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Advanced Drug Delivery Reviews 57 (2005) 1451–1470

Advanced DRUG DELIVERY Reviews

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Bacterial resistance to antibiotics: Enzymatic degradation and modification

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Received 10 December 2004; accepted 11 April 2005
Available online 13 June 2005

Abstract

Antibiotic resistance can occur via three general mechanisms: prevention of interaction of the drug with target, efflux of the antibiotic from the cell, and direct destruction or modification of the compound. This review discusses the latter mechanisms focusing on the chemical strategy of antibiotic inactivation; these include hydrolysis, group transfer, and redox mechanisms. While hydrolysis is especially important clinically, particularly as applied to β-lactam antibiotics, the group transfer approaches are the most diverse and include the modification by acyltransfer, phosphorylation, glycosylation, nucleotidylation, ribosylation, and thiol transfer. A unique feature of enzymes that physically modify antibiotics is that these mechanisms alone actively reduce the concentration of drugs in the local environment; therefore, they present a unique challenge to researchers and clinicians considering new approaches to anti-infective therapy. This review will present the current status of knowledge of these aspects of antibiotic resistance and discuss how a thorough understanding of resistance enzyme molecular mechanism, three-dimensional structure, and evolution can be leveraged in combating resistance.

Keywords: Antibiotic; Resistance; Hydrolase; Acetyltransferase; Kinase; Nucleotidyltransferase; Ribosyltransferase; Redox

Contents

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doi:10.1016/j.addr.2005.04.002

1. Introduction

Ever since the discovery and subsequent clinical use of antibiotics, resistance to these agents has been observed, with a commensurate negative impact on the treatment of infectious disease. In fact, Alexander Fleming, the discoverer of penicillin, commented in the *New York Times* in 1945 that "... the microbes are educated to resist penicillin and a host of penicillin-fast organisms is bred out which can be passed to other individuals and from them to others until they reach someone who gets a septicemia or a pneumonia which penicillin cannot save" (quoted in Ref. [1]). This cautionary note presaged the cycle of antibiotic discovery and subsequent resistance that inevitably arises with the use of these compounds. Antibiotic resistance is now well recognized as a major problem in the treatment of infections in hospitals and, with increasing and alarming frequency, in the community.

Resistance can be active (i.e., the result of a specific evolutionary pressure to adapt a counterattack mechanism against an antibiotic or class of antibiotics) or passive (where resistance is a consequence of general adaptive processes that are not necessarily linked to a given class of antibiotic; e.g., the non-specific barrier afforded by the outer membrane of Gram-negative bacteria). Bacteria achieve active drug resistance through three major mechanisms: (1) efflux of the antibiotic from the cell via a collection of membrane-associated pumping proteins; (2) modification of the antibiotic target (e.g., through mutation of key binding elements such as ribosomal RNA or even by reprogramming of biosynthetic pathways such as in resistance to the glycopeptide antibiotics); and (3) via the synthesis of modifying enzymes that selectively target and destroy the activity of antibiotics. All of these mechanisms require new genetic programming by the cell in response to the presence of antibiotics. In fact, in several cases, the antibiotics or their action actually genetically regulate the expression of resistance genes. Therefore, bacterial cells expend a considerable amount of energy and genetic space to actively resist antibiotics. This review is focused on enzymes that confer resistance to antibiotics. These are a remarkable set of adaptive proteins that utilize a

Table 1
Enzymatic strategies of antibiotic inactivation

Strategy	Type	Antibiotics Affected
Hydrolysis		β-Lactams
		Macrolides
Group transfer	Acyl	Aminoglycoside
		Chloramphenicol
		Type A streptogramin
	Phosphoryl	Aminoglycoside
		Macrolide
		Rifamycin
		Peptide
	Thiol	Fosfomycin
	Nucleotidyl	Aminoglycoside
		Lincosamide
	ADP-ribosyl	Rifamycin
	Glycosyl	Macrolide
		Rifamycin
Other	Redox	Tetracycline
		Rifamycin
		Type A streptogramin
	Lyase	Type B streptogramin

broad cadre of strategies to confer drug resistance (Table 1). The review will make an inventory of these mechanisms and discuss their origins and evolution, focusing primarily (but not exclusively) on clinical resistance mechanisms for the sake of coherence and brevity.

