ANTIBIOTIC RESISTANCE

1. Introduction

It is widely accepted that bacteria as living organisms came to existence over 3.5 billion years ago. As these organisms became compelled to interact with other living entities, they became more complex and evolved the biochemical means for influencing the existence of each other. One of these evolutionary developments was the advent of biochemical pathways for production of antiobiotics. In essence, if growth of a competitor were to be influence, more resources would be available for growth of the original organism. As such, multiple

pathways for generation of "secondary metabolite", which include molecules that have antibacterial properties, have evolved (1,2). A number of these secondary metabolites with antibacterial properties have been discovered over the past few decades. The structures of many of them have been altered by chemists to expand their properties or to impart desirable chemical traits to them. Many of these molecules have found clinical use over the years. In this report, we provide an overview of the important classes of antibiotics of both natural and synthetic origins and we will describe what is known about the mechanisms by which nature gives rise to resistance to them.

2. Molecular Targets for Antibiotics

Many of the first antibiotics discovered in the past 60 years have been natural products from microbial systems. To date, antibiotics that trace their origins to natural products dominate the armamentarium of clinically useful antibiotics. These are molecules that interfere with the biochemical processes of bacteria with some specificity, hence they are useful in mammalian hosts.

A total of over 70 bacterial genomes have been sequenced to date. It would appear that ~1000–5000 genes are found in most of these organisms (3). It has been proposed that somewhere between 20 and 200 or so genes are critical for survival of a broad spectrum of bacteria (3–6). The proteins encoded by these genes are potential targets for antibiotics, if inhibitors for them could be delivered to the site. Furthermore, other type of antibiotics may interfere with assemblies of these gene products or with the structural components that result from their actions, such as the cell wall, bacterial envelope, or ribosome.

Known antibiotics interfere with a small number of biochemical processes coinciding with these critical genes. These processes include metabolic pathways, disruption of the integrity of the cytoplasmic membrane, inhibition of protein biosynthesis, inhibition of DNA biosynthesis, and disruption of the biosynthesis of the cell wall, of which the last three targets are especially important. Whereas it is beyond the scope of this article to discuss all of these processes, we summarize the important processes that are disrupted by the clinically important antibacterials. Figure 1 gives the structures of several important antibacterials.

The bacterial cell wall is an important target for antibacterials, in part because it is a uniquely bacterial structure with a biosynthetic pathway for its assembly that does not find any parallels in other organisms. The cell wall provides structural rigidity and morphology to bacteria. It is a polymeric structure made up of repeat units of *N*-acetyl muramic acid (MurNAc)-*N*-acetyl glucosamine (GlcNAc). Though there are some variations, most bacteria have a five amino acid chain (a pentapeptidyl consisting of L-alanine-D-glutanate-diamino pimelate-D-alanine-D-alanine) attached via the amino group of the L-alanine to the MurNAc segment (Fig. 2). The pentapeptide has uniquely bacterial features such as D-Glu with an amide bond to diaminopimelate (DAP) via its side-chain carboxylate. In turn, DAP is linked to the dipeptide D-Ala-D-Ala (Fig. 2). The substituted MurNAc-GlcNAc disaccharide segments is linked with a neighboring disaccharide segment in a reaction catalyzed by the transglycosylases (TGs). The disaccharide building block as a pyrophosphoryl-undecaprenol ester serves

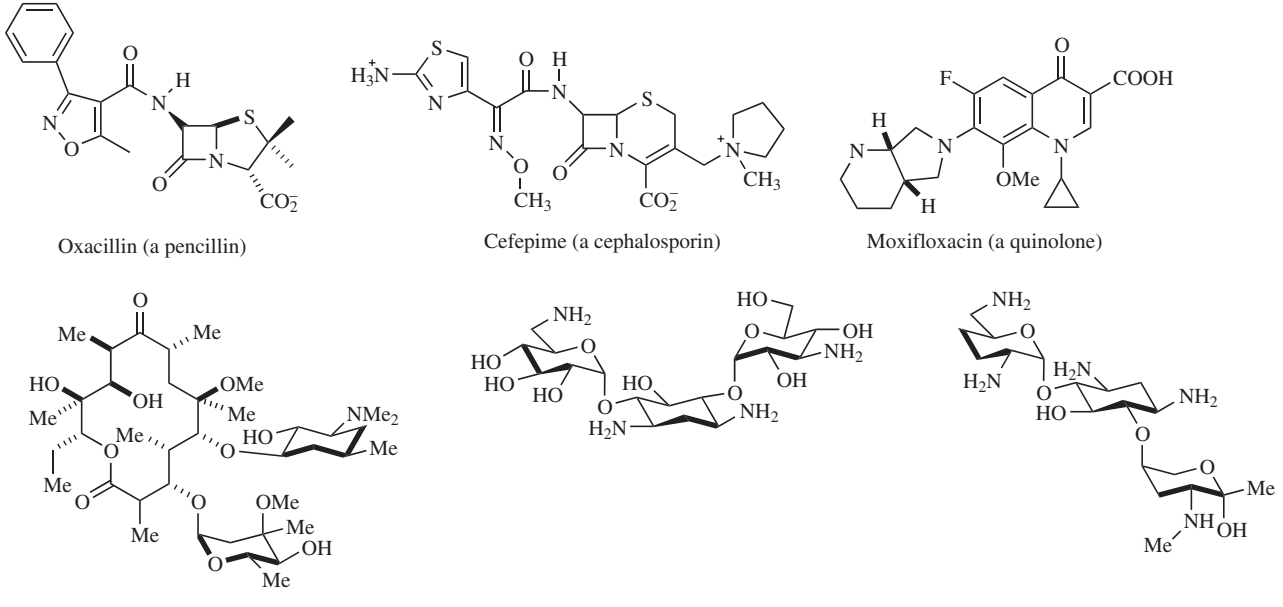
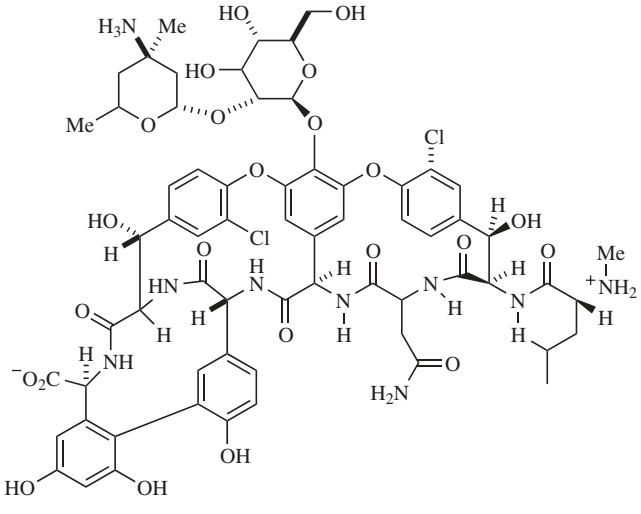
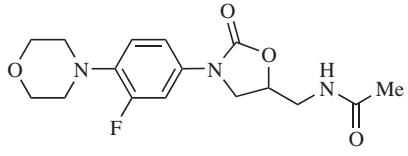


