicillin molecules in a second. Thus, if 10^5 enzymes are secreted per resistant cell, 100 million molecules of penicillin are destroyed every second [7].

Amino acid sequences of PBP active sites contain an active serine in the sequence Ala/Gly-Ser-X-X-Lys, of which the Ser-X-X-Lys sequence is conserved in all PBPs. Moreover, PBP1–3, 5 and 6 of *Escherichia coli* have been shown binding to this conserved serine [10]. Physiologically this serine acts as a nucleophile in the PBP active site and reacts with acyl-D-Ala-D-Ala to yield an acyl-D-alanyl-enzyme intermediate. In penicillin-sensitive bacteria, penicillin binds to this active-site serine forming an acetylated penicilloyl-enzyme intermediate, preventing any

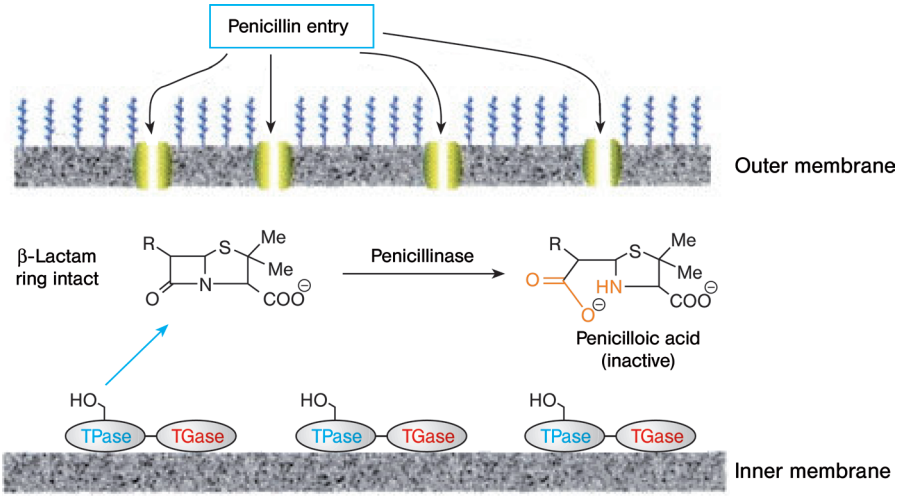

Figure 1.3 Chemical modification of penicillin by penicillinases [7].

normal cross-linking activity. Consistent with their ability to hydrolyze the CO–N bond, some β-lactamases also contain an active-site serine in the Ser-X-X-Lys motif [15] (see also [16] for review). This serine binds penicillin and later releases it as the hydrolyzed penicillin product. Penicillin-bound PBPs are immobilized as the acyl-enzyme because hydrolysis is so slow; however, for active-site serine β-lactamases the acyl-enzyme hydrolysis is rapid. Active-site serine β-lactamases can be divided into three molecular classes (A, C and D) on the basis of their primary structures [14].

The similarity between DD-peptidases and serine β-lactamases with respect to their catalytic pathways has prompted comparison studies between the two molecules, and structural similarities have been shown between class A β-lactamase and the D-Ala-D-Ala penicillin target [17]. Moreover, tertiary structures of class A and C β-lactamases and DD-peptidase hint at an ancestral link between these three enzymes [18]. All have a β-lactam-binding site in their center at the left edge of the five-stranded β-sheet. The reactive serine lies at the N-terminal end of a central α-helix [18] (orange in Figure 1.4). If β-lactamases are ancestrally linked to DD-peptidases, what makes DD-peptidases the unfortunate target of β-lactams, while β-lactamases escape harm and are primed ready to search and destroy β-lactams? Clearly β-lactamases do not catalyze transpeptidation and likewise DD-peptidases do not hydrolyze penicillin as efficiently as β-lactamases. This slow rate of deacylation of penicilloylated PBPs is the basis for β-lactam activity.

Interactions exist between the B3 β-strand of DD-peptidase and the C-terminal carboxylic acid group, the carbonyl group of the C-terminal peptide bond and the amido group of the penultimate peptide bond [18]. If a β-lactam is substituted for the D-Ala-D-Ala peptide, only the first two of these interactions is present. A better interaction between the C6 β-acylamido group of the β-lactam and the β-strand could

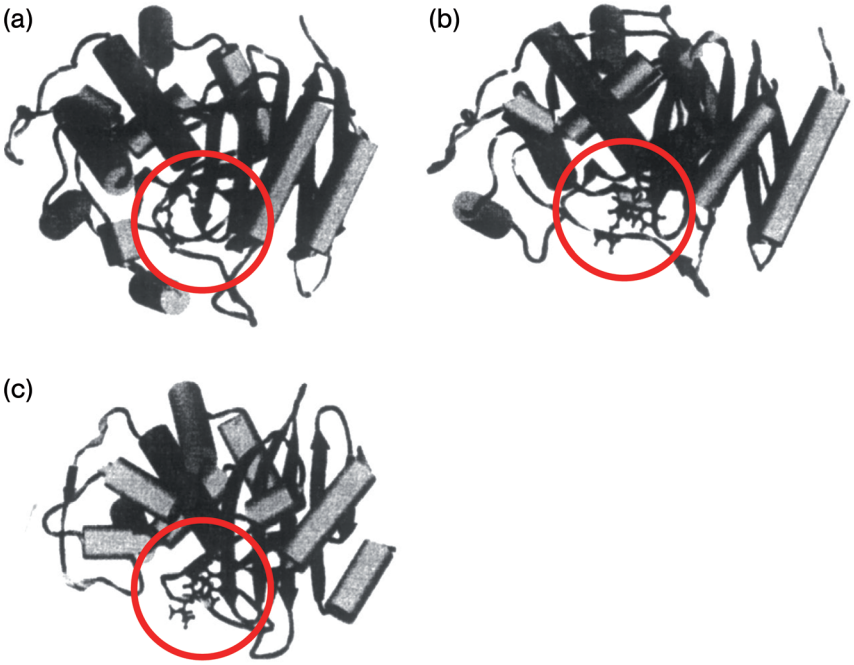

Figure 1.4 Molecular models of (a) D-Ala-D-Ala carboxypeptidase/transpeptidase from *Streptomyces* spp., (b) class C β-lactamase of *Enterobacter cloacae* and (c) class A β-lactamase of *Bacillus licheniformis*. Cylinders and arrows represent α-helices and β-strands, respectively. The reactive serine is at the N-terminus of the central helix H2. The Ω-loop lies at the lower center of each molecule. The red circle denotes the D-Ala-D-Ala-binding site in (a), and the β-lactam-binding site in (b) and (c) [18].

be achieved if the strand was pointed outward to improve hydrogen bonding between the acylamide and the carbonyl group of the β-strand [18]. Class A β-lactamase structure is optimized for hydrolysis of β-lactams and has completely lost its activity on D-Ala-D-Ala peptides. In comparison to class C the B3 β-strand of class A β-lactamases has an accentuated tilt which favors binding and hydrolysis of β-lactams. The Ω-loop protrudes more deeply into the active site allowing the backbone carbonyl group of Asn170 to hydrogen bond with the amide residue on the B3 β-strand. This increases the tilt of B3 β-strand. In contrast to DD-peptidases, class A and C β-lactamases possess a water molecule in the oxyanion pocket which increases the electrophilicity of the oxyanion hole. The absence of a water molecule in the oxyanion hole means DD-peptidases are less able to stabilize the oxyanion β-lactam tetrahedral intermediate [18].

Serine β-lactamases possess four major catalytic elements (Table 1.1), and the active-site serines are Ser70, Ser64 and Ser115 in class A, C and D β-lactamases, respectively [19]. The Ω-loop is also conserved in all serine β-lactamases, suggesting a role in the enzymes function. However, despite this, the exact role of other specific amino acid residues remains unclear.

Table 1.1 Major catalytic elements in class A, C and D β-lactamases [19].

	Group A	Group C	Group D
First element	S70–X–X–K	S64–X–X–K	S67–X–X–K
Second element	S130–X–N	Y150–A–N	S115–X–V
Third element	K234–T/S–G	K314–T–G	K205–T/S–G

The active site of the lactamases can be regarded as consisting of two ensembles of amino acids – a recognition ensemble and a catalytic ensemble. In addition to the active serine, the catalytic ensemble consists of a general base, which receives a proton from the serine side-chain. This activates the serine for nucleophilic addition to the oxygen on the carbonyl group on the β-lactam and allows the nitrogen to accept a proton from an unknown donor. There is also an oxyanion hole consisting of two amino acids that stabilize the tetrahedral oxyanion intermediate through hydrogen bonding and a cationic recognition site for the carboxylate group on the β-lactam [20]. The catalytic ensemble is most probably highly conserved and linked with the major catalytic elements in Table 1.1. The recognition ensemble is suggested to be more variable, consisting of the remaining amino acids and dependent on the specific selective pressures exerted on the bacteria.

Carbapenems are a group of broad-spectrum antibiotics defined by the substitution of the typical sulfur atom at position one with a carbon atom, this structure resists inactivation by most β-lactamases. However, class B β-lactamases can significantly hydrolyze carbapenems [16, 21]. Class B β-lactamases require divalent cations, primarily zinc, and there are three subgroups (B1–B3). The metallo-β-lactamases (MBLs) possess a distinct set of amino acids that define the architecture of their active site which is largely superimposible among the different MBLs. Most MBLs contain the principle binding motif His-X-His-X-Asp [21], although B2 enzymes possess an asparagine instead of a histidine at the first position. B2 enzymes also differ from other MBLs, by only containing a single zinc ion, while other MBLs contain two zinc ions in their active site. Similarly to the serine β-lactamases, the general consensus is that MBL hydrolysis of the β-lactam ring is via nucleophilic attack where their active site orients and polarizes the β-lactam bond to facilitate attack by zinc-activated water/hydroxides [20, 21].

Cefotaxime, cefotriaxone and ceftazidime are extended-spectrum β-lactamase (ESBL) antibiotics introduced in response to growing β-lactamase resistance. Plasmid-encoded class A TEM-1 β-lactamase is a common cause of resistance to penicillins and cephalosporins, but not ESBL antibiotics. However, variants of this class have been found with a G238S substitution displaying increased hydrolysis of ESBL antibiotics. A direct hydrogen bond between the hydroxyl side-chain group of Ser238 and the oxime group of ESBLs is necessary but not sufficient for hydrolytic activity of cefotaxime and ceftazidime [22]. Additionally, an intramolecular hydrogen bond, possibly from Ser238 to the main chain CO group of Asn170 of the Ω-loop, is thought to stabilize the Ω-loop in a new location which enables the enzyme to broaden its substrate profile to include ESBL antibiotics [22]. Fortunately, in terms

of β-lactamase resistance, bacteria have not yet completely undermined our efforts. β-Lactamase inhibitors (e.g. clavulanic acid, sulbactam and tazobactam) are proteins that can be used in combination with β-lactam antibiotics. Despite little by way of antimicrobial activity, the β-lactamase inhibitors bind with higher affinity to the β-lactamases than the β-lactams and can be used to distract the β-lactamases while the β-lactams work without the threat of enzymatic inactivation. However, such a simple strategy only requires the bacteria to learn how to overwhelm the β-lactamase inhibitors with enzymes for them to gain the advantage.

Methicillin is an antistaphylococcal β-lactamase-stable penicillin introduced to overcome β-lactamase resistance. Methicillin resistance is caused by an acquired gene (*mecA*) which results in the synthesis of a fifth PBP (PBP2a), in addition to the intrinsic 1–4 PBPs [23]. Strains of *S. aureus* that acquire the *mecA* gene are MRSA [13]. PBP2a from *S. pneumoniae* exhibits a much lower affinity for β-lactam antibiotics, especially penicillins, when compared with other PBPs [24]. Similarly to other PBPs, PBP2a undergoes acylation with its peptidoglycan substrate at an active-site serine (Ser403) [13].

The stability of the acyl-PBP intermediate (PBP–penicillin) is what conveys the antibiotic action. A very stable intermediate essentially inhibits the transpeptidation activity of PBPs for a long time. Studying the kinetics of this reaction sequence has revealed two aspects that may cumulatively give rise to PBP2a resistance. The first is a significantly reduced rate constant for SauPBP2a acylation (k_2) compared to penicillin-sensitive PBPs. This makes formation of the PBP–penicillin intermediate very unlikely. Moreover SauPBP2a has an elevated dissociation constant (k_d) for the noncovalent preacylation Michaelis complex with the antibiotic, meaning encounters between the antibiotic and PBP2a are not favorable [13]. Interestingly, a resistant form of PBP2x (R-PBP2x) has an overall antibiotic binding efficiency (k_2/k_d) that is over 1000-fold slower than penicillin-sensitive PBP2x. Kinetic studies also noted differences between the deacylation rate constants (k_3) of penicilloyl-R-PBP2x and penicilloyl-S-PBP2x, equating to an increase in the deacylation rate of over 70-fold for R-PBP2x [25].

