

# Antibiotic Resistance: A Survival Strategy

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Antibiotics are natural, semi-synthetic, or synthetic molecules that target the cell wall of bacteria, DNA replication, RNA transcription, or mRNA translation, the cellular machinery responsible for the synthesis of precursor molecules. Bacteria have evolved and adopted numerous strategies to counteract the action of antibiotics. Antibiotic resistance is intrinsic and an inherent characteristic of the microorganism. Intrinsic resistance is due to cell wall impermeability, efflux, biofilm formation, and the expression of genes mediating inactivating enzymes. Antibiotic resistance can also arise by the acquisition of extracellular DNA and is expressed phenotypically as efflux, modification or acquisition of target sites, and enzymatic inactivation of the antibiotic. Not only have bacteria acquired the mechanisms necessary to withstand the effects of antibiotics, they have also acquired elaborate mechanisms to mobilize and disseminate these successful strategies: plasmids, transposons, insertion sequences, and cassettes. Antibiotic resistance is a major worldwide clinical problem of public health concern because of the reduced efficacy caused by the various mechanisms of resistance. Global strategies are emerging to help address this critical problem.

**ABBREVIATIONS:** ABC = ATP-binding cassette; AG = aminoglycoside; CM = cytoplasmic membrane; DNA = deoxyribonucleic acid; ERM = erythromycin ribosome methylase; ESBL = extended-spectrum beta-lactamases; LPS = lipopolysaccharide; MATE = multidrug and toxic effects; MFS = major facilitator subfamily; mRNA = messenger ribonucleic acid; MRSA = methicillin-resistant *Staphylococcus aureus*; OM = outer membrane; PBP = penicillin-binding protein; QRDR = quinolone resistance-determining region; RNA = ribonucleic acid; RND = resistance, nodulation cell division subfamily; SMR = the small multidrug regulator; THF = tetrahydrofolate.

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**INDEX TERMS:** antibiotic resistance; biofilm; efflux; mechanism of action; plasmid; transposon.

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*Focus Continuing Education Credit: see pages 188 to 191 for learning objectives, test questions, and application form.*

## LEARNING OBJECTIVES

1. Identify the mechanism of action and targets of antibiotics.
2. Describe the intrinsic and acquired mechanisms of resistance to antibiotics.
3. Discuss the mechanisms used to disseminate resistant determinants of antibiotics.

Antibiotic resistance emerged soon after the discovery of antibiotics and is associated with the overuse of antimicrobial agents.<sup>1</sup> Although antibiotics allow the medical community to make great strides in human health and welfare, their use also causes selective pressure allowing only the fittest and less susceptible bacterial populations to survive.<sup>2</sup> Antibiotic resistance and its associated clinical failure are of such concern as to unite scientists from across the world to develop strategies to address this issue of public health concern.<sup>3,4</sup>

The adaptive strategies used by microorganisms to survive hostile antibiotic environment are remarkable in their evolution and complexity. This article provides but a brief glimpse of these strategies, using select members of antibiotic classes to illustrate mechanisms of resistance. Intrinsic and acquired mechanisms that facilitate resistance to important therapeutic regimens are included. Many of these resistance

mechanisms may be generalized to other pathogens. Further, multiple mechanisms of resistance are found in the same microorganism resulting in the multi-drug resistant phenotypes observed in clinical settings.

### MECHANISMS OF ACTION IN ANTIBIOTICS

The Clinical and Laboratory Standards Institute currently identifies 23 unique classes and 18 subclasses of clinically useful antibiotics representing approximately 100 antibiotics.<sup>5</sup> Although the classification scheme and number of antibiotics is complex, their mechanisms of action include inhibitors of bacterial cell wall biosynthesis, folate synthesis, DNA replication, RNA transcription, and mRNA translation. These targets are critical to the survival of the organism and the development of antibiotics that affect these targets is logical. Further, by understanding the mechanisms of action of antibiotics,

we gain insight into strategies used by microorganisms to survive their toxic effects. Figure 1 shows the primary sites of antibacterial action for major classes of antimicrobial agents.<sup>6</sup>

#### Bacterial cell-wall biosynthesis

The cell wall of gram-positive and gram-negative bacteria is a multilayered structure composed of a cytoplasmic membrane (CM), which overlies the cytoplasm, the peptidoglycan (PG) layer, and an outer membrane (OM) present only in gram-negative bacteria (Figure 2). The OM is composed of lipopolysaccharides and proteins. In gram-positive bacteria, the PG is substantially thicker and more multi-layered than in gram-negative bacteria. Gram-negative bacteria contain a periplasmic space, which resides between the CM and OM.<sup>7</sup>

