4
Antibiotics and Resistance: A Fatal Attraction

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4.1
To Be or Not to Be Resistant: Why and How Antibiotic Resistance Mechanisms Develop and Spread among Bacteria

The continual battle between humans and the multitude of microorganisms that cause infections and diseases has caused significant morbidity and mortality throughout history. The situation significantly improved when penicillin and other classes of antibiotics were discovered and used to treat infectious diseases. However, almost as soon as antibacterial drugs were introduced in clinics, bacterial resistance spread [1, 2].

Antibiotic resistance can be defined taking into account the pharmacokinetic and pharmacodynamic criteria to determine values above which a therapeutically useful concentration is difficult to obtain. If the minimal inhibitory concentration (MIC) for a bacterium is above those concentration values, a risk exists that the infection cannot be successfully treated. Therefore, the microorganisms are classified as resistant when their MICs are above a predefined threshold.

Bacterial resistance is a concern for several reasons. From a medical, social, and economical viewpoint, resistant bacteria, becoming commonplace in healthcare institutions, often result in treatment failure and this implies an added burden on healthcare costs [3]. In addition, resistant bacteria may also spread and become broader infection-control problems, not only within healthcare institutions but in communities as well [4, 5]. From a biological and microbiological viewpoint, antibacterial drug resistance is a fascinating aspect of molecular evolution and selection of fine mechanisms that allow survival under unfavorable circumstances. In particular, under the selective pressure of antibiotics, bacteria evolve and spread resistance mechanisms that become common to pathogenic and nonpathogenic strains. To fully understand the evolution of resistance, the maintenance of resistance genes within microbial populations and the spread of these genes between species and genera, the concept of ''resistome'' was introduced [6]. The resistome includes the totality of those genetic elements whose function is to counteract toxic effects of antibiotic drugs. Furthermore, the resistome also comprises the collection of genes, called *protoresistance genes*, which have the

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potential to evolve into resistance elements [7]. Many resistance genes have been isolated from clinically relevant strains and from the vast reservoir of environmental nonpathogenic organisms.

Antibiotic-producing environmental bacteria most probably are the original source of many resistance enzymes, reflecting a continuous evolutionary pressure where antibiotic biosynthesis and resistance coevolve [7, 8]. In fact, in soil environments, evolutionary pressure promotes the development and spread of resistance genes among pathogenic and nonpathogenic bacterial genera. This hypothesis is supported by the presence of resistance elements in antibiotic-producing bacteria that have orthologs in clinical isolates [9–12]. Anyway, antibiotic-producing bacteria could not be the sole source of resistance genes. In fact, bacterial genomes contain an unexpected number of genes encoding putative resistance proteins [13–15], which could have originated through amplification and random mutation of genes not originally involved in antibiotic resistance, [16]. Primary sequence analysis of resistance proteins, determination of their molecular mechanisms, and three-dimensional structures revealed homologies to known metabolic and signaling enzymes with no antibiotic-resistance activity [7]. Therefore, it is possible that resistance genes originally derived from elements having other metabolic functions, similar to housekeeping genes encoding enzymes with modest and fortuitous resistance properties, evolved into resistance enzymes as a result of selective pressure of antibiotic exposure. The fact that resistance genes are so widespread in the environment and that even resistance to synthetic antibiotics can be readily selected reveals the plastic nature of the link between molecular evolution and resistome, whose origins may predate the actual antibiotic era [17].

4.1.1 Horizontal and Vertical Transmission of Resistance Genes

Despite the wide range of chemical complexity of antibiotics, there are five major modes of action (interference with cell-wall synthesis, inhibition of protein synthesis, interference with nucleic acid synthesis, inhibition of cofactor biosynthetic pathways and membrane pore formation) and bacteria may manifest resistance to antibacterial drugs through a restricted range of molecular events (Table 4.1; Figure 4.1). In particular, some bacterial species are considered intrinsically resistant to a class of antibiotics because the drug cannot reach its cellular target or because the drug is not able to recognize its target which possesses the same function but a different structure. As a case of intrinsic genetic arrangement conferring resistance to β-lactams, the *Pseudomonas aeruginosa* resistome [18] is described in this chapter (Section 4.5.1). On the other hand, susceptible bacteria may become resistant to a class of antibiotics through two types of genetic events:

1) random spontaneous mutation;
2) acquisition of the genetic information encoding resistance from other bacteria.

Table 4.1 Cellular targeting of antibiotic compounds and resistance strategies.

Mechanism of action	Antibiotic target	Antibiotic class (examples)	Mode of resistance	Resistance gene examples (products)
Interference with cell-wall synthesis	Transpeptidases	β-Lactams (penicillins, cephalosporins, carbapenems, monobactams)	Hydrolysis Efflux Altered target	*ampC*, *blaZ* (β-lactamases) *oprM* *mecA* (low-affinity PBP2a)
	D-Ala-D-Ala	Glycopeptides (vancomycin, teicoplanin)	Altered target	*vanRS* (two-component system) *vanHAX* (dehydrogenase, didpeptidase, ligase) *vanY* (peptidase)
	Lipid II	Lantibiotics (nisin)	Hydrolysis Efflux Binding	*nsr* (protease) *nisEFG* (transporter system) *nisI* (lipopeptide)
	MurA	Epoxide (fosfomycin)	Hydrolysis	*fosA*, *fosX*
Protein synthesis inhibition	50S ribosomal subunit	Macrolides (erythromycin, chloramphenicol, linezolid, tylosin)	Hydrolysis Glycosylation Acetylation Phosphorylation Efflux Altered target	*ereA*, *ereB* (macrolide esterase) *mtg*, *oleI*, and *oleD* (glycosyl-transferases) *cat* (acetyltransferase) *cpt*, *mph* *mtd(A)* *ermE* (23S rRNA methyltransferase)
		Lincosamides (lincomycin, clindamycin)	Nucleotidylation Efflux	*linA*, *linB* *mtd(A)*

(*continued overleaf*)

Table 4.1 (*Continued*)

Mechanism of action	Antibiotic target	Antibiotic class (examples)	Mode of resistance	Resistance gene examples (products)
	30S ribosomal subunit	Aminoglycosides (apramycin, streptomycin, spectinomycin, gentamycin)	Phosphorylation Acetylation Nucleotidylation Efflux Altered target	*aphI*, *neo*, and *km* (phosphotranferases) *aac*(3)IV (apramycin acetyltransferase) *aadA* (streptomycin adenyltransferase) *norM* (transporter) *grmM*, *kamC*, and *kan* (16S rRNA methyltransferases)
		Tetracyclines (chloramphenicol, tigecycline)	Monooxygenation Efflux Acetylation Altered target	*tetX* (monohydroxylase) *tetA*, *mtd(A)* *cat* *tetO*, *tetM*, and *otrA* (ribosome protection proteins)
Nucleic acid synthesis inhibition	DNA synthesis enzymes	Quinolones and fluoroquinolones (ciprofoxacin)	Acetylation Efflux Altered target	*aac*6′-ib *norA*, *norM*, *acrAB* *gyrA*, *parC*
		Aminocoumarins (novobiocyn)	Efflux Altered target	*simX* *gyrB*

	RNA synthesis enzymes	Rifampicin (rifamycin, rifampin)	ADP-ribosylation Monooxygenation Efflux Altered target	*arr* (ADP-ribosyltransferase) *acrAB* *rpoB*
Metabolic pathway inhibition	Folic acid synthesis	Sulfonamides (sulfamethoxazole)	Efflux Altered target	*acrAB* *sul1, sul2* (dihydropteroate synthetase)
Disruption of bacterial membrane	Cell membrane	Polymyxin (colistin)	Efflux Altered target	*acrAB* *pmrAB* (Two component system), *pmrEF* (UDP-glucose dehydrogenase, glycosyltransferase)
		Lipopeptides (daptomycin)	Altered target	*cls* (cardiolipin synthase)

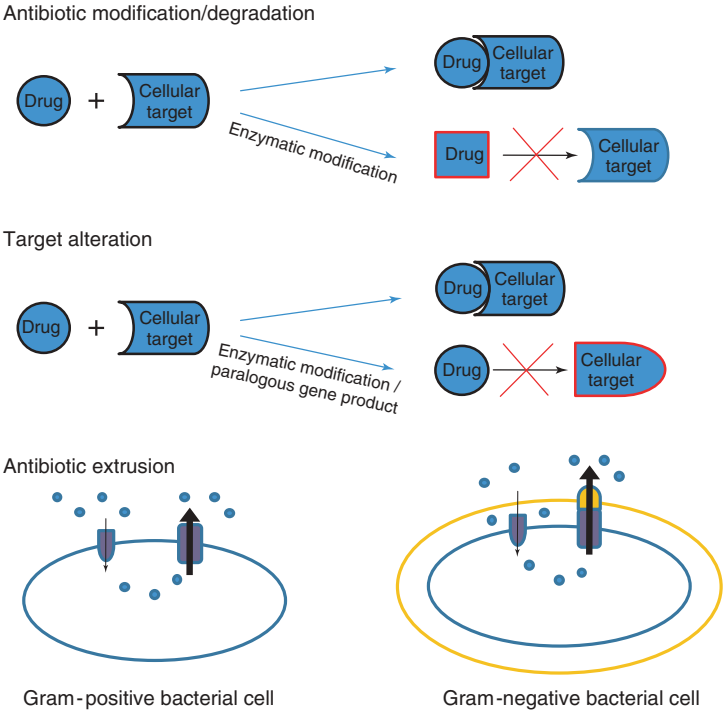

Figure 4.1 Schematic representation of major resistance strategies. Antibiotics can be destroyed or chemically modified by enzymes produced by resistant bacteria. On the other hand, antibiotic targets can be altered to ward off antibiotic recognition. When antibiotic target is located inside cells, antibiotic–target interaction can be prevented by pumping the antibiotic out from cells through efflux pumps to keep low the intracellular drug concentrations.