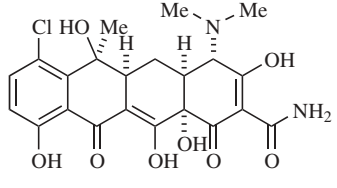
Fig. 1. Chemical structures of some of the antibiotics used for treatment of bacterial infections.



Vancomycin (a glycopeptide)



Linezolid (an oxazolidinone)



Chlortetracycline (a tetracycline)

Fig. 1 (*Continued*)

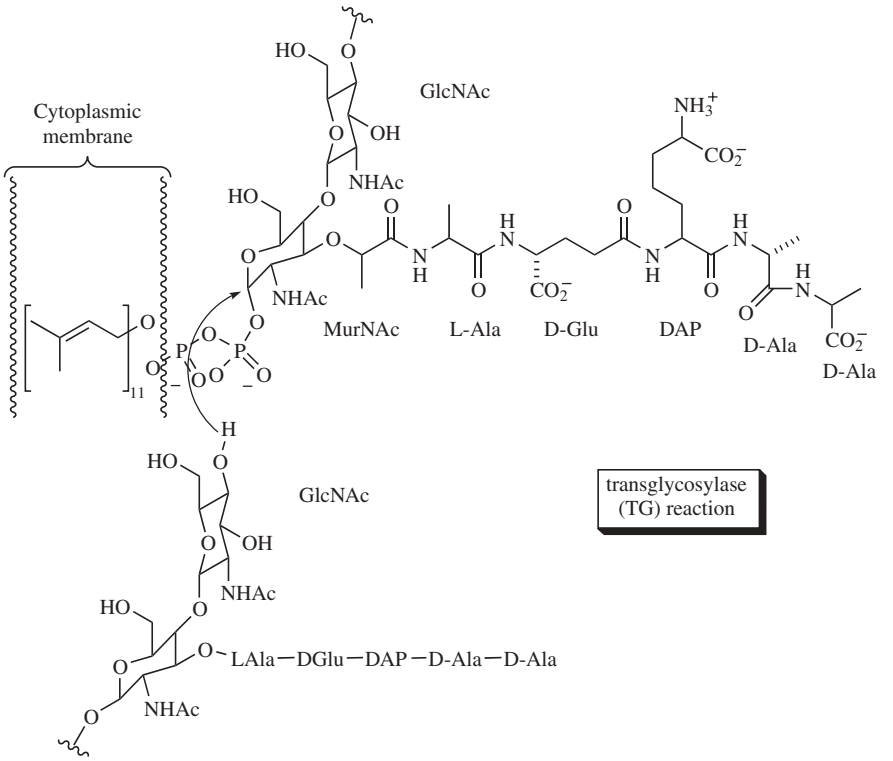


Fig. 2. The mechanism of tranglycosylase results in polymerization of the building blocks for bacterial peptidoglycan.

as a substrate for transglycosylases in this polymerization reaction, the product of which is referred to as the peptidoglycan. Subsequent to polymerization, cross-linking of the cell wall is required, ie, the peptidyl portions are cross-linked to each other via a peptide bond. Since there is no source of energy, such as adenosine triphosphate (ATP), for peptide bond formation outside the cytoplasm, where the cell wall is assembled, nature opted to exchange an amide bond in the peptides. This reaction is carried out by transpeptidases (TP) (Fig. 3), which proceeds through an acyl-enzyme species involving an active site serine residue. How the peptides from the two strands are sequestered in the active site to give the cross-linking reaction was elucidated recently (7). The tranglycosylase and transpeptidase activities are often found in bifunctional enzymes that are anchored to the surface of the cytoplasmic membrane. Both these activities are targets for commonly used antibiotics. For example, penicillin mimics the structure of the acyl-D-Ala-D-Ala portion of peptidoglycan (8). By so-doing, penicillin acylates the same active site serine in transpeptidase, resulting in a stable enzyme-modified species that accounts for the lethal action of these antibiotics (9–11).