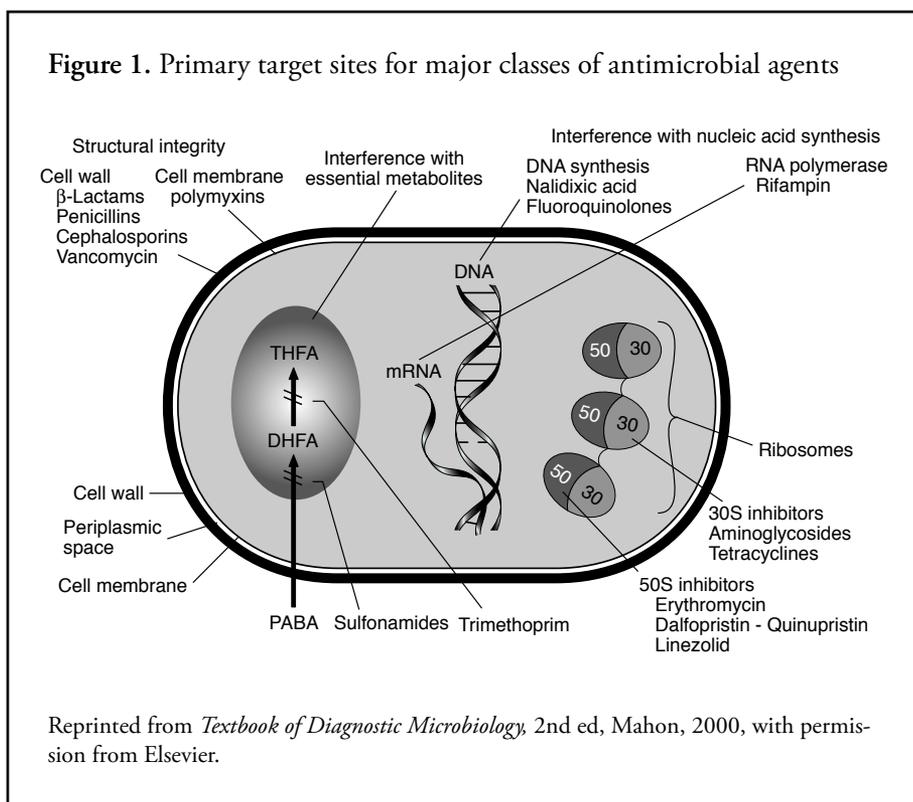
Under normal growing conditions, PG synthesis proceeds by the ligase-

mediated formation of D-ala-D-ala, a precursor used to form UDP-N-acetylmuramyl-pentapeptide. This precursor molecule elongates PG by transglycosylation of the glycan strands and elongates the peptide strands by transpeptidation. So strengthened, the PG layer allows the microorganism to resist osmotic changes in its environment.

$\beta$ -lactam and glycopeptide antibiotics are examples of bacterial cell-wall synthesis inhibitors.  $\beta$ -lactam antibiotics, such as penems, cepems, carbapenems, and monobactams act by binding to penicillin binding proteins (PBP), which are bifunctional transpeptidases/transglycosylases that cross-link peptidoglycan as described in the previous paragraph.<sup>8</sup>

The active moiety of  $\beta$ -lactam antibiotics is the four-member  $\beta$ -lactam ring found in penicillins and cephalosporins. This four-member ring functions as a structural analogue of the normal substrate, acyl D-alanyl-D-alanine, and inhibits the transpeptidation reaction resulting in bacterial lysis and cell death.<sup>7</sup> The bacterial spectrum of  $\beta$ -lactam antibiotics depends on the moieties attached to the penicillin and cephalosporin ring structures and determine narrow to broad-spectrum antimicrobial activity.

Glycopeptides such as vancomycin 'complex' the un-cross-linked peptide strands of the peptidoglycan units that have the pentapeptidyl tails ending in D-ala-D-ala. This prevents their incorporation into the peptidoglycan chain by blocking the transpeptidation step.<sup>9</sup> Glycopeptides bind to the substrate of the transpeptidation enzyme while penicillins bind to the enzyme mediating the transpeptidation reaction. Vancomycin is a narrow-spec-



trum antibiotic and its spectrum and clinical use is limited to gram-positive microorganisms. Glycopeptides cannot penetrate the pores of gram-negative bacteria and are not used to treat such infections. Vancomycin and teicoplanin are glycopeptides used to treat aerobic clinical infections caused by staphylococci, streptococci, and enterococci in the United States.<sup>5</sup>

**Folate synthesis**

Antibiotics are also capable of interfering with anabolism. The folic acid pathway provides the essential precursor molecule, pyridine thymidylate, needed in DNA biosynthesis. The folic acid pathway is mediated by two key enzymes, dihydropteroate synthase and dihydrofolate reductase, which mediate the formation of 7,8 dihydropteroate and tetrahydrofolate (THF) respectively.<sup>10</sup>