Elucidation of the structure of PBP2a provides some explanation for the kinetics. PBP2a from *S. aureus* (SauPBP2a) has an N-terminal extension and transmembrane anchor, a non-penicillin-binding domain (nPD) and a C-terminal transpeptidase domain (see Figure 1.5). The nPD domain positions the transpeptidase domain more than 100 Å away from the transmembrane anchor, suggesting a structural role in giving the transpeptidase domain substantial reach from the cell membrane [26]. The transpeptidase of SauPBP2a shares a similar overall fold with other transpeptidases and the serine β-lactamases. In the apo conformation of the helix α2 N-terminus, Ser403 is in a poor position for nucleophilic attack. Cα, Cβ and Oγ from Ser403 have to move slightly upon acylation. For acylation to occur under normal circumstances, a twisted apo conformation of strand β3 is required to accommodate the helix α2 N-terminus due to steric clash between Ser598 backbone carbonyl in the acyl-PBP complex and the Ser403 Cβ in the apo structure. Twisting of the β3 strand is also required for binding of nitrocefin, due to steric clash between the nitrocefin carboxylate and the Gly599 Cα in the apo structure [26]. Lim

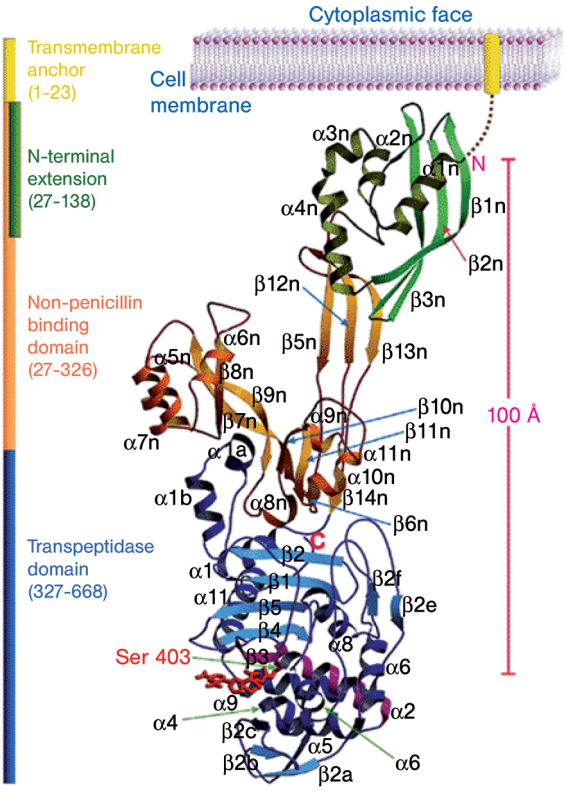

Figure 1.5 Structure of SauPBP2a. The bilobed N-terminal nPB domain is orange and the N-terminal extension is colored green. The transpeptidase domain is blue, with the position of the active site indicated by the red nitrocefin adduct (shown in stick rendering) [26].

and Strynadka describe a closed active-site structure for SauPBP2a, where formation of the Michaelis complex requires a conformation different to the conformation required for acylation. This transition from Michaelis complex to acyl-PBP complex (k_2) is so energetically costly that it does not happen, which is why SauPBP2a is resistant to β-lactams [26].

PBP2a resists modification by β-lactam antibiotics while remaining a competent catalyst in transpeptidation. Coupled with its closed active site [26], this has led to the suggestion that physiological substrates of PBPs interact with the surface of PBP and facilitate opening of the active site, making entry of peptidoglycan possible. Evidence for this is the increased nitrocefin (chromogenic cephalosporin) acylation of PBP2a (k_2) that accompanies increasing concentrations of peptidoglycan fragments.

Moreover, it was proved that the protein undergoes a conformational change consistent with a decrease in helicity [27]. β-Lactam antibiotics are small in comparison to the peptidoglycan substrate and so are not capable of interacting with

SauPBP2a in the same way, explaining why they do not gain access to the active site. The low affinity of PBP2a to β-lactams suggests that it is a naturally resistant form of PBP and therefore was probably never a target of β-lactams [24]. The theory that PBP2a takes over the biosynthesis of the cell wall from the four sensitive native PBPs is unlikely because PBP2a resistance is dependent on continued transglycosylation by native PBP2 [27, 28]. This cooperation may make this mechanism of resistance vulnerable to novel attempts at overcoming resistance in the future.

PBP2x and PBP2b are essential for bacterial growth [30] and are therefore targets of β-lactams [31]. The resistant strain of PBP2x (R-PBP2x) in *S. pneumoniae* has a transpeptidase domain (residues 266–616) carrying numerous substitutions compared to homologous sequences from β-lactam-sensitive streptococci (S-PBP2x) [32]. In recent years there have been exhaustive attempts to identify these mutations and their influence on the resistance profile of PBP2x. Thr338 does not contact the antibiotic directly, however, positioned just after the active-site Ser337, it is the most frequent mutation seen in clinically resistant pneumococci [32]. PBP2x from resistant strains (R-PBP2x) contain a T338A mutation. Imposing this mutation on S-PBP2x reduces the acylation efficiency by a factor of 2 [33] because it abolishes a crucial hydrogen bond between the hydroxyl group of the threonine residue and a buried water molecule [34]. Mutation T338A can coexist in some highly resistant strains with mutation M339F. Replacing methionine with phenylalanine introduces a bulkier side-chain which strains the active-site structure and reorientates the hydroxyl group of Ser337 [26]. It is likely that this distorted catalytic center contributes to the reduction in acylation efficiency seen in clinical strains containing the double mutation compared to those sensitive strains.

Figure 1.6 illustrates the closeness and parallel positioning of cefuroxime in relation to the β3 strand, and also indicates residues T338, T550 and Q552. Replacing Gln552 of S-PBP2x from the R6 strain with glutamic acid, which is commonly found in R-PBP2x, reduces the acylation efficiency by over two-thirds for both penicillin and cefotaxime [32]. Glutamic acid introduces a negative charge into what is normally a

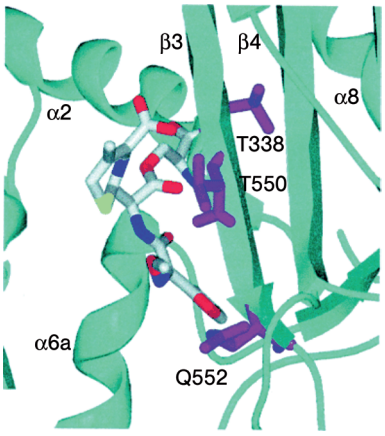

Figure 1.6 View of cefuroxime bound in the active site of S-PBP2x [32].

globally positive active site. This conflicts with the global negativity of β-lactams and reduces the acylation efficiency [32]. Reversion of this mutation confirms its role in the expression of resistance [35]. Crystal structures reveal an overall similarity between the structures of antibiotic-sensitive and antibiotic-resistant strains except in their active-site regions (Figure 1.7a) [34]. Although not disordered, strand β3 (Figure 1.7b), most notably segment Ser548 to Thr550, is displaced by 0.5 Å in resistant strains, while strands β4 and β5 superimpose very well [34].

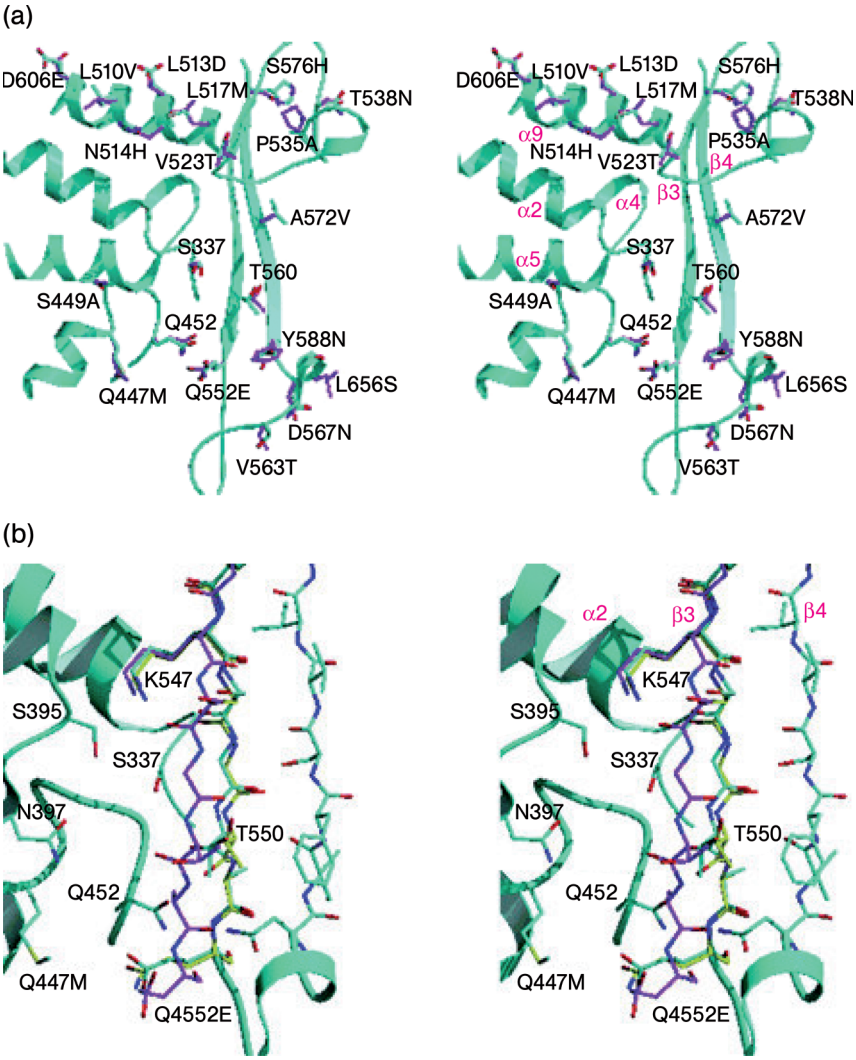

Figure 1.7 (a) Stereo view of the active sites of 5259-PBP2x (cyan) and R6-PBP2x (magenta). (b) View of the displacement of the β3 strands in PBP2a from *S. aureus* (magenta), R6-PBP2x (yellow) and 5259-PBP2x (cyan) [34].

Mutations S389L and N514H with PBP2x of *S. pneumoniae* have two distinct effects on the active site. First, there is steric clash between them which moves the catalytic residue Ser395 to an unfavorable position for enzymatic function. Here, only four of the six stabilizing interactions normally seen between Ser395 and cefotaxime in susceptible strains are formed [36], and these two mutations result in an open active site. The importance of an open active site in the expression of resistance is undetermined; however, it maybe beneficial to resistant strains of *S. pneumoniae* which produce abnormal indirectly cross-linked cell walls.

The *murMN* operon contains *murM* and *murN* genes, and produces branched muropeptides [37]. Instead of the linear-stem peptides (L-Ala-D-iGln-L-Lys-D-Ala) seen in susceptible strains, resistant strains have branched-stem peptides carrying Ala-Ser or Ala-Ala dipeptides on the ε-amino group of the stem peptide lysine residues [25]. Producing branched cell wall precursors is important in the expression of penicillin resistance in *S. pneumoniae* because interrupting the *murMN* operon causes virtually complete inhibition of the expression of penicillin resistance [32]. Although speculative, the theory that branched cell wall precursors are more successful than antibiotics in competition for active-site occupancy is conceivable. It is not implausible to suggest that this is because an open active site is more accessible to branched muropeptides.

Figure 1.7(a and b) shows the close proximity of Thr550 to the active-site Ser337. Alanine is often substituted for threonine at this position in resistant strains and reduces cefotaxime acylation efficiency by almost 20-fold. Interestingly this mutation is neutral for penicillin [32]. Preserving the hydroxyl group, while altering the steric property of the side-chain of position 550, is consistent with a T550S mutation. While this mutant is unable to discriminate between penicillin G and cefotaxime, and shows acylation efficiency values very close to those of Q552E mutant, it is not found clinically.

1.4 Glycopeptides

Glycopeptide antibiotics interfere with the transpeptidation stage of late cell wall biosynthesis. Vancomycin is a clinically used glycopeptide, which targets the terminal Lys (Dap)-D-Ala-D-Ala residues in the mature peptidoglycan intermediate, lipid II, and in unbranched linear peptidoglycan strands. It forms five hydrogen bonds between amides of the cross-linked heptapeptides and the D-Ala-D-Ala dipeptide terminus of each uncross-linked peptidoglycan pentapeptide side-chain (Figure 1.8). The D-Ala-D-Ala terminus is present in lipid I and lipid II; however, vancomycin cannot enter the bacterial cell due to its size and hydrophobicity, and only lipid II and the UDP-MurNAc-pentapeptide are affected [38]. Thus formed, the vancomycin–peptide complex becomes unavailable as substrate for transpeptidases and transglycosylases.