2. Enzymatic strategies of antibiotic inactivation

2.1. Hydrolysis

Many antibiotics have hydrolytically susceptible chemical bonds (e.g., esters and amides), whose integrity is central to biological activity. Not surprisingly then, there are several examples of enzymes that have evolved to target and cleave these vulnerable bonds and, as a result, provide a means of destroying antibiotic activity. Chief among these are a cadre of amidases that cleave the β-lactam ring of the penicillin and cephalosporin classes of drugs. Other examples also include esterases that have been linked to macrolide antibiotic resistance and fosfomycin resistance ring-opening epoxidases. Because these enzymes require only water as a co-substrate, they can often be excreted by the bacteria, thereby intercepting antibiotics before they come into contact with the bacteria.

2.1.1. β-Lactamases

Likely the first antibiotic resistance mechanism reported in the literature was the production of penicillinase by pathogenic *Escherichia coli* [2]. Since then, the study of the β-lactamases has been intense, and there are numerous reviews and monographs that confer the details of this broad family of enzymes (e.g., Refs. [3–7]). For the purpose of this review then, only a brief overview of the resistance chemistry and structure of β-lactamases will be provided.

There are two main molecular strategies employed by β-lactamases to hydrolytically cleave the β-lactam ring of penicillins and cephalosporins: through the action of an active site Ser nucleophile, or through activation of water via a Zn^{2+} centre (Fig. 1). These Ser-β-lactamases or metallo-β-lactamases can be further subdivided based on their three-dimensional structures: amino acid sequences, substrate preferences, and inhibitor sensitivities. The β-lactamase family has been subdivided in a number of ways; however, the nomenclature of Bush et al. [8] has gained prominence. In a study of almost 200 β-lactamases, these authors recognized four main groups of

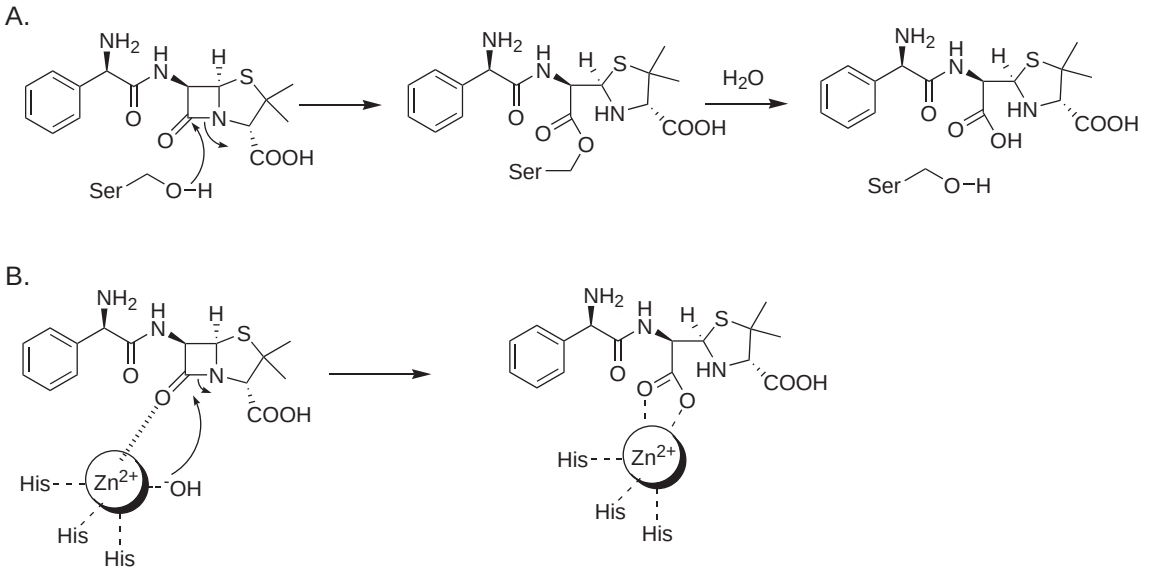

Fig. 1. General mechanism of Ser-β-lactamases and metallo-β-lactamases. The hydrolysis of the penicillin amoxicillin is shown catalyzed by a Ser-β-lactamase (A) and a metallo-β-lactamase (B).