If the pentapeptide was made inaccessible to the transpeptidases, then cross-linking would not take place and bacteria would die. This is the strategy

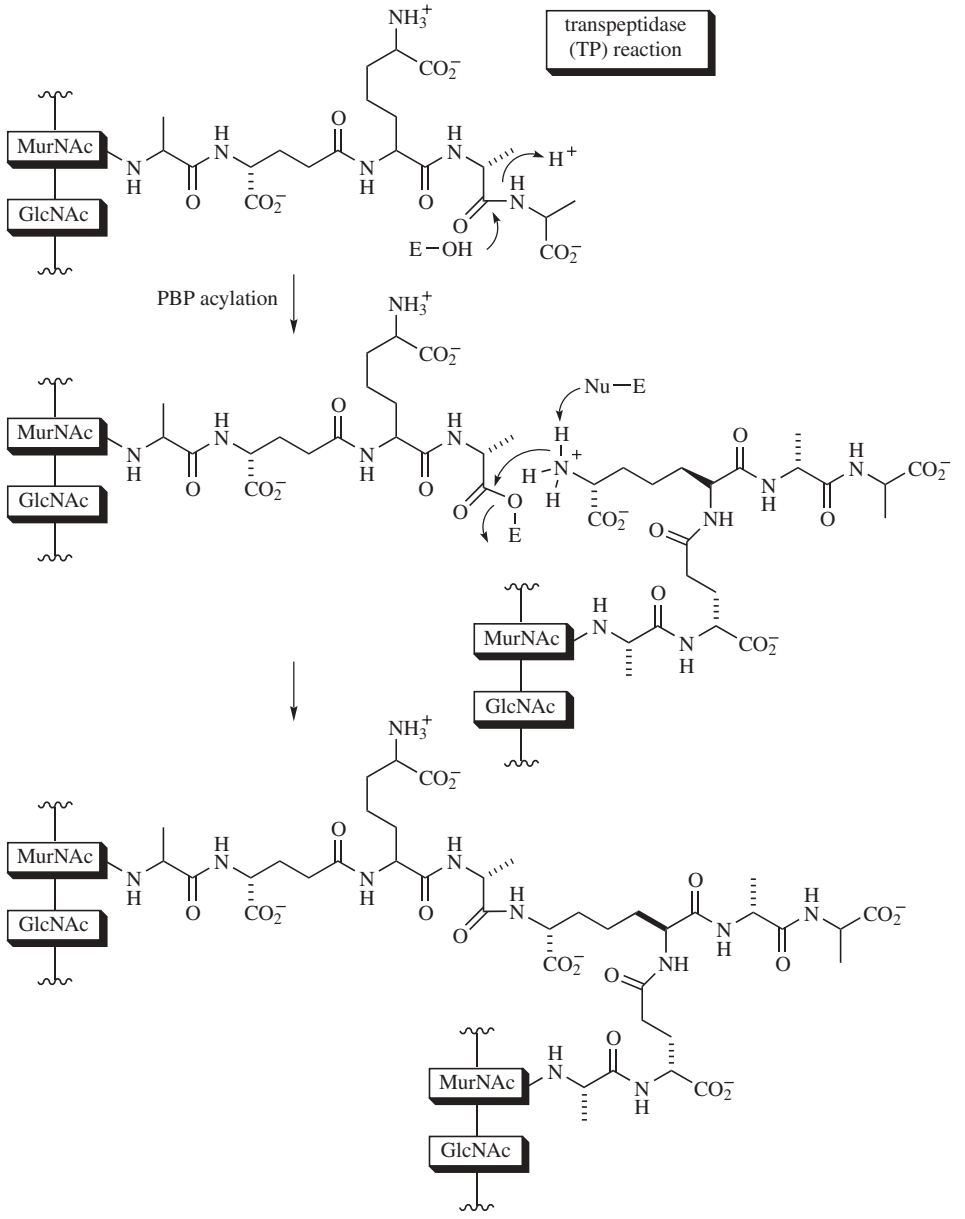


Fig. 3. The two-step reaction of DD-transpeptidases that gives rise to cross-linked cell wall in bacteria.

for glycopeptide antibiotics such as vancomycin, ristocetin, and teicoplanin, which coordinate to the D-Ala-D-Ala portion of the pentapeptide through five hydrogen bonds (Fig. 4) (12). Recent work from the Kahne lab has shown that by modifying the saccharide groups attached to vancomycin's peptide backbone, the target of these derivatives is altered. In contrast to the case of the parental