First produced in 1958, vancomycin is traditionally a last resort antibiotic for treating patients who are gravely ill or infected by organisms resistant to other

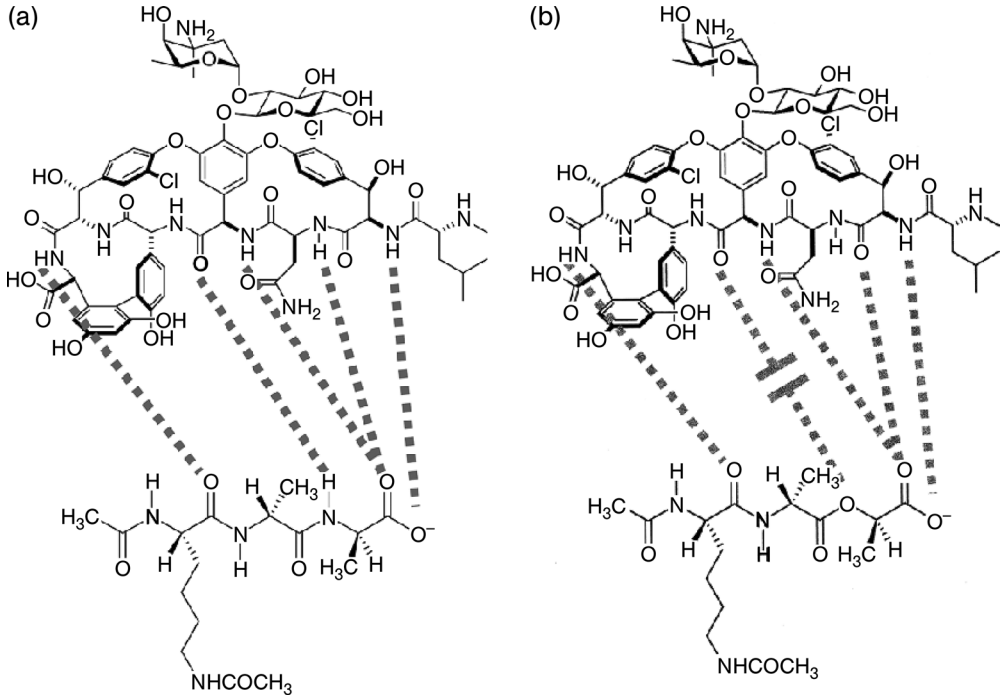

Figure 1.8 (a) Complex formation between vancomycin and *N*-acyl-D-Ala4-D-Ala5. Key hydrogen bonds stabilizing the complex are shown (after (8)). (b) Complex formation between vancomycin and D-Ala-D-Lac does not occur as one of the required hydrogen bonds cannot form [39, 40].

antibiotics such as β-lactams. Due to their unique mode of action, it was considered that development of resistance to glycopeptides was unlikely [8]. That year, nearly 30 years after the introduction of vancomycin, the first vancomycin-resistant enterococci (VRE) in Europe were reported [41]. Since then, VRE have become a major cause of nosocomial infections. Vancomycin-dependent enterococci which require vancomycin for their growth have emerged in patients treated for long periods with vancomycin [42]. Inactivation of the *ddl* gene for D-Ala:D-Ala ligase is responsible and mutants lacking D-Ala:D-Ala ligase are incapable of forming peptidoglycan precursors with complete D-Ala-D-Ala terminals. Such deficiency leads to utilization of an alternative pathway, in which bacteria synthesize peptidoglycan precursors terminating on D-Ala-D-Lac and are not susceptible to vancomycin [43].

Resistance to vancomycin in enterococci is achieved by altering their peptidoglycan precursors so that glycopeptides can no longer bind. Out of the four VanA–D VRE phenotypes, VanA and VanB are the two most clinically relevant. VanA, VanB and VanD have a substitution of the C-terminal D-Ala to D-Lac, whereas VanC and VanE have a D-Ser substituted in this position. D-Lac suppresses a hydrogen bond crucial for antibiotic binding, whereas D-Ser does not alter the hydrogen bonds, but is

responsible for a conformational change which slightly reduces affinity for vancomycin [41]. VanA is characterized by inducible, high-level resistance to both vancomycin and teicoplanin, whereas VanB is resistant to vancomycin but susceptible to teicoplanin [42]. The five *van* genes, *vanRSHAX*, are necessary for both phenotypes.

The resistance mechanism for VRE was first elucidated by Walsh and Courvalin in the 1990s [39]. VanR and VanS proteins form a two component regulator system [40]. VanR senses vancomycin outside the bacterial cell, while VanS activates the transcription of the *VanHAX* genes. VanHAX proteins reprogram the peptidoglycan termini from the *N*-acyl-D-Ala-D-Ala (glycopeptide target) to *N*-acyl-D-Ala-D-Lac. VanH reduces pyruvate to D-lactate, D-Ala-D-Lac is made by a *VanA*-encoded ligase and VanX is a D-Ala-D-Ala dipeptidase which removes the D-Ala-D-Ala intermediate. Additionally, VanY is a DD-carboxypeptidase that cleaves the D-Ala terminal peptide from any normal peptide and VanZ modestly increases the minimum inhibitory concentration (MIC) for teicoplanin, but not for vancomycin, through an unknown mechanism [45]. As a result, D-Ala-D-Lac accumulates sufficiently within the cytoplasm to be added to the UDP-muramyl-tripeptide in a MurF-catalyzed reaction. UDP muramyl-L-Ala-D-γ-Glu-L-Lys-D-Ala-D-Lac is generated, which can then be incorporated into the growing peptidoglycan chain. Organization of the *vanA* operon is similar to that of the *vanB* and *vanD* operons. The mechanisms of VanA-, VanB- and VanD-type resistance are identical, and there is a high degree of similarity in the amino acid sequences of their Van proteins [43].

Figure 1.8 highlights the differences between D-Ala-D-Lac and D-Ala-D-Ala. Resistance is conferred by a 1000-fold decrease in binding affinity between D-Ala-D-Lac and glycopeptides [46]. One of the five hydrogen bonds does not form because of an absent amide group which is replaced by an ester oxygen whose lone pair of electrons exhibit repulsion towards an electronegative oxygen on vancomycin. Many hypotheses have attempted to explain why the VanB phenotype does not confer teicoplanin resistance. One suggests a lipid chain unique to teicoplanin anchors it to the membrane, which enhances antibiotic binding because both antibiotic and target are attached by membrane anchors to the same template [47]. This renders teicoplanin inaccessible to the sensor kinase, so it does not induce resistance [43]. An alternative hypothesis suggests VanS senses peptidoglycan intermediates or degradation products that result from blocking cell wall biosynthesis [48]. Localization of teicoplanin to the membrane means teicoplanin is in a good position to inhibit transglycosylation by binding to lipid II, whereas vancomycin would preferentially act on the nascent peptidoglycan chain to inhibit transpeptidation [8, 49, 50]. The phenotypic difference between VanA and VanB could be a result of differences in gene expression [43] as the specificity of induction in the VanB phenotype is a characteristic of $VanS_B$ [51].

Vancomycin usage has increased with the spread of MRSA, which exerts a greater selective pressure on staphylococcal bacteria and facilitates the development of resistance. Vancomycin intermediate *S. aureus* (VISA) and glycopepetide intermediate *S. aureus* (GISA) strains have been reported in many countries, and the first clinical case of vancomycin-resistant *S. aureus* (VRSA) was documented in the United States in 2002 [52]. VRSA describes *S. aureus* with a MIC of 32 mg/l or less, VISA have

a MIC of 8–16 mg/l and hetero-VRSA (hVRSA) have a MIC of 4 mg/l or less, but which show a population heterogeneity similar to Mu3 (the archetypal hVRSA) when subjected to a full population analysis profile [7]. In one model, vancomycin-susceptible mutants spontaneously emerge due to the slow growth of VRSA strains. These susceptible cultures maintain subpopulations with vancomycin resistance (hVRSA) [53], which produce VRSA mutants when selected for by vancomycin [54]. The model suggests vancomycin and β-lactams maybe involved in vancomycin-sensitive *S. aureus* (VSSA) to hVRSA conversion [53]. Therefore, long-term treatment of MRSA with β-lactams is a possible risk factor for vancomycin resistance.

Enterococci are part of the normal flora of the alimentary canal and are opportunistic, with the capacity to acquire and spread antimicrobial resistant factors [55]. *vanA* genes have been transferred via plasmid-mediated conjugation from enterococci to staphylococci *in vitro* [56], although the mechanism of vancomycin resistance in *S. aureus* appears to be novel. All strains are negative for the *van* genes; however, a thickened cell wall with reduced levels of peptidoglycan cross-linking is common [7, 57]. Decreased cross-linking increases the number of D-Ala-D-Ala side-chains to which glycopeptides bind, therefore VRSA (e.g. Mu50) has an increased binding of vancomycin molecules [57].

Vancomycin-resistant Mu50 cells have an increased proportion of nonamidated muropeptides containing D-glutamate instead of D-glutamine [35, 57]. Pentapeptides containing D-glutamate are poorer substrates for transpeptidases and so consequently fewer cross-links are formed [53]. Additionally, experiments suggest that nonamidated murein monomers may have higher affinity for binding to vancomycin than amidated murein monomers [57]. Cui *et al.* conclude that a unit weight of purified peptidoglycan with a high proportion of nonamidated muropeptides consumes more vancomycin molecules than a unit weight of peptidoglycan with low nonamidated muropeptide content [58]. While nonamidated murein monomers seem to influence vancomycin susceptibility, there is evidence to suggest that absent or greatly reduced levels of PBP4 may also decrease the susceptibility of some strains of staphylococci to vancomycin [59–61]. The introduction of plasmid encoding PBP4 homologs into doubly D-cycloserine/vancomycin-resistant *S. aureus* restores sensitivity to both drugs [62]. PBP4 has transpeptidase activity and so its absence is likely to result in reduced cell wall cross-linking, similar to the effect of nonamidated muropeptides. It has been suggested that this mechanism is strain-specific as not all GISA strains show reduced PBP4 expression [61].

For bacteria, reduced cross-linking comes at a price. Resistance is only conferred if reduced cross-linking is accompanied by an increase in cell wall thickness [7]. Inevitably this increase in cell wall biosynthesis is accompanied by greater nutrient demands. For example, Mu50 cells incorporate 2.3 times more glucose molecules into the cell peptidoglycan compared to VSSA strains [58], suggesting this fitness cost may divert glucose away from other important energy-consuming processes [53]. However, this compromise enabled bacteria to produce many more D-Ala-D-Ala decoys, which positioned at the periphery of the peptidoglycan matrix can act by binding glycopeptides and clogging up their cell walls in an effort to block other glycopeptide molecules from penetrating their sites of cell wall biosynthesis.

1.5 Lantibiotics

Widespread resistance to conventional antibiotics has revived interest in the group of lanthionine-containing peptide antibiotics. These are subdivided into positively charged membrane-disrupting molecules [e.g. nisin (from *Lactococcus lactis*) and subtilin (from *Bacillus subtilis*)], type A and negatively charged, more hydrophobic antibiotics [e.g. mersacidin (from *Bacillus* spp. HILY-85,54728)], classified separately into type B [64, 65]. The best known example, nisin (Figure 1.9), has found use exclusively as a preservative in the food industry and clinical pathogens have not been subjected to sustained antibiotic pressure from molecules in this class. The first reports of an N-type inhibitory substance from *L. lactis* date back to 1928 [66]. Nisin is known to interact with lipid membranes [67, 68] and recently was shown to have multimode action against Gram-positive bacteria, including resistant pathogens MRSA [69] and VRE [70]. The action of nisin is mediated by pyrophosphate recognition [71] of mature cell wall intermediates lipid II and undecaprenyl pyrophosphate, which takes place on the outer leaflet of bacterial plasma membranes. There, lipid II can be engaged in binary membrane-lytic complexes by lantibiotics nisin [72] and subtilin [73]. The increase in membrane permeability leads to deregulation of cell division and cell shape regulation, minicell formation, and bacterial death [74]. Through formation of complexes with the membrane-associated precursors, lantibiotics also inhibit cell wall biosynthesis by removing lipid II and undecaprenyl pyrophosphate from the biosynthetic pathway.

Structural details of target recognition, obtained by nuclear magnetic resonance (NMR) in dimethylsulfoxide from nisin and modified lipid II [75], reveal pyrophosphate engagement, which involves residues from rings A and B. Solid-state NMR studies of nisin/lipid II in membranes [71] and enzymatically digested subtilin/lipid II, also in membranes [73], reveal the key involvement of the N-terminal amino group in nisin and the essential role of residue Trp1 in subtilin to antibiotic function.