enzymes. Groups 1, 2, and 4 are Ser-β-lactamases, while members of group 3 are metallo-β-lactamases. Group 1 is comprised of cephalosporinases that are poorly inhibited by the β-lactamase inhibitor clavulanic acid; group 2 is comprised of clavulanic acid-sensitive penicillinases and includes the so-called extended spectrum β-lactamases (ESBLs) [9]; and group 4 is comprised of lactamases that are poorly inactivated by clavulanic acid and do not fit the other categories.

The Ser-β-lactamases act via a covalent catalytic mechanism reminiscent of Ser proteinases and esterases where an active site Ser performs a ring-opening nucleophilic attack on the lactam ring (Fig. 1). This is then followed by hydrolytic cleavage of the covalent enzyme intermediate. This mechanism parallels the β-lactam inactivation of the extracellular bacterial peptidoglycan transpeptidases, which are the targets for these antibiotics where the drug covalently modifies an active site Ser. However, in this case, the configuration of the active site of these transpeptidases does not permit ready access of water to complete the second half of the lactamase reaction, and therefore the acyl-enzyme intermediate is a dead-end complex resulting in enzyme titration and consequent impairment of cell wall synthesis. The similarity in mechanism between peptidoglycan peptidases and Ser-β-lactamases also extends to their three-dimensional structure and it has been speculated that they are evolutionarily linked [10,11].

The metallo-β-lactamases are members of the broader Zn-dependent hydrolase family [12] and were once thought to be of minimal clinical importance, although they have emerged over the past decade as significant causes of resistance to carbapenems in Gram-negative bacteria [13].

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 via an ester bond that results from the final ring forming step catalyzed by the thioesterase module of the polyketide synthetase responsible for the ring closure step that generates 6-deoxyerythronolide B (for the 15-member erythromycin) macrocyle [14]. It is, therefore, not surprising that this key bond has been targeted by macrolide resistance enzymes operating now in the reverse ring-opening mode. In 1984, the first erythromycin esterase was reported from a macrolide-resistant isolate of *E. coli* [15]. Cloning of the *ereA* gene revealed a sequence encoding a protein of 406 amino acids with a predicted mass of 44.8 kDa [16]. Subsequently, another orthologue, ereB, was cloned from another *E. coli* isolate [17]. ereB is predicted to be 419 amino acids in length (48.2 kDa) and is 23% identical (43% similar) to ereA. Both proteins are predicted to cleave the macrocycle ester, which is followed by (likely) non-enzymatic intramolecular hemiketal formation, followed by a second internal cyclization event via intramolecular condensation, followed by dehydration (Fig. 2).

While not a common drug resistance mechanism, the presence of erythromycin esterases results in very high levels of resistance (MIC $\geq$ 1600 μg/ml) in *E. coli* [18] and the genes have been located to a class 2 integron in *E. coli* [19] and a class 1 integron in *Providencia stuartii* [20]. The presence of these genes on mobile genetic elements implies the ability to become widespread in the microbial community, and the presence of esterases has been confirmed in at least one clinical isolate of *Staphylococcus aureus* [21] and in environmental isolates of *Pseudomonas* sp. [22].