In susceptible bacteria that acquire resistance by spontaneous mutations resistance may be conferred by:

1) modification or loss of the target with which the antibiotic interacts (e.g., change in penicillin-binding protein 2b in *Pneumococci*, which results in penicillin resistance);
2) upregulation of enzymes that inactivate the antimicrobial agent (e.g., β-lactamases that destroy the β-lactame antibiotics) or that modify the antibiotic target (e.g., ribosomal methylase in *Staphylococci* preventing erythromycin binding);
3) downregulation or inactivation of the outer membrane protein channel required by the drug for cell entry (e.g., OmpF in *Escherichia coli*);
4) upregulation of pumps that expel the drug from the cell (e.g., efflux of fluoroquinolones in *Staphylococcus aureus*).

In all these cases, strains of bacteria carrying chromosomal mutations conferring resistance survive and grow under the selective pressure of antibiotic use, which

instead kills the susceptible strains and promotes spreading of resistant genotypes. This kind of selection is named *vertical evolution* because resistance-associated genetic elements are transmitted from cell to cell through cell duplication [2, 19].

Bacteria also develop resistance through the acquisition of new genetic material from resistant organisms. This kind of selection is termed *horizontal evolution*, and may occur in an intra- or interspecific way or even among different genera and may be facilitated by transposable elements such as transposons, which contain resistance genes [19]. Genetic exchange mechanisms include events such as conjugation, transduction, and transformation [2, 19]. During conjugation, a gram-negative bacterium transfers a plasmid carrying resistance genes to a recipient bacterium through a mating bridge, which joins the two bacteria. In gram-positive bacteria, exchange of DNA by conjugation is usually triggered by sex pheromones, which facilitate the clumping of donor and recipient cells. During transduction, resistance genes are transferred via bacteriophage. Finally, the so-called competent bacteria may acquire and incorporate resistance genes from other bacteria that have released their DNA into the environment after cell lysis, by transformation [2, 19]. Through genetic exchange mechanisms, many bacteria become resistant to multiple classes of antibacterial agents, and these multidrug-resistant (MDR) bacteria (e.g., resistant to at least three antibacterial drug classes) are a serious problem, particularly in hospitals and other healthcare institutions where they occur very commonly.

Mutation, genetic exchange, and selection cause quick adaption to the introduction of antibiotic drugs into their environment. In rare cases, a single mutation may be sufficient to confer high-level resistance on an organism (e.g., high-level rifampicin resistance in *S. aureus* or high-level fluoroquinolone resistance in *Campylobacter jejuni*). In most cases, a single event, even if in a key bacterial gene, may only slightly reduce the susceptibility to an antibiotic, but it may be just enough to allow its initial survival until it acquires additional mutations or additional genetic information resulting in a high resistance level [2]. As an example of a gene acquisition/mutation series conferring resistance, the organism may first acquire gene-encoding enzymes that destroy the antibiotic, thus reducing its overall concentration; then, bacteria may acquire efflux pumps that extrude the antibacterial agent from the cell. Finally, bacteria may acquire several genes or accumulate mutations that produce a product not recognized by the antibiotic agent, or in the case of gram-negative bacteria, may acquire mutations that limit access to the intracellular target via downregulation of porin genes. As a real case of gene acquisition series, resistance mechanisms of *S. aureus* [20] are described in this chapter (Section 4.5.2).

4.2 Bacterial Resistance to Antibiotics by Enzymatic Degradation or Modification

Antibiotic resistance coevolved with biosynthesis as a means of bacterial self-immunity strategies for the production of toxic secondary (e.g., dispensable for

bacterial growth, at least under laboratory conditions) metabolites in antibiotic-producing bacteria [7, 8]. This coevolution strategy could have independently evolved *de novo* in nonproducing organisms or could be imported via horizontal gene transfer. The genes for resistance, stably integrated into the genome under selective pressure, reflect prior exposure during the evolution of the species. This idea is also consistent with the hypothesis that naturally produced antibiotics do not exert antibiotic activity at the concentrations present in the environment, but rather they play a role as signaling molecules [7, 21] and resistance elements could have evolved as receptors or mediators of such signaling molecules. Furthermore, antibiotic inactivation mechanisms share many similarities with well-characterized enzymatic reactions involved in primary metabolism [7]. Enzymes that confer resistance by destroying or modifying antibiotics utilize a set of chemical strategies that can be functionally grouped into hydrolysis, group transfer, and redox mechanisms (Table 4.2) [7, 12].

4.2.1 Antibiotic Resistance by Hydrolytic Enzymes

The integrity of chemical structure is essential for antibiotic activity. Thus, several kinds of enzymes confer resistance by targeting and cleaving chemical bonds that are hydrolysis prone. The best-known examples are the amidases that cleave the β-lactam ring of the penicillin and cephalosporin classes of drugs. Other examples include esterases (macrolide resistance) and ring-opening epoxidases (fosfomycin resistance). These enzymes require water for catalysis and are excreted by bacteria, so that they intercept the antibiotics before they come into contact with their bacterial target [7, 12].

Table 4.2 Antibiotic resistance by enzymatic modification.

Strategy	Type	Example enzymes	Targeted antibiotic classes
Hydrolysis		BlaZ	β-Lactams
		EreA, EreB	Macrolides
		FosA, FosX	Epoxides
Group transfer	Phosphoryl	APH(3′)	Aminoglycoside
		MPH	Macrolide
	Acyl	CAT	Chloramphenicol
		AAC(6′)	Aminoglycoside
	Nucleotidyl	ANT(2′)	Aminoglycoside
		LinA, LinB	Lincosamide
	ADP-ribosyl	ARR	Rifamycin
	Glycosyl	Mtg	Macrolide
		Not characterized	Rifamycin
Redox	Oxidation	TetX	Tetracycline
		Iri	Rifamycin

4.2.1.1 β-Lactamases

The first antibiotic-resistance strategy reported in the literature is the production of the β-lactamase penicillinase by pathogenic *E. coli* [1]. There are two main classes of β-lactamases based on the molecular mechanism of hydrolysis of the β-lactam ring: (i) Ser-β-lactamases, such as BlaZ, that work through the action of a Ser nucleophile active site and (ii) metallolactamases that activate water through a Zn^{2+} center [7, 12]. β-Lactams bind peptidoglycan transpeptidase preventing cross-linking, eventually compromising cell-wall integrity (Figure 4.2). Indeed, there is a similarity between peptidoglycan transpeptidases and Ser-β-lactamases concerning molecular mechanism of action and three-dimensional structure. Therefore, it has been speculated that peptidoglycan transpeptidases and BlaZ-like lactamases are evolutionarily linked [7, 12, 22]. The *blaZ* gene is present in plasmids and its expression is under the control of two regulatory genes, *blaI* and *blaR1*. The product of the latter gene is a sensor-transducer, which, in the presence of penicillin, initiates a cascade of events that leads to enhanced penicillinase expression. Metallo-β-lactamases are members of the Zn-dependent hydrolase family and are a significant cause of resistance to carbapenems in gram-negative bacteria [7, 12, 23].

4.2.1.2 Macrolide Esterases

The macrolide antibiotics, such as erythromycin, block the peptide exit tunnel of the large subunit of the ribosome and, as a result, interfere with protein synthesis. Macrolides are cyclized by a thioesterase responsible for the ring closure step that generates 6-deoxyerythronolide B (for the 15-member erythromycin) macrocyle [12, 24]. Therefore, this key bond is targeted by macrolide-resistance enzymes operating in reverse ring-opening mode. Two erythromycin esterases, encoded by *ereA* [25] and *ereB* [26] genes and first isolated from two different *E. coli* strains, share 43% similarity [12]. Both proteins result in very high levels of resistance in *E. coli* [27]. The presence of these genes on mobile genetic elements [28] implies their ability to become widespread in the microbial community and the presence of esterases has been confirmed in at least one clinical isolate of *S. aureus* [29] and in environmental isolates of *Pseudomonas* sp. [30].

4.2.1.3 Epoxidases

The epoxide antibiotic fosfomycin covalently modifies the enzyme MurA, an essential protein required for the synthesis of *N*-acetylmuramic acid, one of the sugarbuilding blocks of cell-wall peptidoglycan. Enzymatic resistance to this antibiotic occurs through destruction of the reactive epoxide by ring opening [12]. The enzyme FosX, whose gene was first isolated from the nonpathogenic soil bacterium *Mesorhizobium loti* [31] and FosA, a metalloenzyme found in gram-negative bacteria [32], catalyze epoxide ring opening through water- and glutathione-dependent reactions, respectively [12]. Both enzymes require a catalytically important divalent metal cation (Mn^{2+}) [12].

