



Mechanisms of Antibiotic Resistance

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ABSTRACT Emergence of resistance among the most important bacterial pathogens is recognized as a major public health threat affecting humans worldwide. Multidrug-resistant organisms have not only emerged in the hospital environment but are now often identified in community settings, suggesting that reservoirs of antibiotic-resistant bacteria are present outside the hospital. The bacterial response to the antibiotic "attack" is the prime example of bacterial adaptation and the pinnacle of evolution. "Survival of the fittest" is a consequence of an immense genetic plasticity of bacterial pathogens that trigger specific responses that result in mutational adaptations, acquisition of genetic material, or alteration of gene expression producing resistance to virtually all antibiotics currently available in clinical practice. Therefore, understanding the biochemical and genetic basis of resistance is of paramount importance to design strategies to curtail the emergence and spread of resistance and to devise innovative therapeutic approaches against multidrug-resistant organisms. In this chapter, we will describe in detail the major mechanisms of antibiotic resistance encountered in clinical practice, providing specific examples in relevant bacterial pathogens.

INTRODUCTION

The discovery, commercialization, and routine administration of antimicrobial compounds to treat infections revolutionized modern medicine and changed the therapeutic paradigm. Indeed, antibiotics have become one of the most important medical interventions needed for the development of complex medical approaches such as cutting-edge surgical procedures, solid organ transplantation, and management of patients with cancer, among others. Unfortunately, the marked increase in antimicrobial resistance among common bacterial pathogens is now threatening this therapeutic accomplishment,

jeopardizing the successful outcomes of critically ill patients. In fact, the World Health Organization has named antibiotic resistance as one of the three most important public health threats of the 21st century (1).

Infections caused by multidrug-resistant (MDR) organisms are associated with increased mortality compared to those caused by susceptible bacteria, and they carry an important economic burden, estimated at over 20 billion dollars per year in the United States alone (2–4). The Centers for Disease Control and Prevention conservatively estimates that at least 23,000 people die annually in the United States as a result of an infection with an antibiotic-resistant organism (5). Moreover, according to a recent report, antibiotic resistance is estimated to cause around 300 million premature deaths by 2050, with a loss of up to \$100 trillion to the global economy (6). This situation is worsened by a paucity of a robust antibiotic pipeline, resulting in the emergence of infections that are almost untreatable and leaving clinicians with no reliable alternatives to treat infected patients.

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To understand the problem of antimicrobial resistance, it is useful to discuss some relevant concepts. First, antimicrobial resistance is ancient, and it is the expected result of the interaction of many organisms with their environment. Most antimicrobial compounds are naturally produced molecules, and as such, coresident bacteria have evolved mechanisms to overcome their action to survive. Thus, these organisms are often considered to be intrinsically resistant to one or more antimicrobials. However, when discussing the antimicrobial resistance conundrum, bacteria harboring intrinsic determinants of resistance are not the main focus of the problem. Rather, in clinical settings, we are typically referring to the expression of acquired resistance in a bacterial population that was originally susceptible to the antimicrobial compound. As will be discussed later in the chapter, the development of acquired resistance can be the result of mutations in chromosomal genes or be due to the acquisition of external genetic determinants of resistance, likely obtained from intrinsically resistant organisms present in the environment.

Second, it is important to recognize that the concept of antimicrobial resistance/susceptibility in clinical practice is a relative phenomenon with many layers of complexity. The establishment of clinical susceptibility breakpoints (susceptible, intermediate, and resistant) mainly relies on the *in vitro* activity of an antibiotic against a sizeable bacterial sample, combined with some pharmacological parameters (e.g., blood and infection site concentrations of the antimicrobial, among others). Thus, when treating antibiotic-resistant bacteria, the interpretation of susceptibility patterns may vary according to the clinical scenario and the availability of treatment options. For instance, the concentration of gentamicin achieved in the urine may be sufficiently high to treat a lower urinary tract infection caused by an organism reported as gentamicin resistant. Similarly, different penicillin breakpoints have been established for *Streptococcus pneumoniae* depending on if the isolate is causing meningitis as opposed to other types of infections, taking into account the levels of the drug that actually reach the cerebrospinal fluid (7). In addition, the *in vivo* susceptibility of an organism to a particular antibiotic may vary according to the size of the bacterial inoculum, a situation that has been well documented in *Staphylococcus aureus* infections treated with some cephalosporins. Indeed, there is evidence to suggest that some cephalosporins (e.g., cefazolin) may fail in the setting of high-inocula, deep-seated infections caused by cephalosporin-susceptible *S. aureus* (8). Therefore, in the following sections, we will focus on the molecular and

biochemical mechanisms of bacterial resistance, illustrating specific situations that are often encountered in clinical practice.

GENETIC BASIS OF ANTIMICROBIAL RESISTANCE

Bacteria have a remarkable genetic plasticity that allows them to respond to a wide array of environmental threats, including the presence of antibiotic molecules that may jeopardize their existence. Bacteria sharing the same ecological niche with antimicrobial-producing organisms have evolved ancient mechanisms to withstand the effect of the harmful antibiotic molecule and, consequently, their intrinsic resistance permits them to thrive in its presence. From an evolutionary perspective, bacteria use two major genetic strategies to adapt to the antibiotic “attack”: (i) mutations in gene(s) often associated with the mechanism of action of the compound and (ii) acquisition of foreign DNA coding for resistance determinants through horizontal gene transfer (HGT).

Mutational Resistance

In this scenario, a subset of bacterial cells derived from a susceptible population develops mutations in genes that affect the activity of the drug, resulting in preserved cell survival in the presence of the antimicrobial molecule. Once a resistant mutant emerges, the antibiotic eliminates the susceptible population, and the resistant bacteria predominate. In many instances, mutational changes leading to resistance are costly to cell homeostasis (i.e., decreased fitness) and are only maintained if needed in the presence of the antibiotic. In general, mutations resulting in antimicrobial resistance alter the antibiotic action via one of the following mechanisms: (i) modifications of the antimicrobial target (decreasing the affinity for the drug; see below), (ii) a decrease in the drug uptake, (iii) activation of efflux mechanisms to extrude the harmful molecule, or (iv) global changes in important metabolic pathways via modulation of regulatory networks. Thus, resistance arising due to acquired mutational changes is diverse and varies in complexity. In this chapter, we will give several examples of antimicrobial resistance arising through mutational changes (see below).

HGT

Acquisition of foreign DNA material through HGT is one of the most important drivers of bacterial evolution, and it is frequently responsible for the development of

antimicrobial resistance. Most antimicrobial agents used in clinical practice are (or derive from) products naturally found in the environment (mostly soil). Bacteria sharing the environment with these molecules harbor intrinsic genetic determinants of resistance, and there is robust evidence suggesting that such “environmental resistome” is a prolific source of the acquisition of antibiotic resistance genes in clinically relevant bacteria. Furthermore, this genetic exchange has been implicated in the dissemination of resistance to many frequently used antibiotics.

Classically, bacteria acquire external genetic material through three main strategies: (i) transformation (incorporation of naked DNA), (ii) transduction (phage mediated), and (iii) conjugation (bacterial “sex”). Transformation is perhaps the simplest type of HGT, but only a handful of clinically relevant bacterial species are able to naturally incorporate naked DNA to develop resistance. Emergence of resistance in the hospital environment often involves conjugation, a very efficient method of gene transfer that involves cell-to-cell contact and is likely to occur at high rates in the gastrointestinal tract of humans under antibiotic treatment. As a general rule, conjugation uses mobile genetic elements (MGEs) as vehicles to share valuable genetic information, although direct transfer from chromosome to chromosome has also been well characterized (9). The most important MGEs are plasmids and transposons, both of which play a crucial role in the development and dissemination of antimicrobial resistance among clinically relevant organisms.

Finally, one of the most efficient mechanisms for accumulating antimicrobial resistance genes is represented by integrons, which are site-specific recombination systems capable of recruiting open reading frames in the form of mobile gene cassettes. Integrons provide an efficient and rather simple mechanism for the addition of new genes into bacterial chromosomes, along with the necessary machinery to ensure their expression: a robust strategy of genetic interchange and one of the main drivers of bacterial evolution. For details on the mechanisms of HGT, readers are directed to a recent state-of-the-art review (10).

MECHANISTIC BASIS OF ANTIMICROBIAL RESISTANCE

Not surprisingly, bacteria have evolved sophisticated mechanisms of drug resistance to avoid killing by antimicrobial molecules, a process that has likely occurred over millions of years of evolution. Of note, resistance to

one antimicrobial class can usually be achieved through multiple biochemical pathways, and one bacterial cell may be capable of using a cadre of mechanisms of resistance to survive the effect of an antibiotic. As an example, fluoroquinolone resistance can occur by three biochemical routes, all of which may coexist in the same bacteria at a given time (producing an additive effect and, often, increasing the levels of resistance): (i) mutations in genes encoding the target site of fluoroquinolones (DNA gyrase and topoisomerase IV), (ii) overexpression of efflux pumps that extrude the drug from the cell, and (iii) protection of the fluoroquinolone target site by a protein designated Qnr (see below for details on each of these mechanisms). However, bacterial species seem to have evolved a preference for some mechanisms of resistance over others. For example, the predominant mechanism of resistance to β -lactams in Gram-negative bacteria is the production of β -lactamases, whereas resistance to these compounds in Gram-positive organisms is mostly achieved by modifications of their target site, the penicillin-binding proteins (PBPs). It has been argued that this phenomenon is likely due to major differences in the cell envelope between Gram-negative and Gram-positive organisms. In the former, the presence of an outer membrane permits “control” of the entry of molecules to the periplasmic space. Indeed, most β -lactams require specific porins to reach the PBPs, which are located in the inner membrane. Therefore, the bacterial cell controls the access of these molecules to the periplasmic space, allowing the production of β -lactamases in sufficient concentrations to tip the kinetics in favor of the destruction of the antibiotic molecule. Conversely, this “compartmentalization” advantage is absent in Gram-positive organisms, although production of β -lactamases also seems to be successful in certain scenarios (e.g., staphylococcal penicillinase).

To provide a comprehensive classification of the antibiotic resistance mechanisms, we will categorize them according to the biochemical route involved in resistance, as follows: (i) modifications of the antimicrobial molecule, (ii) prevention of the compound reaching the antibiotic target (by decreasing penetration or actively extruding the antimicrobial compound), (iii) changes to and/or bypassing of target sites, and (iv) resistance due to global cell-adaptive processes. Each of these mechanistic strategies encompasses specific biochemical pathways that will be described in detail in the remainder of the chapter. Of note, we will focus the discussion on the most important mechanisms, giving examples that have relevant clinical impact.

Modifications of the Antibiotic Molecule

One of the most successful bacterial strategies to cope with the presence of antibiotics is to produce enzymes that inactivate the drug by adding specific chemical moieties to the compound or that destroy the molecule itself, rendering the antibiotic unable to interact with its target.

Chemical alterations of the antibiotic

The production of enzymes capable of introducing chemical changes to the antimicrobial molecule is a well-known mechanism of acquired antibiotic resistance in both Gram-negative and Gram-positive bacteria. Interestingly, most of the antibiotics affected by these enzymatic modifications exert their mechanism of action by inhibiting protein synthesis at the ribosome level (11). Many types of modifying enzymes have been described, and the most frequent biochemical reactions they catalyze include (i) acetylation (aminoglycosides, chloramphenicol, streptogramins), (ii) phosphorylation (aminoglycosides, chloramphenicol), and (iii) adenylation (aminoglycosides, lincosamides). Regardless of the biochemical reaction, the resulting effect is often related to steric hindrance that decreases the avidity of the drug for its target, which in turn, is reflected in higher bacterial MICs.

One of the best examples of resistance via modification of the drug is the presence of aminoglycoside modifying enzymes (AMEs) that covalently modify the hydroxyl or amino groups of the aminoglycoside molecule. Multiple AMEs have been described to date, and they have become the predominant mechanism of aminoglycoside resistance worldwide. These enzymes are usually harbored in MGEs, but genes coding for AMEs have also been found as part of the chromosome in certain bacterial species, as seen with some aminoglycoside acetyltransferases in *Providencia stuartii*, *Enterococcus faecium* and *Serratia marcescens* (12). The nomenclature to classify the multiple AMEs considers their biochemical activity (acetyltransferase [ACC], adenyltransferase [ANT] or phosphotransferase [APH]), the site of the modification, which is depicted by a number from 1 to 6 corresponding to the particular carbon on the sugar ring, and a single or double prime symbol to symbolize that the reaction occurs in the first or second sugar moiety, respectively. In addition, whenever there is more than one enzyme catalyzing the exact same reaction, a roman numeral is used to differentiate them (Fig. 1).