2.1.3. Epoxidases

The epoxide antibiotic, fosfomycin, covalently modifies the enzyme *MurA*, an essential gene required for the synthesis of *N*-acetylmuramic acid, one of the essential sugar building blocks of cell wall peptidoglycan. Enzymatic resistance to this antibiotic occurs through destruction of the reactive epoxide by ring opening by either a thiol-containing co-substrate (see Section 2.2.3 below) or water. The later mechanism is catalyzed by the enzyme FosX from the non-pathogenic soil bacterium *Mesorhizobium loti* [23]. The gene encoding this enzyme has orthologues in the chromosomes of number of bacteria indicating that resistance may be widespread in the environment. The crystal structure of FosX resembles that of FosA, a glutathione-dependent fosfomycin resistance metalloenzyme found in Gram-negative bacteria [24], with which it shares a catalytically important divalent metal cation (Mn^{2+}). A conserved Glu residue (Glu44) in FosX orthologues may be a catalytic base important

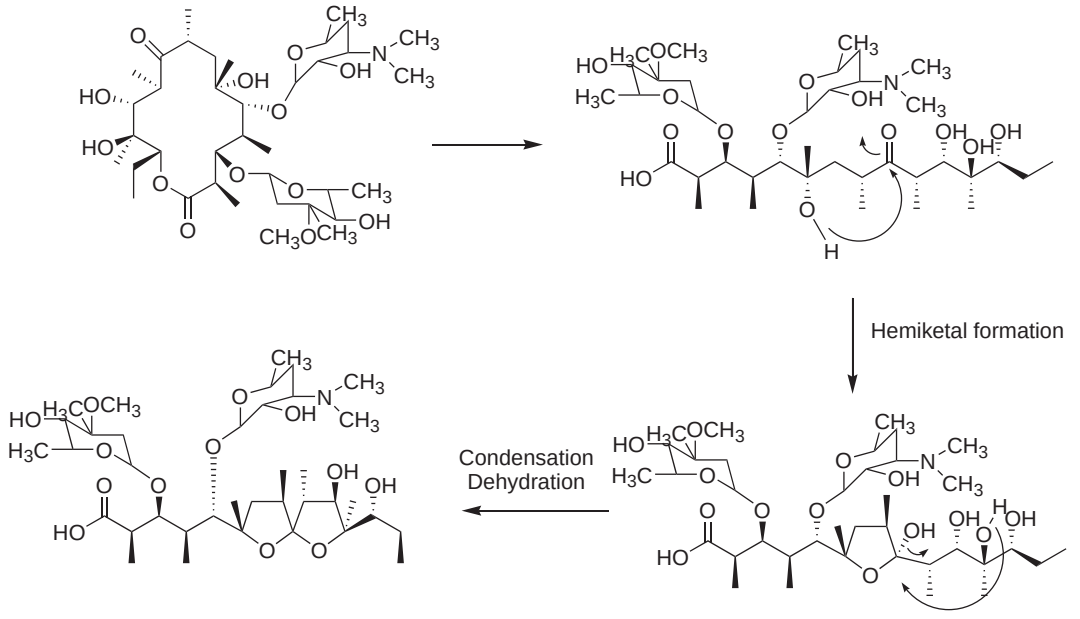

Fig. 2. Reaction and downstream products of macrolide esterases.

for the activation of water for attack on the epoxide ring, which occurs at position 1 (Fig. 3).

2.2. Group transfer

The most diverse, and consequently the largest, family of resistance enzymes is the group transferases. These enzymes covalently modify antibiotics resulting in structural alterations that impair target binding. Chemical strategies include *O*-acylation and *N*-acylation, *O*-phosphorylation, *O*-nucleotidylation, *O*-ribosylation, *O*-glycosylation, and thiol transfer. These covalent modification strategies all require a co-substrate for activity, including ATP, acetyl-CoA, NAD^+, UDP glucose, or glutathione. Consequently, these enzymes are only active in the cytosol.