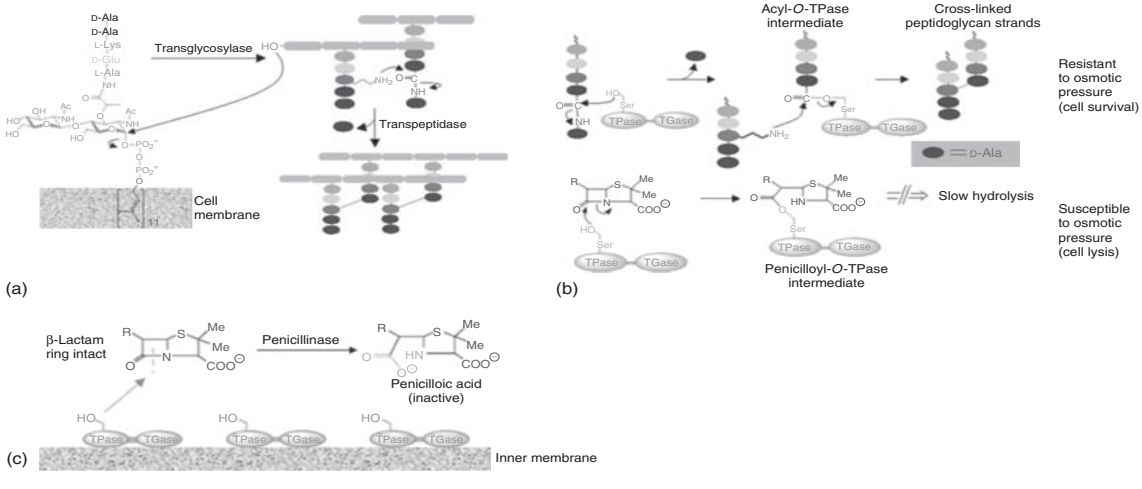

Figure 4.2 Transpeptidation inhibition during cell-wall biosynthesis by β-lactam antibiotic penicillins. (a) Cell-wall biosynthesis steps. (b) Inhibition of transpeptidase activity by penicillins through formation of a slowly hydrolyzing penicilloyl-O-TPase intermediate. This intermediate, deacylating very slowly, is unable to cross-linking promptly peptide chains in the peptidoglycan layer, leaving it mechanically weak and susceptible to lysis due to osmotic pressure changes. (c) Hydrolysis of β-lactam ring by β-lactamase penicillinase. TGase, transglycosylase; TPase, transpeptidase. *Source*: Modified from Ref. [11] with permission.

4.2.1.4 **Proteases**

Lantibiotics (i.e., lanthionine-containing antibiotics) are antimicrobial peptides, produced by a large number of gram-positive bacteria, which exert their antibiotic activity mainly by inhibiting bacterial cell-wall biosynthesis. The lantibiotic nisin, a 34-residue peptide produced by *Lactococcus lactis* strains, is widely used as a food preservative because of its potent bactericidal activity. In nisin-producer *L. lactis* strains, the lipoprotein NisI and the ABC tranporter system NisEFG prevent nisin toxic effect. In non-nisin-producing *L. lactis*, nisin resistance (*nsr*) could be conferred by *nsr* gene, which encodes a 35-kDa protein (NSR) able to digest nisin, thus reducing its affinity for its cellular target (the membrane-anchored cell-wall precursor lipid II) and, thus, its bactericidal activity [33].

4.2.2
Antibiotic Transferases Prevent Target Recognition

Transferases represent the largest family of resistance enzymes [7, 12]. These enzymes covalently modify antibiotics, impairing target binding. Their activities include *O*- and *N*-acylation, *O*-phosphorylation, *O*-nucleotidylation, *O*-ribosylation, *O*-glycosylation and thiol transfer. All these reactions require a cosubstrate, including adenosine triphosphate (ATP), acetyl-CoA, nicotinamide adenine dinucleotide (NADH) uridine diphosphate (UDP) glucose or glutathione, and, consequently, all these enzymes work only in the cytosol [7, 12].

4.2.2.1 **Acyltransfer**

Covalent modification by acyltransfer, in particular acetyltransfer, is a common mechanism of antibiotic inactivation employed by bacteria. Acetyltransferases target hydroxyl (for *O*-acetylation) and/or amine groups (for *N*-acetylation) on antibiotics and the resulting ester or amide is biologically stable and essentially irreversible without the action of a cognate esterase or amidase [7, 12].

Aminoglycoside Acetyltransferases The aminoglycoside antibiotics impair the codon–anticodon decoding mechanism by binding to 16S rRNA at the A-site of the ribosome. This interaction causes the inhibition of translation and also the synthesis of aberrant proteins as a consequence of translational infidelity (miscoding). The aminoglycoside acetyltransferases (AACs) modify the key hydroxyl and amine groups of the aminoglycoside antibiotics (Figure 4.3), blocking the interaction with the rRNA and resulting in resistance [12]. Aminoglycoside inactivation via AAC enzymes was the second bacterial-resistance mechanism discovered after that of penicillinases [34]. The AACs are classified according to their regiospecificity of acetyltransfer on the aminoglycoside structure [7, 12]. For example, the AAC(6′) acts by *N*-acetylating the aminoglycoside on the amine group frequently found at position 6′ of the aminohexose linked to position 4 of the central 2-deoxystreptamine ring, while the AAC(3) *N*-acetylates the amine group linked to position 3 of the 2-deoxystreptamine ring [7, 12]. Genes encoding these enzymes are widespread both in clinics (as a result of their frequent association with resistance

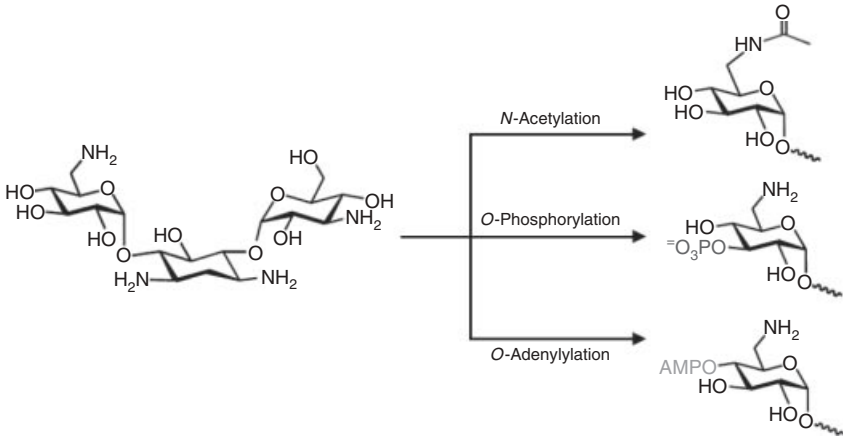

Figure 4.3 Aminoglycoside enzymatic modifications negatively affect ribosomal target recognition. Kanamycin can be chemically modified by three kinds of enzymatic processing: *N*-acetylation; *O*-phosphorylation; and *O*-adenylylation. *Source*: Modified from Ref. [11] with permission.

plasmids, transposons and integrons) and in the environment (as orthologs have been identified in many of bacterial genomes) [12].

Chloramphenicol Acetyltransferases Chloramphenicol prevents protein chain elongation by specifically binding to the 23S rRNA of the 50S ribosomal subunit, thereby inhibiting peptidyl transferase activity of the bacterial ribosome. Chloramphenicol acetyltransferases (CATs) are trimeric enzymes that have two distinct structural types: class A and class B (also known as the *xenobiotic CATs*) [7, 12, 35]. CATs inactivate chloramphenicol by covalently linking one or two acetyl groups, derived from acetyl-S-coenzyme A, to the hydroxyl groups. The chloramphenicol acetylation inhibits binding to the 23S rRNA.

4.2.2.2 Phosphotransferases

Aminoglycoside Phosphotransferases Kinases are enzymes catalyzing phosphate transfer from nucleotide triphosphates (NTPs), typically ATP, to a diverse set of substrates. Aminoglycoside phosphotransferases (APHs) (Figure 4.3), widely distributed among bacterial pathogens, are classified on the basis of their regiospecificity of phosphoryl transfer and substrate specificity [7, 12]. Thus, each APH is specific to a given range of aminoglycosides, which become unable to bind to their target on the A-site of the ribosome after phosphorylation. The genes encoding APH are frequently found on multidrug resistance R plasmids, transposons and integrons; therefore, the resistance genes are very often present in bacterial populations [7, 12]. The APH(3′) family is ubiquitous and is widely used as resistance markers in molecular biology research (e.g., the *neo* cassette).

Macrolide Phosphotransferases Phosphate transfer is also adopted by bacteria to block the effects of macrolides such as erythromycin. Characterization of the product of inactivation revealed that phosphorylation occurs on the free hydroxyl (site 2′ in the macrolide nomenclature) of the desosamine sugar that interacts directly with the 23S rRNA [7, 12]. Genes encoding macrolide phosphotransferase (MPH) enzymes have been isolated from *E. coli* (*mphA* and *mphB*) [36, 37] and from *S. aureus* (*mphC*) [38]. The presence of these genes results in very high MIC values (2 mg ml^{-1}) for 14- and 16-member macrolides [12].