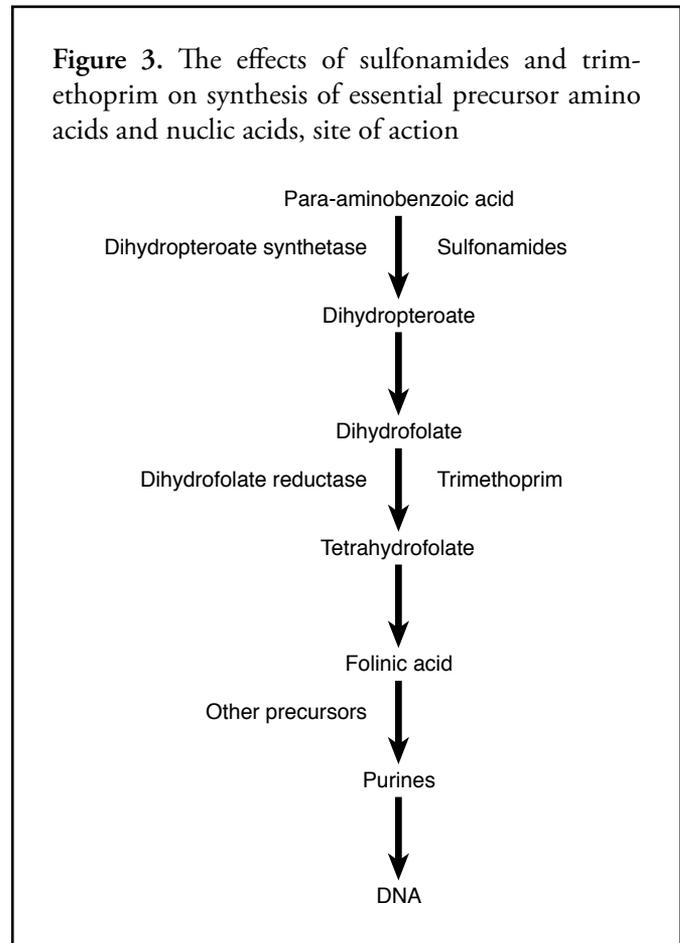
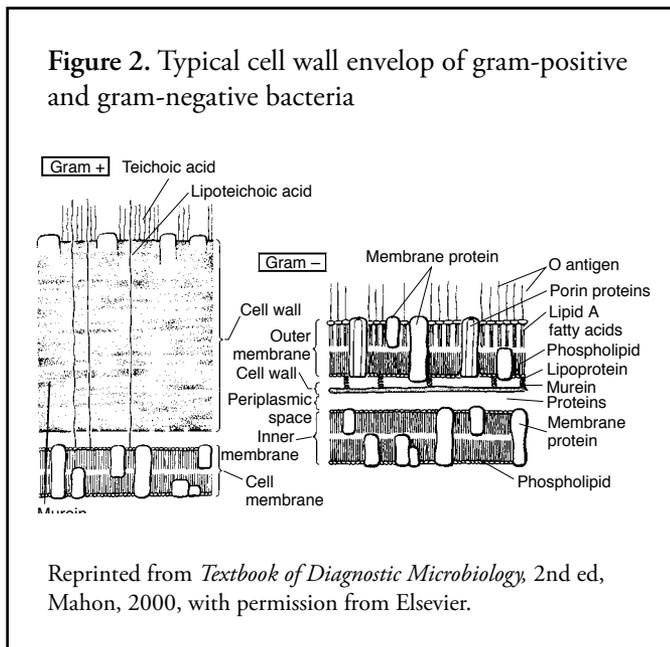
Sulfamethoxazole and trimethoprim are synthetic molecules that block key anabolic steps of the folic acid pathway, preventing the formation of THF (Figure 3). Sulfamethoxazole blocks the step leading to the formation of 7,8 dihydropteroate by competitively inhibiting the binding of the structural analogue para-aminobenzoic acid with dihydropteroate synthase. Trimethoprim blocks the step leading to formation of THF by preventing the dihydrofolate reductase mediated recycling of folate coenzymes. The folate pathway inhibitors are used to treat enterobacteriaceae that cause urinary tract infections.<sup>9</sup>

**DNA replication**

The enzymes necessary for DNA replication are topoisomerases

I, II, III, and IV. Quinolones affect DNA replication by targeting topoisomerases II (DNA gyrase) and IV, enzymes considered important in controlling DNA topology, replication, and decatenation at the end of bacterial DNA synthesis. DNA gyrase and topoisomerase IV are tetramers composed of two A and B subunits each. The subunits of DNA gyrase are encoded by *gyrA* and *gyrB* respectively while the subunits of topoisomerase IV are encoded by *parC* and *parE*.<sup>11</sup> The tetramers of DNA gyrase and topoisomerase IV have been shown to be highly homologous; with *gyrA* homologous to *parC* and *gyrB* homologous to *parE*.<sup>12</sup> In general, the targets of quinolones appear to be selective, targeting DNA gyrase in gram-negative bacteria and topoisomerase IV in gram-positive bacteria, but newer quinolones appear to have high affinity for both targets.<sup>13</sup>

Analyses of quinolone mechanism of action suggest they interact with DNA gyrase-DNA complexes and topoisomerase IV-DNA complexes to trap the enzymes as stabilized reaction intermediates that form a barrier to DNA replication.<sup>14</sup> The quinolones and fluoroquinolones are used to treat the enterobacteriaceae, pseudomonas, and other non-



enterobacteriaceae, staphylococci, enterococci, neisseria, and streptococci species other than *S. pneumoniae*.<sup>5</sup>

### RNA transcription

The transcription of DNA into RNA is mediated by RNA polymerase; bacterial RNA polymerase is a core tetramer composed of an  $\alpha$  subunit, two  $\beta$  subunits ( $\beta\beta'$ ), a  $\gamma$  subunit, and a dissociable  $\sigma$  subunit that controls transcription of particular gene classes.<sup>9</sup>

Rifampin, a synthetic derivative of rifamycin B is used in combination with other antibiotic classes to treat *Mycobacterium tuberculosis* by targeting transcription of RNA. The target of rifampin in *M. tuberculosis* is the RNA polymerase subunit at an allosteric site, with the subsequent blocking of RNA chain elongation.<sup>15</sup> Aerobic species treated with rifampin include staphylococci, enterococci, Haemophilus, and *Streptococcus pneumoniae*.<sup>5</sup>

### mRNA translation

The translational machinery decodes mRNA during protein synthesis. The protein biosynthetic step is mediated by the sequential binding of the 30S and 50S ribosomes to mRNA leading to translation of the genetic code into protein product. The initiation step requires binding of the 30S subunit to the ribosomal binding site of mRNA, followed by binding of formylmethionyl tRNA to the P site of the 30S ribosomal subunit. Next, the 50S subunit binds to form the preinitiation complex. The codon immediately following the initiation codon dictates binding of the next tRNA to the ribosomal A site. Since protein synthesis is central to cellular function, it is an excellent target for antibiotic drug product development. Thus, the bacteria ribosome is a primary target of numerous antibiotics with some targeting the 30S subunit, e.g., aminoglycosides, tetracyclines, and others, the 50S subunit, e.g., macrolides, oxazolidinones, and streptogramins.