2.2.1. Acyltransferases

Acyltransfer, and specifically acetyltransfer, is a common mechanism of antibiotic inactivation employed by bacteria. Covalent modification of vulnerable hydroxyl and/or amine groups on antibiotics results in compounds that lose their ability to bind target and, therefore, become inactive. The resulting ester (for *O*-acetylation) or amide (for *N*-acetylation) is biologically stable and, therefore, essentially irre-

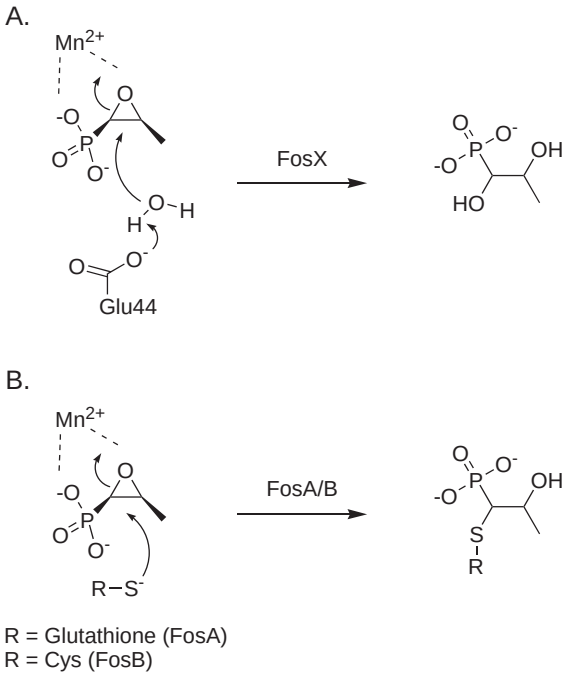

Fig. 3. Proposed ring opening reactions catalyzed by fosfomycin resistance enzymes FosX (A) and FosA and FosB (B).

versible without the action of a cognate esterase or amidases enzyme. The high concentration of acetyl-CoA (>0.3 mM in *E. coli* growing on glucose-containing media [25]) in the cell ensures that the enzymes have a ready supply of co-substrate.

2.2.1.1. Aminoglycoside acetyltransferases. The aminoglycoside antibiotics bind to the A-site of the ribosome and, as a result, impair the codon–anticodon decoding mechanism. This results in the synthesis of aberrant proteins and consequent blocking of translation fidelity. The interaction of the cationic aminoglycoside antibiotics with the A-site is primarily through the 16S rRNA. Disruption of key hydroxyl and amine groups on the aminoglycoside antibiotics therefore blocks the interaction with the rRNA, resulting in resistance. The aminoglycoside acetyltransferases (AACs) are members of the GCN5 superfamily of proteins that include the histone acetyltransferases among others. The AACs are classified based on their regiospecificity of acetyltransfer on the aminoglycoside structure. For example, the AAC(6′) *N*-acetylate aminoglycoside on the amine group that is frequently found on position 6′ of the aminohexose linked to position 4 of the central 2-deoxystreptamine ring, while AAC(3) *N*-acetylate linked to position 3 of the 2-deoxystreptamine ring (Fig. 4). Genes encoding these enzymes are widespread both in the clinic (as a result of their frequent association with resistance plasmids, transposons, and integrons) and in the environment (as orthologues have been identified in a number of sequenced bacterial genomes).