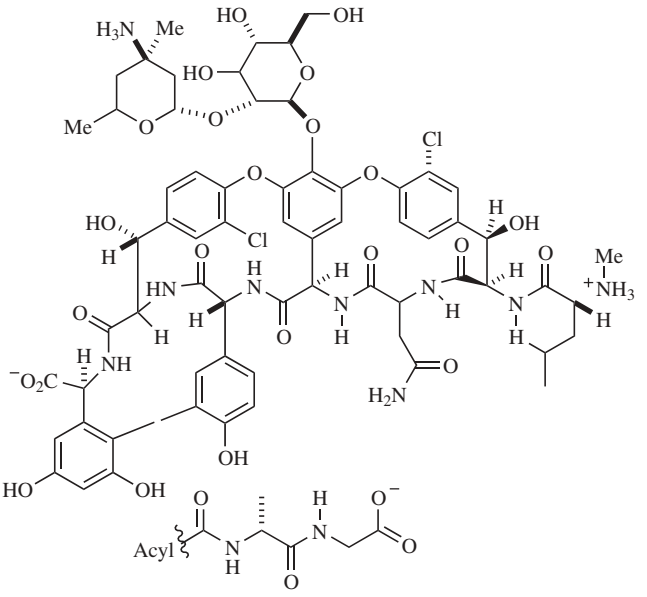


Fig. 4. The binding interactions between vancomycin (top) and the acyl-D-Ala-D-Ala portion of the pentapeptide of the bacterial peptidoglycan. The hydrogen bonds are indicated by dotted lines.

molecule, they seem to kill bacteria by inhibiting the transglycosylation step and furthermore, the modified saccharide groups themselves also posses substantial antibacterial activity (13,14).

Transglycosylases and transpeptidases are clearly important targets for antibiotics, and since they are present on the surface of the cytoplasmic membrane, they are more readily accessible by the respective inhibitors. However, assembly of MurNAc-GlcNac-pyrophosphorylundecaprenol ester, the substrate for transglycosylase, takes place in the cytoplasm. This process requires 10 enzymes, which themselves are targets for antibiotic development (15,16). However, there are not many examples of inhibitors for these enzymes that show antibacterial property, since the molecules should traverse the bacterial envelope to reach their target, which is often difficult.

Biosynthesis of DNA and its repair processes have been targeted by the quinolone class of antibiotics. The quinolones form a stable ternary complex with DNA and an enzyme, the DNA gyrase. When this stable complex is encountered by the replication fork, DNA replication cannot proceed further (17–20). It is worth noting that the parental member of the quinolone class, nalidixic acid, was not isolated as an antibiotic, but rather was a synthetic compound that was shown to possess antibacterial property. A number of derivatives of nalidixic acid, such as the fluoroquinolones ciprofloxacin, moxifloxacin (Fig. 1), and sparfloxacin have become important clinically used drugs.

Protein biosynthesis and the ribosomal assembly have been the target of many different classes of antibiotics, principally because their functions are

very central to the life processes of bacteria. The protein biosynthetic process involves multiple steps that take place in the ribosome. Much of the surface of the ribosome is involved in these processes, which gives opportunities for binding of many different classes of small molecules to the same sites to interfere with the biochemical events. Nature has done so aptly, as there are multiple classes of antibiotics that are known to bind to ribosome. A full survey of these antibiotics is beyond the scope of this article, but the important classes of these antibiotics are macrolides, aminoglycosides, tetracyclines, and oxazolidinones. The first three are of natural product origin, whereas the oxazolidinones (eg, linezolid) were synthetic molecules discovered to have antibacterial activity during directed screening (Fig. 1).

The recent determination of the X-ray structure of the ribosome has been a major advance in understanding the protein synthesis machinery (21–25). A few publications have addressed the structural aspects of complexes of antibiotics with ribosome (26,27). These studies have revealed that antibiotics (eg, chloramphenicol, clindomycin) and macrolides (eg, erythromycin, clarithromycin, and roxithromycin) interact with the residues of 23S ribosomal RNA at the peptidyl transferase cavity. On the other hand, tetracycline interacts mainly with the small ribosomal subunit (30S) at the decoding center.

3. Biochemical Strategies for Resistance to Antibiotics

Development of antibiotic resistance is very complex. It is the result of a series of genotypic and phenotypic interactions of the biological systems of the host, pathogen, and antibiotic. Mutagenesis and gene acquisition are two important mechanisms in bacterial survival in the face of antibiotic or other life threatening challenges. There are many factors that effect the appearance and spread of acquired antibiotic resistance. Among these, the mutation frequency and the biological cost of resistance have become of increasing importance in understanding antibiotic resistance. The mutation frequency measures all the mutations present in a given population regardless of the status of the bacterial growth at which the mutation appears. Mutations happen randomly throughout the genome, and the rate by which the resistant mutants form will depend on the size of the genome and the bacterial population. When the mutation impairs a given gene product, the organism may die. However, should the mutation not be lethal, then it creates an incremental change in the organism. In a recent report, we have reported that as much as 10^6 or more mutants per milliliter of growth might exist in actively growing populations of bacteria (28). The resistance mutations that occur during antibiotic-induced stress generally are associated with loss of bacterial fitness (biological cost) (29). For these mutants to be selected in the face of the antibiotic challenge, other mutations, second-site mutations, are needed to counterbalance the effects of the resistance mutations (30). These second-site mutations usually compensate the biological cost on bacterial fitness without loss of resistance and are distinct under different growth conditions (31–33). There are several cases in the literature that demonstrate that antibiotic resistance is associated with a biological loss of the bacterial fitness. The emergence of *Staphylococcus aureus* gentamicin-resistant small colony

variants was shown to be the result of selection for gentamicin resistance. When these variants were exposed to cycles of antibiotic-free medium, they returned to a sensitive parental phenotype (34). Also, it is believed that resistance, rather than virulence, selects for the clonal spread of methicillin-resistant *S. aureus* (35). In the same context, because only certain Salmonella strains express resistance to cephalosporins and β-lactamase inhibitors mediated by AmpC-type enzymes, Morosini and co-workers (36) argued that the maintenance and expression of the *ampC* gene may be too costly for Salmonella to support its normal growth and virulence.