There are important differences in the geographical distribution, the bacterial species in which these enzymes

disseminate, and the specific aminoglycosides they affect. For instance, the APH(3) family is widely distributed in Gram-positive and Gram-negative bacteria and alters kanamycin and streptomycin but spares gentamicin and tobramycin. On the other hand, AAC(6')-I is mainly found in Gram-negative clinical isolates including *Enterobacteriaceae*, *Pseudomonas*, and *Acinetobacter* and affects most aminoglycosides including amikacin and gentamicin (12). In addition, the activity and distribution of AMEs from the same family also varies. For instance, among the adenyltransferases, which classically affect both gentamicin and tobramycin, the genes encoding ANT(4'), ANT(6'), and ANT(9') are usually harbored in MGEs of Gram-positive bacteria, and ANT(2") and ANT(3") are more prevalent in Gram-negative organisms (12).

Finally, it is worth mentioning that some of these enzymes have evolved more than a single biochemical activity. Indeed, AAC(6')APH(2"), which is mainly found in Gram-positive organisms, is a bifunctional enzyme (with acetylation and phosphotransferase activities) that likely arose from the fusion of two AMEs encoding genes. This protein confers high-level resistance to all aminoglycosides except for streptomycin and is located on a Tn4001-like transposon that is widely distributed among enterococci and staphylococci. Furthermore, the presence of this bifunctional enzyme accounts for most of the high-level gentamicin resistance detected in enterococci (including in vancomycin-resistant strains) and methicillin-resistant *S. aureus* worldwide (13).

Another classical example of enzymatic alteration of an antibiotic involves the modification of chloramphenicol, an antibiotic that inhibits protein synthesis by interacting with the peptidyl-transfer center of the 50S ribosomal subunit. The chemical modification of chloramphenicol is mainly driven by the expression of acetyltransferases known as CATs (chloramphenicol acetyltransferases). Multiple *cat* genes have been described in both Gram-positive and Gram-negative organisms, and they have been classified into two main types: type A, which usually results in high-level resistance, and type B, which confers low-level chloramphenicol resistance (14). Although these determinants are usually harbored in MGEs such as plasmids and transposons, they have also been reported as being part of the core genome (chromosome) of certain bacteria.

Destruction of the antibiotic molecule

The main mechanism of β -lactam resistance relies on the destruction of these compounds by the action of β -lactamases. These enzymes destroy the amide bond of

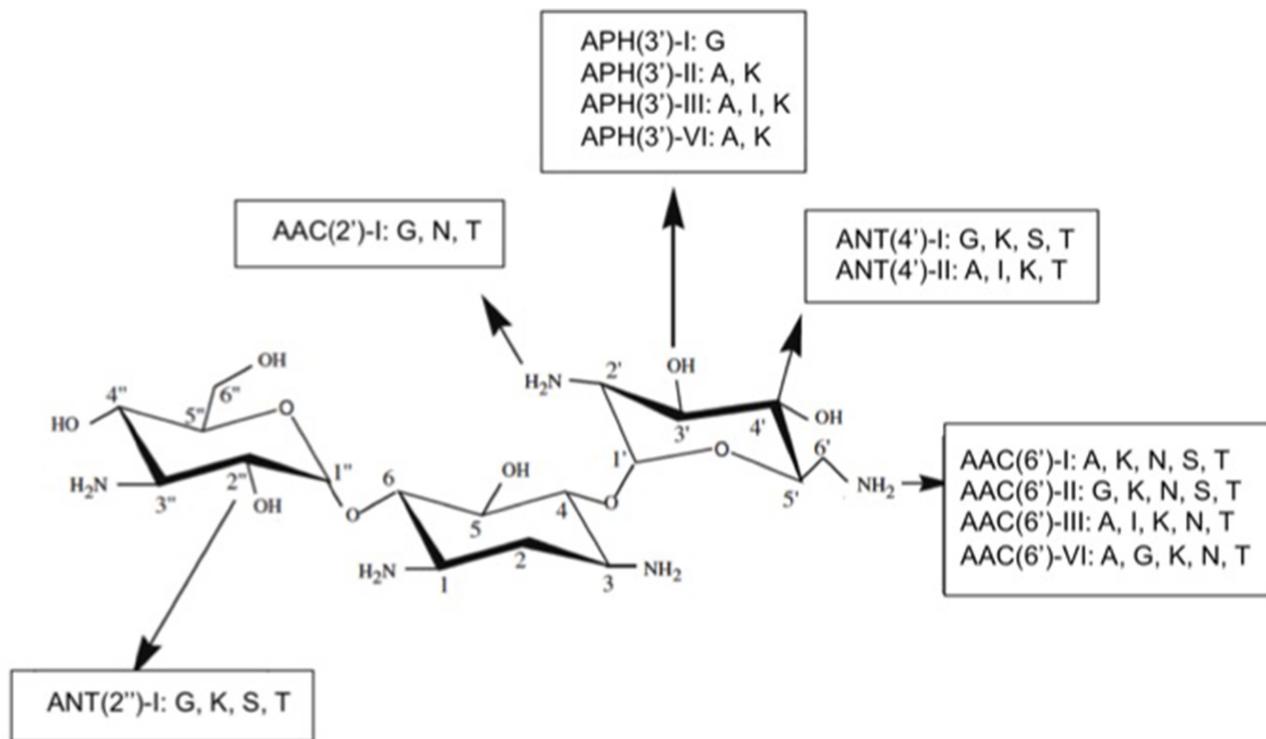


FIGURE 1 Representation of different types of aminoglycoside-modifying enzymes and their nomenclature. Each group of enzymes is identified by their biochemical activity as follows: acetyltransferase (AAC), adenyltransferase (ANT), and phosphotransferase (APH). Next in the enzyme name, an algebraic number in parentheses indicates the number of the carbon that is inactivated. The ring of the sugar in which the reaction takes place is symbolized by one (first sugar moiety) or two apostrophes (second sugar moiety). Roman numerals are used to differentiate distinct isoenzymes acting in the same site. Not all existing enzymes are shown. A, amikacin; G, gentamicin; I, isepamicin; K, kanamycin; N, netilmicin; S, sisomicin; T, tobramycin. Modified from reference [106](#).

the β -lactam ring, rendering the antimicrobial ineffective. β -lactamases were first described in the early 1940s, one year before penicillin was introduced to the market, but there is evidence of their existence for millions of years ([15](#), [16](#)). Infections caused by penicillin-resistant *S. aureus* became clinically relevant after penicillin became widely available and the mechanism of resistance was found to be a plasmid-encoded penicillinase that was readily transmitted between *S. aureus* strains, resulting in rapid dissemination of the resistance trait ([17](#)). To overcome this problem, new β -lactam compounds with a wider spectrum of activity and less susceptibility to penicillinases (such as ampicillin) were manufactured. However, during the 1960s a new plasmid-encoded β -lactamase capable of hydrolyzing ampicillin was found among Gram-negative organisms (termed TEM-1 after the name of the patient in which it was originally found [Temoneira]) ([18](#)). From then on, the development of newer generations of β -lactams has systemati-

cally been followed by the rapid appearance of enzymes capable of destroying any novel compound that reaches the market, in a process that is a prime example of antibiotic-driven adaptive bacterial evolution.

Genes encoding for β -lactamases are generally termed *bla*, followed by the name of the specific enzyme (e.g., *bla_{KPC}*), and they have been found in the chromosome or localized in MGEs as part of the accessory genome. These genes can also be found forming part of integrons, a situation that facilitates their dissemination. In terms of their expression, transcription of these genes can be constitutive, or it may require an external signal to induce their production.

To date, more than 1,000 β -lactamases have been described (www.lahey.org/studies), and many more are likely to continue to be reported as part of the normal process of bacterial evolution. Two main classification schemes have been proposed in an attempt to group this large number of enzymes. First, the Ambler classification

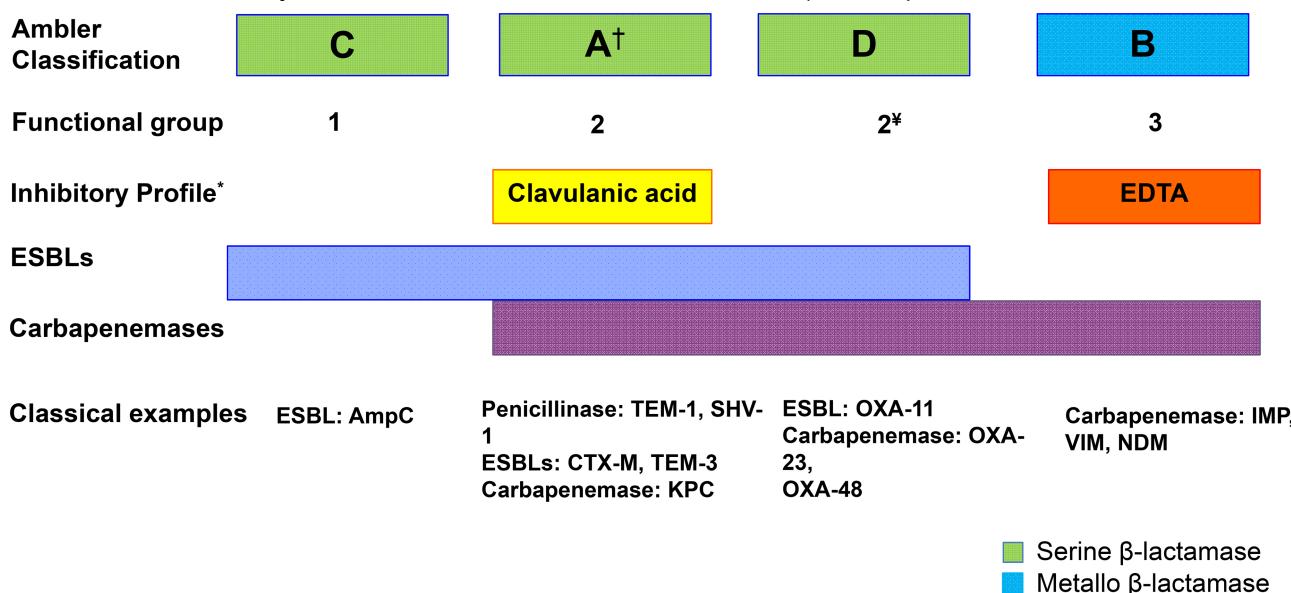
relies on amino acid sequence identity and separates β -lactamases into four groups (A, B, C, and D). On the other hand, the Bush-Jacoby classification divides β -lactamases into four categories (each with several subgroups) according to their biochemical function, mainly based on substrate specificity (19, 20). A summary of the most important enzymes and their classification is presented in Fig 2.

It is important to note that both of these classification schemes have caveats and that they do not fully overlap. For instance, Ambler class A and D enzymes are all considered to be within group 2 in the Jacoby-Bush system. In addition, while the Ambler classification seems to be easier to follow, the lack of correlation with functional characteristics of the enzymes may lead to confusion. As an example, the Ambler class A group encompasses enzymes with a wide range of biochemical activities, from narrow-spectrum β -lactamases to enzymes capable of destroying almost all available β -lactams, including carbapenems. Moreover, enzymes originally classified within a group harboring a particular biochemical profile can evolve into novel enzymes with different substrate specificities, usually due to mutations in the active site. A good example of this process

is TEM-3, an enzyme that evolved from the original TEM-1 penicillinase after acquiring the ability to hydrolyze third-generation cephalosporins and aztreonam (a functional profile that defines it as an “extended spectrum β -lactamase” [ESBL]) due to the development of two amino acid substitutions that altered its function (18, 21).