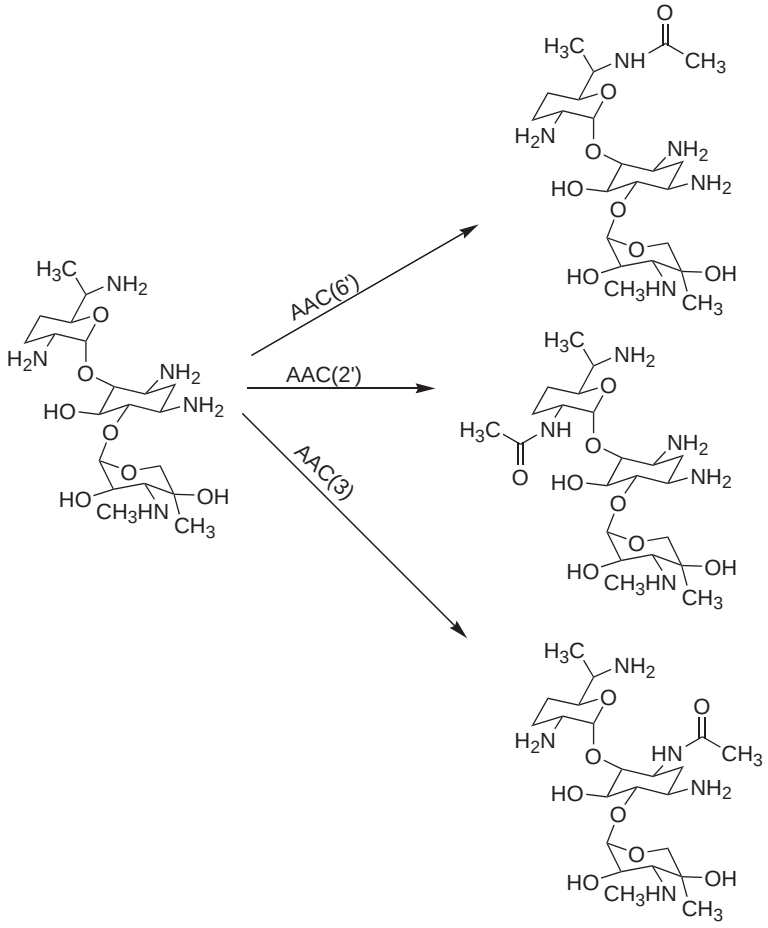

Fig. 4. Reactions catalyzed by AACs.

Three-dimensional structures for four members of the class have been reported [26–29] and, as noted above, these show structural homology to the GCN5 superfamily of acyltransferases [30]. There are no common active site catalytic residues among all AACs, although analysis of the active site region where aminoglycosides bind reveals a highly negatively charged surface that serves as a docking platform for these basic antibiotics.

2.2.1.2. Chloramphenicol acetyltransferases. Chloramphenicol acetyltransferases (CATs) are trimeric enzymes that have two distinct structural types: class A and class B (also known as the xenobiotic CATs) [31]. The three-dimensional structures of the class A CAT, CAT-III from *E. coli* [32] and class B CAT from *Pseudomonas aeruginosa* [33], have been determined. As noted above, these enzymes share a quaternary trimeric organization and have three active sites at the interface of the monomers, although their primary sequences and 3D structures are dissimilar. Nonetheless, it is likely that they share similar molecular strategies of *O*-acetylation. The role of an active site His residue of CAT-III as an active site base required to deprotonate the nucleophilic hydroxyl group of chloramphenicol has been well characterized [34], and the active site of the class B CAT also has a conserved His residue that likely plays a similar role (Fig. 5).

2.2.1.3. Streptogramin acetyltransferases. The streptogramin acetyltransferases (VATs for *v*irginiamycin *a*cetyl*t*ransferase) inactivate the type A streptogramins by acetylation of the free hydroxyl group at position 14 (Fig. 6). The genes encoding these enzymes have been identified in a number of Gram-positive pathogens including the staphylococci and enterococci [35–39]. Five of these enzymes, VatA–E, have been cloned and shown to share significant amino acid similarity. One of these, VatD, has been crystallized and its three-dimensional structure solved [40,41]. The structure revealed a similar fold to the class B CAT family with a trimeric enzyme with three active sites at the interfaces of the monomers. Like the CATs, the binding sites for acetyl-CoA and the streptogramins, virginiamycin M1 and dalfopristin, on VATs suggested a mechanism whereby an invariant His residue deprotonates the hydroxyl to enhance nucleophilicity and facilitate attack at the thioester of acetyl-CoA (Fig. 6). This proposed mechanism is consistent with site-directed mutagenesis and steady-state kinetic studies on VatA (N. Johnston and G. Wright, unpublished).