Aminoglycosides are cationic carbohydrate-containing molecules. Their positive charge provides the basis for their interaction with a specific region of the 16S rRNA in the 30S ribosomal subunit. This interaction is mediated by hydrogen bonding to the various substituents in the aminoglycoside cyclitol rings thus providing a high-affinity docking site. The binding of aminoglycosides to the A-binding site on the 30S subunit prevents the docking of aminoacyl-tRNA resulting in mistranslation and subsequent production of aberrant proteins. The incorporation of aberrant proteins into the cell wall results in cell leakage and enhanced cellular penetration.<sup>16</sup>

Macrolides such as erythromycin, clarithromycin, and azithromycin bind the 50S subunit at the peptidyltransferase cavity in the proximity of the A and P loops, near adenine 2058 of 23S rRNA. By blocking the elongating peptides' exit tunnel, premature release of peptidyl-tRNA intermediates occurs and polypeptide translation ceases. Macrolides also prevent assembly of the 50S ribosomal subunit by binding to 23S rRNA.<sup>17</sup>

### MECHANISMS OF ANTIBIOTIC RESISTANCE

Resistance to antibiotics is conveniently divided into mechanisms that are an innate characteristic, described as intrinsic, and those that result from the acquisition of DNA by transformation and recombination or by acquisition of extrachromosomal DNA. The latter include acquisition of plasmids and transposons capable of disseminating resistant determinants. Where feasible, this article uses members of the antibiotic classes previously discussed to exemplify intrinsic or acquired mechanisms of resistance.

#### Intrinsic resistance (chromosomal)

Bacteria differ widely in their cell wall composition and hence their intrinsic susceptibility or resistance to antibiotics. Intrinsic resistance of bacteria to the action of antibiotics depends on the hydrophobic or hydrophilic nature of the antibiotic and by impermeability of the antibiotic through the cell wall. Impermeability is mediated by cell wall composition, formation of biofilms, efflux, or by chromosomally mediated enzymatic inactivation.

#### Impermeability

In order for antibiotics to affect an internal cellular process, they must penetrate the cell wall. Influx of antibiotics depends on the chemical nature of the antibiotic and the characteristic structure of the cell wall. There are two fundamental structures of the cell wall that lead to impermeability, lipopolysaccharide composition and the expression of OM proteins, porins that restrict inward inflow of antibiotics and biocides.<sup>6</sup>

Lipopolysaccharide (LPS) and proteins are critical in maintaining the integrity of the OM of gram-negative bacteria as a permeability barrier. The core region of the LPS is strongly negatively charged and functions as a selective permeability barrier for negatively charged antibiotics resulting in decreased susceptibility.

Porins are OM channels that permit the influx of nutrients and efflux of waste products. Antibiotics can function as

substrates for porin proteins. Alterations of porin production and structure may result in a resistant phenotype. Nosocomial pathogens showing decreases or loss of porin synthesis are observed in combination with other resistance mechanisms resulting in multidrug resistant pathogens.<sup>18</sup> In addition, the molecular size and lipophilicity affect the rate at which antibiotics transverse porins.

The OM barrier explains in part the intrinsic resistance of gram-negative bacteria to antibiotics and is only clinically significant in the context of other resistance mechanisms, such as efflux, that work synergistically to mediate survival of the organism.<sup>19,20</sup>

### Biofilms

Biofilms are bacterial sessile communities irreversibly attached to a substrate and embedded in a matrix of extracellular polymeric substances, the glycocalyx.<sup>21</sup> Resistance is attributable to a growth rate significantly slower than planktonic cells, the physiological state of the microorganism associated with expression of genes responding to stress, and delayed penetration and interaction of the antibiotic through the extracellular glycocalyx. Several studies evaluated the interaction or penetration of antibiotics through the extracellular material and found that diffusion of  $\beta$ -lactam and macrolide antibiotics is more rapid than are aminoglycosides.<sup>22,23</sup>

### Efflux

It is now widely accepted that the 'intrinsic resistance' of gram-negative bacteria to certain antibiotics is a result of the activity of efflux systems and impermeability.<sup>24-26</sup> Efflux pumps are transporter proteins involved in the removal of toxic substances from the interior of the cell to the external environment. Efflux pumps are found in gram-positive and gram-negative bacteria. Some efflux pumps are specific for a single drug or substrate while others are capable of transporting multiple substrates. Increased efflux results in subtherapeutic intracellular concentrations of antibiotics and subsequent therapeutic failure. Five major efflux pump protein families that mediate resistance to antibiotics have been described. They are the major facilitator subfamily (MFS), resistance nodulation cell division subfamily (RND), the small multidrug regulator subfamily (SMR), the ATP-binding cassette (ABC) family, and multidrug and toxic effects (MATE) family. A proton motive force mediated by the counter flow of protons drives the MFS, RND, MATE, and SMR subfamilies. The ABC family uses the hydrolysis of ATP by ATPase to provide the energy for active transport of antibiotics and other toxic molecules.<sup>27</sup> The intrinsic

efflux mechanism of resistance is chromosomally located and is activated by environmental signals or by mutation in regulatory genes.<sup>28</sup>