Deciphering the role of the different types of enzymes and their characteristics is a complex task that requires understanding some of the terminology frequently used in the literature. As mentioned above, an ESBL enzyme has the ability to hydrolyze penicillins, third-generation cephalosporins (the hallmark characteristic), and monobactams, but it harbors modest (or no) activity against cephemycins and carbapenems. Most of the ESBLs belong to Ambler class A and, as such, they are generally inhibited by clavulanic acid or tazobactam. This property distinguishes them from AmpC enzymes, which are class C β -lactamases that also hydrolyze third-generation cephalosporins but are not inhibited by clavulanic acid or tazobactam. Of note, a subgroup of class D OXA enzymes capable of destroying third-generation cephalosporins is also considered within the ESBL group (see below and Fig. 2). Another clinically

FIGURE 2 Schematic representation of β -lactamases. Molecular classification of β -lactamases follows the Ambler classification. Correlation with the main functional group of the Bush and Jacobi classification is also shown. Of note, the latter classification has several subgroups that are not shown. Representative examples of each group of enzymes are provided. [†]Class A enzymes are the most diverse and include penicillinases, ESBLs, and carbapenemases. [‡]Ambler class D enzymes belong to the functional group/subgroup 2d. *Class A enzymes belonging to the subgroup 2br are resistant to clavulanic acid inhibition. EDTA, ethylenediaminetetraacetic acid; ESBLs, extended-spectrum β -lactamases.



relevant group of enzymes is the carbapenemases, a diverse group of β -lactamases with the ability to hydrolyze carbapenems, the most potent β -lactams available in clinical practice. These enzymes can be divided into serine carbapenemases (Ambler class A or D) and metallo-carbapenemases (Ambler class B). In the rest of this section, we will provide examples of the different types of β -lactamases using the Ambler classification as the backbone for discussion.

Class A β -lactamases have a serine residue in the catalytic site, a property that they share with class C and D enzymes. Most class A enzymes are inhibited by clavulanic acid, and their spectrum of activity includes monobactams but not cephamycins (cefoxitin and cefotetan). Class A enzymes include a wide range of proteins with very different catalytic activities, from penicillinases (TEM-1 and SHV-1, which only hydrolyze penicillin) to ESBLs (such as CTX-M) to carbapenemases such as KPC (*Klebsiella pneumoniae* carbapenemase), an enzyme that is currently prevalent in several Gram-negative species. We will discuss details of CTX-M (ESBL) and KPC carbapenemases, both class A enzymes with high clinical impact.

CTX-M is a plasmid-encoded ESBL commonly found in *K. pneumoniae*, *Escherichia coli*, and other *Enterobacteriaceae* around the world. In contrast to other Ambler class A ESBLs such as TEM-3, this enzyme did not derive from TEM or SHV; rather, the current evidence suggests that it was likely acquired from *Kluyvera* spp. (an environmental bacterium with no major human pathogenic significance) through HGT (22). Genes encoding CTX-M enzymes have been found in association with insertion sequences (IS_{Ecp1}) and with transposable elements such as Tn402-like transposons. These mobile elements can be captured by a broad range of conjugative plasmids or phage-like sequences that can serve as vehicles for dissemination (23). Consequently, CTX-M enzymes have become the most prevalent ESBL worldwide and are responsible for a large proportion of cephalosporin resistance in *E. coli* and *K. pneumoniae*.

To date, five families of class A carbapenemases have been described, of which three are typically chromosomally encoded (IMI [imipenem-hydrolyzing enzyme], SME [*S. marcescens* enzyme], and NMC [not-metallocarbenzyme carbapenemase]), and the remaining two (KPC and GES) are classically harbored in plasmids or other MGEs (24). As with other class A enzymes they are all inhibited by clavulanic acid and tazobactam, and they hydrolyze aztreonam but not cephamycins. KPC was first reported in 1996 from a *K. pneumoniae* strain recovered from a patient in North Carolina (25). Although

these enzymes are predominantly found in *Klebsiella* spp. (thus its name, *K. pneumoniae* carbapenemase), they have been reported in several other Gram-negative organisms, including *Enterobacter* spp., *E. coli*, *Proteus mirabilis*, and *Salmonella* spp., among others. Furthermore, they have also been found in nonlactose fermenter organisms such as *Pseudomonas aeruginosa*. A total of 22 variants of the *bla_{KPC}* gene have been described to date, most of them located in plasmids harboring transposable elements (e.g., Tn4401) or in association with insertion sequences such as ISKpn6 and ISKpn7 (26).

Class B enzymes are also known as metallo- β -lactamases because they utilize a metal ion (usually zinc) as a cofactor (instead of a serine residue) for the nucleophilic attack of the β -lactam ring. They are inhibited by the presence of ion-chelating agents such as EDTA and, similar to class A carbapenemases, they are active against a wide range of β -lactams, including carbapenems. Metallo- β -lactamases are not inhibited by clavulanic acid or tazobactam, and while they efficiently hydrolyze cephamycins, aztreonam is typically a poor substrate. These enzymes were discovered over 50 years ago encoded by genes usually located in the chromosome of nonpathogenic bacteria. However, the situation dramatically changed during the 1990s, when enzymes such as IMP and VIM were increasingly reported in clinical strains of *Enterobacteriaceae*, *Pseudomonas* spp., and *Acinetobacter* spp. (24). Indeed, genes encoding these enzymes have been found as part of the accessory genome of pathogenic bacteria, which suggests HGT. There are about 10 types of metallo-carbapenemases, but most of the clinically important ones belong to 4 families: IMP, VIM, SPM, and NDM. Considering their high frequency and worldwide spread, we will briefly discuss IMP, VIM, and NDM.

The first IMP-type enzymes were described in Japan in the early 1990s in *S. marcescens*, and since then, more than 20 subtypes have been described worldwide in *Enterobacteriaceae*, *Pseudomonas* spp., and *Acinetobacter* spp., among other organisms. The *bla_{IMP}* genes have been found on large plasmids and forming part of class 1 integrons (27). The VIM-type enzymes were first described in the late 1990s in Verona, Italy (Verona integron-encoded metallo β -lactamase) and have since spread throughout the globe. These enzymes were initially found in *P. aeruginosa*, but their association with class 1 integrons, along with reports locating them in different types of MGEs, has likely contributed to their dissemination to many different bacterial species, making them a major concern around the globe. Among the many variants of VIM described to date, VIM-2 is the

most widely distributed enzyme, with reports from Europe, Asia, Africa, and the Americas (28).

More recently (2008), a new carbapenemase was identified in a *K. pneumoniae* isolate recovered from a Swedish patient who had been previously admitted to a hospital in New Delhi, India. The enzyme was designated NDM-1, in reference to its origin (New Delhi metallo β-lactamase) (29). NDM-1 shares little amino acid identity with other members of the Ambler class B enzymes (e.g., 32% with VIM-1), but its hydrolytic profile is very similar to all of them. The *bla_{NDM}* gene has been found in several types of plasmids that are readily transferable among different species of Gram-negative organisms, and it has also been associated with the presence of insertion sequences such as the *ISAb125*. In contrast to other genes encoding metallo-enzymes, *bla_{NDM}* is not usually related to integron-like structures (30). Nevertheless, NDM-1 rapidly spread around the globe, becoming a prime example of how a resistance determinant can readily disseminate worldwide despite many efforts to avoid its transmission. Moreover, MGEs containing genes coding for NDM enzymes generally carry multiple other resistance determinants such as genes encoding other carbapenemases (e.g., VIM-type and OXA-type enzymes), ESBLs, AMEs, methylases conferring resistance to macrolides, the quinolone resistance Qnr protein, enzymes that modify rifampin, and proteins involved in resistance to sulfamethoxazole, among others. Thus, the presence of NDM-1 is frequently accompanied by an MDR phenotype.

The emergence of NDM-1 is particularly concerning because the *bla_{NDM}* gene has been shown to be readily transmissible among different types of Gram-negative organisms, spreading to many countries in a short span of time and becoming one of the most feared resistance determinants in several parts of the world (28). In addition, in the Indian subcontinent (i.e., India and Pakistan), the *bla_{NDM}* gene is not only extensively disseminated among nosocomial pathogens, but it is frequently found in community-associated isolates. Furthermore, several reports have found NDM-1-producing Gram-negative bacteria in soil and in drinking water, suggesting that these genes may be disseminating through the human microbiota (31).

Class C β-lactamases confer resistance to all penicillins and cephalosporins (although cefepime is usually a poor substrate), including cephamycins. They do not reliably hydrolyze aztreonam and are not inhibited by clavulanic acid. The most clinically relevant class C enzyme is AmpC, which is a cephalosporinase that is generally encoded on the chromosome (although the

bla_{AMPc} gene has also been found in plasmids). Production of chromosomal AmpC is a hallmark of *Enterobacter cloacae*, *Enterobacter aerogenes*, *Citrobacter freundii*, *S. marcescens*, *Providencia* spp., *Morganella morganii*, and *P. aeruginosa*, among others. In contrast, *Proteus mirabilis*, *Proteus vulgaris*, *Klebsiella* spp., and *Stenotrophomonas* spp. are classical examples of species in which the *bla_{ampC}* gene is absent from the core genome (32).

The expression of *ampC* is generally inducible and is under strict control of a complex regulatory mechanism that has been best studied in *Enterobacter* spp. AmpR is a transcriptional regulator of the LysR family that acts as a repressor of the transcription of *bla_{AMPc}*. Under noninducing conditions (absence of β-lactams), AmpR is bound to peptidoglycan precursors (UDP-MurNAc pentapeptides), and interaction of AmpR with its cognate promoter does not occur (resulting in absence of *bla_{AMPc}* transcription). In contrast, in the presence of β-lactams, the alterations in cell wall homeostasis result in accumulation of peptidoglycan byproducts such as anhydro-muropeptides that compete for the same AmpR binding site with the UDP-MurNAc pentapeptides. As a result of this competition, AmpR is released and is able to interact with the *bla_{AMPc}* promoter, activating transcription of the gene (33, 34).

Another mechanism by which *ampC* is overexpressed is through AmpD, a cytosolic amidase that recycles muropeptides. AmpD effectively reduces the concentration of anhydro-UDP-MurNAc tri-, tetra-, and pentapeptides, preventing displacement of UDP-MurNAc pentapeptide from AmpR and, therefore, *ampC* overexpression. Mutations in *ampD* are often seen in isolates that constitutively overproduce AmpC, affecting the clinical efficacy of cephalosporins. As mentioned, cefepime is not a good substrate for AmpC enzymes; however, high-level production of AmpC may markedly increase cefepime MICs (32, 35).

Class D β-lactamases include a wide range of enzymes that were initially differentiated from the class A penicillinases due to their ability to hydrolyze oxacillin (hence their name) and because they were poorly inhibited by clavulanic acid. Many OXA variants have been described, including enzymes with the ability to degrade third-generation cephalosporins (ESBLs) (e.g., OXA-11 from *P. aeruginosa*) and carbapenems (e.g., OXA-23 from *Acinetobacter baumannii*). For example, OXA-48 is a widely disseminated class D carbapenemase which was originally described in 2001 in Turkey from an MDR isolate of *K. pneumoniae*. OXA-48 and its variants are now widely spread in clinical

isolates of *K. pneumoniae* and other *Enterobacteriaceae* and have also been found in *A. baumannii* (36). Many other types of OXA enzymes have been described to date, possessing a variety of hydrolytic profiles and encoded by genes that are often found in a wide range of MGEs. In certain instances, the OXA-containing MGE inserts in the chromosome result in core-genome genes encoding OXA enzymes. This phenomenon has often been described in *Acinetobacter*, with both OXA-51 and OXA-69 encoded by genes located in the chromosome (36).

Although class D enzymes are particularly prevalent in *A. baumannii*, they have been reported in many other clinically relevant organisms, such as *E. coli*, *Enterobacter* spp., *K. pneumoniae*, and *P. aeruginosa*, among others. Furthermore, intra- and interspecies transmission of some of these genes has been particularly successful, with enzymes such as OXA-23 and OXA-58 currently being spread around the globe.

Decreased Antibiotic Penetration and Efflux

Decreased permeability

Many of the antibiotics used in clinical practice have intracellular bacterial targets or, in the case of Gram-negative bacteria, targets located in the cytoplasmic membrane (the inner membrane). Therefore, the compound must penetrate the outer and/or cytoplasmic membrane to exert its antimicrobial effect. Bacteria have developed mechanisms to prevent the antibiotic from reaching its intracellular or periplasmic target by decreasing the uptake of the antimicrobial molecule. This mechanism is particularly important in Gram-negative bacteria (for the reason specified above), limiting the influx of substances from the external milieu. In fact, the outer membrane acts as the first line of defense against the penetration of multiple toxic compounds, including several antimicrobial agents. Hydrophilic molecules such as β -lactams, tetracyclines, and some fluoroquinolones are particularly affected by changes in permeability of the outer membrane since they often use water-filled diffusion channels known as porins to cross this barrier (37). The prime example of the efficiency of this natural barrier is the fact that vancomycin, a glycopeptide antibiotic, is not active against Gram-negative organisms due to the lack of penetration through the outer membrane. Likewise, the innate low susceptibility of *Pseudomonas* and *A. baumannii* to β -lactams (compared to *Enterobacteriaceae*) can be explained, at least in part, to a reduced number and/or differential expression of porins (38).