4.2.2.3 Nucleotidyltransferases

Nucleotidyltransferases, transferring nucleotide monophosphate moiety from NTPs to an accepting hydroxyl group on the antibiotic, are grouped in two major classes according to specificity of their target: (i) the ANTs that modify aminoglycosides (Figure 4.3) and (ii) the Lin proteins that inactivate the lincosaminide antibiotics that include lincomycin and its semisynthetic derivative, clindamycin [7, 12].

The aminoglycosides gentamicin and tobramycin, widely used in clinics, are both modified by ANT(2′) whose encoding gene is distributed among pathogenic bacteria [39].

Clindamycin is the lincosamide antibiotic most often used clinically. It binds to the peptide exit tunnel of the bacterial ribosome in the same region as the macrolide antibiotics [7]. There are three characterized lincosaminide nucleotidyltransferase genes, *linA* from *Staphylococcus haemolyticus*, *linA′* from *S. aureus* and *linB* from *Enterococcus faecium* [12, 40–42]. LinA and LinB do not show sequence homology and LinB modifies lincomycin and clindamycin at same position, while LinA modifies lincomycin and clindamycin at different positions [12].

4.2.2.4 ADP-Ribosyltransferases

Adenosine diphosphate (ADP)-ribosyl transfer, requiring NAD as ADP-ribosyl donor, is a common mechanism of protein posttranslational modification in both eukaryotes and prokaryotes. However, ADP-ribosylation of the RNA polymerase inhibitor rifampin (rifampicin), used in the treatment of infections caused by *Mycobacterium tuberculosis*, is so far the only well-documented example of this kind of modification in antibiotic resistance [12]. In mycobacteria, a unique rifampin ADP-ribosyltransferase (ARR) interferes with the activity of this drug [12, 43]. ARR-2, another enzyme with similar activity, is associated with multidrug resistance integrons in gram-negative bacteria [12, 44]. These enzymes, sharing about 55% identity to each other, are unique among ARRs for their small size and for their sequence differences with respect to other ARRs [7, 12].

4.2.2.5 Glycosyltransferases

Glycosyltransfer is a widespread mechanism of antibiotic resistance among soil bacteria, both producer and nonproducer strains, but infrequently encountered among pathogens [7, 12]. The soil bacterium *Streptomyces lividans* possesses the *mtg* gene, which is an example of this class of resistance [45]. The Mtg enzyme catalyzes glucosylation of erythromycin and other macrolides at position 2′ of

the desosamine sugar using UDP glucose as the glucose donor. Glycosylation of rifampin at position 23 by pathogenic *Nocardia* spp. is also reported but the enzyme has not yet been characterized [46].

4.2.3 Redox Enzymes

Oxidation is a common mechanism for mammalian detoxification of xenobiotics by a membrane-bound cytochrome P-450, which possesses broad substrate specificity. In contrast, the oxidation or reduction of antibiotics has not been frequently exploited by pathogenic bacteria [12]. The best-studied example of this strategy is the oxidation of tetracycline antibiotics by TetX, an enzyme that catalyzes the monohydroxylation of tetracycline antibiotics in an oxygen-dependent manner [47]. The gene encoding TetX was found on conjugative transposons in the obligate anaerobe *Bacteroides fragilis* and its role was only uncovered when the gene was cloned into *E. coli* [48, 49]. TetX acts on first- and second-generation tetracyclines and it is also active against the third-generation antibiotic tigecycline. Under aerobic conditions, TetX utilizes nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of magnesium and converts tigecycline to 11a-hydroxytigecycline. The modified molecule binds weakly magnesium, which is essential for its binding to ribosome [50].

Another predicted monooxygenase with antibiotic inactivation properties was cloned from rifampin-resistant *Rhodococcus equi*. Expression of the gene in *E. coli* resulted in rifampin resistance by an uncharacterized mechanism [12, 51].

4.3 Antibiotic Target Alteration: The Trick Exists and It Is in the Genetics

Alteration of the antibiotic target as a result of mutation, chemical modification, substitution, and masking of key binding elements, is a widespread strategy to elude antibiotic action.

4.3.1 Low-Affinity Homologous Genes

Spontaneous mutation is the driving force of molecular evolution. As a consequence of selective pressure in the modern antibiotic era, many cases of mutation not affecting bacterial fitness in housekeeping genes are reported to lead to resistance in previously susceptible strains. In addition, many strains are reported to be resistant to a class of antibiotics as a result of sequence differences in the target gene, which makes the product unable to interact with the antibiotic. This may be the case of antibiotic producer bacteria, such as actinomycetes, which have to protect themselves from the killing activity of their own product [52]. However, it is quite surprising that paralogous genes encoding products not susceptible to

antibiotics are found much more frequently in nonproducer bacterial strains [52]. In this case, the binomial chance-necessity concept (e.g., random mutations spread by means of selective pressure) could justify the hypothesis of an early exposure to toxic compounds in an early phase of bacterial molecular evolution. In this context, the isolation of environmental bacterial strains, not producing antibiotics and carrying paralogous genes whose products are not affected by the drugs, may be considered as a strong indication of the occurrence of a molecular struggle that started outside clinics [17].

4.3.1.1 Rifamycin Low-Affinity RpoB

Rifampicin inhibits DNA-dependent RNA polymerase in bacterial cells by binding its β-subunit, thereby compromising messenger RNA synthesis. In particular, rifampicin interacts with the β-subunit when the RNA polymerase is an $\alpha_2\beta$ trimer. Thus, rifampicin-resistant bacteria, including the producer strain *Amycolatopsis mediterranei*, posses RNA polymerases with different β subunit structures that are not readily inhibited by the drug [53]. In particular, most mutations map to the N-terminal region of resistant RpoB spanning amino acids 505–537 (*E. coli* numbering). The mutations are mainly point mutations resulting in single amino acid substitutions, with few deletions or insertions, causing poor binding of rifampicin to the RNA polymerase [53].

4.3.1.2 Mutated Genes Conferring Resistance to Quinolone, Fluoroquinolone and Aminocoumarins

Quinolone and fluoroquinolone interfere with DNA replication. A high level of resistance to this class of antibiotics is associated with mutations in the *gyrA gene*, encoding a subunit of DNA gyrase, in gram-negative bacteria and in *gyrA* and *parC* (a subunit of topoisomerase IV) in gram-positive bacteria [54, 55]. A 41 amino acid sequence, corresponding to amino acids 67–106 in *E. coli* GyrA, was identified in both gram-negative and gram-positive organisms as the quinolone-resistance-determining region [55].

Aminocoumarins, such as novobiocin, are inhibitors of bacterial DNA gyrase. In particular, aminocoumarins target the GyrB subunit, necessary for energy transduction. Resistance to this class of antibiotics usually results from genetic mutation in the *gyrB* subunit [56].

4.3.1.3 PBP2a: A Low-Affinity Penicillin-Binding Protein

The *mecA* gene encodes the penicillin-binding protein 2a (PBP2a) a transpeptidase membrane protein that possesses a low affinity for β-lactam antibiotics, such as methicillin and penicillin, and is responsible for β-lactam resistance in methicillin-resistant *S. aureus* (MRSA) [54]. The *mecA* gene expression is controlled by *mecI*, encoding a negative regulator, and *mecR1*, encoding a sensor protein, which derepress *mecA* expression inactivating MecI in the presence of β-lactam. The *mecA* gene is placed in the staphylococcal chromosomal cassette *mec* (SCC*mec*), which is a mobile genetic element of the *Staphylococcus* bacterial species that contains the *ccr* genes coding for recombinases required for horizontal transfer [2, 20, 54].

4.3.1.4 Dihydropteroate Synthases Not Inhibited by Sulfonamide

Sulfonamides, synthetic antimicrobial agents that contain the sulfonamide group, act as competitive inhibitors of dihydropteroate synthetase (DHPS), an enzyme involved in folate synthesis. Sulfonamide resistance in gram-negative bacilli generally arises from the acquisition of genes encoding dihydropteroate synthase variants such as *sul1, sul2* and *sul3* that are not inhibited by the drug [57]. The *sul1* and *sul3* genes are normally found linked to other resistance genes in class 1 integrons, while *sul2* is usually located in small nonconjugative plasmids or in large transmissible multiresistance plasmids [57].

4.3.2 Chemical Modification of Antibiotic Target

The capability to chemically modify a molecular target of an antibiotic is mainly attributed to gene products expressed in antibiotic-producing bacteria such as actinomycetes. Nevertheless, homologous genes were recently found in resistant strains from clinical isolates. The spreading of such genes represents a problem from a nosocomial viewpoint and poses intriguing questions concerning the evolutionary history of resistance genes.

4.3.2.1 23S rRNA Modification

Erythromycin, a natural product of *Saccharopolyspora erythraea*, was the first macrolide to be advanced to medical use in the early 1950s for the treatment of infections due to gram-positive pathogenic bacteria [58]. Macrolides inhibit bacterial growth by binding to the ribosome and blocking the nascent polypeptide chain in the early rounds of protein synthesis [59] or in some cases macrolides with extended side chains reach close to the catalytic center and stop peptide bond formation from the beginning [59]. Erythromycin methyltransferases (Erms) from macrolide-resistant bacteria, including ErmE from erythromycin producer *S. erythraea*, can methylate adenine at position 2058 of 23S rRNA (*E. coli* numbering) [60]. The *ermA* gene, carried by Tn554-like transposons, is widespread in MDR-MRSA strains while *ermC*, usually plasmid-located, is more common among methicillin-sensitive *Staphlococcus aureus* (MSSA) strains [20]. In *Staphylococci*, *erm* genes can also confer resistance to a broader group of antibiotics such as lincosamide and streptogramin in addition to macrolides (macrolide-lincosamide-streptogramin B, MLSB resistance) [20].