For example in the RND superfamily, the *mexAB-oprM* operon in *Pseudomonas aeruginosa* regulates porin and pump genes. Mutations in the gene *mexR* coding for the repressor protein result in reduced affinity for the promoter target and upregulation of the *mexAB-oprM* operon. This three-component efflux pump provides an exit portal for quinolones, tetracycline, chloramphenicol, beta-lactams, and meropenem but not imipenem.<sup>29</sup>

Efflux pumps capable of using various substrates mediate cross-resistance to multiple antibiotics. Exposure of an efflux pump to any one substrate belonging to a similar or different substrate profile used by that pump may result in over-expression and consequent cross-resistance to all other substrates. Antibiotics are no exception; in the *mexAB* system of *P. aeruginosa*, mutants that over-produce *mexAB* are less susceptible to fluoroquinolones,  $\beta$ -lactams, chloramphenicol, trimethoprim, and triclosan.<sup>30</sup>

### Enzymatic inactivation

$\beta$ -lactam antibiotics are the most common treatment for bacterial infections and consist of four major groups: penicillins, cephalosporins, monobactams, and carbapenems. All four groups have a four-member  $\beta$ -lactam ring that is susceptible to the hydrolytic activity of  $\beta$ -lactamases, the most wide spread mechanisms of bacterial resistance to this class of antibiotics.<sup>31</sup> This antibiotic class is used to discuss intrinsic chromosomal mechanisms of resistance.

$\beta$ -lactamases hydrolyze  $\beta$ -lactam antibiotics using two distinct mechanisms; those having a serine-based mechanism of action and those having a metallo based mechanism of action. These mechanisms of action allow classification of  $\beta$ -lactamases into four major classes with classes A, C, and D containing active-site serine enzymes and class B  $\beta$ -lactamases exhibiting metallo based mechanisms of action.<sup>31</sup>

$\beta$ -lactam antibiotics act by binding to PBPs, bifunctional transglycosylases/transpeptidases responsible for cross-linking of glycan strands and backbone peptide strands, respectively.<sup>6</sup> The mechanistic and architectural similarities of class A, C, and D  $\beta$ -lactamases to PBPs suggest a common evolution of serine-based  $\beta$ -lactamases from PBPs. Thus, PBPs and  $\beta$ -lactamases are both receptors for the antibiotic entering into the cell. The binding equilibrium between PBP,  $\beta$ -lac-

tamases and the antibiotic determines the fate and ultimate survival of the microorganism. It is this observation that led to the development of  $\beta$ -lactamase inhibitors, e.g., clavulanic acid sulbactam and tazobactam, that are used jointly with susceptible antibiotics. The  $\beta$ -lactamase inhibitors are structural analogues of the  $\beta$ -lactam antibiotics and function as substrates for  $\beta$ -lactamase.

Class A and C are considered the most clinically important  $\beta$ -lactamases with class A enzymes usually found on plasmids and constitutively expressed, and class C enzymes usually found on the chromosome and inducible by exposure to  $\beta$ -lactams.<sup>32</sup> In gram-negative bacteria, the  $\beta$ -lactamases are localized to the periplasmic space where they act on incoming  $\beta$ -lactam antibiotics. In gram-positive bacteria,  $\beta$ -lactamases are secreted as exoenzymes and offer less protection to the pathogen.

Intrinsic chromosomal resistance mediated by enzymatic inactivation of penicillin class antibiotics is exemplified by the class C  $\beta$ -lactamases produced by virtually all gram-negative bacteria. Clinically important nosocomial pathogens encoding chromosomal versions of class C  $\beta$ -lactamases include *Citrobacter freundii*, *Enterobacter aerogenes*, and *P. aeruginosa*. Most class B metallo-dependent enzymes are chromosomally encoded cephalosporinases and are constitutive or inducible. They are expressed in clinically important nosocomial pathogens such as *Stenotrophomonas maltophilia*, *Klebsiella pneumoniae*, and *P. aeruginosa*.<sup>33</sup>

#### Acquired resistance

Resistance mechanisms and the genes that mediate resistance have presumably evolved in organisms that produce antibiotics. Further, normal housekeeping genes may acquire new functions to detoxify antibiotics in organisms that coevolved with antibiotic producing organisms. Thus, target organisms may both acquire or disseminate resistance determinants. Microorganisms use multiple combinations of intrinsic and acquired antibiotic resistance strategies to survive. These include efflux, modification of existing antibiotic targets, acquisition of new targets, and production of enzymes that inactivate the antibiotic.