2.2.2. Phosphotransferases

Kinases are ubiquitous enzymes that catalyze phosphate transfer from a nucleoside trinucleotide, typically ATP, to a diverse set of substrates. Most are *O*-phosphotransferases (e.g., Ser, Thr, and Tyr kinases), sugar kinases (e.g., hexokinase), and amino acid kinases (e.g., aspartate kinase), although *N*-phosphotransferases are known, notably creatine kinase, which phosphorylates the *N*-methylguanidine group of creatine and is essential for high-energy phosphate storage in muscles, and His kinases, which are key components of (mostly) bacterial two-component signal transduction pathways. The known antibiotic kinases involved in resistance are exclusively *O*-phospho-

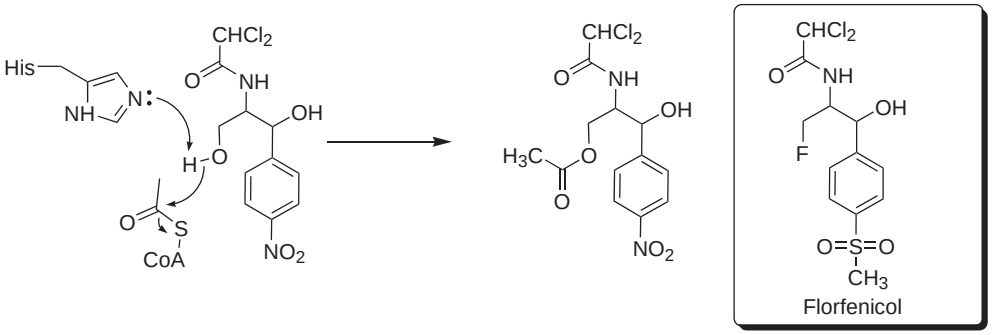

Fig. 5. Mechanism of chloramphenicol acetyltransferases and structure of florfenicol, which is not susceptible to CAT-mediated resistance.

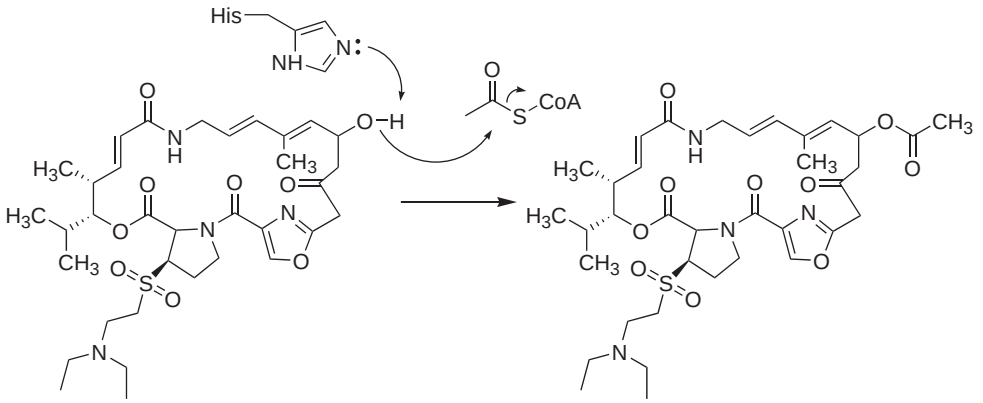

Fig. 6. Mechanism of acetylation of type A streptogramins by VAT.

transferases (Table 1), and many share structural and mechanistic details with other kinases such as the protein kinases.

2.2.2.1. Aminoglycoside kinases. Aminoglycoside kinases, termed APHs for *a*minoglycoside *ph*osphotransferases, are widely distributed among bacterial pathogens. Phosphorylation of the antibiotics results in a dramatic effect on their ability to bind to their target on the A-site of the ribosome [42]. The genes encoding these enzymes are frequently found on multi-drug resistance R plasmids, transposons, and integrons; therefore, even if aminoglycosides are not widely used in a given setting, the resistance genes are very often present in bacterial populations. Like the AACs discussed above, APHs are classified based on their regiospecificity of phosphoryl transfer, their substrate specificity, and the specific gene sequence in question. The APH(3′) family is especially ubiquitous and has, in fact, been widely used as traceable resistance marker in molecular biology research (e.g., the *neo* cassette).