The high activity of nisin and subtilin against Gram-positive organisms requires effective self-protection of the producer organisms, which belong to the target group. Resistance is coencoded with lantibiotic production and in nisin it is dependent on the expression of *nisI*, as well as the genes *nisE*, *nisF* and *nisG*. The *nisI*-encoded protein reduces nisin binding to membranes, while the proteins produced from *nisE*, *nisF* and *nisG* expression appear to be homologous to ATP-binding cassette (ABC)

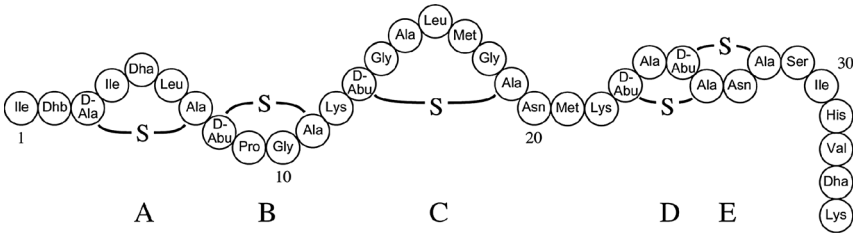

Figure 1.9 Structure of nisin. The five lanthionine/methyllanthionine rings are labeled A–E.

multidrug pumps [76]. Subtilin resistance is encoded by analogous genes in *Bacillus*, *spaIFEG*. [77]. However, the mechanism of self-immunity relies on highly specific antibiotic recognition, and cross-resistance of *L. lactis* to subtilin and of *B. subtilis* to nisin is not observed [78].

1.6 Aminoglycosides

Aminoglycosides are a large group of naturally occurring or semisynthetic polycationic compounds that inhibit translation in bacteria. Their structure consists of amino sugars linked via glycosidic bonds to amino-substituted cyclic alcohols, aminocyclitols [79].

Translation in bacteria requires specific interactions between aminoacyl-tRNA anticodons and mRNA codons. This occurs in a highly conserved rRNA sequence of the 30S ribosomal subunit. Aminoglycosides target a major groove in the model A-site of bacterial 30S rRNA. Binding within a pocket created by an AA base pair and a single bulged adenine, they induce codon misreading [80]. The most common mechanism of aminogylcoside resistance is enzymatic modification. However, bacteria can also reduce their susceptibility through target modification of the ribosomal-binding site or defects within uptake and efflux processes.

Specific nucleotides in the aminoacyl-tRNA site (A-site) region of *E. coli* 16S rRNA are protected by paramomycin from dimethyl sulfate (DMS), suggesting that this is a site of action for aminoglycosides [80]. These include the C1407–G1494 base pair, A1408, A1493 and U1495. Base pairing in the lower stem and asymmetry of the internal loop resulting from the presence of a nucleotide at position 1492 are also required for specific binding [81]. The base pair 1409–1491 forms the floor of the antibiotic-binding pocket and mutations affecting the secondary structure of this base pair confer resistance to aminoglycosides such as paromomycin [82]. Streptomycin also protects specific bases within the A-site region of *E. coli* 16S rRNA [83]. Moreover, mutations corresponding to this highly conserved region in *M. tuberculosis* confer resistance to streptomycin [84]. Target modification also extends beyond the-binding site of streptomycin. High-level streptomycin resistance is frequently due to mutations within the 530 loop region [85]. Although it may be possible for such a conformational change to result so that aminoglycoside binding is affected, it is more likely that the resulting conformational change prevents bound streptomycin-induced codon misreading. Mutations within ribosomal protein S12 also confer aminoglycoside resistance [86]. This highlights the importance of the 530 loop in aminoglycoside resistance, as ribosomal protein S12 helps stabilize this region [83, 87]. The evidence strongly implicates the A-site in aminoglycoside binding and shows that it is amenable to target modification in order to express resistance.

Structural differences between bacterial and human ribosomes concerning base pairs involved in antibiotic binding allow aminoglycosides to target selectively bacterial cells without harming human cells. The structure of the A-site of *E. coli* 16S ribosomal RNA reveals important interactions between the A-site RNA and rings I and II of

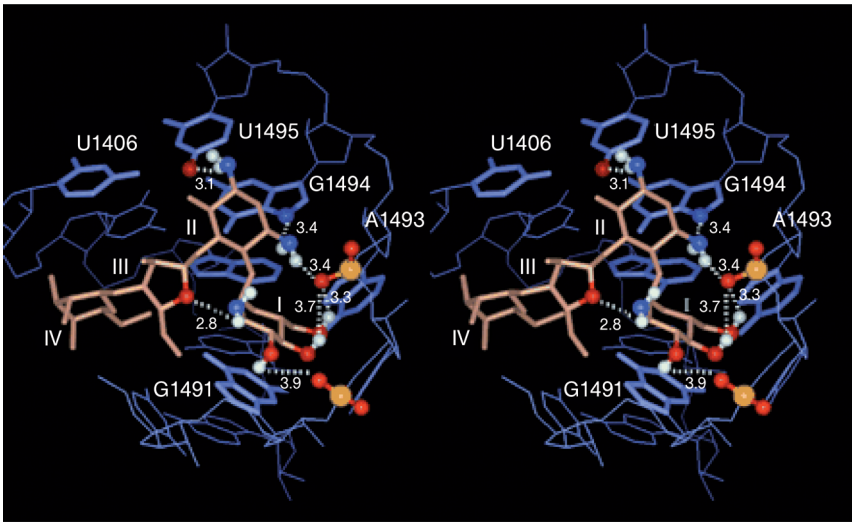

Figure 1.10 Stereo projection of paromomycin in the 16S rRNA-binding site showing specific contacts between rings I and II of paromomycin and the A-site RNA. The RNA is blue; paromomycin is tan; the view is into the major groove of the RNA core [80].

paromomycin that can be seen in Figure 1.10 [80]. The exocyclic amine and hydroxyl groups of rings I and II make specific contacts that stabilize the antibiotic–RNA complex and are primary targets for aminoglycoside-modifying enzymes conferring resistance. Rings III and IV contribute weakly to specific antibiotic binding and function [80].

Ribosomal RNA methylase enzymes are expressed in many aminoglycoside-producing actinomycetes to protect them from antibiotics of their own or from other microorganisms. For example *Streptomyces tenjimariensis* methylates A1408 and confers high-level resistance to aminoglycosides [88, 89]. In the base pair A1408–A1493, essential in aminoglycoside binding to the 16S rRNA, A1408 is the hydrogen acceptor and its loss leads to kanamycin resistance [80]. This target modification is also apparent in *Micromonospora purpurea*, which methylates G1405 and confers resistance to gentamicin through the resulting steric clash with ring III of the antibiotic [88–90]. Interestingly, clinical isolates of *Pseudomonas aeruginosa* and *Serratia marcescens* have also been found with genes encoding 16S rRNA methylase activity [91, 92]. Sharing considerable primary sequence similarity to aminoglycoside producers suggests possible gene transfer from actinomycetes to Gram-negative pathogens [93].

Antibiotic deactivation is a major contributor to aminoglycoside resistance. Enzymatic acetylation of the 3-amino group of paromomycin disrupts hydrogen bonding with the target, which involves the amino group and the N7 of G1494, as well as specific electrostatic and hydrogen-bonding contacts with the A1493 phosphate [80]. Ring I of paromomycin fits tightly in a pocket within the bacterial 16S

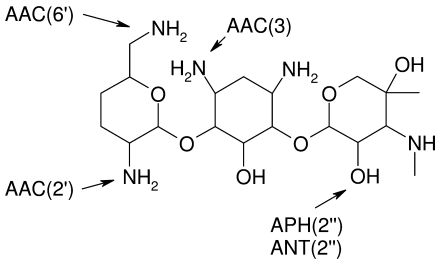

Figure 1.11 Structure of gentamycini and sites of enzymatic attack following [95].

rRNA and changes to the exocyclic groups of this ring interfere with binding. Acetylation of the 6′-amino also group gives rise to aminoglycoside resistance, and phosphorylation and adenylation of the 3′- and 4′-OH groups, respectively, leads to steric and electrostatic penalties to complex formation [80].

Enzymatic modifications of hydroxyl or amino groups on the aminocyclitol residues lead to reduced antibiotic activity. Modified aminoglycosides have diminished bacterial 16S rRNA A-site binding and lose their ability to inhibit protein synthesis [94]. Aminoglycoside *N*-acetyltransferases (AACs) acetylate the amino groups (*N*-acetylation) of the antibiotic and are dependent on acetyl-CoA. *O*-Phosphotransferases (APHs) phosphorylate the hydroxyl groups (*O*-phosphorylation). *O*-Nucleotidyltransferases (ANTs) add AMP moieties to hydroxyl groups (*O*-adenylation). The function of APHs and ANTs is ATP-independent. The position of the group attacked and the ring that carries it are indicated by the number of the enzyme (Figure 1.11).

Acquisition of Gcn5-related *N*-acetyltransferases (GNATs) provides bacteria with resistance to gentamicin, tobramycin and netilmicin. The crystal structure of plasmid-encoded AAC(3)-Ia from *Serratia marcescens* (SmAAC) has been determined [96] and the chromosomal gene *aac(6′)-Ii* from *Enterococcus faecium* has been characterized [97]. The four motifs C, D, A and B are characteristic of GNATs. Motif A seems to be critical for activity of NATs because site-directed mutations to Arg101, Gly104 and Gly106 of the invariant segment Arg/Gln-X-X-Gly-X-Gly/Ala, resulted in human spermidine/spermine NAT having no measurable effect [98]. AAC(3)-Ia contains all of the motifs (C, D, A and B) common to GNATs.

Cofactor binding and acetyl transfer catalyzed by 3-NAT I from *P. aeruginosa* (PsAAT) suggests acetyl-CoA binds to the enzyme, followed by aminoglycoside binding [99]. The acetyl group is only attached to CoA via a relatively weak thioester linkage and can easily be transferred to the aminoglycoside, while the acetylated product is released followed by CoA. Structural similarities between SmAAT and PsAAT hint at a common mechanism of acetyl transfer [96]. The acetyl group from acetyl-CoA lies above the side-chain of Gln145. Gln145 forms part of the floor of the active site, placing the acetyl group within close proximity of the aminoglycoside [96] (Figure 1.12). The thumb-like structure of motif B is a β-hairpin projection made up of S5 and S6. Asp147, Asp150 and Asp151 from the S5/S6 loop create one wall of a narrow canyon-like feature with negative electrostatic potential that extends away from the acetyl-CoA-binding surface toward the convex face of the enzyme. Asp53 from the acidic H1/H2 loop forms the

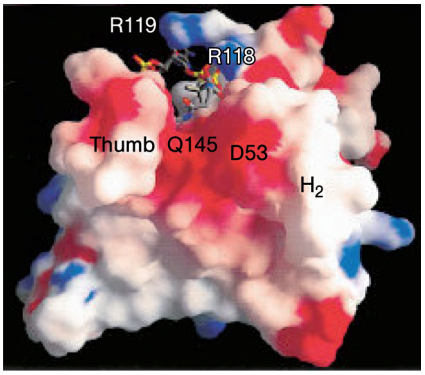

Figure 1.12 Active-site surface of SmAAT, showing CoA in the cofactor-binding notch and the negatively charged gentamicin-binding slot [96].

other wall [96]. The importance of negatively charged residues in the aminoglycoside-binding site shown here is supported by similar findings from work on the molecular structure of kanamycin nucleotidyltransferase [100].

APHs transfer a γ-phosphate group from ATP to the 3′- and/or 5′-hydroxyl group of aminoglycosides such as amikacin. APH(3′)-IIIa is a 264-residue enzyme existing as a monomer or covalent dimer linked via disulphide bridges, and is carried by opportunistic enterococci and staphylococci [101]. The monomers within the dimer are arranged in a head-to-tail/tail-to-head fashion. The disulphide bond joins Cys19 located in a β-sheet (β1) of one monomer to Cys156, which is part of a loop region between the helices αA and αB of the other monomer and vice versa. It is proposed that there is movement around this linkage, which is supported by the fact that there are few other significant bonds between the two monomers, except for hydrogen bonding between Asp150 and the side-chains of Arg5 and Trp85 of the partner molecule. The binding sites of each monomer face each other but are more likely to be independent due to the 20 Å distance between the two [101].

According to the crystal structure of the APH(3′)-IIIa monomer [101], there is a 94-residue N-terminal lobe and a 157-residue C-terminal lobe, tethered by a 12-residue stretch containing a short β-strand and α-helix. Between the two lobes is a deep cleft containing the ATP-binding site. Akin to the other aminoglycoside modifying enzymes mentioned here, APH(3′)-IIIa operates a Theorell–Chance kinetic mechanism [102] where phosphorylation follows binding of ATP and binding of the aminoglycoside. Release of the modified drug is followed by the rate-limiting dissociation of ADP. There is a dramatic similarity in structure between APH(3′)-IIIa and protein kinases [101], which suggests a mechanistic and evolutionary link between these antibiotic-resistance enzymes and Ser/Thr/Tyr protein kinases [11]. This relationship is strengthened by the report that aminoglycoside kinases can act as serine protein kinases [103].