#### Efflux

In bacteria, the genes of efflux pumps can be acquired, such as *mef* in *S. pneumoniae*, or intrinsic, like *acrAB* in gram-negative bacteria. Acquired macrolide resistance mediated by efflux has recently been described in streptococci and is encoded by *mefE* in *S. pneumoniae*, and by *mefA* in *Streptococcus pyogenes*.<sup>34</sup> Both *mefA* and *mefE* are now referred to as

*mefA* because of their close homology. The genetic element carrying *mefA* appears to be widely disseminated in *S. pneumoniae* and transferable to other streptococcal species and various other genera.<sup>35,36</sup>

*S. pneumoniae* isolates with efflux resistance are susceptible to clindamycin and usually have erythromycin a minimum inhibitory concentrations (MICs) ranging from 1 to 16  $\mu\text{g}/\text{mL}$ . Since the *mef* phenotype demonstrates resistance to erythromycin but susceptibility to clindamycin, surveillance studies monitoring susceptibility patterns of erythromycin and clindamycin were used to deduce that the most prevalent mechanism of macrolide resistance in the U.S. in *S. pneumoniae* is efflux.<sup>37</sup>

#### Target site modification

Modification of a target reduces the affinity of the target for the antibiotic. Modification of a target site occurs primarily by chromosomal mutation as in quinolone antimicrobials, and by enzymatic alteration of macrolide, glycopeptide, and  $\beta$ -lactam antibiotic target sites.

#### Mutation

Quinolones target DNA gyrase and topoisomerase IV. The primary mechanism of resistance to this antimicrobial class is modification by mutations encoding single amino acid changes in these targets. Mutations are generally localized to the amino terminal domains of *gyrA* and *parC*, termed the quinolone resistance-determining region (QRDR).<sup>38</sup> These mutations occur equally in both the *gyrA* and *gyrB* subunits but isolates from clinical settings demonstrate an exclusive prevalence for mutations in *gyrA*.<sup>39</sup> In *E. coli*, alterations in *gyrA* resulting in amino acid substitutions occur predominantly in the QRDR between positions 67 and 106.<sup>37</sup>

Although the presence of a single mutation in the QRDR of *gyrA* usually results in high-level resistance to nalidixic acid, the presence of additional mutation(s) in *gyrA* and/or another target such as *parC* is required to produce high levels of resistance to fluoroquinolones.<sup>40</sup> Conversely, in gram-positive bacteria, first step mutations leading to fluoroquinolone resistance occur in *parC* or *parE* subunits of topoisomerase IV and second step mutations occur in *gyrA*.<sup>41</sup> Interestingly, first step mutations in *gyrA* do not cause an elevated MIC in *S. aureus*, suggesting that the primary target of the quinolone is topoisomerase IV.<sup>42</sup>

$\beta$ -lactam antibiotics kill *S. pneumoniae* by targeting endogenous high-molecular weight penicillin binding proteins PBP1A, 1B,

2A, 2B, and 2X. Mutations in these PBPs confer low-level to high-level resistance to  $\beta$ -lactam antibiotics depending on the number of mutations and PBPs involved.<sup>43,44</sup> Mutations in PBP2 or PBP2X mediated by amino acid changes in close proximity to the active-site region of the PBP result in low level resistance.<sup>43</sup> Mutations in any of these transpeptidases/transglycosylases result in elevated MICs. High-level resistance is the result of mutation in all five PBPs.<sup>44</sup> In *S. pneumoniae*, evidence suggests that PB2B and 2X high-level resistance is also mediated by horizontal acquisition of DNA fragments via transformation and by homologous recombination of these fragments with the coding sequences of the PBP proteins.<sup>45</sup>

### Enzymatic alteration

Enzymatic alterations of antibiotic targets result in reduced affinity of antibiotics for their microbial targets and are exemplified by erythromycin ribosomal methylase, and by reprogramming of the peptidoglycan termini.

Macrolides such as erythromycin bind the 50S subunit of the ribosome at the peptidyltransferase cavity in the proximity of the A and P loops, and near adenine 2058 of 23S rRNA.<sup>16</sup> Mono or dimethylation of the amino group in the adenine residue of 23S rRNA results in reduced affinity of the macrolide for its target site and in elevated MICs. Resistance to macrolides is mediated by an erythromycin ribosome methylase (ERM), which is found on plasmids, and transposons that allow broad dissemination to many bacterial species. Methylation does not affect the function of the adenine residue other than to help the organism become less susceptible to the macrolide class of antibiotics. Methylation of the 23S rRNA also results in cross-resistance to the lincosamide family and streptogramin B class of antibiotics. Cross-resistance to all three antibiotics groups is known as the MLS<sub>B</sub> resistance phenotype.<sup>13</sup> The *erm* gene conferring the MLS<sub>B</sub> may be constitutively or inducibly expressed.

Vancomycin is a glycopeptide used to treat gram-positive bacteria, such as enterococci, that cause endocarditis. It is the drug of choice for the treatment of methicillin resistant *S. aureus*. Although vancomycin was used sparingly during its introduction, increased use resulted in the emergence of vancomycin resistant enterococci (VRE). Currently there are two choices for the treatment of VRE, dulfopristin-quinupristin and linezolid.

The phenotypes conferring resistance to vancomycin in clinically important enterococci are known as VanA and VanB. Enterococci containing the VanA phenotype, unlike

VanB, are highly resistant to vancomycin (MICs  $\geq 64$   $\mu\text{g/mL}$ ) and resistant to teicoplanin (MICs  $\geq 16$   $\mu\text{g/mL}$ ).<sup>46</sup> Strains carrying the VanB phenotype range from vancomycin susceptible to resistant but remain sensitive to teicoplanin. The VanA phenotype is resident on plasmids and on transposons that mediate spread of the determinant while VanB appears to be on large chromosomal elements. VanA is inducible by vancomycin and teicoplanin and is controlled by *VanS* and *VanR*, a regulatory pair that combine sensor and response regulators. These are responsible for control and expression of inducible vancomycin resistance.<sup>45</sup> The transmissibility of the vancomycin resistant elements is of great concern in the medical community, particularly to methicillin resistant *S. aureus* since vancomycin remains the treatment of choice for MRSA. Recent genetic analysis of a high-level vancomycin resistant *S. aureus* isolates (MIC = 1024  $\mu\text{g/mL}$ ) of clinical origin revealed the presence of a multiresistant conjugative plasmid harboring Tn 1546 (VanA).<sup>47</sup> Potential spread of this strain in the healthcare settings would have grave consequences.