Several types of porins have been described, and they can be classified according to their structure (trimeric or monomeric), their selectivity, and the regulation of their expression. Among the best-characterized porins, the three major proteins produced by *E. coli* (known as OmpF, OmpC, and PhoE) and the *P. aeruginosa* OprD (also known as protein D2) are classical examples of porin-mediated antibiotic resistance. Alterations of porins can be achieved by three general processes: (i) a shift in the type of porins expressed, (ii) a change in the level of porin expression, and (iii) impairment of the porin function. Changes in permeability through any of these mechanisms frequently result in low-level resistance and are often associated with other mechanisms of resistance, such as increased expression of efflux pumps (see below) (39).

One classic example of porin-mediated resistance is the aberrant production of OprD in *P. aeruginosa*, which is normally used for the uptake of basic amino acids and antibiotics (i.e., imipenem, a potent antipseudomonal antibiotic from the carbapenem class). Mutations in the *oprD* gene have been shown to arise in clinical isolates of *P. aeruginosa* during therapy (40). Furthermore, clinical and *in vitro* studies have shown that these changes can produce resistance alone or in conjunction with overexpression of an efflux pump and/or the production of a carbapenem-hydrolyzing enzyme, resulting in high levels of resistance to carbapenems.

Another example relates to clinical isolates of *K. pneumoniae* recovered before and after antimicrobial therapy. The posttherapy isolates were found to exhibit a shift in porin expression from OmpK35 to OmpK36 (the latter possessing a smaller channel size). This alteration in the type of porin expressed correlated with a 4- to 8-fold decrease in susceptibility for a wide range of β -lactam antimicrobials (41, 42). Similar examples are found in other bacterial species of clinical importance such as *E. cloacae*, *Salmonella* spp., *Neisseria gonorrhoeae*, and *A. baumannii*.

Efflux pumps

The production of complex bacterial machineries capable of extruding a toxic compound out of the cell can also result in antimicrobial resistance. The description of an efflux system able to pump tetracycline out of the cytoplasm of *E. coli* dates from the early 1980s and was among the first to be described (43). Since then, many classes of efflux pumps have been characterized in both Gram-negative and Gram-positive pathogens. These systems may be substrate-specific (for a particular antibiotic such as *tet* determinants for tetracycline and *mef* genes for macrolides in pneumococci) or have broad

substrate specificity, which is usually found in MDR bacteria (44). This mechanism of resistance affects a wide range of antimicrobial classes including protein synthesis inhibitors, fluoroquinolones, β -lactams, carbapenems, and polymyxins. The genes encoding efflux pumps can be located in MGEs (as initially described for the *tet* gene) or in the chromosome. Chromosomally encoded pumps can explain the inherent resistance of some bacterial species to a particular antibiotic (e.g., *Enterococcus faecalis* intrinsic resistance to streptogramin A; see below) (45).

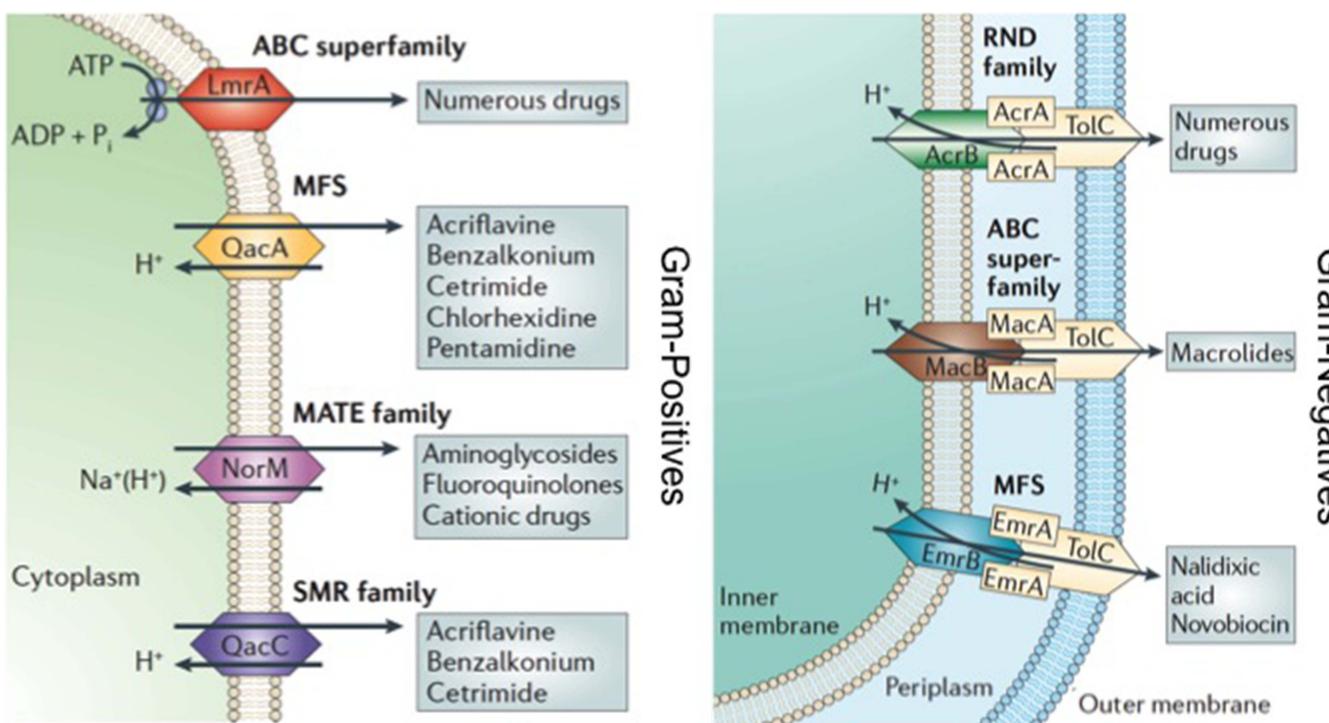
There are five major families of efflux pumps: (i) the major facilitator superfamily, (ii) the small multidrug resistance family (SMR), (iii) the resistance-nodulation-cell-division family (RND), (iv) the ATP-binding cassette family, and (v) the multidrug and toxic compound extrusion family. These families differ in terms of structural conformation, energy source, range of substrates they are able to extrude, and the type of bacterial organisms in which they are distributed (46) (Fig. 3).

Tetracycline resistance is one of the classic examples of efflux-mediated resistance, where the Tet efflux

pumps (belonging to the major facilitator superfamily family) extrude tetracyclines using proton exchange as the source of energy. Currently, more than 20 *tet* genes have been described, most of which are harbored in MGEs. The majority of these pumps are preferentially found in Gram-negative organisms, with *Tet*(K) and *Tet*(L) being among the few exceptions that predominate in Gram-positive organisms. Many of these pumps affect tetracycline and doxycycline but do not decrease minocycline or tigecycline susceptibility, because they are not able to use these compounds as substrates (44, 47). In addition to the tetracycline-specific transport systems, several MDR efflux pumps such as *AcrAB*-*TolC* in *Enterobacteriaceae* and *MexAB*-*OprM* in *P. aeruginosa* (both belonging to the RND family) are able to extrude tetracyclines (including tigecycline) as part of their contribution to multidrug resistance (48, 49).

Of note, MDR pumps belonging to the RND family are frequently found in the chromosome of clinically relevant Gram-negative bacteria and determine varying degrees of intrinsic resistance to several antimicrobials. Efflux pumps that belong to this family are organized

FIGURE 3 Representation of different types of efflux pumps in Gram-positive and Gram-negative bacteria. The five major families of efflux pumps are shown: ATP-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the multidrug and toxic-compound extrusion (MATE) family, and the resistance nodulation division (RND) family. A diagrammatic comparison of all the families showing their source of energy and examples of drugs and compounds that serve as a substrate are shown. Modified from reference 107 with permission.



as tripartite structures spanning the width of the Gram-negative cell envelope and selectively communicating the cytoplasm with the external environment. Among them, one of the best studied is the AcrAB-TolC system (classically found in *E. coli*), which is composed of a transporter protein located in the inner membrane (AcrB), a linker protein located in the periplasmic space (AcrA), and a protein channel located in the outer membrane (TolC) (108). RND pumps function as proton antiporters and are able to transport a wide array of substrates, conferring resistance to tetracyclines, chloramphenicol, some β -lactams, novobiocin, fusidic acid, and fluoroquinolones. In addition, they are capable of extruding several toxic compounds such as bile salts, cationic dyes, and disinfectants, among many others. Crystallographic studies have provided insight on the structure and function of these pumps, improving our understanding of how these systems operate. Indeed, they have shown that AcrB has two binding pockets with different substrate preferences and that compounds are moved out of the cell through a series of conformational changes in a functionally rotating mechanism that finishes with the substrate being extruded via TolC (a process that requires an interaction with the periplasmic accessory protein AcrA) (109). Of note, recent investigations have described a small protein named ArcZ, which has been shown to modulate and enhance the affinity of AcrB for certain molecules such as chloramphenicol and tetracycline through a mechanism that is yet to be determined (110).

Another important phenotype of clinical relevance mediated by the efflux mechanism is that of resistance to macrolides. The best-characterized efflux pumps are encoded by the *mef* genes (*mefA* and *mefE*) that extrude the macrolide class of antibiotics (e.g., erythromycin). The Mef pumps are mainly found in *Streptococcus pyogenes* and *S. pneumoniae*, along with other streptococci and Gram-positive organisms. MefA is usually carried in a transposon (Tn1207) located in the chromosome, and MefE is harbored in the so called “MEGA-element,” a fragment of DNA known as the macrolide efflux genetic assembly element that has been found inserted in different regions of the bacterial chromosome. Macrolide resistance due to these pumps does not result in cross-resistance to lincosamides and streptogramins (the so called MLS_B group) (50).

Other efflux pumps resulting in macrolide resistance in Gram positive organisms include MsrA and MsrC, which belong to the ATP-binding cassette transporter family. MsrA is a plasmid-borne determinant that was initially described in *Staphylococcus epidermidis*.

MsrC is a chromosomally encoded protein described in *E. faecalis* that produces low-level resistance to macrolides and streptogramin B. Finally, another predicted efflux pump is Lsa (encoded by the chromosomal gene *lsa*), which is responsible for the intrinsic resistance of *E. faecalis* to lincosamides and streptogramin A (LS_A phenotype) (44, 45).

Changes in Target Sites

A common strategy for bacteria to develop antimicrobial resistance is to avoid the action of the antibiotic by interfering with their target site. To achieve this, bacteria have evolved different tactics, including protection of the target (preventing the antibiotic from reaching its binding site) and modifications of the target site that result in decreased affinity for the antibiotic molecule.

Target protection

Although some of the genetic determinants coding for proteins that mediate target protection have been found in the bacterial chromosome, most of the clinically relevant genes involved in this mechanism of resistance are carried by MGEs. Examples of drugs affected by this mechanism include tetracycline (Tet[M] and Tet[O]), fluoroquinolones (Qnr), and fusidic acid (FusB and FusC).

One of the classic and best-studied examples of the target protection mechanism is the tetracycline resistance determinants Tet(M) and Tet(O). Tet(M) was initially described in *Streptococcus* spp. and Tet(O) in *Campylobacter jejuni*, but they are now both widely distributed among different bacterial species, likely because they have been found in several plasmids and in broad-range conjugative transposons (51). These proteins belong to the translation factor superfamily of GTPases and act as homologues of elongation factors (EF-G and EF-Tu) used in protein synthesis. TetO and TetM interact with the ribosome and dislodge the tetracycline from its binding site in a GTP-dependent manner. Dönhöfer et al. recently showed that TetM directly dislodges and releases tetracycline from the ribosome by an interaction between the domain IV of the 16S rRNA and the tetracycline binding site. Furthermore, this interaction alters the ribosomal conformation, preventing rebinding of the antibiotic (52). Similarly, TetO has also been shown to compete with tetracycline for the same ribosomal space and to alter the geometry of the binding site of the antibiotic, displacing the molecule from the ribosome and allowing protein synthesis to resume (53).