Asp166 in cAPK has been implicated as the catalytic base required for the deprotonation of the substrate hydroxyl group for efficient attack at the γ-phosphate

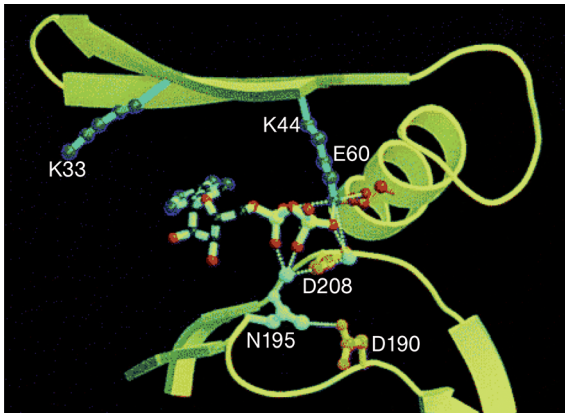

Figure 1.13 Nucleotide-binding site of APH(3′)-IIIa located in the cleft between the N- and C-terminal lobes. Conserved residues are shown interacting with ADP and the two magnesium ions. Although in the binding pocket, Lys33 does not interact directly with the nucleotide [101]

of ATP. The structure of the nucleotide-binding site of APH(3′)-IIIa (Figure 1.13) suggests a similar role for Asp190 because it is in the correct position to interact with the incoming hydroxyl. Site-directed mutagenesis of Asp190 causes a minimum 650-fold decease in k_{cat} [101] confirming this role. Affinity labeling demonstrates that the conserved Lys44 is close to the triphosphate-binding pocket [104]. Figure 1.13 shows Lys44 located on β-strand 3 and positioned directly over the ATP triphosphate-binding site, where it interacts with α- and β-phosphates. Site-directed mutagenesis also supports the role of Lys44 in ATP binding [101].

Le Goffic *et al*. originally isolated kanamycin nucleotidyltransferase (KNT) from *S. aureus* and showed it could catalyze the transfer of a nucleoside monophosphate group from a nucleotide to the 4′-hydroxyl group of kanamycin [104]. The enzyme utilizes ATP, GTP or UTP and can inactivate a wide range of aminoglycosides [100]. Contrary to nondenaturing gel electrophoresis findings [105], crystallographic studies suggest that KTNase is a dimer, the interface between the monomers of which is formed by electrostatic interactions. KNTase possibly operates a Theorell–Chance kinetic mechanism like nucleotidyltransferase 2″-I. Direct in-line displacement of pyrophosphate from P1 (nucleotide) by C2″-OH (aminoglycoside) is believed to produce nucleotidylaminoglycoside in a single step, accompanied by inversion of the phosphorous [106]. Enzyme turnover is controlled by the rate-limiting step of product release after pyrophosphate release [100].

Figure 1.14(a) shows the pronounced basket-like cleft formed by the two subunits of the dimmer, which accommodates different aminoglycosides. The N-terminal domain is delineated by Met1 to Glu127 and characterized by a five-stranded mixed β-pleated sheet, whereas the C-terminal domain contains five α-helices and is formed by Ala128 to Phe253 [79]. The subunits wrap round each other to form the aminoglycoside-binding site. The adenine portion of the nucleotide is involved in

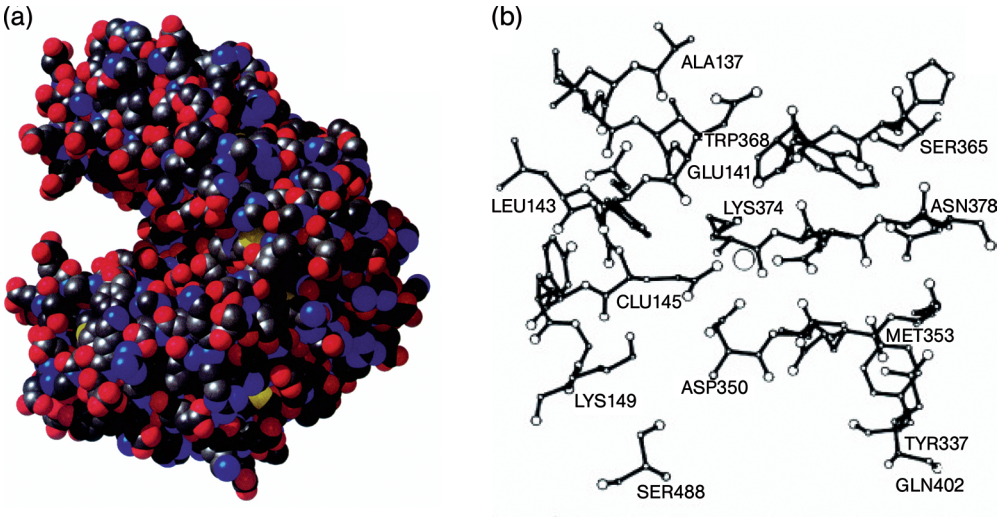

Figure 1.14 (a) Space-filling representation of all the atoms in the KNTase model. (b) Close-up view of the putative zinc-binding site [100].

few specific interactions with the protein, which explains why ATP, GTP and UTP are all substrates for the enzyme [79]. Close-up views of the zinc-binding site (Figure 1.14b) show it surrounded by a ring of seven negatively charged amino acid residues: Glu141, Glu142 and Glu145 from subunit 1, and Glu52, Glu67 Glu76 and Asp50 from subunit 2. This crown of negativity would bind kanamycin and other positively charged aminoglycosides, implicating the zinc-binding region as the active site.

KNTase bound with kanamycin and the nonhydrolyzable nucleotide analog AMPCPP also suggests a group of seven negatively charged amino acid residues form a portion of the binding site [79]. Consistent with other research on aminoglycosides [80], the amino sugar of kanamycin interacts most extensively with KNTase. It is wedged against the adenine ring of AMPCPP, while the third amino sugar has few specific interactions with the protein [79]. The exocylic hydroxyl and amino groups on this amino sugar can form hydrogen bonds with the side-chains of Glu67, Glu76 and Lys74 from subunit I, and Glu141 and Glu145 from subunit II. Ser94 and Glu141 from subunit II interact with the aminocyclitol moiety. Of particular interest is Glu145, whose carboxylate group is within hydrogen-bonding distance of the 4′-hydroxyl group. A resistance mechanism involving nucleophilic attack of the α-phosphorous on the nucleotide by activated kanamycin has been proposed [79]. Glu145 acts as a general base abstracting a proton from the 4′-hydroxyl group, which activates kanamycin for subsequent attack at the α-phosphate of the nucleotide. Both the nucleotide and the antibiotic are in the proper orientation for a single in-line displacement reaction. Additionally, Lys149 is within close proximity of the α-phosphoryl oxygens and would increase the electrophilic character of the

phosphorous center, making it more susceptible for nucleophilic attack [107]. This mechanism is limited by the 5.0 Å distance for nucleophilic attack, which is too long. However, Pedersen *et al.* emphasize that the mechanism is based on a nucleotide analogue and enzyme catalysis may induce conformational changes that reduce this distance [79].

Aminoglycoside uptake involves three consecutive steps [107]. The first is adsorption of the cationic aminoglycoside to the surface, facilitated by electrostatic interactions with the negatively charged lipopolysaccharides in outer membranes of Gram-negative bacteria. The second and third steps are dependent on transmembrane potentials generated by the respiratory chain. Thus, anaerobic bacteria are intrinsically resistant [108] and strains with mutations in ATP synthetases have reduced susceptibility [107]. Some bacteria seem to have also developed permeability mechanisms linked to uptake and efflux in order to counteract the accumulation of aminoglycosides intracellularly. Models for this have been proposed; however, the exact mechanics of this have yet to be elucidated.

Multidrug efflux is another component of aminoglycoside resistance. An important structural example is AcrB from *E. coli* reveals a multiprotein complex involving AcrB, AcrA and TolC, which enable drug translocation across the plasma membrane followed by transport via TolC across the periplasm and the outer membrane [109, 110]. In this model, AcrB facilitates proton counterflow-driven drug transport from the plasma membrane inner leaflet into a protein cavity on the outer side of the membrane, as well as sequestering of drug form the membrane outer leaflet. From there, TolC provides a route to the bacterial exterior.

While AcrB does not efflux aminoglycosides [93], the model based on AcrB (Figure 1.15) can be applied to AcrD-mediated aminoglycoside efflux. Localized in the cytoplasm, resistance nodulation cell division (RND) proteins use membrane proton-motive force as their energy source. In Gram-negative bacteria they interact with a membrane fusion protein located in the periplasmic space and an outer membrane protein (OMP) to form a continuous tripartite channel. Trimeric TolC, the OMP that interacts with AcrB, forms a barrel composed of 12 outer membrane-spanning β-strands and 12 α-helices extending into the periplasmic space for over 100 Å. The internal cavity is open to the external environment to provide solvent access. Each monomer of AcrB contains 12 transmembrane domains (TMDs) and two large periplasmic domains. Substrates bind to the ring-like arrangement of TMDs connected to the periplasmic funnel [93]. There is an opening between the two periplasmic domains connecting the periplasm with the central channel. These 'vestibules' can channel substrates selected from the outer leaflet of the cell membrane or from the periplasmic space into the efflux apparatus for exportation out of the cell.

There are more acidic residues at the entrance of the AcrD vestibules compared to vestibules of AcrB [111]. These extra acidic residues may give AcrD the capacity to efflux aminoglycosides which are attracted towards the phospholipid through electrostatic interactions. Aminoglycoside hypersusceptibility accompanying the disruption or deletion of genes encoding AcrB in *E. coli* [112] implicates RND protein efflux in aminoglycoside resistance. Resistance to aminoglycosides, due to efflux, can

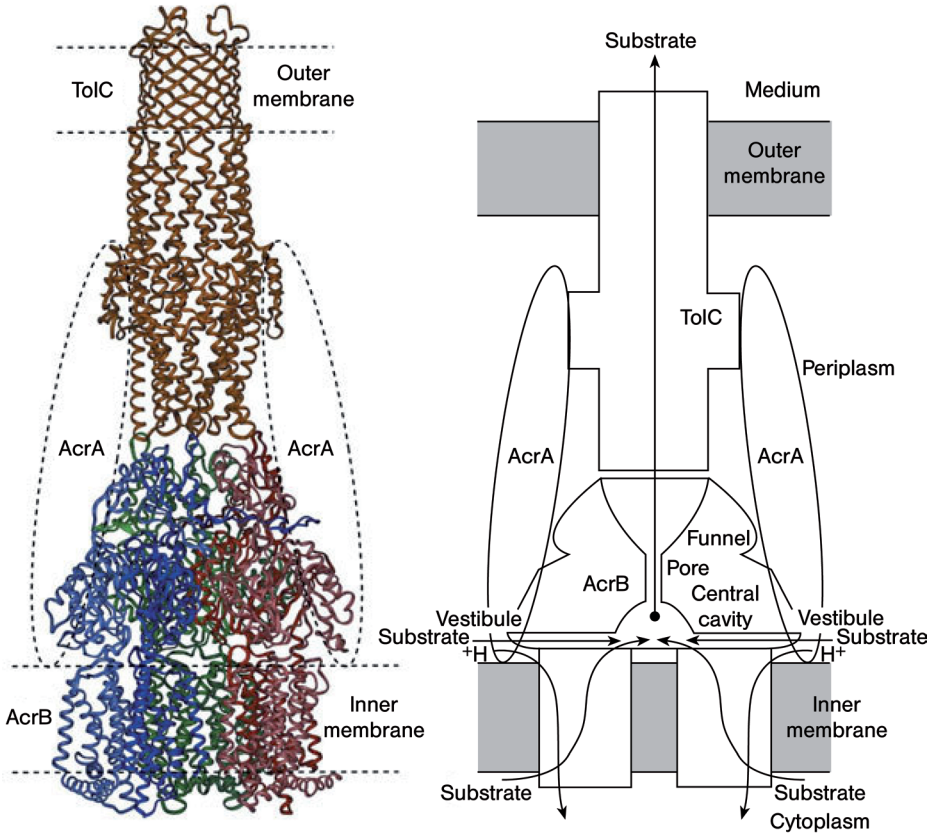

Figure 1.15 Model of multidrug efflux based on *E. coli* AcrB, AcrA and TolC complexes [110].

be mediated by the major facilitator superfamily (MFS) proteins. This has been shown in mycobacteria [113, 114], where resistance is encoded by *tap* and *P55* genes. Genes and proteins homologous to *P55*/P55 have been detected in many mycobacteria, including *M. tuberculosis* [93].