4.3.2.2 16S rRNA Modification

Resistance to aminoglycosides is frequently due to the acquisition of modifying enzymes such as acetyltransferases, phosphorylases and adenylyltransferases (Section 4.2). Other mechanisms of aminoglycoside resistance include single-step mutations in chromosomal genes encoding ribosomal proteins, impaired antibiotic uptake and ribosomal protection by methylation of 16S rRNA [61]. Methylation of bases involved in the binding of aminoglycosides to 16S rRNA leads to a reduction in binding affinity, thereby causing high-level resistance to

aminoglycosides. Methylases, such as KamB and KamC, are intrinsically produced by some aminoglycoside-producing organisms such as *Streptomyces* spp. and *Micromonospora* spp. [62]. Recently, several plasmid-encoded 16S rRNA methylases have emerged in clinical isolates of gram-negative bacilli [61]. The ArmA, RmtA and RmtB methylases were detected in *P. aeruginosa* strains and a *Serratia marcescens* strain, respectively [63].

4.3.2.3 Reprogramming Chemical Composition of a Bacterial Cell-Wall Precursor

Glycopeptides, nonribosomally synthesized peptides, target the D-Ala-D-Ala end of uncross-linked pentapeptide side chain in nascent peptidoglycan chains. The interaction, preventing the transpeptidase recognition, inhibits peptide cross-linking, causing the formation of a weak cell wall that is not able to withstand the osmotic pressure (Figure 4.4) [10, 11]. A sophisticated example of the strategy to escape the glycopeptide effect was revealed in both glycopeptide-producing and in nonproducing bacteria, such as vancomycin-resistant enterococci (VRE) [10, 11]. In these strains, the *vanHAX* operon genes encode a set of enzymes that reduces pyruvate to D-lactate (VanH), adds D-alanine and D-lactate together to produce D-Ala-D-Lac (VanA) and then hydrolyses the D-Ala-D-Ala (VanX) (Figure 4.4 and Figure 4.5) [10, 11]. The resistance mechanism is positively regulated by a two-component signal transduction system (*vanS* and *vanR* genes) in the presence of vancomycin (Figure 4.5) [10, 11]. The D-Ala-D-Lac is incorporated into the end of the peptidoglycan strands instead of D-Ala-D-Ala and this substitution, having no effect on the cross-linking efficiency, lowers the binding affinity of vancomycin by 1000-fold and enables the VRE to grow at 1000-fold higher levels of antibiotic (Figure 4.4) [10, 11]. The high homology between glycopeptide-resistance determinants suggests horizontal transfer events from producer to nonproducer strains.

4.3.3 Ribosomal Protection and Tetracycline Resistance

Resistance to tetracycline may be mediated by inactivation by TetX (Section 4.2.3) or by the integral membrane efflux protein tetracycline (TetA) (Section 4.4.2) or by a mechanism known as *ribosomal protection* mediated by a soluble protein [64]. Ribosomal protection proteins (RPPs) are 72.5 kDa proteins belonging to a widely distributed class of tetracycline resistance determinants. There are 11 different types of RPPs in both gram-positive and gram-negative bacteria [64]. TetO and TetM are the most prevalent and the best-studied classes of RPPs, while OtrA is believed to be the ancestor of some other RPPs found in pathogens such as *Mycobacteria*. RPPs display high homology to translation elongation factors EF-Tu and EF-G, which are ribosome-dependent GTPases. Therefore, it has been suggested that RPPs are EF paralogs that have evolved through duplication and divergence of an ancestral GTPase [64]. RPPs were earlier proposed to work as tetracycline-resistant elongation factors capable of carrying out protein synthesis in the presence of tetracycline, but now it is believed that RPPs displace tetracycline from the ribosome so that

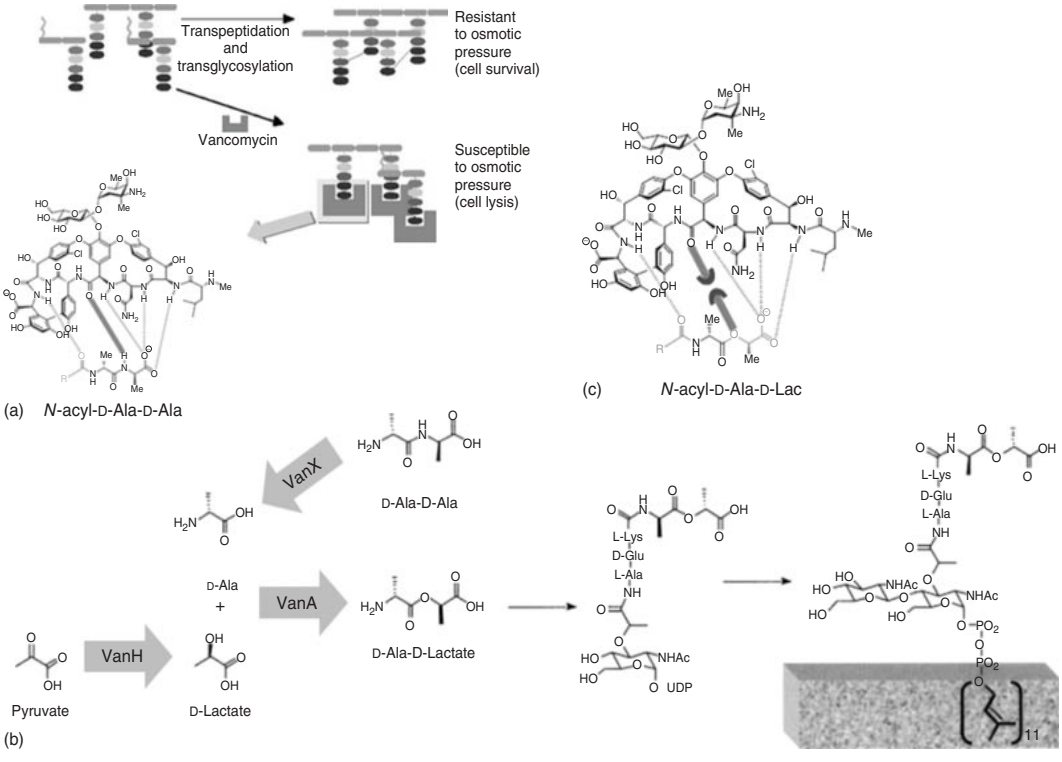

Figure 4.4 Transpeptidation inhibition during cell-wall biosynthesis by vancomycin. (a) Inhibitory effect of vancomycin on cross-linking and strength-conferring transpeptidation by sequestering of substrate. (b) Reprogramming of biochemical composition of bacterial cell-wall precursors by *vanHAX* gene products. (c) Complexation of the D-Ala-D-Ala termini of peptidoglycans by vancomycin in a network of five hydrogen bonds. *Source*: Modified from Refs. [10, 11] with permission.

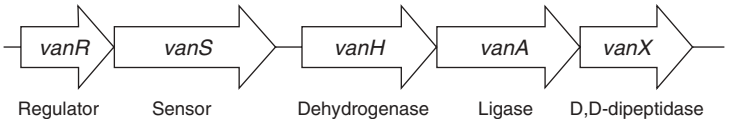

Figure 4.5 Schematic representation of *van* genes in vancomycin-resistant enterococci.

the tetracycline-free ribosome can bind the aminoacyl-tRNA (aa-tRNA) in the A-site and protein synthesis can continue [64]. RPPs are effective against first- and second-generation tetracyclines but not against tigecycline, which is a third-generation compound probably because this drug has a stronger binding affinity for its target [64].

4.3.4 Chromosomal Mutations in Genes Required for Membrane Phospholipid Metabolism: Lipopeptide Resistance

The lipopeptide antibiotic daptomycin, produced by actinomycete, is used to treat gram-positive bacterial infections, including those caused by enterococci and staphylococci [65]. Daptomycin is approved to treat complicated skin and skin structure infections and has been used to treat VRE bacteremia and endocarditis, among other infections [20, 66]. It is proposed that daptomycin kills cells by a calcium-dependent insertion into the cell membrane followed by oligomerization that causes pores, allowing ion leakage from the cell and rapid depolarization of the bacterial cell membrane [67]. Daptomycin resistance has been extensively studied in *S. aureus*, where it results from chromosomal mutation. Microarray-based comparative genome analyses of *S. aureus* and *E. faecalis* strains subjected to *in vitro* daptomycin serial passage revealed that certain genes and intergenic regions (such as *mprF*, *rpoB*, *yycG* and *cls*) acquired mutations during the evolution of daptomycin resistance. Mutations in these regions in many, but not all, daptomycin-resistant *S. aureus*, *E. faecalis* and *E. faecium* clinical isolates have also been detected. The impact of these genetic changes has not been fully delineated [66, 68]. MprF catalyzes the lysinylation of phosphatidylglycerol (PG), generating lysylphosphatidylglycerol (Lys-PG). As daptomycin seems to interact preferentially with PG, the binding of lysine, which would convert negatively charged PG to positively charged Lys-PG, may interfere with daptomycin-membrane interactions [64]. Cls catalyzes reversible transphosphatidylation of cardiolipin (CL; bis-PG), a negatively charged phospholipid associated with septal and polar membrane protein-lipid microdomains in *B. subtilis* and other bacteria. CL has the potential to significantly impact local membrane structure and charge–charge interactions at the membrane. Thus, *cls* mutations observed in the daptomycin-resistant strains theoretically could result in decreased CL synthesis or increased CL degradation, thus changing CL amount in membranes of daptomycin-resistant enterococci [66]. Therefore, membrane composition seems to be critical for daptomycin antibiotic activity and, therefore, an improved understanding of how membrane compositions

change in resistant strains would be critical for unraveling the precise mechanism of daptomycin resistance.