Another example of target protection is the quinolone resistance protein Qnr, which is a plasmid-mediated

fluoroquinolone resistance determinant frequently found in clinical isolates. Initially described in a clinical isolate of *K. pneumoniae* in the mid-1990s (54), Qnr belongs to the pentapeptide repeat protein family, and it acts as a DNA homologue that competes for the DNA binding site of the DNA gyrase and topoisomerase IV. It is thought that this reduction in the DNA gyrase-DNA interaction decreases the opportunities of the quinolone molecule to form and stabilize the gyrase-cleaved DNA-quinolone complex that is lethal for the cell (55). Several different *qnr* alleles have been described to date, namely *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, and *qnrVC*, all of which have a similar mechanism of action. Notably, the presence of Qnr confers low-level quinolone resistance. However, harboring Qnr-encoding genes has been shown to promote the emergence of highly resistant isolates by facilitating the selection of mutants with point mutations in genes encoding the DNA gyrase and/or topoisomerase IV (56) (the predominant target of the fluoroquinolone class of antibiotics; see below).

Modification of the target site

Introducing modifications to the target site is one of the most common mechanisms of antibiotic resistance in bacterial pathogens affecting almost all families of antimicrobial compounds. These target changes may consist of (i) point mutations in the genes encoding the target site, (ii) enzymatic alterations of the binding site (e.g., addition of methyl groups), and/or (iii) replacement or bypass of the original target. As mentioned, regardless of the type of change, the final effect is always the same: a decrease in the affinity of the antibiotic for the target site. Classical examples of each of these strategies will be detailed below.

Mutations of the target site

One of the classical examples of mutational resistance is the development of rifampin resistance. Rifampin is a rifamycin that blocks bacterial transcription by inhibiting the DNA-dependent RNA polymerase, which is a complex enzyme with a $\alpha_2\beta\beta'\sigma$ subunit structure. The rifampin binding pocket is a highly conserved structure located in the β subunit of the RNA polymerase (encoded by *rpoB*), and after binding, the antibiotic molecule interrupts transcription by directly blocking the path of the nascent RNA (57). High-level rifampin resistance has been shown to occur by single-step point mutations resulting in amino acid substitutions in the *rpoB* gene, and many different genetic changes have been reported. Of note, while these mutations result in decreased affinity of the drug for its target, they usually

spare the catalytic activity of the polymerase, permitting transcription to continue (58).

Another well-characterized example of mutational resistance involves the mechanism of fluoroquinolone resistance (as briefly mentioned above). Fluoroquinolones kill bacteria by altering DNA replication through the inhibition of two crucial enzymes, DNA gyrase and topoisomerase IV. Development of chromosomal mutations in the genes encoding subunits of the above-mentioned enzymes (*gyrA-gyrB* and *parC-parE* for DNA gyrase and topoisomerase IV, respectively) is the most frequent mechanism of acquired resistance to these compounds. Importantly, since fluoroquinolones interact with two enzymes (DNA gyrase and topoisomerase), and both of them are essential for bacterial survival, the level of resistance achieved by developing changes in one of the enzymes depends on the potency with which the antimicrobial inhibits the unaltered target. Thus, in contrast to rifampin, clinically relevant fluoroquinolone resistance frequently requires an accumulation of genetic changes over time, with the first mutation producing minor increases in the MIC (59).

Finally, another good example of antibiotic resistance arising due to mutational changes is resistance to oxazolidinones (linezolid and tedizolid). These drugs are synthetic bacteriostatic antibiotics with broad Gram-positive activity that exert their mechanism through an interaction with the A site of bacterial ribosomes. Such interaction inhibits protein synthesis by interfering with the positioning of the aminoacyl-tRNA. Linezolid is the most widely used antibiotic of this class, because tedizolid was only recently approved for clinical use. Although linezolid resistance remains an uncommon phenomenon, it has been well described in most clinically relevant Gram-positive organisms. The most commonly characterized mechanisms of linezolid resistance include mutations in genes encoding domain V of the 23S rRNA and/or the ribosomal proteins L3 and L4 (*rplC* and *rplD*, respectively), and methylation of A2503 (*E. coli* numbering) in the 23S rRNA mediated by the Cfr enzyme (see below) (Fig. 4) (60).

Mutations in genes encoding the central loop of domain V of the 23S rRNA in the 50S ribosomal subunit are the most frequent determinants of linezolid resistance. A number of mutations have been described to date, and the most frequent change found in clinical isolates appears to be the transition G2576T (*E. coli* numbering). Regardless of the position and type of genetic change, these mutations result in decreased affinity of the drug for its ribosomal target. Since bacteria carry multiple copies of the 23S rRNA genes, mutations need

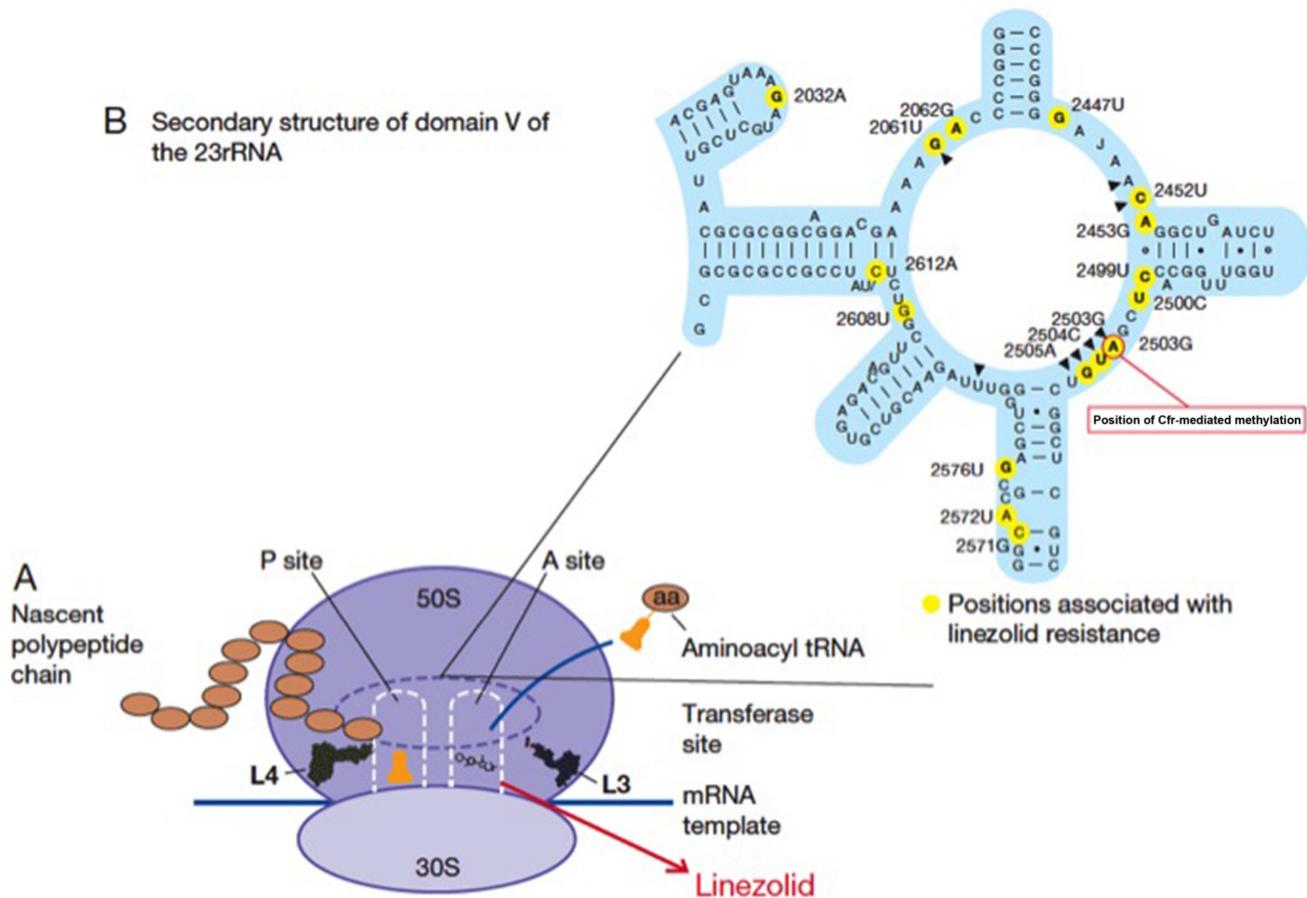


FIGURE 4 Schematic representation of the mechanism of action and resistance to linezolid. **(A)** Linezolid interferes with the positioning of aminoacyl-tRNA by interactions with the peptidyl-transferase center (PTC). Ribosomal proteins L3 and L4 associated with resistance are shown. **(B)** Representation of domain V of 23S rRNA showing mutations associated with linezolid resistance. Position A2503, which is the target of Cfr methylation, is highlighted. Adapted from reference 92.

to accumulate in multiple alleles to yield a clinically relevant phenotype (gene-dose effect) (61). In addition, substitutions in the L3 and L4 ribosomal proteins have also been associated with development of linezolid resistance *in vivo* and *in vitro*, both alone and in combination with other resistance determinants (60).

Enzymatic alteration of the target site

One of the best-characterized examples of resistance through enzymatic modification of the target site is the methylation of the ribosome catalyzed by an enzyme encoded by the *erm* genes (erythromycin ribosomal methylation), which results in macrolide resistance. These enzymes are capable of mono- or dimethylating an adenine residue in position A2058 of domain V of the 23rRNA of the 50S ribosomal subunit. Due to this

biochemical change, the binding of the antimicrobial molecule to its target is impaired. Since macrolides, lincosamides, and streptogramin B antibiotics have overlapping binding sites in the 23S rRNA, expression of the *erm* genes confers cross-resistance to all members of the MLS_B group (62, 63). More than 30 *erm* genes have been described, many of them located in MGEs, which may account for their ample distribution among different genera, including aerobic and anaerobic Gram-positive and Gram-negative bacteria. In staphylococci, the most important *erm* genes are *erm(A)* (mostly distributed in a transposon in methicillin-susceptible *S. aureus* [MRSA]) and *erm(C)* (found in plasmids in MRSA). On the other hand, *erm(B)* has been more frequently reported in enterococci and pneumococci (where it was first described), located in plasmids and

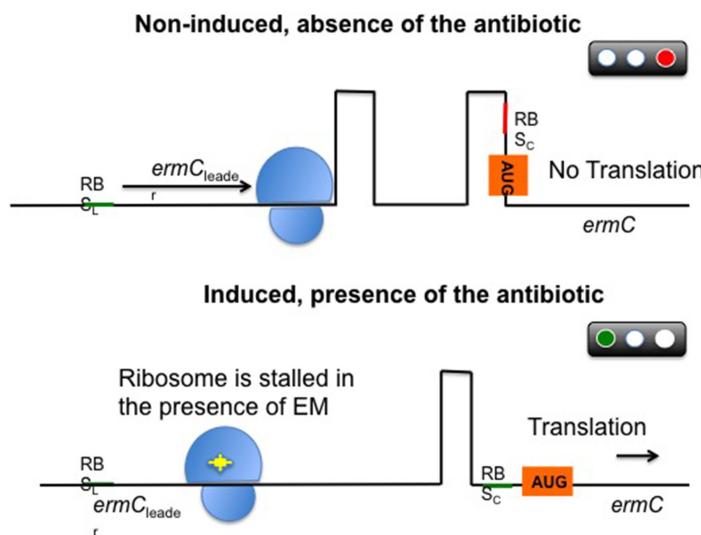
conjugative and nonconjugative transposons such as Tn917 and Tn551. These genes are widely distributed and have now been found in over 30 bacterial genera (64).