1.7 Macrolides

Macrolides are another class of antibiotics, structurally distinct from the aminoglycosides, that inhibit bacterial protein synthesis. Macrolides are characterized by a 14-, 15- or 16-membered macrolactone ring. The structure of erythromycin (Figure 1.16) consists of a 14-membered macrolactone ring with a cladinose sugar and a desosamine sugar attached. The 16-membered ring macrolides have two sugars attached through an amino sugar. Telithromycin lacks an α-L-caladinose at position 3 on the erythronolide A-ring and is a semisynthetic macrolide from the ketolide class, which are active against bacteria resistant to erythromycin A [95].

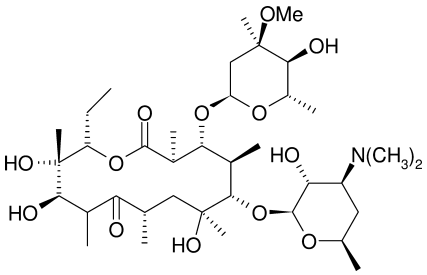

Figure 1.16 Structure of erythromycin.

While macrolides are the focus here, lincosamides and type B streptogramins will be referred to regarding their overlapping functions (the MLS_B phenotype) and their common 50S target. Erythromycin halts protein synthesis after formation of the initiation complex, probably by interfering with the translocation reaction [95]. Erythromycin, clarithromycin and roxithromycin bind to the entrance of the tunnel which channels nascent peptides away from the peptidyltransferase center [115]. While binding of macrolides may not block peptidyltransferase activity, erythromycin bound to the tunnel would interfere with channeling of nascent peptides (Figure 1.17).

Widespread use of macrolides has led to the emergence of resistance in *S. aureus* and *Streptococcus pyogenes*. Impermeability and efflux has provided Gram-negative bacilli with intrinsic macrolide resistance, except the azalides [95]. The three types

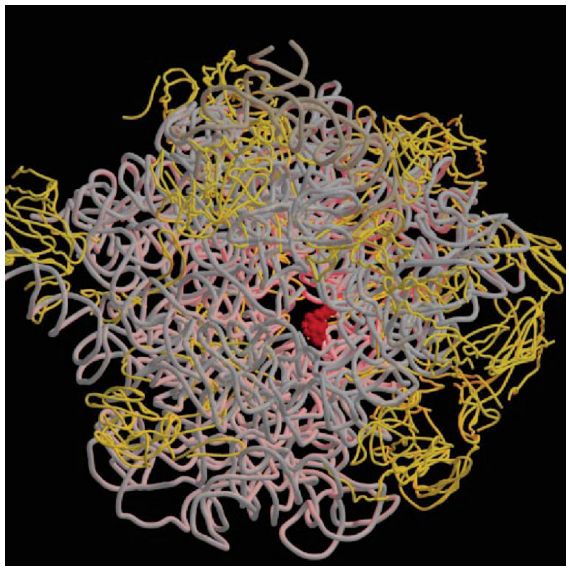

Figure 1.17 Top view of the *Deinococcus radiodurans* 50S subunit showing erythromycin (red) bound at the entrance of the tunnel [115].

of macrolide resistance are target modification conferring the MLS_B phenotype, active efflux and macrolide-inactivating enzymes. The lactone ring and the desosamine sugar are the two reactive components of macrolides which form hydrogen bonds with the peptidyltransferase cavity of 23S rRNA [115]. The 6-OH group is within hydrogen-bonding distance of N6 of A2062Ec, and the 11-OH group and 12-OH group hydrogen bond with O4 of U2609Ec. The 2′-OH group of the desosamine sugar forms hydrogen bonds with N6 and N1 of A2058Ec and N6 of A2059Ec [113]. Dimethylation of the N6 amino group of A2058 located in domain V of 23S rRNA is consistent with this, and confers resistance to erythromycin and its derivatives (except 16-membered macrolides). This post-transcriptional modification is mediated by adenine-specific *N*-methyltransferase activity and is encoded for by *erm* (erythromycin ribosome methylation) genes. Dimethylation of N6 adds a bulky substituent to the residue, causing steric hindrance for binding and abrogating the hydrogen bonding with the 2′-OH group of the macrolide [115]. A substitution of guanine for adenine at 2058 has been described in staphylococci and streptococci [95]. This change confers resistance because it would disrupt hydrogen bonding therefore prevent macrolides from binding to the 23S rRNA. Acquisition of mutant A2059G gives rise to macrolide and lincosamide resistance despite increasing the generation time of R6. This ML phenotype is not conferred by any other existing determinants known [116].

Ribosomal proteins L4 and L22 have been implicated in resistance [117] and mutations in which have been reported in clinical isolates of *S. pneumoniae* and *S. pyogenes* [95]. However, the distances between erythromycin and L4 and L22 are too great for meaningful interactions [115]. Multiple changes in reactivity to chemical probes indicate that both mutations disrupt the conformation of specific residues in domains II, III and V. Domain V includes residue A2058; however, neither mutation shows any effects at or within its vicinity [118]. The MLS_B phenotype is associated with mutations or methylation of A2058, so mutations L4 and L22 do not give rise to MLS. Rather, they confer resistance to erythromycin, spiramycin and tylosin, but not lincosamides [118]. Research suggests mutations in L4 confer an MS_B phenotype implicating a 3-amino-acid substitution and an 18-bp insertion [116]. Supporting ribosomal proteins binding at multiple rRNA sites, perturbations of the 23S rRNA structure have been observed with mutations within ribosomal proteins L4 and L22. The resultant 23S rRNA is resistant to macrolides.

Efflux is clinically relevant in *S. pneumoniae*, *S. pyogenes* [119, 120] and most Gram-negative bacteria. Acquired *mefE* and *mefA* encode the membrane protein Mef, an active efflux pump accounting for over 50% of the resistance seen in *S. pneumoniae* and *S. pyogenes* [95]. *MefE* and *mefA* are 90% identical, and are now referred to collectively as *mef*(*A*) [121]. *mef*(*A*) confers resistance to macrolides, but not lincosamides (the M-R phenotype). The macrolide efflux pump, AcrAB-TolC of *H. influenzae* and *E. coli* is an example of an intrinsic efflux pump that acts in synergy with slow penetration of outer cell walls to give the high MICs seen in Gram-negative bacteria [122].

The ribosomes of *E. coli* K12 strain expressing *mefA* display no evidence of methylated 23S rRNA and are more sensitive to macrolides that macrolide-suscepti-

ble strains [123], confirming a novel gene unrelated to *erm* genes [120]. Erythromycin inactivation and *msrA*-encoded efflux have been ruled out as potential candidates for the M-R phenotype seen in streptococci [123]. Moreover, discernible regions of MefA show no homology to the Walker motifs A and B which characterize ABC transporters. Similarities in tertiary structure exist between MefA and Tet antiporters of Gram-negative bacteria, consistent with it being a member of the MFS.

As a 12-transmembrane secondary transporter with H^+ antiporter activity, MefA couples drug efflux to a downhill electrochemical gradient of protons. MefA has a simple 12-transmembrane segmented structure possible resulting from duplication of a gene encoding a six-transmembrane segment protein [124]. The N-terminal halves of MFS proteins exhibit greater sequence similarity than the C-terminal halves and so form the site of variable ligand binding, whereas the C-terminal is probably involved in energization of the protein [125, 126]. Macrolide recognition is explained using a model based on the C-terminal half of the BmrR protein (BRC) [127]. BRC (Figure 1.18) consisting of eight β-strands and three α-helices is only active as a dimer. Binding of the ligand is governed by a buried charged acidic residue, and the α2 helix periodically undergoes a change to expose the binding site and facilitate binding of the ligand [128]. Binding of electroneutral ligands is governed by the size of the opening to the binding site as well as hydrophobic forces. Once bound, hydrophobic and aromatic residues stabilize the ligand until a second conformational change, presumably linked to proton translocation, results in expulsion of the ligand [126].

Extrusion of macrolides can be explained using the hydrophobic 'vacuum cleaner model' or the 'flippase model'. The 'vacuum cleaner model' [124] proposes macrolides move freely into the lipid phase of the membrane and on reaching MefA are actively expelled out of the cell. The 'flippase model' proposes that on reaching the protein within the membrane, the drug is 'flipped' to the outer layer. Structural characteristics of multidrug-resistant proteins favor the vacuum cleaner model [124]. The 12 TMDs of MefA would form a central aqueous pore open to the environment, closed to the cytosol and lined with aromatic amino acids which could facilitate the

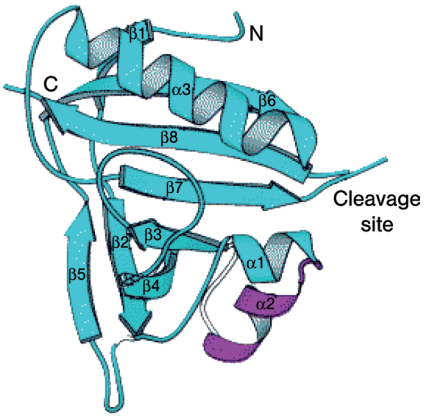

Figure 1.18 Monomer of BRC. The essential acid residue is indicated as Glu134 in β7 [128].

transport of hydrophobic substrates [129]. In contrast to MefA, MsrA/B in staphylococci acts as an ATP-dependent efflux pump conferring the MS phenotype. *S. aureus* cells expressing MsrA accumulated significantly less erythromycin than cells without MsrA [120]. MsrA efflux is abolished by arsenate and strongly inhibited by dinitrophenol [130] consistent with its dependence on ATP. MsrA/B-mediated efflux is relatively uncommon compared to methylase-mediated resistance in staphylococci [131].

As a member of the ABC family, the structure of MsrA consists of two TMDs. These typically comprise of six transmembrane-spanning α-helices and two nucleotide-binding domains (NBDs), located on the inner surface of the bacterial cell membrane as sites of ATP hydrolysis (Figure 1.19). Deletion of 42 C-terminal codons from a fragment of *S. epidermidis msrA* results in loss of the MS resistance phenotype demonstrating the importance of the C-terminal domain [132, 133]. All NBDs possess a Walker A motif (P-loop) and a Walker B motif which hydrogen bond extensively with the nucleotide. The Walker B motif provides the catalytic base. The ABC signature motif, H-loop, Q-loop and stacking aromatic are only characteristic of transport ABCs and coordinate the bound nucleotide or nucleophile water [134]. NBDs couple conformational changes induced by ATP binding, hydrolysis and

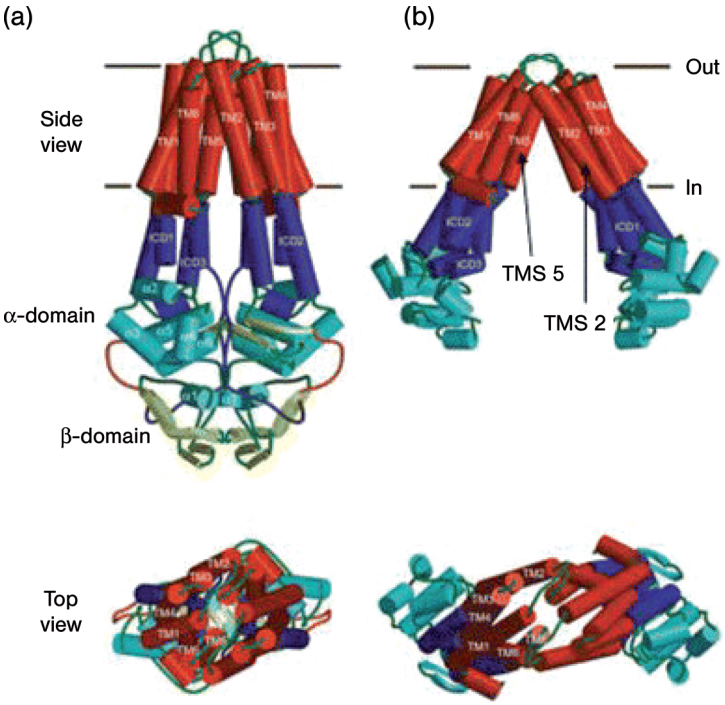

Figure 1.19 Structure of (a) MsbA from *Vibrio cholerae* and (b) MsbA from *E. coli*, showing the location of the transmembrane domains (red), the intracellular domain (blue) and the nucleotide-binding domain (cyan) [126].

release to the transport process [134] in order to energize transport of macrolides and type B streptogramins. Figure 1.19 shows the tilt of the monomers of a typical MDR, away from the normal of the membrane forming a cone-shaped chamber that excludes access from the outer leaflet of the membrane so a ligand can only bind from the inner leaflet [126].

An ATP switch model has been proposed as the first coherent model for ABC transport [134]. Binding of ligands such as macrolides induces a conformational change in the TMD which is then relayed via intracellular domains to the NBD. The conformational change here enhances the binding of ATP and lowers the activation energy required for 'closed' dimer formation. ATP binding is associated with a 'closed' NBD dimer conformation, whereas dissociation of the NPD dimer to an 'open' conformation is caused by ATP hydrolysis. This acts as a switch for conformational changes back in the TMDs that mediate transport of the macrolide. The 'closed' dimer conformation may expose the macrolide to unfavorable positively charged residues lining the inner leaflet half of the TMD chamber. This prompts the macrolide to 'flip' to the more favorable, hydrophobic environment of the outer leaflet half of the chamber. This flip induces a conformational change in TMDs, especially TMS-2 and -5 and the NBDs which would lead to expulsion of the macrolide [126]. ATP hydrolysis appears to initiate resetting of the transporter to it basal 'open' conformation [134].