Bateria have evolved many mechanisms for acquiring resistance genes. These mechanisms enable bacteria to move DNA sequences from cell to cell via conjugation and transformation, or from one genome to another via classical recombination, transposition, and site-specific recombination (37). The site-specific recombination mechanism is important in acquisition and spread of the bacterial resistance. It involves dissemination of resistance genes via gene cassettes and integrons, a very common process in gram-negative bacteria (38). Gene cassettes [500–1000 base pairs (bp) in size] generally consist of a target recombination sequence (*attC* site) normally associated with a single reading frame coding for an antibiotic resistance determinant. Resistance gene cassettes have been found for each class of known antibiotics. They quite often are acquired from more sophisticated genetic structures such as integrons (39). Integrons possess a recombination site (*aatI*) at which the gene cassettes are integrated. This site contains the gene for an integrase, a promoter, and a ribosome-binding site much like a cloning and expression vector (40). In many instances, several resistant gene cassettes are found in an integron. These genetic structures are known as multiresistant integrons. To date, there are 63 antibiotic-resistance gene cassettes identified in multiresistant integrons (41). The number of the gene cassettes organized in an integron can be as many as 200 (41). Such genetic structures are known as superintegrons and were first identified in the *Vibrio cholerae* genome (42). Super integrons also contain genes with other functions beyond resistance. Recent studies have revealed that super integrons are ancient structures, widespread in proteobacteria, serving as gene acquisition machines and most likely they are the source of modern gene cassettes and multiresistant integrons (41).

Since there are many different antibiotic agents, and each organism may experience a different selection event, there are multiple and disparate mechanisms for resistance. However, the most common mechanisms for selection fall into several categories, as listed in Table 1. For the purpose of this article we will describe the resistance to the most commonly used antibiotics.

The first β-lactam antibiotic to be used clinically was penicillin G (mid-1940s). This molecular class, including other β-lactam antibiotics, has enjoyed exceptional success clinically because it inhibits a step such as cross-linking of the cell wall, that is unique to bacteria. Barring the allergic response by a small fraction of the population to these antibiotics, these molecules generally are not toxic to the host. However, their clinical success resulted in extensive use of these antibiotics, which in turn contributed to the appearance and dissemination of mechanisms of resistance. It is important to note that at least four distinct mechanisms for resistance to β-lactam antibiotics have been documented.

Table 1. **Major Bacterial Resistance Mechanisms Identified to Date**

Resistance mechanisms	Class of antibiotics	Examples
reduced permeability	β-lactams, fluoroquinolones, folate inhibitors	penicillins, cephalosporins, norfloxacin, ofloxacin, ciprofloxacin, trimethoprim, sulfamethoxazole, fosfomycin
efflux mechanism	tetracyclines, fluoroquinolones, chloramphenicol, macrolides, aminoglycosides, β-lactams, quinolones, novobicin	tetracycline, minoclycline, doxycycline, ciprofloxacin, ofloxacin, chloramphenicol, erythromycin, lincosamide, penicillin, cephalosporin, imipenem
target modification	β-lactams, fluoroquinolones, aminoglycosides, tetracyclines, folate inhibitors, glycopeptides	penicillins, cephalosporins, norfloxacin, ofloxacin, ciprofloxacin, gentamycin, to bramycin, amikacin, streptomycin, rifamycin, tetracycline, doxycycline, trimethoprim, sulfamethoxazole, vancomycin, teicoplanin, mupirocin, fusidic acid
target bypass	sulfonamides, trimethoprim	sulfamethoxazole, sulfadiazine
target amplification	β-lactams,	penicillins, cephalosporins
resistance enzyme	β-lactams, aminoglycosides, macrolides, chloramphenicol	pencillins, cephalosporins, carbapenems, neomycin, tobramycin, amikacin, gentamycin, lincosamide, erythromycin, clindamycin, chloramphenicol
biofilm formation	most classes of antibiotics	

The most common mechanisms of resistance to β-lactams is through the expression of β-lactamases. These enzymes hydrolyze the β-lactam moiety of the drug, rendering it inactive. The success of this strategy is underscored by the fact that over 350 such enzymes have been identified from clinical strains (43). These enzymes fall into four structural classes, all of which appear to follow a distinct catalytic mechanism (44–46). It has been argued that four distinct progenitor proteins gave rise to the four classes of β-lactamases in disparate evolutionary steps (47). Golemi and co-workers (48) documented that the OXA-10 β-lactamase from *Pseudomonas aeruginosa* is sequestered in the periplasmic space of this organisms in a minimum concentration of 4 μM. This concentration is produced by ~1200 molecules of the enzyme per bacterial cell, each of which is able to turn over ~1500 molecules of cloxacillin—a penicillin—per second (a total of 1.8×10^6 cloxacillin molecules are turned over per second per resistant bacterium). It is self-evident that these enzymes are formidable barriers to the antibiotics effects of these pharmaceutical agents.