Erm-mediated resistance carries an important bacterial fitness cost due to less efficient translation by the methylated ribosome. Hence, although the MLS_B phenotype can be constitutively expressed, in most cases it is subject to strict control via complex posttranscriptional gene regulation. Through this mechanism, bacteria growing in the absence of antibiotics produce an inactive mRNA transcript that cannot be translated into the desired protein (in this case a methylase). Conversely, in the presence of antibiotic, the transcript becomes active and the system is primed to confer rapid resistance. This is best characterized by the inducible MLS_B phenotype of the *erm(C)* operon in *S. aureus*, which is conformed by the *erm(C)* gene, an upstream gene encoding a leader peptide and an intergenic region (Fig. 5). In the absence of an inducer, transcription of the operon generates an mRNA with a secondary structure that conceals the ribosomal binding site upstream of

erm. Translation proceeds through the leader peptide and then terminates, preventing the production of ErmC. In the presence erythromycin (but also other macrolides), the ribosome stalls due to inhibition by the antibiotic during translation of the leader peptide, allowing a conformational change in the *ermC* mRNA that unmasks its ribosomal binding site, resulting in efficient translation of *erm(C)* (65). Thus, bacteria have evolved a sophisticated mRNA-based control mechanism to tightly regulate the expression of these methylases, ensuring a high efficiency of action in the presence of the antibiotic while minimizing the fitness costs for the bacterial population. The array of compounds capable of inducing the MLS_B phenotype varies among different *erm* genes, but as a general rule the best inducer is erythromycin, while the inducing ability of other macrolides varies. Similarly, the system is usually not induced by lincosamides or streptogramins. However, the use of these agents against isolates carrying inducible *erm* genes may result in the selection of constitutive mutants *in vivo* (particularly in severe infections), leading to therapeutic failures.

Another relevant example of enzymatic alteration of the target is Cfr-mediated linezolid resistance. The *cfr* gene is a plasmid-borne determinant initially described in 2000 in a bovine isolate of *Staphylococcus sciuri* and first reported in humans in 2005 in an *S. aureus* strain isolated from a patient in Colombia (66). Since then, it has been found in several species of human pathogens, including *S. aureus*, *E. faecalis*, *E. faecium*, and some Gram-negative bacteria. This gene encodes the Cfr enzyme, which is a member of the S-adenosyl-L-methionine (SAM) methylase family, which also confers resistance to phenicols, lincosamides, pleuromutilins, and streptogramin A. Moreover, *cfr* has been associated with various MGEs, suggesting that it has an enhanced potential to spread and to cause transferable linezolid resistance in the future. Carriage of *cfr* does not appear to confer resistance to the recently FDA-approved oxazolidinone tedizolid (67).

FIGURE 5 Schematic representation of the posttranscriptional control of the *ermC* gene. Under noninducing conditions, the ErmC leader peptide is produced and the *ermC* mRNA forms two hairpins, preventing the ribosome from recognizing the ribosomal binding site (RBS) of *ermC*. As a result, translation is inhibited. After exposure to erythromycin (EM, yellow star), the antibiotic interacts with the ribosome and binds tightly to the leader peptide, stalling progression of translation. This phenomenon releases the *ermC* RBS and permits translation. RBS_L, ribosomal binding site of the leader; RBS_C, ribosomal binding site of *ermC*; AUG, initiation codon. Ribosome represented in blue and erythromycin in yellow.



Complete replacement or bypass of the target site
Using this strategy, bacteria are capable of evolving new targets that accomplish similar biochemical functions of the original target but are not inhibited by the antimicrobial molecule. The most relevant clinical examples include methicillin resistance in *S. aureus* due to the acquisition of an exogenous PBP (PBP2a) and vancomycin resistance in enterococci through modifications of the peptidoglycan structure mediated by the *van* gene clusters. Finally, another route to avoid the

antimicrobial action is to “bypass” the metabolic pathway that the antibiotic inhibits by overproducing the antibiotic target. A relevant example of this mechanism is resistance to trimethoprim-sulfamethoxazole (TMP-SMX). In the remainder of this section we will provide further details of the examples mentioned above.

The antibacterial activity of β -lactams relies on their ability to disrupt cell wall synthesis through inhibition of PBPs, which are important enzymes responsible for the transpeptidation and transglycosylation of peptidoglycan units emerging from the cytoplasm. Resistance to methicillin (a semisynthetic penicillin that is stable against the staphylococcal penicillinase) in *S. aureus* results from the acquisition of a foreign gene (likely from *S. sciuri*) designated *mecA* that is often located in a large DNA fragment designated staphylococcal chromosomal cassette *mec* (SCC*mec*). The *mecA* gene encodes PBP2a, a PBP that has low affinity for all β -lactams, including penicillins, cephalosporins (except for last-generation compounds), and carbapenems. Acquisition of *mecA* renders most β -lactams useless against MRSA, and alternative therapies need to be used in serious infections. Of note, PBP2a carries a transpeptidase domain, but it does not function as a transglycosylase (class B PBP), so it requires the activity of other native PBPs to perform the latter function and fully cross-link peptidoglycan. Specifically, the penicillin-insensitive transglycosylase domain of PBP2 (a class A PBP) is particularly important to achieve transglycosylation of peptidoglycan in the presence of β -lactams in *mecA*-carrying MRSA isolates.

The *mecA* gene is usually found as part of a gene cassette inserted into a larger MGE (SCC*mec*), whose basic components include *meca*, *mecR1* (encoding the signal transducer protein MecR1), *mecI* (encoding the repressor protein Mecl), and *ccr* (encoding a recombinase: cassette chromosome recombinase). To date, 11 SCC*mec* allotypes have been described with varying degrees of genetic homology and different sizes, insertion sequences, and accompanying resistance genes (68, 69). SCC*mec* types seem to differ among different MRSA clones. Indeed, community-associated MRSA strains appear to harbor shorter SCC*mec* cassettes (e.g., SCC*mec* type IV) and carry fewer antibiotic resistance determinants, whereas hospital-associated isolates possess longer elements (e.g., SCC*mec* type II) and are usually MDR (70).

A two-component regulatory system that includes the repressor protein Mecl and the signal transducer MecR1 regulates the expression of *mecA*. Once MecR1 senses the presence of β -lactams in the environment, it triggers

a signal transduction cascade that removes the Mecl repressor from its DNA binding site, resulting in transcription of *mecA* and its regulatory genes. These events culminate with the production of PBP2a, which is the hallmark of methicillin resistance in *S. aureus* (70, 71).

Another important example of the replacement and bypass strategy to achieve resistance is related to vancomycin resistance. Similar to β -lactams, glycopeptides (i.e., vancomycin and teicoplanin) kill bacteria by inhibiting cell wall synthesis. However, unlike β -lactams, glycopeptides do not directly interact with PBPs. Instead, they bind to the terminal D-alanine-D-alanine (D-Ala-D-Ala) of the pentapeptide moiety of the nascent peptidoglycan precursors (lipid II), preventing PBP-mediated cross-linking and resulting in inhibition of cell wall synthesis. It has been postulated that the main effect of the binding of vancomycin to D-Ala-D-Ala-ending precursors emerging from the cytoplasm is alteration of transglycosylation (presumably due to steric hindrance) preventing further processing of the cell wall and leading to bacterial death (72).

Vancomycin resistance is especially relevant in enterococci (particularly *E. faecium*), and it is usually accompanied by the presence of other resistance determinants, making the treatment of infections caused by these organisms an important clinical challenge (73). Vancomycin resistance in enterococci involves the acquisition of a group of genes (designated *van* gene clusters) that code for a biochemical machinery that remodels the synthesis of peptidoglycan by (i) changing the last D-Ala for either D-lactate (high-level resistance) or D-serine (low-level resistance) and (ii) destroying the “normal” D-Ala-D-Ala-ending precursors to prevent vancomycin from binding to the cell wall precursors. The change of D-Ala for D-lactate removes a single hydrogen bond between the vancomycin molecule and its target (D-Ala-D-Ala moiety), decreasing the antibiotic affinity for the precursor about 1,000-fold. Although the change of D-Ala for D-Ser does not remove any of the five hydrogen bonds between vancomycin and its target, the presence of the hydroxyl group of serine affects the interaction of the antibiotic with the precursors, reducing its affinity, albeit less markedly than with the D-Lac replacement (74).

The origin of the *van* genes has been a topic of intense investigation. Genes nearly identical to those of the *vanA* gene cluster (the most prevalent in clinical enterococcal strains) have been found in soil organisms such as *Paenibacillus thiaminolyticus* and *Paenibacillus apiarius* (75). To date, nine distinct enterococcal *van* clusters

have been described (*vanA*, *vanB*, *vanC*, *vanD*, *vanE*, *vanG*, *vanL*, *vanM*, and *vanN*). The *vanADLM* clusters synthesize precursors ending in D-Lac, whereas the *vanCEGN* produce D-Ser-ending peptidoglycan. Most clinical vancomycin-resistant enterococci (VRE) isolates carry the *vanA* or *vanB* gene clusters, which are usually found in MGEs either associated with plasmids or inserted in the chromosome. We will provide further details of the biochemical mechanism of VanA-mediated resistance (involving both vancomycin and teicoplanin). The reader is referred to other comprehensive reviews for additional details on glycopeptide resistance (13, 74, 76).

The *vanA* gene cluster is usually located on a Tn3-family transposon designated Tn1546, which has been found on both conjugative and nonconjugative plasmids. This gene cluster consists of seven genes coding for three groups of proteins: (i) a classical two-component regulatory system that regulates the expression of resistance (VanS is the histidine kinase and VanR the response regulator of the system), (ii) enzymes necessary for the synthesis of new peptidoglycan precursors, namely a dehydrogenase (VanH) and an amino acid ligase with altered substrate specificity (VanA) capable of producing D-Ala-D-Lac, and (iii) enzymes that destroy the normal D-Ala-D-Ala-ending precursors (VanX and VanY). Of note, an additional gene, *vanZ*, is present in Tn1546, but its function remains unknown.

Induction of the *vanA* gene cluster appears to involve initial sensing by VanS of the accumulation of substrates resulting from inhibition of glycosyltransferase activity (77). This initial step results in an ATP-dependent phosphorylation of the response regulator VanR, which subsequently binds to two promoters, one of them located upstream of its own gene (*vanR*) and the other upstream of *vanH* in Tn1546 (78). The *vanH* gene encodes a dehydrogenase enzyme necessary for the production of D-lactate using pyruvate as the substrate. D-Lac is then bound to a molecule of D-Ala by the VanA ligase, and the D-Ala-D-Lac dipeptide is subsequently added to the nascent tripeptide (MurNAc-L-Ala¹-γ-D-Glu²-L-Lys³) to form the altered peptidoglycan unit (UDP-MurNAc-pentadepsipeptide; Mur-NAc-L-Ala¹-γ-D-Glu²-L-Lys³-D-Ala⁴-D-Lac⁵) (Fig. 6).

The other genes of Tn1546 code for enzymes that destroy D-Ala-ending precursors. The *vanX* gene encodes a D,D-dipeptidase that hydrolyzes any D-Ala-D-Ala produced in the “normal” peptidoglycan synthesis pathway, and *vanY* codes for a membrane-bound D,D-carboxypeptidase that removes the last D-Ala of normal ending precursors, ensuring that no D-Ala-D-Ala-ending

pentapeptides (which could potentially bind vancomycin) are exposed on the cell surface. Finally, Tn1546 harbors another gene (designated *vanZ*) that appears to be involved in teicoplanin resistance (but not vancomycin) and whose function is unknown (76).

Development of high-level vancomycin resistance in *S. aureus* (vancomycin-resistant *S. aureus*) was first described in 2002 and was the result of acquisition by an MRSA strain of the *vanA* gene cluster from a VRE (*E. faecalis*) isolate (79). However, occurrence of this phenomenon continues to be rare. Although transfer of an enterococcal plasmid containing the *vanA* gene cluster in Tn1546 to *S. aureus* has been shown to occur *in vitro*, the efficiency of this mechanism is low since replication of enterococcal plasmids in staphylococci is frequently suboptimal. However, a potentially more worrisome scenario is the acquisition of the *vanA* gene cluster by community-associated strains using native staphylococcal plasmids. Indeed, a recent report described such a phenomenon where the *vanA* gene cluster was harbored in a highly transferable staphylococcal plasmid originally identified in community-associated *S. aureus* isolates. The isolate was found in a bloodstream isolate of MRSA recovered from a Brazilian patient (80), and transfer to a methicillin-susceptible isolate within the same patient was also documented.