4.3.5
Covalent Modifications on Lipopolysaccharide Core Conferring Polymixine Resistance

The PmrA–PmrB two-component system governs resistance to antimicrobial peptide compounds including polymyxin, polylysine, protamine and neutrophil antimicrobial peptides CAP37 and CAP57 [69]. The genes encoding these peptides have been shown to be activated *in vivo* and are regulated by PhoP–PhoQ system, but can also be activated under mild acidic conditions in a PhoP–PhoQ independent manner. PmrA–PmrB activation results in the modification of phosphate groups of the lipopolysaccharide (LPS) core and lipid A with ethanolamine and modification of the 4′ phosphate of lipid A with aminoarabinose. These covalent modifications, seen in resistant bacteria including *Yersinia enterocolitica*, *Proteus vulgaris*, *E. coli*, *K. pneumoniae* and *Burkholderia* (*Pseudomonas*) *cepacia*, reduce electrostatic interactions and, hence, weaken binding between the peptide and the cell surface [69]. PmrA–PmrB are involved in the regulation of the *pmrA-pmrB* operon itself and of *pmrE* and *pmrF* loci that are necessary for resistance to polymyxin [69]. The *pmrE* locus contains a single gene previously identified as *pagA* (or *ugd*), which encodes a UDP-glucose dehydrogenase [69]. The *pmrF* locus comprises the second gene of a putative operon predicted to encode seven proteins, some with similarity to glycosyltransferases and other complex carbohydrate biosynthetic enzymes involved in lipid A aminoarabinose modification. The activity of these enzymes can promote resistance to cationic antimicrobial peptides [69]. In addition, genes flanking this putative operon are also regulated by PmrA–PmrB and/or have been associated with *S. typhimurium* polymyxin resistance with a mechanism that is still to be investigated [69].

4.4
Efflux Systems

Efflux pumps are major players in bacterial MDR and pose major hurdles in the drug discovery process [70–76]. They typically export structurally different organic compounds including antibiotics, environmental toxic compounds, or molecules produced by the host organism such as bile, indicating that these systems could allow bacteria to survive in their ecological niche.

Efflux pumps can be specific to one substrate or can transport a range of unrelated substances; the efflux pumps that transport multiple, structurally dissimilar toxic agents can be associated with MDR. Therefore, MDR efflux pumps are of clinical relevance because they can render a bacterial infection untreatable by the antibiotics of choice. MDR efflux pumps are found in all bacteria and their primary functions could be other than antibiotic resistance such as maintenance of cell homeostasis

or intracellular solute concentrations, extrusion of toxic by-products of metabolism and transport of nucleotides or amino acids.

Efflux pumps reduce the intracellular antibiotic concentration and often act synergistically with other resistance mechanisms to provide a high level of resistance to antibiotics. Efflux-pump genes are mostly located on the chromosome, although they can also be carried by plasmids, and are frequently subjected to both specific and global regulation.

Bacterial efflux pumps are grouped into five families (Figure 4.6) according to their primary structure and mode of energy coupling:

1) ATP-binding cassette (ABC) superfamily;
2) major facilitator superfamily (MSF);
3) small multidrug-resistance family (SMR);
4) resistance-nodulation-division (RND) superfamily;
5) multidrug and toxic compound extrusion (MATE) family.

These families, except for the ABC family, are secondary transport systems and utilize an electrochemical gradient of cations across the membrane for drug transport.

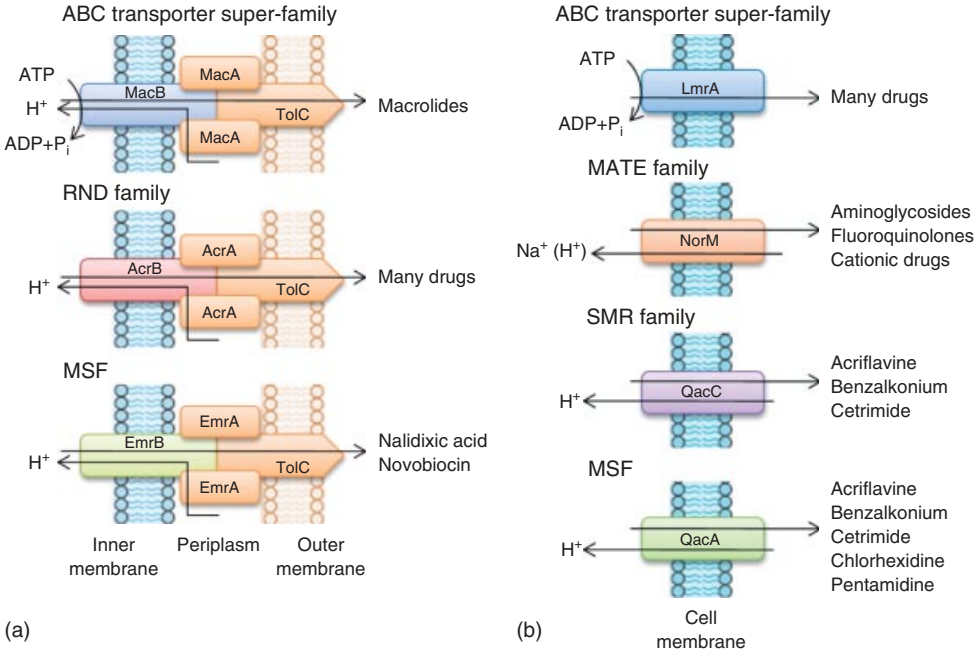

Figure 4.6 Diagrammatic representation of the structure and membrane location of members of the five characterized families of multidrug resistance efflux pumps in gram-negative (a) and gram-positive (b) bacteria. IM, inner membrane; P, periplasm; OM, outer membrane; CM, cell membrane.

Efflux pumps usually consist of a monocomponent protein with transmembrane spanning domains; however, in gram-negative bacteria an efflux pump, located in the inner membrane, works together with a periplasmic protein named membrane-fusion protein (MFP) and an outer membrane channel protein.

A bacterial cell can express efflux pumps from more than one family and/or more than one type of pump belonging to the same family.

4.4.1 The ATP-Binding Cassette (ABC) Superfamily

The transporters of the ABC family are conserved from humans to bacteria and export a wide array of substrates in a process driven by ATP hydrolysis [77, 78]. ABC transporters consist of a transmembrane domain (TMD) and a nucleotide-binding domain (NBD).

LmrA from *L. lactis* is the first member of the ABC transporter family discovered in bacteria, whose TMD and NBD are expressed as a single polypeptide. LmrA catalyzes the extrusion of many hydrophobic compounds including antibiotics through the cell membrane. ATP binds to the NBD, where binding and hydrolysis induce conformational changes that lead to the extrusion of the substrate via the TMD [79, 80].

S. aureus Sav1866 exporter protein is a homolog of LmrA that contains two nucleotide-binding domains in close contact and two TMDs; by simultaneous hydrolysis of two molecules of ATP, this protein opens a transmembrane channel and pumps drugs out of the cell, thereby conferring MDR [81–83].

E. coli MacB (Figure 4.6) is an ABC-type macrolide efflux transporter with four transmembrane segments and one nucleotide-binding domain, which functions by cooperating with the MFP MacA and the multifunctional major outer membrane channel TolC [84–86]. TolC plays an important role in the excretion of a wide range of molecules, including antibiotics, bile salts, organic solvents, enterobactin, several antibacterial peptides, and virulence factors [87].

4.4.2 The Major Facilitator Superfamily (MSF)

The MSF is a very large, ancient group of proteins consisting of secondary integral membrane transporters driven by chemiosmotic energy [88] and includes proton (H^+)/drug antiporters such as QacA, NorA, NorB, NorC and LmrS of *S. aureus*, Mdt(A) of *L. lactis*, MdfA and EmrA of *E. coli* and TetA family of efflux pumps from gram-negative and gram-positive bacteria. These proteins span the lipid bilayer of the cell membranes 12–14 times.

The QacA efflux pump [89–92] spans the membrane 14 times and is energized by H^+ to extrude structurally diverse monovalent and divalent cationic substrates, in particular quaternary ammonium compounds. In MDR *S. aureus*, QacA is encoded by plasmid-borne genes and the expression of *qacA* genes is regulated by QacR, a transcription regulator belonging to the *tetR* family [93].