The second mechanism of resistance to β-lactam antibiotic is the evolutionary acquisition of DD-transpeptidases—the target enzymes—with reduced affinity for these drugs. The prime example of these is the case of methicillin-resistant *S. aureus* (MRSA), which is a scourge of hospitals. The aforementioned transglycosylases and transpeptidases are collectively referred to as penicillin-binding proteins (PBPs). In a single acquisition event of unknown origin, a DD-transpeptidase was introduced to *S. aureus* that has the ability to perform the functions of other PBPs in this organism (49). Hence, inhibition of the four known native staphylococcal PBPs in *S. aureus* by a β-lactam atibiotic is overcome by the availability of this new enzyme, PBP2′, which is not readily inhibited by these drugs (50,51). Other examples of low-affinity PBPs, are the chromosomal PBPs found in *enterococci*: PBP3r and PBP5 in *Enterococci hirae*, PBP in *Enterococci faecalis* and PBP4 *Enterococci faecium*.

β-Lactam antibiotics must reach the outer surface of the cytoplasmic membrane to inhibit the PBPs. Hence, in gram-negative bacteria, β-lactam antibiotic has to penetrate the outer membrane to reach its target. This penetration takes place through the channel-forming proteins, namely, porins. These proteins transverse the outer membrane and are the portals through which the nutrients enter the cell. Porins hav been known to undergo mutations such that penetration by the antibiotic is slowed down. This is a means for resistance to imipenem, a member of the carbapenem class of β-lactam antibiotics (52,53). In some other cases, the decrease in permeability of β-lactams has been related to the mutational loss of major porins (54). This mechanism for resistance is also seen in combination with hyperexpression of antibiotic-modifying enzymes (54) and is not common, as alteration in these protein portals into the bacterium would have implications for penetration of nutrients and the survival of the organism.

The fourth mechanism of resistance to β-lactam antibiotics was discovered only recently (55). It has been reported that there exists an LD-transpeptidase that is capable of carrying out the cross-linking reaction not with the penultimate D-Ala residue, but rather with the third amino acid (DAP, in Fig. 2). Such cross-linking occurs at low levels in sensitive strains of *Escherichia coli* and *Escherichia faceium* but Mainardi and co-workers (55) reported that *in vitro* selection for resistance to ampicillin in *E. faecium* has shifted the

dependence on transpeptidases in favor of β-lactam-insensitive LD-transpeptidase (100%), hence bypassing the function of the ubiquitous β-lactam-sensitive DD-transpeptidases, the traditional targets of these antibiotics (9).

Vancomycin has been in use since 1958, but only in the early 1980s did clinicians start using it heavily against hospital-acquired (nosocomial) infections. Vancomycin has been considered to be the antibiotics of last resort against gram-positive infections, especially the ones caused by the methicillin-resistant *S.aureus* (56,57). Over the past 10 years, a number of *Enterococcus* strains with high-level inducible resistance to vancomycin and its analogues have been identified (58). Five plasmid-borne genes are found to be necessary to induce high level of vancomycin resistance, *vanR, vanS, vanH, vanA*, and *vanX* (59). These genes are responsible for the alteration of vancomycin target: the peptidoglycan precursors in resistant strain end in D-Ala-D-Lac (Lac for lactate) instead of D-Ala-D-Ala as in sensitive strains. The conversion of the amide bond to ester entails the loss of an important hydrogen bond to the glycopeptide in the complex, which has been estimated to result in a 1000-fold reduced affinity for complex formation (60). The VanR and VanS proteins comprise a two-component regulatory system that regulates the transcription of *vanRS* and *vanHAX* genes. VanA, gene product of *vanA*, is a ligase that synthesizes D-Ala-D-Lac, which is added to the UDP-MurNAc-tripeptide. VanH reduces pyruvate to D-Lac, the substrate for VanA. VanX hydrolyzes D-Ala-D-Ala produced by the chromosomally D-Ala-D-Ala ligase, thereby reducing the pool of D-Ala-D-Ala, which would otherwise compete with D-Ala-D-Lac for incorporation into the peptidoglycan precursor.

Vancomycin resistance is also seen in methicillin-resistant *S. aureus* clinical strains (VRSA). Such strains would appear to lack the enterococcal *van* genes, which suggest the possibility for other mechanisms in resistance to vancomycin (61). Studies on vancomycin resistance in MRSA have associated this resistance with overproduction of penicillin-binding protein 2 (PBP2) and/or cell-wall thickening (61,62). Overexpression of PBP2 would allow more peptidoglycan precursors to be incorporated into the cross-linked cell wall synthesis (ie, higher degree of cross-linking), thus less amount of this precursor will be available as a target of vancomycin binding. Cell-wall thickening, would also prevent penetration of vancomycin into the cell wall, thus the level of the antibiotic reaching the target would be less than is needed to kill the bacteria.