The *vanB* gene cluster harbors genes similar to those carried by the *vanA* cluster, with the difference that the VanS_B sensor kinase does not appear to be activated by the presence of teicoplanin. Thus, isolates harboring the *vanB* cluster remain susceptible to this glycopeptide. The *vanB* gene cluster is also carried by mobile elements in Tn1547 or related conjugative transposons and has been identified in pheromone-responsive plasmids. In addition, the *vanB* cluster lacks the *vanZ* gene and carries an additional gene (designated *vanW*) whose function remains to be established.

The prototypical gene cluster responsible for low-level vancomycin resistance and the production of D-Ala-D-Ser peptidoglycan precursors is *vanC*. Of note, enterococci carrying the *vanEGLN* clusters also produce D-Ala-D-Ser, exhibit a low level of vancomycin resistance, and carry genes similar to those described in *vanC*. The main differences in terms of gene content and biochemical activities of VanC-type mediated resistance compared with VanA and VanB are that (i) they encode a unique racemase (VanT) capable of producing D-serine using L-serine as a substrate, (ii) they possess a ligase (*vanCEGLN*) with the ability to synthesize D-Ala-D-Ser dipeptides, and (iii) they often harbor a single gene (*vanXY*) encoding both D-Ala-D-Ala dipeptidase and

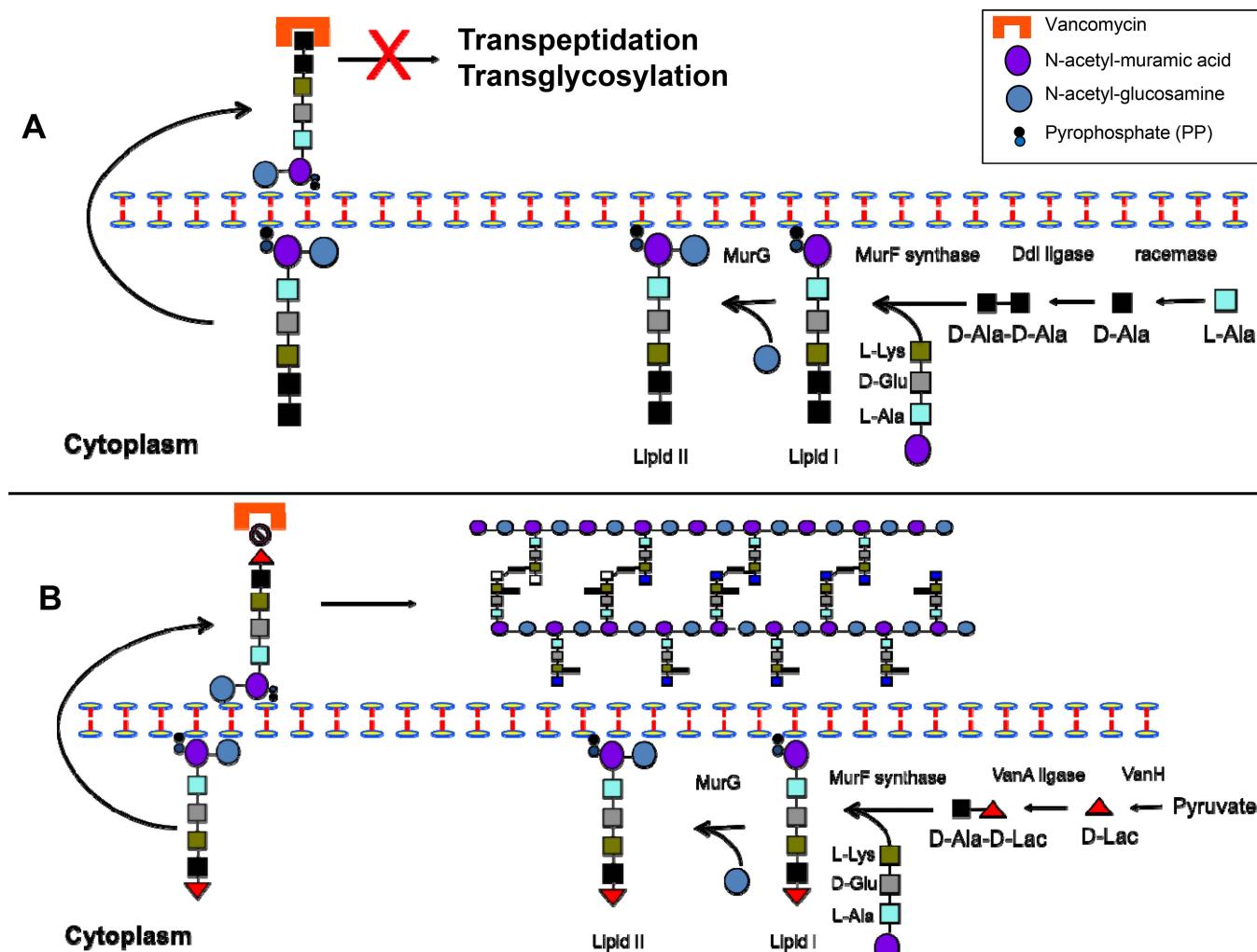


FIGURE 6 Schematic representation of peptidoglycan biosynthesis and mechanisms of vancomycin action (A) and resistance (B). (A) Normal peptidoglycan production. Binding of the antibiotic to the terminal D-Ala-D-Ala of the peptidoglycan precursors prevents transpeptidation and transglycosylation, interrupting cell wall synthesis and resulting in bacterial death. (B) The change in peptidoglycan synthesis produced by the expression of the *vanA* gene cluster. Change of the terminal dipeptide from D-Ala-D-Ala to D-Ala-D-Lac markedly reduces the binding of vancomycin to the peptidoglycan target permitting cell wall synthesis to continue.

carboxypeptidase activities that are normally coded for two distinct genes in the other clusters (*vanX* and *vanY*; see above).

On rare occasions, VRE strains can develop null mutations in the native D-Ala-D-Ala ligase (*ddl*) that abolish the normal production of D-Ala-D-Ala for peptidoglycan synthesis. Thus, strains harboring such mutations rely on the production of altered peptidoglycan precursors for cell wall synthesis by the inducible *van* clusters (e.g., *vanA*). Therefore, cell survival depends on the permanent presence of the antibiotic to induce the

system (hence, these isolates are designated vancomycin-independent enterococci). This phenotype seems to be unstable since mutations in the VanS sensor or promoter regions frequently revert the phenotype (81).

As mentioned above, another well-described strategy of “target bypass” is to increase the production of the antimicrobial target with the objective of overwhelming the antibiotic by increasing the amount of targets available. One of the best examples of this mechanism is the development of resistance to TMP-SMX. This drug impairs bacterial synthesis of purines

and some important amino acids by altering the production of folate, exploiting the fact that most bacteria are unable to incorporate folate from external sources. Therefore, bacteria rely on their own biochemical machinery for folate synthesis. The synthetic pathway of folate involves two major enzymes: (i) dihydropteroic acid synthase (DHPS), which forms dihydrofolate from para-aminobenzoic acid (inhibited by SMX), and (ii) dihydrofolate reductase (DHFR), which catalyzes the formation of tetrahydrofolate from dihydrofolate (inhibited by TMP).

Although development of resistance to TMP-SMX can be achieved through several strategies including amino acid changes in the above enzymes (decreasing their affinity for the antibiotic molecules, target modification) and acquisition of external genes encoding DHPS or DHFR that are less sensitive to inhibition by TMP-SMX (target bypass), a clever bypass strategy is the overproduction of DHFR or DHPS through mutations in the promoter region of the DNA encoding these enzymes. These mutations result in the production of increased quantities of the above enzymes, overwhelming the ability of TMP-SMX to inhibit folate production and permitting bacterial survival (82, 83). Interestingly, enterococci use another bypass strategy by incorporating exogenous tetrahydrofolic acid and folic acid when added to the media. This ability to use folate from different sources is correlated with an up to 25-fold increase in the MICs of TMP-SMX and is thought to impair the antimicrobial activity *in vivo* (84, 85).

Resistance Due to Global Cell Adaptations

Through years of evolution, bacteria have developed sophisticated mechanisms to cope with environmental stressors and pressures in order to survive in the most hostile environments, including the human body. Bacteria need to compete for nutrients and avoid the attack of molecules produced by rival organisms to gain the “upper hand.” Inside a particular host, bacterial organisms are constantly attacked by the host’s immune system, and to establish themselves in particular biological niches, it is crucial that they adapt and cope with these stressful situations. Thus, bacterial pathogens have devised very complex mechanisms to avoid the disruption of pivotal cellular processes such as cell wall synthesis and membrane homeostasis. Development of resistance to daptomycin (DAP) and vancomycin (low-level in *S. aureus*) are the most clinically relevant examples of resistance phenotypes that are the result of a global cell-adaptive response to the antibacterial attack.

DAP is a lipopeptide antibiotic related to cationic antimicrobial peptides produced by the innate immune system that exerts its bactericidal effect by altering cell envelope homeostasis. The bactericidal activity of DAP requires four important steps (Fig. 7). First, DAP is complexed with calcium (rendering the molecule positively charged) and, subsequently, is directed to the cell membrane (CM) target by electrostatic interactions with the usually negatively charged CM. Of note, recent evidence suggests that DAP mainly targets the CM at the level of the division septum (86). Second, once the antibiotic molecules reach the CM, they initially oligomerize at the outer leaflet of the CM and, subsequently, these DAP oligomers reach the inner CM leaflet. DAP’s oligomerization at the outer leaflet of the CM appears to be dependent on the presence of the phospholipid phosphatidylglycerol (PG) (87). Additionally, another phospholipid (cardiolipin), seems to play an important role in the translocation of DAP oligomers from the outer to the inner leaflet, but its contribution is not completely understood. In fact, there is evidence to suggest that the presence of cardiolipin in high concentrations may prevent translocation of DAP oligomers into the inner leaflet of the phospholipid bilayer (87). Third, once the DAP oligomers reach the inner leaflet of the CM, they organize and form transmembrane pore-like structures that are likely to alter the physicochemical properties of the CM and promote leakage of ions (e.g., potassium) from the cytoplasm, causing important electrochemical alterations. Finally, these structural and functional CM alterations lead to bacterial death in the absence of cell lysis by mechanisms that are not fully understood.

Bacteria have developed ancient systems of defense to withstand cAMP action and possess a cadre of regulatory systems that are involved in protecting the cell envelope when under attack by cationic antimicrobial peptides. In enterococci, work using whole-genome sequencing of a clinical strain-pair of *E. faecalis* that developed DAP resistance (DAP-R) over the course of therapy revealed that changes in a three-component regulatory system designated LiaFSR (which orchestrates the cell-envelope stress response in Gram-positive organisms) are paramount in the development of DAP-R (88). In *Bacillus subtilis*, where the system was first characterized, and other Gram-positive bacteria, LiaFSR (and the homolog system VraTSR in *S. aureus*) is composed of three proteins: (i) LiaF (VraT), a transmembrane protein that appears to negatively regulate the system, (ii) LiaS (VraS), a classical sensor-histidine kinase protein that phosphorylates the response regulator,