Characterization of the VanA determinant shows five genes in tandem array; three of which are involved in target modification. These *VanH*, *VanA*, and *VanX* genes sequentially modify the peptidoglycan termini, N-acyl-D-Ala-D-Ala, involved in cross-linking to N-acyl-D-Ala-D-lactate. The result is uncross-linked peptidoglycan termini and resistance to vancomycin. Van H codes for a D-hydroxy acid dehydrogenase that synthesizes the D-lactate used by VanA, a ligase that mediates the preferential production of D-Ala-D-lac. Van X is a protein that acts specifically to cleave the natural peptidoglycan termini D-Ala-D-Ala thus preventing the competing synthesis of vancomycin susceptible peptidoglycan. Other Van type phenotypes mediate resistance to vancomycin using analogous mechanisms.<sup>48</sup>

### Acquisition of new targets

Microorganisms also adapt to the toxic effects of antibiotics by the acquisition of less susceptible targets; targets with reduced affinity to the antibiotic. Methicillin resistant *Staphylococcus aureus* (MRSA) emerged soon after the introduction of methicillin into clinical medicine in the 1960s and is an example of a pathogen that solved the toxic effects of antibiotics by the acquisition of a new target. Resistance to methicillin is mediated by the acquisition of a mobile element carrying a staphylococcal cassette chromosome *mec* (SCC*mec*).<sup>49</sup> The MRSA phenotype arises from the transfer of a mobile DNA element encoding a triad of genes: *mecR1-mecI-mecA*. The gene responsible for methicillin resistance is *mecA*, a gene that codes for a

new penicillin binding protein, PBP2A (also PBP2A'), a bifunctional transglycosylase/transpeptidase with reduced affinity for  $\beta$ -lactam antibiotics including penicillins, cephalosporins, carbapenems, and penems.<sup>50</sup> The origin of the *mecA* gene remains unknown, as is the molecular basis of insensitivity to  $\beta$ -lactams.

#### Inactivation of antibiotic

Another strategy used by bacteria to survive the action of antibiotics is the acquisition of enzymes that inactivate the antibiotic directly.  $\beta$ -lactams and aminoglycosides are examples of antibiotics inactivated by these acquired enzymatic mechanisms of resistance.

Class A  $\beta$ -lactamases are primarily penicillinases produced by gram-negative and gram-positive bacteria capable of hydrolyzing penicillin class antibiotic substrates. Class A  $\beta$ -lactamases are active-site serine enzymes and are structural analogues of PBPs so they are receptors for  $\beta$ -lactam antibiotics. The most important clinical Class A  $\beta$ -lactamases are TEM-1 and SHV-1, usually found in gram-negative bacteria. TEM-1 is typically found in *E. coli*, *K. pneumoniae*, *Enterobacter aerogenes*, and *Haemophilus influenzae* and is typically located on transmissible plasmids.<sup>51</sup> SHV-1 is usually found in clinical isolates of *K. pneumoniae* and appears to be the most common of the chromosomal  $\beta$ -lactamases found in this species.<sup>50,52</sup>

With the emergence of plasmid mediated beta-lactamase resistance, such as mediated by TEM-1 and SHV-1, new oxyimino- $\beta$ -lactam parenteral antibiotics resistant to the hydrolysis of these  $\beta$ -lactamases were introduced into clinical practice. However, with continued use of these parenterally administered cephalosporins and monobactams, variants of existing  $\beta$ -lactamases emerged that could hydrolyze the new antibiotics.<sup>53,54</sup> These extended spectrum  $\beta$ -lactamases (ESBLs) are now known to be derivatives of common TEM, SHV, and OXA type  $\beta$ -lactamases that differed by one or more amino acid substitutions near the reactive sites of the enzymes.<sup>53</sup> The modified amino acid sequence yielded a protein capable of extending substrate utilization and greater affinity for the antibiotic target molecule.<sup>55</sup> Interestingly, the enhanced spectrum of ESBLs also makes them more susceptible to the inhibitory action of the  $\beta$ -lactamase inhibitors.<sup>53</sup> Also, ESBLs have specific sets of penicillins, cephalosporins, and monobactams they can hydrolyze. Not all ESBLs are capable of hydrolyzing all cephalosporins equally well.<sup>54</sup>

As previously discussed, the amino and hydroxy radicals of aminoglycosides form hydrogen bonds with the 30S ribosomal subunit, thus preventing mRNA translation. Resistance to aminoglycosides is mediated by efflux, changes in target site, impermeability, or by enzymatic modification of the amino and hydroxy moieties appended to the cyclitol rings. The most clinically relevant is enzymatic modification, thus preventing recognition of the 16S binding sites and subsequent inhibition of mRNA translation.<sup>56</sup>