Inactivating enzymes are important resistance mechanisms in *Nocardia* spp., and include esterases which hydrolyze the lactone ring and enzymes which fix a glucose or a phosphate at the 2′-OH group of D-desosamine [95]. Until recently there had been no reports of esterase activity in Gram-positive organisms; however, strain 01A1032 of *S. aureus* exhibits esterase activity of 14- and 16-membered macrolides, and also the ability to efflux azithromycin [133]. Ribosomes from strain 01A1032 demonstrated 100% inhibition by erythromycin and azithromycin, ruling out any activity of Erm methylases and ribosomal mutations. Polymerase chain reaction (PCR) results gave a PCR product consistent with an *msrA*-like gene, which implicates an ABC protein in mediating the efflux resistance mechanism. It remains unclear if efflux and enzymatic inactivation are part of one mechanism, act synergistically or are mutually exclusive [133].

1.8 Tetracyclines

Tetracyclines are polycyclic broad-spectrum antibiotics with activity against both Gram-positive and Gram-negative organisms. The main structural feature of a tetracycline molecule is a linear fused nucleus of four rings (Figure 1.20). In the 1950s and 1960s tetracycline was one of the most widely used antibiotics [135] due to its broad spectrum of activity, few side-effects, low toxicity, low production costs and the possibility for oral administration. Widespread resistance to tetracycline now limits its use, with it being discontinued as first-line therapy for the treatment of sexually transmitted diseases, after the appearance of tetracycline-resistant *Neisseria*

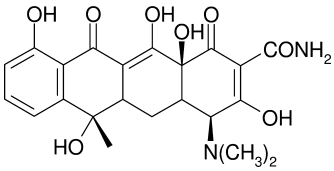

Figure 1.20 Structure of tetracycline.

gonorrhoeae [136]. Tetracyclines target both mammalian (80S) and bacterial (70S) ribosomes, although 70S ribosomes are more sensitive to tetracycline, which is also found in higher concentrations in bacterial cells compared to mammalian cells. Tetracycline disrupts the codon–anticodon interaction between mRNA and tRNA by inhibiting the binding of aminoacyl-tRNA to the A-site. The initial stages of ternary complex (EF-Tu·GTPaa·tRNA) binding such as decoding and GTP hydrolysis are still possible; however, accommodation of the tRNA into the 50S A-site is blocked, preventing further extension of the peptide chain. The ribosome becomes locked in a nonproductive and energetically expensive cycle of ternary complex binding and release (Figure 1.21) [137].

Tetracycline binds to a single site on the 30S ribosomal subunit, which includes a region of 16S rRNA containing base A892. The anticodon of bound aminoacyl-tRNA is positioned very close to a 16S rRNA region which includes a base at 1400. This suggests that tertiary folding of 16 rRNA brings bases 892 and 1400 very close to each other and that bound tetracyclines block aminoacyl-tRNA binding by disturbing this

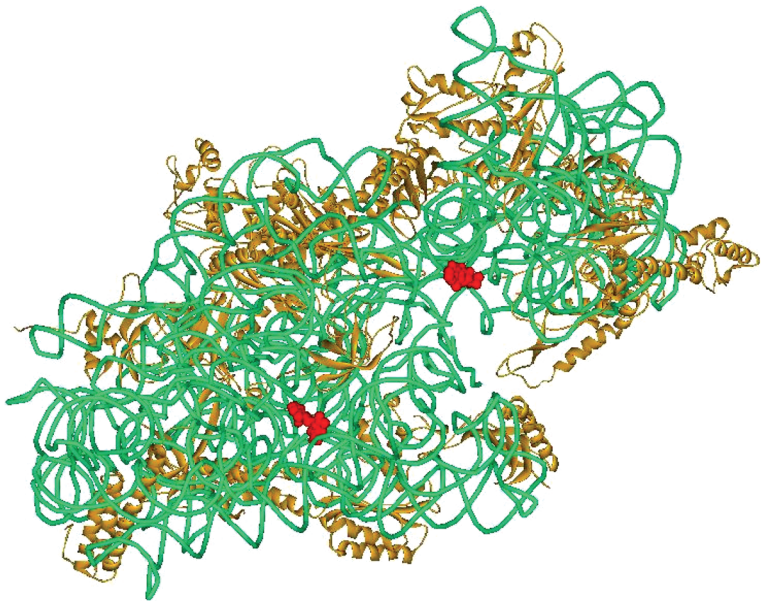

Figure 1.21 Crystal structure of tetracycline (red) bound to the 30S ribosomal subunit from *Thermus thermophilus* [137] (PDB code: 1HNW). Visualized using Discovery Studio (Accelrys).

folding [10]. Even though ribosomal protein S7 is not associated with 16S rRNA, tetracycline binds strongly to both the 30S ribosomal subunit and S7 ribosomal protein [138]. It is suggested that binding of tetracycline to 16S rRNA causes such a distortion in RNA structure that ribosomal protein S7 is presented to the 892–1400 domain [10].

Crystallographic study reveals two tetracycline-binding sites [137]. The primary site (20 Å wide and 7 Å deep) is formed by an irregular minor groove of H34 (RNA residues 1196–1200:1053–1056) in combination with residues 964–967 from the H31 stem loop. Hydrophobic interactions are apparent between bases 1054 and 1196 of H34 and the fused-ring system of tetracycline. However, tetracycline interacts primarily through hydrogen bonding between oxygen atoms of one side of the tetracycline molecule and the exposed sugar phosphate backbone oxygen atoms of H34. There is also a hydrogen bond to the oxygen phosphate of G966 from H31. Important salt bridges exist between a putative magnesium ion on the hydrophilic side of tetracycline and phosphate oxygen atoms of G1197 and G1198. Tetracycline bound to the primary site sterically interferes with aminoacyl-tRNA binding to the A-site [137]. The secondary binding site is associated with the H27 Switch region (residues 891–894:908–911) and H11 (residues 242–245) [132]. Binding of tetracycline here cannot directly interfere with tRNA binding; however, it could interfere with the transition between open and closed states of the 30S ribosomal subunit [139], which is important for the decoding reaction [140]. Pioletti *et al.* identified six (Tet-1–6) tetracycline-binding sites on the 30S subunit [141]. Tet-1 corresponds with the same residues from H34 and H31 as the primary site location and Tet-5 is associated with the H27 switch region of the secondary site [141]. The roles of the four other binding sites are less clear cut. Consistent with this is enhanced DMS modification of C1054 and the protection of A892 from this modification, by tetracycline [142].

Tetracycline uptake is energy dependent [143] and mediated by a change in pH rather than a transport protein [144]. Existing in protonated form (TH2) and magnesium-chelated form (THMg), TH2 is free to diffuse into the cell through the phospholipid membrane, whereas THMg is not. Intracellular pH is high compared to the pH outside the cell and so is trapped within the bacterial cell is a greater proportion of THMg [144]. Altering porin proteins such as OmpF can confer resistance by limiting tetracycline diffusion [135]; however, efflux and ribosomal protection are the most common resistant determinants [145].

There are tetracycline-specific and multidrug efflux pumps conferring tetracycline resistance [146, 147]. Tetracycline and a narrow spectrum of related antibiotics is exported in *E. coli* by TetB and other proteins encoded by the *tet* genes [145]. TetB alone normally does not give rise to resistance, but is usually associated with TetA or OtrA efflux pumps [148]. Tet proteins belong to the major facilitator superfamily and have a predicted six-plus-six transmembrane domain organization, which provides the structural basis underlying an electrically neutral proton tetracycline antiport system [149, 150]. A model for the 'tetracycline transport cycle' in resistant cells proposes tetracycline is probably effluxed as a metal–chelate complex coupled with proton influx [151].

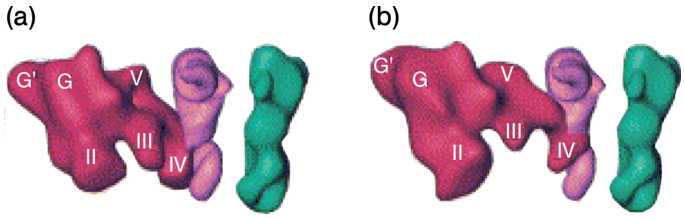

Figure 1.22 (a) Positioning of EF-G in GTP form (red) together with A-site (pink)- and P-site (green)-bound tRNAs. (b) Positioning of Tet(O)·GTPγS (red), the P-site-bound fMet-tRNA (green) together with tRNA in a position equivalent to the observed position of the A-site-bound tRNA in the pretranslocational ribosome [152].

Ribosomal protection is conferred by ribosomal protection proteins (RPPs) that associate with the ribosome and make it insensitive to tetracycline inhibition. Tet(O) and Tet(M) are well-studied examples, sharing 75% amino acid sequence identity [153] and other members of RPPs [Tet(S), Tet(T), Tet(Q), TetB(P), Tet(W) and OtrA] function through similar mechanisms to Tet(O) and Tet(M) [139]. Tet(M) binds to ribosomes, shares considerable amino acid homology to EF-G and exhibits similar ribosome-dependent GTPase activity to this promoter of translocation [154]. Moreover, binding of Tet(O) or EF-G enhances the reactivity of A1408 to DMS modification [142]. Both Tet(M) and Tet(O) catalyze the release of tetracycline from the ribosome in a GTP-dependent manner.

The tip of domain IV of Tet(O) is positioned to interact with the noncovalent junction between the shoulder and head of the 30S subunit (Figure 1.22). This region is made up of helices 18 and 33/34 of 16S rRNA. H34 contributes to the Tet-1/primary tetracycline-binding site [152] and is involved in maintenance of translational fidelity, particularly in stop codon decoding and frame shifting [155]. The interaction between the tip of domain IV of Tet(O) and the junction linking the head and shoulder of the 30S subunit (Figure 1.23) is consistent with its role in chasing tetracyclines from the

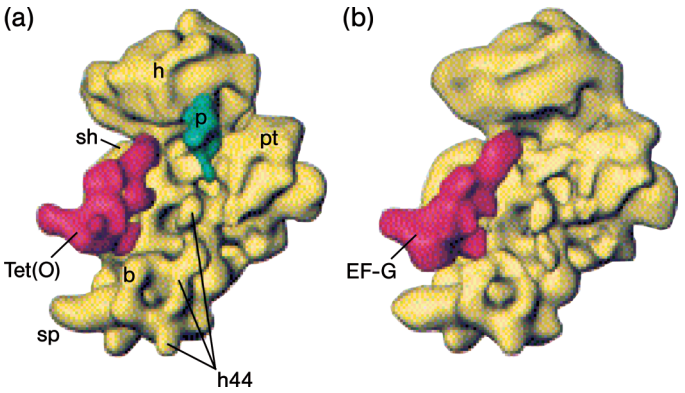

Figure 1.23 The 30S parts of the fMET-tRNA·70S·Tet(O) map. Landmarks: sh, shoulder; h, head [152].

primary binding site [142]. However, Tet(O) binding does not promote translocation and leaves the conformation of the ribosome virtually unchanged [148]. An interaction between domain III of Tet(O) and S17 ribosomal protein could also be relevant in Tet(O)-mediated resistance [152].

Binding of Tet(O) protects C1214 from DMS chemical modification and enhances the reactivity of A1408 [142]. Interestingly, C1214 can be found on helix 34 (h34) and is close but does not overlap the tetracycline-binding site. Located on h44, A1408 is distinct from the tetracycline-binding site. Enhanced DMS modification of C1054 upon tetracycline binding is inhibited by Tet(O); however, protection from DMS modification of A892 by tetracycline binding is unaffected by Tet(O) binding. This suggests Tet(O) only prevents tetracycline from binding to the primary site C1054 [142]. Protection of C1214 from chemical modification indicates the base is being shielded directly or it undergoes a conformational change resulting in decreased accessibility [142]. Tet(O) has close proximity with h34 (Figure 1.24) suggesting a direct interaction or indirect through other contacts in h34, however it does not approach h44. The enhancement of reactivity of A1408 indicates a Tet(O)-induced conformational change which has been suggested to involve ribosomal protein S12, whose core makes contact with the backbone of h44 around residues 1491 and 1492 [142].

Tet(O) intercalates into the elongation cycle of the post-translocational ribosome (POST complex) [156]. The PRE complex is characterized by deacyl-tRNAf^{MET} in the P-site and AcPhe-tRNA^{Phe} in the A-site, whereas the POST complex has an empty A-site. Tet(O) and EF-Tu cannot simultaneously bind to the ribosome because they occupy overlapping sites. Instead, Tet(O) binding was found to stabilize a conformation of the GAR (L11 region, i.e. H42/43/44) which left the ribosome in a conformation favorable for interaction with EF-TU [156].