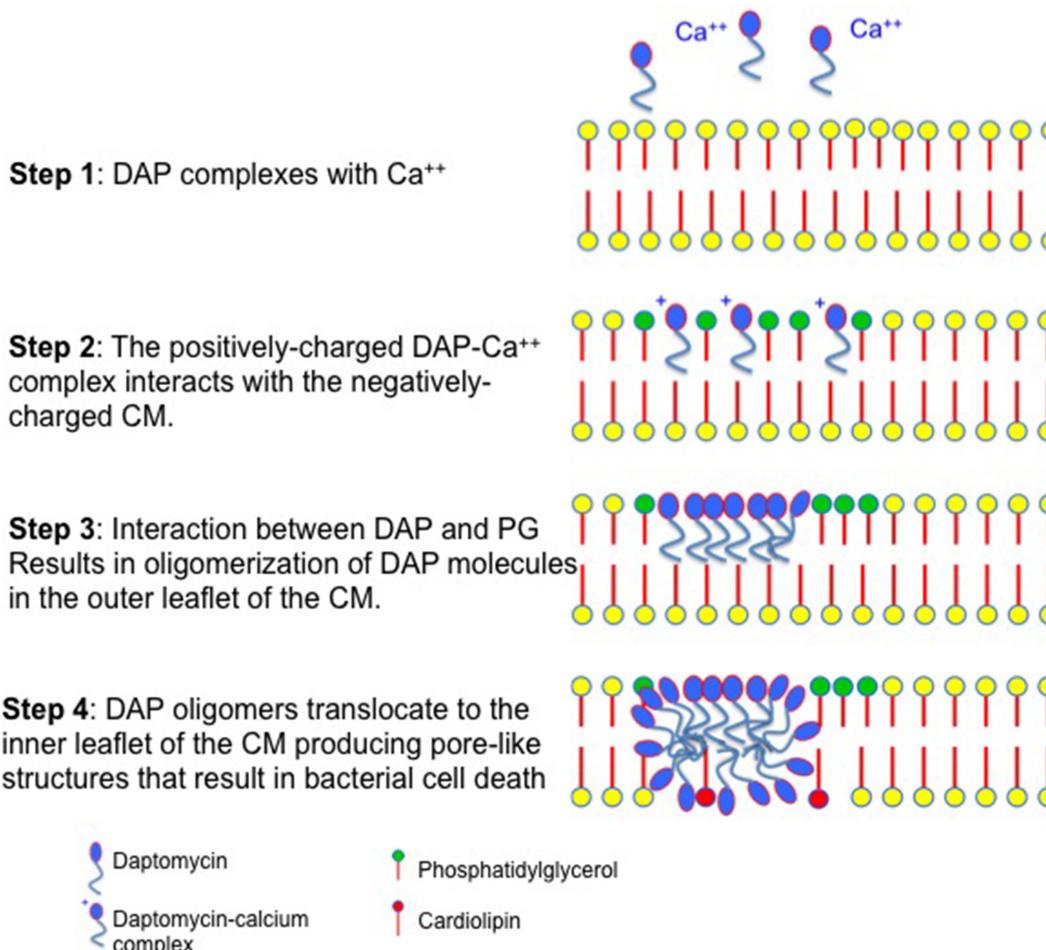


FIGURE 7 Diagrammatic representation of the mechanism of action of daptomycin. DAP, daptomycin; PG, phosphatidylglycerol; CM, cell membrane.

and (iii) LiaR (VraR), the response regulator of the system. Indeed, a single deletion of an isoleucine at position 177 of LiaF increased the DAP MIC from 1 to 4 µg/ml (established clinical breakpoint is 4 µg/ml) and, more importantly, was sufficient to abolish the bactericidal activity of DAP (89). Moreover, in recent genomic analyses of 19 DAP-nonsusceptible *E. faecium* (DAP MICs from 3 to 48 µg/ml; clinical breakpoint is 4 µg/ml), the most frequently identified mutations were in *liaFSR*, supporting the hypothesis that changes in this system are a pivotal step toward DAP-R in enterococci (90). Furthermore, the majority (75%) of DAP-susceptible *E. faecium* isolates recovered from bacteremic patients whose MIC was in the higher range of susceptibility (i.e., between 3 and 4 µg/ml) harbored mutations in *LiaFSR*. Conversely, none of the isolates of the same collection with DAP MIC ≤ 2 µg/ml exhibited changes in this system (91). More importantly, these changes were sufficient to abolish the *in vitro* bactericidal activity of DAP

and were associated with a clinical failure in a neutropenic patient with VRE bacteremia (92).

The mechanism by which *LiaFSR* results in DAP-R is not fully understood. Furthermore, the specific mechanism through which this system orchestrates the cell-envelope response to stress is still a matter of active research. In *B. subtilis*, the *lia* locus consists of six genes, *liaIH-liaGFSR*, of which *liaGFSR* are constitutively expressed at a low basal level due to the presence of a weak constitutive promoter upstream of *liaG*. In contrast, expression of *liaIH* is completely *LiaR*-dependent. Although *LiaR* regulates other genes, Wolf et al. provided evidence indicating that *liaIH* is the only relevant target of *LiaR*-dependent gene expression in wild-type cells. The physiological role of *LiaI* and *LiaH* are not completely understood. *LiaI* is a small hydrophobic protein of unknown function with two putative transmembrane helices, and *LiaH* is a member of the phage-shock protein family that forms large oligomeric

ring-like structures (resembling what has been reported with PspA in *E. coli*). The LiaFSR system constitutes a cell envelope stress-sensing/response system that is highly conserved in *Firmicutes* bacteria (93).

In enterococci, recent evidence suggests that LiaR mediates a reorganization of anionic phospholipids (i.e., cardiolipin) in the CM associated with DAP-R. In a clinical strain of DAP-R, development of resistance was clearly associated with redistribution of cardiolipin microdomains from the septum to other CM areas (94). This mislocalization seems to divert DAP from its principle CM septal target, resulting in bacterial survival of the DAP attack. Furthermore, deletion of LiaR completely reverted DAP susceptibility and restored the organization of cardiolipin domains (95).

Other regulatory systems involved in cell envelope homeostasis have also been associated with DAP-R. For example, YycFG (WalKR), an essential two-component regulatory system that has been implicated in cell-wall synthesis and homeostasis, has been found to be important to DAP-R in both enterococci and *S. aureus* (96, 97). Although the exact mechanism mediating this phenomenon has not been fully elucidated, it appears to involve alteration in cell wall metabolism that results in changes in surface-charge-producing electrostatic repulsion of the positively charged calcium-DAP complex from the cell envelope.

A second group of genes that have been shown to contribute to the development of DAP-R correspond to enzymes involved in the metabolism of CM phospholipids. For example, two enzymes, a glycerol-phosphodiester phosphodiesterase (GdpD) and cardiolipin synthase (Cls), were found to enhance the DAP-R phenotype in the background of *liaFSR* mutations in *E. faecalis* (88). These changes seem to alter the CM phospholipid composition mainly by decreasing the amount of PG. Other enzymes such as MprF, PG synthase (PgsA), cyclic fatty acid synthase (Cfa), and geranyltransferase (98) involved in CM phospholipid homeostasis have also been linked to DAP-R. In *S. aureus*, MprF (a lysyl-PG synthase) has been one of the most studied enzymes, and inactivation of this protein reversed DAP-R. This enzyme harbors two domains: (i) a lysyl-transferase domain that transfers the positively charged amino acid lysine from its tRNA carrier to PG (lysyl-PG) in the inner leaflet of the CM and (ii) a flipase domain, through which newly synthesized lysyl-PG is translocated from the inner to the outer leaflet of the CM (99). Mutations in *mprF* appear to produce a gain of function of the enzyme and, as a result, the cell surface becomes more positively charged, repelling the DAP-calcium complex (also with positive charge). Of interest,

a homolog of LiaFSR (VraTSR) seems also to contribute to the DAP-R phenotype mediated by changes in MprF in *S. aureus* (97).

The development of high-level vancomycin resistance mediated by the acquisition of the *van* gene cluster is a rare event in *S. aureus*. However, a much more common problem is the finding of *S. aureus* isolates with intermediate susceptibility to vancomycin (known as VISA isolates), exhibiting MICs between 4 to 8 µg/ml. This phenomenon was first reported in Japan in 1997 and led to therapeutic failure (100). The isolate, designated Mu50, was derived from a vancomycin-susceptible strain known as Mu3 (\leq 2 µg/ml). Population analyses of Mu3 later confirmed that this strain contained a subpopulation of bacterial cells capable of surviving at concentrations of vancomycin above 2 µg/ml (clinical breakpoint for susceptibility), a phenomenon that has now been designated the heterogenous-VISA (hVISA) phenotype. Because the resistant subpopulation is difficult to identify, detection of hVISA becomes very challenging, and some *S. aureus* strains reported as susceptible to vancomycin by standard susceptibility testing may still exhibit this phenotype (101). Indeed, *S. aureus* within the range of susceptibility, with vancomycin MICs >1 and \leq 2 µg/ml have been more frequently associated with the hVISA phenotype. Due to the difficulties in detecting these strains, vancomycin failures have been increasingly reported in deep-seated infections. Published studies estimate that the overall prevalence of MRSA strains with an hVISA/VISA profile range between 0 and 8.24%, but it can be as high as 30% in selected populations (e.g., patients with MRSA infective endocarditis) (102, 103).

The hVISA/VISA isolates usually emerge *in vivo* in patients with a history of an MRSA infection that failed to respond to a prolonged course of vancomycin therapy. From a mechanistic point of view, the development of the hVISA/VISA does not occur by the acquisition of foreign DNA material (as seen in vancomycin-resistant *S. aureus*); rather, the phenotype appears to be the result of sequential and ordered genetic changes that usually involve genes forming part of regulatory systems controlling cell envelope homeostasis (similar to that described above for DAP). The specific mechanisms that lead to the hVISA/VISA phenotype remain to be completely understood. However, the available evidence shows that the regulatory systems most consistently implicated in this mechanism of resistance are YycFG (WalKR), VraSR (homolog of LiaFSR), and GraRS (104). Interestingly, these two- and three-component regulatory systems are involved in cell wall homeostasis,

supporting the notion that selection of the hVISA/VISA phenotype involves important remodeling of the cell wall to survive the antimicrobial attack.

Apart from the above regulatory systems, another change that has been frequently associated with the VISA phenotype is mutations in *rpoB* (encoding the B subunit of the RNA polymerase). Indeed, Watanabe et al. analyzed 38 VISA isolates from 10 countries and demonstrated that mutations in the *rpoB* gene were present in the majority (71%) of the isolates (105). However, the mechanisms by which mutation of *rpoB* led to reduced vancomycin and DAP susceptibility are unclear.

Phenotypically, hVISA/VISA strains exhibit distinct metabolic characteristics that may include (i) increased fructose utilization, (ii) increased fatty acid metabolism, (iii) impaired acetate metabolism and tricarboxylic acid cycle, (iv) decreased glutamate availability, and (iv) increased expression of cell wall synthesis genes. These global homeostatic changes appear to lead to a reduced autolytic activity with a thickened cell wall and an increased amount of free D-Ala-D-Ala dipeptides, with less peptidoglycan cross-linking (101, 104). In addition, VISA strains bind vancomycin more avidly than their non-VISA counterparts, but diffusion of the antibiotic molecule into the inner part of the cell wall appears to be impaired. Hence, it has been postulated that these changes result in trapping of vancomycin in the outer layers of the peptidoglycan, preventing the antibiotic molecule from reaching its target of precursors emerging from the cytoplasmic membrane. As a result, cell wall synthesis and peptidoglycan cross-linking continue to be uninterrupted.

Finally, a striking feature of many hVISA/VISA strains is their ability to revert from one phenotype to another (or even to a fully vancomycin-susceptible phenotype) in the absence of vancomycin exposure. Therefore, there seems to be a price to pay for developing resistance, and this is yet another example of the ability of bacteria to adapt to the environment by means of their remarkable genetic plasticity.

CONCLUDING REMARKS

The use of antimicrobials in clinical practice is a recent development compared to the emergence of bacterial organisms on our planet. Therefore, the development of antibiotic resistance should be viewed as a normal adaptive response and a clear manifestation of Darwin's principles of evolution. Arguably, the implementation of antimicrobial therapy in clinical practice has been one of

the most successful advances of modern medicine, paving the way for complex and highly sophisticated medical interventions that have allowed us to significantly prolong the life span of the population around the globe. To survive, bacteria, in a process likely pressed by the increased use of antimicrobials in clinical practice, have developed complex and creative strategies to circumvent the antibiotic attack. Antibiotic resistance has rapidly evolved in the past few decades to become one of the greatest public health threats of the 21st century. Indeed, infections that are untreatable due to multidrug resistance of the infecting organism have become more common in clinical settings. This dire scenario has been worsened by a shortage of research and development on antibiotics. The "golden" pipeline of antibiotic discovery (1960s and 1970s) rapidly dried up as the identification of new compounds became more challenging. Big pharma concentrated their efforts on other more profitable and rewarding areas, leaving the wave of resistance to grow unabated.

If we are to tackle this problem, efforts in research and development need to be heavily increased and supported. A complete understanding of the mechanisms by which bacteria become resistant to antibiotics is of paramount importance to designing novel strategies to counter the resistance threat. We are in need of developing antibiotics, with the understanding that the microorganism will respond to them and resistance will develop (an evolutionary fact). Therefore, efforts to develop antibiotics and study mechanisms of resistance should be continuous, resilient, and steady. This is likely to be a long haul "war" against living entities with a major ability to adapt and survive.

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