S. aureus NorA efflux pump extrudes the quinolone drug norfloxacin and several antimicrobial agents including chloramphenicol [94, 95]. NorA, possessing 12 TM and being chromosomally encoded, is partly homologous to tetracycline resistance and sugar transport proteins [96, 97]. The *norA* cloned from chromosomal DNA of quinolone-resistant *S. aureus* TK2566 conferred relatively high resistance to hydrophilic quinolones such as norfloxacin, enoxacin, ofloxacin and ciprofloxacin, but only low or no resistance to hydrophobic drugs such as nalidixic acid, oxolinic acid and sparfloxacin in *S. aureus* and *E. coli*. NorB [98] and NorC [99], organized into 14 transmembrane segment (TMS), confer resistance to quinolones, such as norfloxacin, ciprofloxacin and sparfloxacin. The expression of *norA*, *norB* and *norC* together with *tet38*, which encodes tetracycline resistance, is under the control of MgrA, a global regulator that also affects diverse virulence factors [100, 101].

LmrS was identified in a clinically isolated MRSA strain [102]. Proteins homologous to LmsrS are widely distributed among gram-positive bacterial genera such as *Staphylococcus*, *Bacillus*, *Lactobacillus*, *Listeria* and *Enterococcus*. LmrS confers resistance to linezolid and fusidic acid, two antimicrobials with strong activity against MRSA. This efflux pump, with 14 TM, is encoded by a chromosomal *lmrS* gene. The cloned *lmrS* gene confers resistance to kanamycin, lincomycin, fusidic acid, linezolid, chloramphenicol, erythromycin, streptomycin, trimethoprin and florfenicol.

The plasmid-specified multiple drug transporter Mdt(A) contains 12 TMS and is a member of the MSF with some interesting structural differences; it has two antiporter motifs and a putative ATP-binding site [103]. Mtd(A) confers resistance to lincosamides, 14-, 15- and 16-membered macrolides, streptogramins and tetracyclines. The molecular mechanism responsible for drug transport by Mdt(A) remains to be elucidated.

MdfA [104, 105], identified in *E. coli*, contains 12 TMS. *E. coli* cells expressing MdfA from a multicopy plasmid exhibit resistance not only to lipophilic compounds including ethidium bromide, daunomycin, tetraphenyphosponium, rhodamine, rifampin, tetracycline, puromycin but also to chemically unrelated clinically important antibiotics such as erythromycin, choramphenicol, some aminoglycosides and fluoroquinolones. In addition, MdfA is involved in maintaining the physiological pH of the cell.

EmrB is a membrane protein with 14 TM domains, while the MFP EmrA has a large soluble C-terminal domain with a single N-terminal TM domain; together with the outer membrane channel TolC, EmrAB forms a tripartite efflux system [106].

Efflux of tetracyclines predominantly occurs via proteins that are members of the major facilitator superfamily (MFS) group of integral membrane transporters. There are 26 different classes of MFS tetracycline transporters present in gram-negative and gram-positive bacteria.

The *tetA* family pumps are grouped into two major groups [107, 108]. The first group comprises chromosomally encoded efflux pumps possessing 12-TMS, found in gram-negative bacteria. The second group comprises plasmid-encoded

efflux pumps having 14-TMS identified in *S. aureus*, *Bacillus*, *Staphylococcus* and *Streptococcus* spp. [73, 109].

In *E. coli*, tetracycline enters cells by simple diffusion through the lipid bilayer region of the plasma membrane as a protonated neutral form. Then it loses a proton and chelates with Mg^{2+}. The resulting monovalent cation is exported by TetA coupled with H^+ influx. Thus, this pump functions as a metal-tetracycline/H^+ antiporter [110].

4.4.3 The Small Multidrug-Resistance Family (SMR)

This family of transporters is represented by EmrE of *E. coli* and QacC of *Staphylococcus epidermidis* [111–114]. The SMR are small (about 12 kDa) integral inner membrane proteins conferring resistance to lipophilic compounds, like quaternary ammonium compounds, and to a wide range of antibiotics, such as β-lactams, cephalosporins and aminoglycosides [74].

These proteins span the cytoplasmic membrane as four transmembrane α-helices with short hydrophilic loops, making them hydrophobic and permitting their solubilization in organic solvents. Similar to the MSF superfamily proteins, the SMR proteins perform drug efflux via an electrochemical H^+ gradient. The SMR family contains more than 250 annotated members and is grouped into three subclasses: (i) the small multidrug pumps (SMPs), (ii) the paired small multidrug-resistance proteins (PSMR) and (iii) the suppressors of GroEL mutant proteins (SUG). The latter do not carry out drug efflux but their overaccumulation suppresses GroEL mutations, suggesting that SUG proteins may play an important role in the uptake of chaperone regulatory compounds. The peptide methionine sulfoxide reductase (PMSR) proteins are distinct from SMP and SUG subclass proteins because they are constituted by two SMR homologs that must be simultaneously expressed to confer drug resistance. PMSR protein pairs generally consist of one protein with typical SMR protein length and of a second longer protein, for example, *E. coli* YdgE and YdgF or *B. subtilis* EbrA and EbrB [115–117]. PSMR proteins are structurally different from other SMR homologs owing to the presence of longer hydrophilic loops and of a large hydrophilic C-terminus in one of the two proteins. SMR proteins may be encoded on the chromosomes or on plasmids and may be associated with integrons.

4.4.4 The Resistance-Nodulation-Division (RND) Superfamily

Efflux pumps of the RND family, which function as H^+/drug antiporters, are mainly found in gram-negative bacteria and catalyze the active efflux of many antibiotics and chemotherapeutic agents [118]. RND transporters are protein complexes that span both cytoplasmic and outer membrane. The complex comprises a cytoplasmic membrane transporter protein, a periplasmic-exposed membrane adaptor protein classified as MFP, and an outer membrane channel protein. Importantly, each of

these three component proteins is essential for drug efflux and the absence of even one component makes the complex nonfunctional.

The *E. coli* AcrAB-TolC and the *P. aeruginosa* MexAB–OprM complexes are well characterized [119–121]. AcrAB-TolC can handle a very wide range of compounds. These include cationic dyes, detergents, bile acids and antibiotics such as penicillins, cephalosporins, fluoroquinolones, macrolides, chloramphenicol, tetracyclines, novobiocin, fusidic acid, oxazolidinones and rifampicin. The MexAB-OprM complex exports antimicrobial compounds, such as fluoroquinolones, β-lactams, tetracycline, macrolides, choramphenicol, novobiocin, trimetropin and sulphonamides, and also exports dyes, detergents, disinfectants, organic solvents and acylated homoserine lactones involved in quorum sensing.

The AcrB or MexB transporter protein captures its substrates, either from within the phospholipid bilayer of the inner membrane or from the cytoplasm, and then transports them to the extracellular medium through TolC or OprM, respectively, which form a channel in the outer membrane. Cooperation between the inner membrane transporter proteins and outer membrane channel proteins is mediated by periplasmic accessory proteins AcrA and MexA, respectively. Thus, in enterobacteriaceae TolC can function as a channel for different RND-family efflux pumps and can interact with ABC and MFS transporters. Similarly, OprM of *P. aeruginosa* can interact with various RND-family proteins.

4.4.5
The Multidrug and Toxic Compound Extrusion (MATE) Family

The MATE family is the most recently categorized among the five efflux transporter families [122, 123]. The MATE family has been shown to be ubiquitously distributed and extremely flexible in function. MATE efflux pumps utilize Na^+/H^+ gradient for transport of metabolic and xenobiotic organic cations and have been reported to contain three branches: the NorM branch, a branch containing several eukaryotic proteins and a branch containing *E. coli* DinF.

These proteins are predicted to have 12 α-helical transmembrane regions. The X-ray structure of the NorM revealed an outward-facing conformation with two portals open to the outer leaflet of the membrane and a unique topology of the predicted 12 transmembrane helices distinct from any other known MDR transporter.

NorM, a multidrug Na^+-antiporter, was the first MATE family pump identified from *Vibrio parahaemolyticus*. It confers resistance to dyes, fluoroquinolones and aminoglycosides. NorM homologs have recently been characterized in many species such as *E. coli, N. gonorrhoeae, V. cholerae* and *Erwinia amilovora* [124–127].

The DinF protein is an uncharacterized member of this family of transporters. Expression of the *dinF* gene is DNA damage (UV or mitomycin C) inducible. The *dinF* gene is located downstream of the *lexA* gene, which encodes the global repressor of the SOS regulon. On the basis of sequence similarity, DinF may function as a proton-driven efflux system, possibly for nucleotides, given its potential role in response to DNA damage [128, 129].

As the majority of the bacterial MATE transporters have been identified by expression in *E. coli*, the functional role of these pumps in the native hosts is unclear.