Tetracycline binds to the A-site on the post-translocational ribosome where it sterically interferes with the accommodation of aminoacyl-tRNA. A conformational

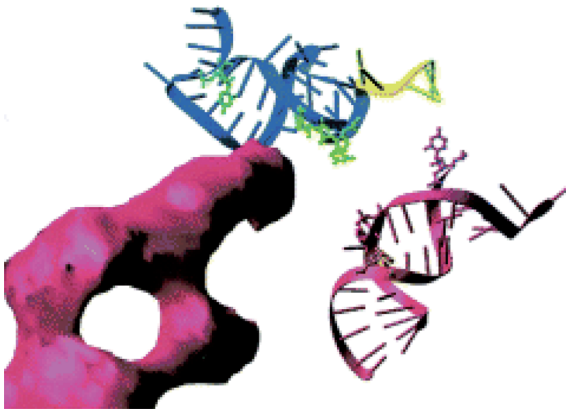

Figure 1.24 Interaction of domain IV of Tet(O) (red density) with the region around the primary tetracycline-binding site. Blue ribbon: helix 34; red ribbon: helix 44 [142].

change in the decoding site follows tetracycline binding without release of tRNA bound to the E-site accompanies. Tet(O) present in low abundance in the cell recognizes the blocked ribosome by virtue of its open A-site, prolonged pausing in the POST state and possibly the drug induced conformational change [156]. The interaction between Tet(O) and the ribosome triggers release of tetracycline prior to GTP hydrolysis [157] and induces rearrangements in the A-site [142]. Tet(O) hydrolyzes GTP leaving the ribosome in a conformation which is compatible with EF-TU binding. The conformational changes prolong at the A-site after Tet(O) release enhancing the ability of the ternary complex to compete with tetracycline [156].

1.9 Fluoroquinolones

The development of quinolones began with the introduction of nalidixic acid in 1962, they are synthetic compounds based on the 4-quinolone nucleus (Figure 1.25). First-generation quinolones include cinoxacin and oxolinic acid, which despite a limited spectra, had potent Gram-negative activity. Second-generation quinolones were fluorinated (fluoroquinolones), and included ciprofloxacin which was introduced in 1987 and was the first widely use quinolone. Fluoroquinolones have excellent Gram-negative activity and can be administered orally [9]. The late 1990s saw the introduction of the third generation of quinolones (e.g. levofloxacin and moxifloxacin) with increased Gram-positive activity [158]. Quinolones inhibit DNA replication by targeting DNA gyrase and topoisomerase IV. The proposed model of quinolone action on DNA gyrase in Figure 1.26 shows quinolones binding to the enzyme–DNA complex through significant hydrogen bonds between the 3-carboxy and 4-oxy groups common to quinolones and the unpaired bases of the unwound single strand. This binding prevents DNA gyrase from relegating the cleaved DNA and double-strand breaks accumulate, setting off the SOS repair system which ultimately leads to bacterial cell death. In response to this threat, bacteria have developed resistance mechanism, which include altering the target enzymes (DNA gyrase and topoisomerase IV), changing cell wall permeability and quinolone efflux mechanisms [159].

Quinolones do not bind directly to either the individual subunits of type II topoisomerases or the complete tetramers. Instead, they act on type II topoisomerases

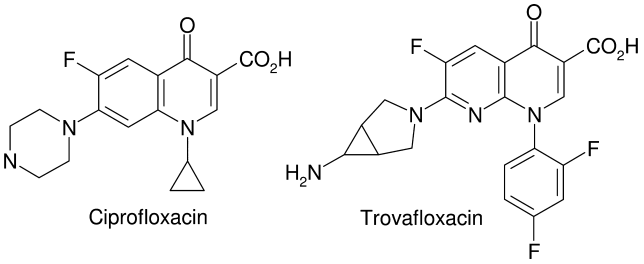

Figure 1.25 Second- and third-generation fluoroquinolones.

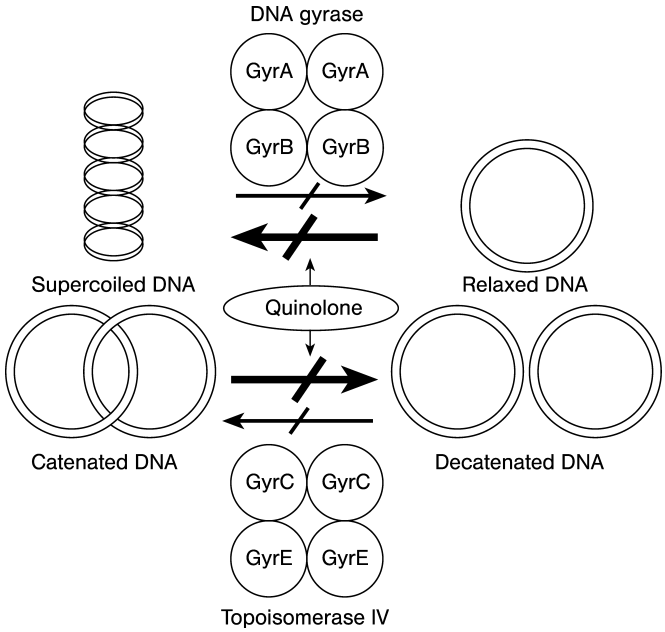

Figure 1.26 Quinolones block DNA gyrase and topoisomerase activity by stabilizing the enzyme–DNA complex. This inhibits the movement along the DNA of other proteins such as DNA and RNA polymerase [165].

by trapping or stabilizing an enzyme reaction intermediate in which both DNA strands are covalently linked to the breakage reunion subunits (GyrA from DNA gyrase or GrlA from topoisomerase IV) [160, 161]. Stabilization of this cleavage complex initiates a series of events leading to cell death [162]. Mutations in type II topoisomerases are often found in a discrete sequence of genes called the quinolone-resistant determinant region (QRDR) [163]. For example, determination of the nucleotide sequence for the QRDR of *gyrA* from codon 55 to 138 revealed that three (C5, C14 and L1) out of 12 of the clinical isolates of *Bacteroides fragilis* tested demonstrated the same Ser82Phe mutation in GyrA. All three of these clinical isolates showed the highest MIC of 4 μg/ml out of the clinical strains tested [164].

Mutations in type II topoisomerases are not just restricted to the QRDRs and the spontaneous *S. aureus* mutant, MT52244c9 is evidence for this. MT5224c9 was obtained in one step by plating MT5 *gyrB142* on ciprofloxacin. Compared to its parental strain, MT5224c9 showed an approximate 4-fold increase in MIC of ciprofloxacin yet an approximate 8-fold decrease in MIC of the courmarin novobiocin. The mutation is localized to *Sma*I chromosomal fragment A by a linkage to a *Tn*551 insertion in this fragment, which encodes topoisomerase IV *grlB* and *grlA* genes [166, 167]. The mutation in question is Asn470Asp and is actually confined to the GrlB subunit of topoisomerase IV [162], which hydrolyzes ATP for the energy-dependent introduction of negative supercoils into the DNA during replication.

Mutations in *grlA* and *gyrA* genes have also been shown to confer resistance to ciprofloxacin. Substitution of Ser80 with Phe or Tyr in the *grlA* gene was found to be the principle mutation (39.8%), while substitution of Ser84 with Leu in the *gyrA* gene was the principle mutation (35.2%) in *S. aureus* isolates [168].

Interestingly, the Asn470Asp mutation is located just outside the region of amino acids 451–458 which is highly conserved between GrlB and GyrB [162]. An homologous mutation (Asn493) in the yeast species *Saccharomyces cerevisiae* has been located through crystal structures to B′α3 helix, which is some distance from the active-site tyrosine involved in DNA strand breaking [169]. This raises the question as to how such a mutation can confer resistance. Despite the absence of structural data on the topoisomerase–DNA–quinolone complex, Fournier and Hooper suggest that resistance is unlikely to result from the fact that the Asn470Asp mutation introduces a negative charge because this would actually increase its affinity for the positively charged piperazinyl group on ciprofloxacin. In light of the fact that mutant enzymes exhibit reduced relaxation of supercoiled DNA [166], it is further suggested that the *grlB* mutation reduces the intrinsic catalytic efficiency of the enzyme. This could be true if quinolones only bind to specific steps in the catalytic cycle of the enzyme–DNA complex. Mutation could induce conformational changes which slow down the catalytic cycle, meaning that there would be fewer opportunities where the enzyme–DNA complexes are in an appropriate conformation for quinolones to bind to [162].

The mechanism shared by proteins which belong to the MFS has already been dealt with in detail in the macrolide chapter. Of course there are many other proteins which are part of this family. Fluoroquinolone resistance, for example, is associated with over expression of one such multidrug efflux protein, NorA [168]. Ciprofloxacin-resistant strains of *Mycobacterium smegmatis* have been shown to have an increased initial phosphate uptake [170]. Uptake of phosphate is mediated by the phosphate-specific transporter Pst which possesses a nucleotide-binding subunit (PstB) reported to exhibit ATPase activity [171]. This is consistent with Pst as a member of the ABC family of transport proteins. Monitoring phosphate uptake and fluoroquinolone sensitivity in strains with a disrupted *pstB* gene (WT^d) showed that there was a remarkable reduction in phosphate scavenging ability. Also compared to wild-type cells, the sensitivity of WT^d cells to ciprofloxacin, ofloxacin and sparfloxacin was increased by approximately 2-fold [170].

High-level fluoroquinolone resistance in *Coxiella burnetii* has been associated with two distinct nucleotide mutations in the *gyrA* gene [172]. Interestingly, a pH-related mechanism appears to contribute to perfloxacin resistance seen in *C. burnetii* [173]. Spyridaki *et al.* found that at pH 7.2 susceptible strains (SCB2 and SCB4), demonstrated a higher penetrability of pefloxacin than perfloxacin-resistant strains of *C. burnetii* (RCB2 and RCB4). The suggestion that an energy-dependent process such as active efflux may play a role in the reduced accumulation of perfloxacin was dismissed after the difference in accumulation of pefloxacin was not abolished by the addition of carbonyl cyanide *m*-chlorophenylhydrazone. However, the study showed that in an acid environment of pH 4.5, all strains demonstrated reduced intracellular concentration of pefloxacin. This provides some evidence to suggest a

pH-related mechanism maybe involved in reducing the pefloxacin concentration in *C. burnetii* [173].

1.10 Conclusions

The effectiveness and remarkable success of antibiotics in controlling bacterial infections put them forward amongst the most successful examples of chemotherapeutic intervention. Between 1900 and 1980 the death rate from infectious diseases fell from 797 per 100 000 to 36 per 100 000 of the population [7]. However, the very success of antibiotic chemotherapy brought about the widespread concerns surrounding their use. The 'golden age' of antibiotic therapy, which characterized the 1950s and 1960s, has come to an end. In fact, within the last 40 years we have struggled in a ceaseless war with resistance where bacteria have adapted quickly to new antibiotic developments, threatening a return to the 'dark ages' of the preantibiotic era.

Infections resistant to antibiotic treatment present a major and increasing problem to hospital and community care. Resistance to all antibiotic classes has been reported and is easily transferred within mixed bacterial populations. Over the past 70 years a vast body of research has been dedicated to the elucidation of the molecular mechanisms of antibiotic action and bacterial resistance to antibiotics. Some of the molecular and structural determinants underpinning resistance to major antibiotic classes have been outlined here, with special attention to target modification, antibiotic deactivation and drug efflux.

The action of β-lactam and glycopeptides antibiotics, which target cell wall biosynthesis, is evaded through target modifications and antibiotic deactivation. β-Lactamases can be deployed to deactivate β-lactam antibiotics, while penicillin-binding proteins with altered binding sites provide an example of modification of the antibiotic target. Alteration of peptidoglycan peptide termini helps protect cell wall synthesis from glycopeptides, while reduced cross-linking coupled to a grossly thickened wall essentially stalls glycopeptides at the cell wall periphery. Specific molecular changes within ribosomal structures can foil aminoglycoside, tetracycline or macrolide attack. Further protection can be achieved by positioning proteins to protect protein synthesizing machinery from the action of tetracyclines. Important resistance mechanisms protecting bacterial protein synthesis include enzymatic deactivation of aminoglycosides and efflux systems expelling incoming macrolides, aminoglycosides and tetracyclines.

The dependence of our society on the use of antimicrobial chemotherapy to control infections maintains the evolutionary pressure on bacteria and drives the development of antibiotic resistance. This emphasizes the urgent necessity of a sustained effort to explore new antibiotic targets and mechanisms of action. Renewed efforts to explore alternative strategies for infection management have brought to the top of scientific agenda defense peptides, lanthionine antibiotics, phage therapies and other antimicrobial techniques, which have not seen wide use against clinically significant pathogens and resistance to which remains uncommon.

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