Enzymatic modification results from N-acetylation, O-phosphorylation, and O-adenylation of aminoglycoside radicals. Therefore, aminoglycoside-modifying enzymes are classified by their modifying reaction, their regiospecificity on the aminoglycoside ring structure, and by their specific isozyme sequence.<sup>57</sup> The classification system incorporates the type of enzyme mediating the reaction, i.e., AAC, N-acetyltransferase; ANT, O-adenyltransferase, and APH, O-phosphotransferase; the regiospecific site of modification based on aminoglycoside ring numbering convention, and the distinct phenotype. Thus, APH (3'') Ib is an O-phosphotransferase that phosphorylates the 3'' position of the double prime ring resulting in resistance to streptomycin.<sup>58</sup>

Inactivation of the aminoglycoside by the aminoglycoside-modifying enzymes is mediated by the transfer of a functional group to the aminoglycoside: AAC transfers the acetyl group from acetyl-CoA to the NH<sub>2</sub> group, ANT transfers the nucleotide triphosphate, and APH transfers the phosphoryl group from ATP to the OH or NH<sub>2</sub> group.<sup>55,56</sup>

#### DISSEMINATION

What is the evolutionary strategy that allows microorganisms to obtain and exchange resistance determinants? The elements that evolved to allow the horizontal and vertical exchange and dissemination of genetic material, and that play a significant role in adaptation and evolution are plasmids, transposons, insertion sequences, and integrins.<sup>59</sup> Plasmids are extrachromosomal, circular structures present in bacterial cells. They contain genes encoding proteins and RNA, are capable of self-replication, and are partitioned into daughter cells during cellular division. Plasmids also acquire and exchange information with the chromosome and other resident plasmids including antibiotic resistance genes. They may be conjugative and mediate their own dissemination or non-conjugative and require mobilization by conjugative plasmids. Transposons (Tn) are DNA elements that can transpose from one place on the chromosome to another. Transposition is mediated by a transposase, an enzyme that

facilitates non-homologous recombination. Transposons are also capable of carrying antibiotic resistance genes and function as shuttles, carrying these determinants among plasmids, and between plasmids and chromosomal DNA. Some transposons are conjugative and mediate their own transfer between bacteria. The archetype plasmid responsible for the dissemination of antibiotic resistance utilizing these elements is exemplified by plasmid NR1 isolated from *Shigella flexneri* and is used to discuss the relationship and structures of these elements.<sup>60</sup>

NR1 is a 94.5kb multiple antibiotic resistant plasmid and carries the genes for self-transmissibility and autonomous replication. NR1 also carries a resistance-determining region bound by direct repeats and is self-transmissible as Tn2670. Nested within Tn2670 is Tn21. Tn21 is of particular interest not only because it exhibits the transpositional characteristic of transposons and the presence of resistance determinants, but also because it contains a potentially mobile DNA element, the integron. Integrons are genetic elements capable of integrating resistance genes (cassettes) by an integron-encoded site-specific recombinase. The integron in Tn21 does not code for its own mobilization but can move when transposition proteins are provided by another genetic element containing the mobilization function; is bound by imperfect terminal inverted repeats; contains the *aad*A1 resistant determinant on a cassette. The integron also provides a promoter that directs transcription of any cassette inserted into its 5' conserved sequence. Gene cassettes, flanked by the 59-base element recombination site, are recognized by the integron-encoded site-specific recombinase, which mediates insertion into the integron. Over 60 new cassettes conferring resistance to a range of antimicrobial agents have been identified.<sup>61</sup>

NR1 also carries Tn10, which confers resistance to tetracycline and is bound by insertion sequences IS10L and IS10R.<sup>58</sup> Insertion sequences (IS) are transposons found in bacteria that carry genes only for the enzymes needed to promote their own transposition. IS elements can form composite transposable genetic elements, such as Tn10, with the IS elements forming the proximal and distal ends and genetic material that codes for antibiotic resistance located in-between.

As stated previously, vancomycin is an important antibiotic in the treatment of MRSA. Recently, an *S. aureus* isolate of clinical origin was found to contain a conjugative multidrug resistant plasmid that encoded resistance to

vancomycin, trimethoprim, beta-lactams, aminoglycosides, and disinfectants. Genetic analysis of a clinical *S. aureus* isolate mediating high-level vancomycin resistance revealed the presence of a multiresistant conjugative plasmid harboring Tn 1546 (VanA). A Tn-1546-like transposable genetic element encoding for transposition, regulation of VanA expression, and resistance is also found in enterococci. The data suggest the interspecies transfer of Tn1546 from a co-isolate of *Enterococcus faecalis* to MRSA.<sup>45</sup> Resistance to vancomycin can be spread by the transposition of the Tn-1547 to a conjugative plasmid and transferred by conjugation to recipient strains, or by excision and circularization of the transposons followed by conjugation.<sup>47</sup>

## CONCLUSIONS

Two factors contribute to resistance; antibiotics that contribute selection pressure and the emergence of organisms that phenotypically demonstrate resistance.<sup>62</sup> We now know that resistance may be intrinsic or acquired; each providing similar phenotypic characteristics mediated by related genotypic characteristics. Within the acquired mechanisms of resistance are also adaptive mechanisms that enhance the survival strategy of resistance pathogens. These are elements that allow inter and intraspecies exchange of resistance determinants via plasmids, transposons, and insertion elements. The continued overuse of antibiotics and emergence of new variants of existing adaptive mechanisms assure the evolution and dissemination of resistance determinants and survival of human pathogens. Thus, we must remain vigilant regarding the rational use of these extremely valuable clinical agents to assure their continued efficacy.

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