4.5 The Case Stories of Intrinsic and Acquired Resistances

4.5.1 β-Lactam Resistome of *P. aeruginosa*: Intrinsic Resistance Is Genetically Determined

P. aeruginosa is an opportunistic pathogen showing low intrinsic antibiotic susceptibility. Intrinsic resistance is attributed to the low permeability of cellular envelopes together with the presence of chromosomally encoded multidrug efflux pumps or antibiotic-inactivating enzymes that resemble those present in transposable elements and usually acquired by horizontal transferring. However, further intrinsic mechanisms act in synergy as many chromosomal genes that contribute to β-lactam resistance of *P. aeruginosa* were identified using a comprehensive library of transposon-tagged insertion mutants [18]. In particular, genes whose inactivation resulted in changes in antibiotic resistance encode proteins that belong to a variety of functional groups, including cell division (FtsK), metabolic enzymes such as phosphoenolpyruvate carboxikinase, elements involved in cell attachment and motility such as fimbrial proteins or chemotaxis proteins, elements involved in the biosynthesis of LPS and in alginate production, and transcriptional regulators like GlnK (involved in nitrogen metabolism) [18]. Other resistance elements such as transporters, porins and regulatory proteins involved in the expression of chromosomally encoded β-lactamases (similar to those encoded by *dacB, mpl, ampR*, and *ampD*) were also identified. Altogether, these results indicate that the intrinsic resistome of *P. aeruginosa* might be considered a property highly dependent on the metabolic networks and biochemical characteristics of cells and not just the consequence of bacterial adaptation to the presence of antibiotics [18].

4.5.2 Acquired Antibiotic Resistance in *S. aureus*

The rapid acquisition of resistance determinants in *S. aureus*, starting with penicillin and methicillin, up to the most recent linezolid, is an example of bacterial adaptive evolution of bacteria in the antibiotic era. Resistance mechanisms in *S. aureus* include enzymatic inactivation of the antibiotic (penicillinase and aminoglycoside-modification enzymes), modification of antibiotic target (PBP2a of MRSA and D-Ala-D-Lac of peptidoglycan precursors of vancomycin-resistant strains), trapping of the antibiotic (vancomycin) and efflux pumps (fluoroquinolones and tetracycline) [20].

4.5.2.1 Acquired Resistance to β-Lactams and Glycopeptides

When penicillin first entered into clinical use in the 1940s, all *S. aureus* isolates were virtually susceptible to this antibiotic. However, within 10 years, *S. aureus* strains resistant to penicillin appeared and soon spread to become the most frequently isolated strains. Since then, several new antibiotic classes have been used, but *S. aureus* has shown a unique ability to quickly respond to each new challenge with the development of a new resistance mechanism. *S. aureus* resistance is mostly acquired via horizontal DNA transfer. Penicillin resistance is due to the production of β-lactamase, whose encoding gene is carried by a plasmid. Plasmids encoding penicillinase production also carry other resistance genes, such as resistance to disinfectants (quaternary ammonium compounds), dyes (acriflavine and ethidium bromide) and heavy metals (lead, mercury and cadmium), as well as to other antibiotics (erythromycin, fusidic acid and aminoglycosides) [20]. After the emergence of β-lactam-resistant strains, methicillin was designed to be invulnerable to the hydrolytic activity of the staphylococcal enzyme [20]. However, some strains of *S. aureus* developed resistance to this antibiotic very soon after its use. Unlike MSSA strains, MRSA strains are often MDR ones, being resistant also to a number of antibiotics of different classes, including macrolides, aminoglycosides and fluoroquinolones [20]. As described in Section 4.3.1.3, methicillin resistance is due to the production of an additional penicillin-binding protein named PBP2a, which possesses a reduced affinity for penicillin and β-lactams. As described in Section 4.3.1.3, PBP2a is the product of the *mecA* gene, which is controlled by regulatory genes *mecI* and *mecR1*. The *mecA* complex, whose origin is unknown, is found within a 30–60 kb mobile genetic element, denominated SCCmec in which is also found a *ccr* gene complex containing two recombinase genes (*ccrA* and *ccrB*), which mediate site-specific integration/excision of the element from the staphylococcal chromosome. SCCmec is an antibiotic resistance island as it can integrate additional mobile elements or resistance genes including insertion sequences, transposons, such as Tn554, which carries resistance genes for spectinomycin and erythromycin, integrated plasmids, such as pUB110, which encodes tobramycin and kanamycin resistance, mercury operons and more [20]. After the widespread emergency of MRSA, vancomycin has represented the cornerstone of therapy for MRSA infections. Over the past decade, a long-feared event has occurred: the appearance of strains that are not susceptible to vancomycin, showing either intermediate resistance (vancomycin-intermediate *S. aureus* (VISA)) or, worse, full resistance to this antibiotic (vancomycin-resistant *S. aureus* (VRSA)) [20]. The intermediate resistance in VISA has been associated with the presence of a thickened cell wall rich in peptidoglycan chains that are not cross-linked. Thus, vancomycin bound to the terminal dipeptide D-Ala-D-Ala is unable to reach the inner cell-wall layers, where vancomycin can exert its inhibitory action, blocking the incorporation of the precursors into the nascent peptidoglycan [20]. No characteristic genetic trait has been associated with VISA, although a relationship was observed with the loss of the accessory gene regulator (*agr*) locus, a quorum-sensing gene cluster that regulates virulence, conferring a selective survival advantage in the presence of vancomycin [20]. Unlike VISA, VRSA are usually high-level vancomycin resistant. VRSA strains

have acquired the *vanHAX* operon (Figure 4.5) that confers high-level resistance to both glycopeptides, vancomycin and teicoplanin, from VRE [20]. Therefore, the genetic and biochemical bases of resistance are the same as those of VRE and have been completely elucidated [10, 11, 20].

4.5.2.2 Acquired Resistance to Fluoroquinolones

In *S. aureus*, resistance to fluoroquinolones is conferred by point mutations occurring primarily in the subunit ParC (also named GrlA) of topoisomerase IV and secondarily in the subunit GyrB of DNA gyrase [20]. In addition, in some strains, overexpression of an efflux pump termed *NorA* contributes to the resistance phenotype. Multiple mutations and combination of resistance mechanisms also confer cross-resistance to newer fluoroquinolones, including those with increased activity against gram-positive bacteria [20]. New antibiotics such as linezolid, which is very active against MRSA strains, have been recently used to treat MRSA infections. Linezolid is an antibiotic belonging to the new class of the oxazolidinones that inhibits protein synthesis by binding to domain V of the 23S subunit of the bacterial ribosome [20]. As the chemical structure and/or the mechanism of action of this new drug are novel, the occurrence of natural resistance or cross-resistance was not anticipated. However reports of resistance developing during linezolid treatment are increasing [20].

4.6 Strategies to Overcome Resistance

The molecular struggle between antibiotic resistance and susceptibility is an evolutionary force that speeded up in the clinical experience of the past 50 years. Anyway, the understanding of the dynamics driving the molecular evolution of antibiotic-resistance genes can be used to survey clinically relevant organisms for the emergence of resistance during therapy and/or to improve the strategies leading to drug discovery and optimization. In this context, databases unifying resistance gene information, such as the Antibiotic Resistance Genes Database (ARDB, *http://ardb.cbcb.umd.edu/index.html*), would be helpful for facilitating studies of antibiotic resistance genes and for developing strategies to overcome the antibiotic resistance emergency. In fact, the extensive knowledge of resistance mechanism can be exploited to chemically modify promising molecules in such a way as to avoid enzymatic modification in vulnerable hot spots or to codevelop enzyme-specific inhibitors of resistance.

For example, chemical modifications of hydroxyl groups that can be targeted by kinases which inactivate aminoglycoside antibiotics led to the development of aminoglycosides such as tobramycin and gentamicin that lacked sites of inactivation [12, 130]. A similar strategy was adopted for florfenicol to overcome CAT-mediated resistance by acetylation at the hydroxyl linked to C3 [35]. Chemical modification of molecular structure has also driven the development of new β-lactamase-insensitive semisynthetic β-lactams, such as penems and

carbapenems [12, 131]. These antibiotics possess broad-spectrum activity and enhanced stability to β-lactamases.

Another application of a thorough understanding of resistance mechanisms is the development of resistance enzyme inhibitors. These inhibitors can be coadministered with the antibiotics to maintain antimicrobial activity. This approach has been highly successful in clinics as exemplified by the use of the β-lactamase inactivators of clavulanic acid, sulbactam and tazobactam to overcome resistance to the β-lactamase penicillinases [12, 131]. Interestingly, inhibitors blocking eukaryotic Ser- , Thr-, Tyr-kinases were also able to interfere with aminoglycoside kinases [7, 133]. In addition, many regulatory proteins activating resistance genes are two-component systems where His-kinase is the sensor membrane protein activating the transcriptional regulatory proteins as in the case of VanS-VanR. The use of His-kinase inhibitors, which lack targets in eukarya cells, may result in decreased resistance toward glycopeptides with the absence of unwanted collateral effects. Many pharmaceutical companies possess chemical libraries of protein kinase inhibitors that could be readily screened for infectious disease therapy. In addition, chemical families of efflux pump inhibitors, specifically targeting active transport in the bacterial cell, have been described and characterized [134]. Among them, several inhibitor compounds, such as arylpiperidines [120], demonstrate efficient blocking of the efflux pump activity involved in the MDR phenotype as observed in many gram-negative clinical isolates [134].

Given the continuing emergence of MDR pathogens, the need for new antibiotics is acute and growing. The antibiotic drug discovery pipeline may be supported by creative approaches based on the understanding of antibiotic-resistant molecular mechanisms. Therefore, resistance gene products, their origins, evolution, distribution throughout bacterial populations and mode of action may provide new insights for the development of alternative strategies having a significant impact on the treatment of infectious diseases.

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