Aminoglycosides are another class of antibiotics used against the infections caused by gram-positive and gram-negative bacteria. They bind to the 30S subunit of the bacterial ribosome. As with any other class of antibiotics, their antimicrobial properties are compromised by bacterial resistance. Methylation of the ribosomal binding site is known to cause resistance to gentamicin, as an example of altered target (63). This mechanism is observed only in aminoglycoside-producing organisms. Altered uptake of aminoglycoside, a rare resistance mechanism, is exhibited by anaerobes and organisms such as *P. aeruginosa* (64). However, the most common mechanism for resistance to aminoglycosides is by their structure modification by three families of enzymes collectively referred to as aminoglycoside-modifying enzymes (66). As shown in Figure 5, three types of reactions have been documented for these activities, namely, *N*-acetyltransferase, *O*-phosphotransferase, and *O*-adenyltransferase reactions. In each case, the

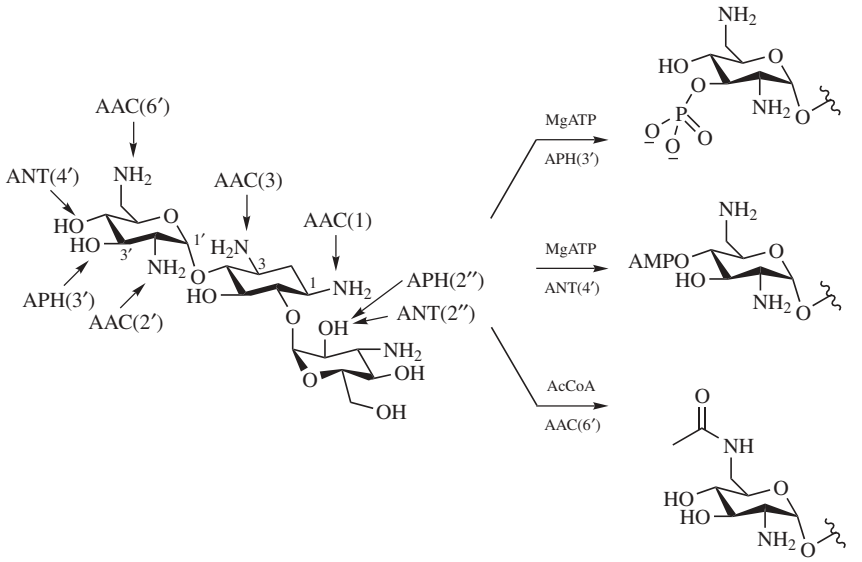


Fig. 5. Sites of modification in aminoglycosides antibiotics, as depicted for kanamycin B.

multifunctional aminoglycoside is chemically modified to derivatives that are devoid of antimicrobial activity, in light of the fact that the affinity of the ribosomal site for each is dramatically reduced (67). Note that these enzymes are cytoplasmic, as is the ribosome. However, the enzymes are produced in high concentrations (68), and they often carry out their respective reactions in rapid reactions (64,65).

Erythromycin, a macrolide antibiotic, is commonly used against gram-positive bacteria. Macrolide antibiotics inhibit bacterial protein synthesis by binding to the 23S rRNA of the 50S ribosomal subunit (26). Resistance to macrolides developed soon after the introduction of erythromycin to the clinic in 1953. The first resistant clinical isolates to macrolides were *S. aureus*, but subsequently resistance transferred to other organisms. Bacteria have developed three mechanisms that protect them from the action of the macrolides: target site alteration (69), antibiotic modification (70), and altered antibiotic transport (71). Target site alteration is the most common mechanism of resistance in the organisms that produce this antibiotic (eg, *Streptomyces erythreus*). In these organisms the 23S rRNA is posttranscriptionally modified by an adenine-specific *N*-methyltransferase (methylase). These enzymes are encoded by a class of genes known as *erm* (erythromycin ribosome methylation), which mono- and dimethylate the exocyclic *N*-6 position of a highly conserved adenine nucleotide (A2058 according to *E. coli* numbering) within the peptidyl transferase loop (72,73), which is important for binding of the macrolide antibiotics. To date, >30 *erm*-related genes have been identified from different bacterial sources that range from clinical pathogens to actinomycetes (71). The methyltransferase enzymes have been classified into two classes. The first class includes the Erm enzymes that only monomethylate, such as Lrm from *Streptomyces lividans*, Clr from *Streptomyes caelestis*, and TlrD from *Streptomyces fradiae*. The second class

includes those that predominately dimethylate adenine, such as ErmC from *S. aureus*, ErmE from *Saccharopolyspora erythrea*, and ErmSF from *S. fradiae*. Erm proteins can be expressed constitutively or induced by the presence of low levels of the antibiotics. It is interesting to note that resistance to erythromycin has always been seen associated with resistance to chemically distinct, but functionally overlapping antibiotic families such as lincosamide and streptogramin B. This type of resistance has been referred to as MLS_B resistance, with the initials referring to the three types of antibiotics (71). Removal of macrolides from the cytoplasm of bacteria by efflux pumps is another mechanism of low-level resistance.

Since the discovery of the *erm* genes, another means of resistance involving alteration of rRNA structure has been identified. This mechanism involves single-base substitution at A2058 in the 23S rRNA. Generally, pathogenic bacteria that develop macrolide resistance through mutations at this position possess only one or two rRNA operons (*rrn*,) as in the case of *Helicobacter pylori* and *Mycobacterium* species. Species with more copies of *rrn* operons, such as *Enterococcus, Streptococcus*, and *Staphylococcus* confer resistance by expression of Erm enzymes or efflux pumps (69).

Linezoild (Zyvox-Pharmacia) is the first oxazolidinone antimicrobial approved by the U.S. FDA (April 2000) for clinical use against infections caused by multiresistant gram-positive bacteria, including MRSA, vancomycin-resistant enterococci (VRE) and penicillin-resistant *Streptococcus pneumonia* (74). This antibiotic inhibits initiation of protein synthesis by preventing the formation of a ternary complex among $tRNA^{fMet}$, mRNA, and the ribosome (75). Spontaneous resistance to linezolid in *S. aureus* and *S. epidermis* develops at a rate of $<10^{-9}$ (76). Studies of linezolid-resistant clinical isolates and laboratory-derived linezolid-resistant strains of MRSA and VRE have revealed single-point mutations clustered in the DNA region encoding the central loop of domain V of 23S rRNA (77–79). MRSA resistant to linezolid has been shown too possess either G2576T/U or G2447U mutations (78,80). The VRE strains resistant to linezolid have developed single-point mutations at positions G2528U (*E. faecalis*), G2576U (*E. faecalis* and *E. faecium*, and G2505A (*E. faecium*) (79). Interestingly, a laboratory-developed linezolid-resistant *E. faecalis* strain had acquired three other mutations in addition to C2512U, G2513U, and C2610G (79). In laboratory studies, *E. coli* has also been shown to develop resistance to linezolid attributed to G2032A/U/C mutations in the 23S rRNA.

Bacteria have an intrinsic mechanism for protection from any toxic compounds in their environment. The gram-negative bacteria and gram-positive mycobacteria combine two mechanisms of resistance. First, the outer membrane and the mycolate-containing cell wall, respectively, produce effective permeability barriers. Second, the antibiotics that make it through the first outer membrane barrier are pumped out by the multidrug resistance efflux pumps (MDR). In gram-negative bacteria, MDR pumps interact with outer membrane channels and accessory proteins, forming multisubunit complexes that extrude antibiotics directly into the medium, bypassing the outer-membrane barrier.

The outer membrane of gram-negative bacteria is a barrier to many antibiotics. It consists of an inner leaflet of glycerophospholipids, which has high fluidity owing to the presence of unsaturated fatty acids, and an outer leaflet

of lipopolysaccharides. The lipopolysaccharides lack unsaturated fatty acids, hence they are more rigid and less permeable. The outer membrane is traversed by proteins known as porins, through which nutrients enter the cell. These porin channels have a limited opening with an exclusion limit of 600–1000 Da.

The implication of the reduced cell wall permeability alone in bacterial resistance could not justify the high level of resistance observed for many antibiotics. Rather, the synergistical action of outer-membrane barrier and the active efflux pumps, such as ArcB of *E. coli*, MexB of *P. aeruginosa*, and MtrD of *N. gonorrohoeae*, produces effective drug resistance. By actively pumping out antibiotic molecules, these systems prevent intracellular accumulation of the antibiotics in such levels that is necessary to exert the lethal activities. The efflux pumps have a broad range of substrates (Table 1), thus they have become a serious problem in the treatment of many infectious diseases (81). They are associated with both intrinsic and acquired resistance to antibiotics. MDR efflux pumps such as MexAB-OprM and MexXY-OprF in *P. aeruginosa* are reported to be constitutively expressed in wild-type strains, thus contributing to the intrinsic resistance of this organism to a number of antimicrobial agents, including tetracyclines, chloramphenicol, quinolones, novobiocin, macrolides, trimethoprim, β-lactams, and β-lactamase inhibitors (82). Recently, it has been reported that MDR pump AmrAB-OprA in *Burkholderia pseudomallei* and MexXY-OprF in *P. aeruginosa* are involved in extrusion of aminoglycosides directly into the external medium (83,84). Acquired MDR can arise via three mechanisms: (*1*) amplification and mutation of genes encoding the MDR proteins that alters expression and the activity of the transporters; (*2*) mutations in the regulatory proteins that lead to the increased expression of multidrug transporters, eg, mutations in a repressor gene *mex*R leads to the hyperexpression of *mexAB-oprM* in MDR clinical isolates; and (*3*) transfer of resistance genes on transposons or plasmids (85).

Microorganisms have the ability to irreversibly attach to and grow on a surface and produce extracellular polysaccarides that facilitate attachment and matrix formation (86). Such matrix association of cells is known as biofilms. Biofilms may form on any surface, but their formation on the surface of indwelling medical devices, tooth enamel, heart valves or the lung, and middle ear is of biomedical concern. Many different organisms develop biofilms, including pathogenic bacteria such as *K. pneumonia*, *P. aeruginosa*, *S. aureus*, *E. faecalis*, and fungi (87).

Biofilm-associated organisms have altered phenotypes with respect to growth rate and gene transcription (88) due to biofilm composition and structure (87). Biofilm consist of microcolonies held together by an extracellular matrix (polysaccharides). Its structure is hetrogeneous, with water channels that allow transport of essential nutrients and oxygen to the cells within the biofilm. Studies have shown that growth of biofilm causes a decrease in antimicrobial susceptibility, which might be intrinsic or acquired (89). Intrinsic resistance of biofilm can be related to the multicellular structure of the biofilm, which can slow down drug diffusion or possibly the matrix itself may react with the drug. Furthermore, biofilm-associated organisms have reduced growth rates that might as well minimize antimicrobial intake rate. Plasmid transfer through conjugation allows acquisition of antimicrobial resistance mechanisms, but several

studies have shown that mechanisms of antibiotic resistance such as the efflux pumps, modifying enzymes, and target alteration do not seem to have a major impact on biofilm antibiotic resistance. Clearly, resistance in biofilms is more complicated: Multiple resistance mechanisms can act in concert. Adherence of bacteria to implanted medical devices or damaged tissues in the form of biofilm and inherent resistance contribute to duration of bacterial infections. As a result, biofilm formation has become a serious clinical problem (90).

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