The best studied APH is APH(3′)-IIIa, which has both 3′- and 5″-regiospecific phosphoryl transfer capacities [43,44], and serves as a useful example to discuss the class. The enzyme is primarily found in Gram-positive cocci such as staphylococci and enterococci, and confers resistance to a broad range of aminoglycosides, but not to gentamicin or tobramycin, which both lack the critical 3′-hydroxyl group that accepts the phosphate group donated by ATP (Fig. 7) [43,45]. Extensive steady-state kinetic analysis demonstrated that the enzyme requires formation of a ternary complex with ATP and the aminoglycoside substrate and, like all other kinases, divalent cations are essential for activity [46–48]. The three-dimensional structure of the enzyme revealed a remarkable similarity with Ser, Thr, and Tyr protein kinases, which was not evident from the primary amino acid sequence [49]. This similarity in structure also extends to similarity in mechanism, demonstrable protein and peptide kinase activity of APHs [50], and common sensitivity to inhibitors [51].

Other aminoglycoside kinases include the spectinomycin-modifying enzyme APH(9) [52], and APH(3″) (StrA) and APH(6) (StrB), which both modify streptomycin [53]. The APH(2″) enzymes are highly problematic in the clinic as they confer resistance against most of the clinically used aminoglycosides such as gentamicin and tobramycin. The enzyme can be expressed either as the C-terminal domain of a bifunctional enzyme that also includes an AAC(6′) in the N-terminus [54], or independently as a single enzyme [55]. The bifunctional enzyme, AAC(6′)–APH(2″), is widely distributed among pathogenic bacteria and confers high level resistance to virtually all aminoglycosides except streptomycin and spectinomycin [56,57]. The AAC(6′) domain of this bifunctional enzyme has overlapping aminoglycoside modification capacity with APH(2″) domain and aminoglycosides can be doubly modified [56] as a result, possibly contributing to the very high MICs associated with this enzyme. The AAC(6′) domain also confers resistance to the antibiotic, fortimicin. This AAC is unusual, too, in that it has measurable *O*-acetyl-

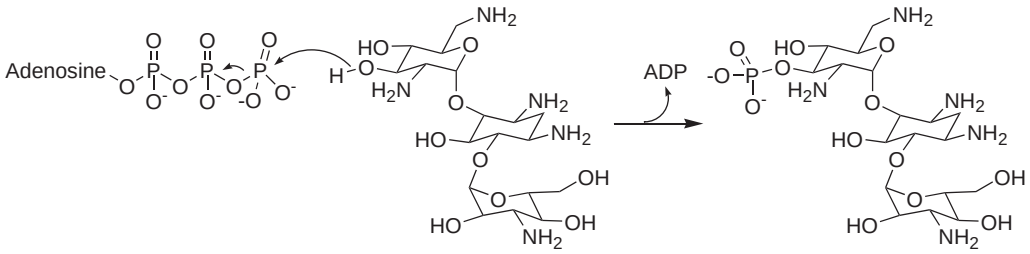

Fig. 7. Action of APH(3′)-IIIa on kanamycin.

transferase activity and can, therefore, modify aminoglycosides such as paromomycin [57].

2.2.2.2. Other antibiotic kinases. The molecular strategy of covalent modification by phosphate to provide resistance to antibiotics has also been exploited by bacteria to block the effects of macrolides such as erythromycin and peptide antibiotics such as viomycin (Fig. 8). The macrolide kinases, MPHs, and viomycin kinase (Vph) have been cloned and characterized, although our understanding of mechanisms is not as thorough as for the APHs. The MPHs were reported in Japan to be linked to macrolide resistance in *E. coli* [58–61] and have also been detected in *S. aureus* [62] and *P. aeruginosa* [63]. Characterization of the product of inactivation revealed that phosphorylation was occurring on the free hydroxyl (site 2′ in the macrolide nomenclature) of the desosamine sugar (Fig. 8) that interacts directly with the 23S rRNA [64]. Three genes encoding MPH enzymes have been sequenced, *mphA* [60] and *mphB* [61] from *E. coli*, and *mphBM* (also known as *mphC*) from *S. aureus* [62]. The presence of these genes results in very high MIC values (>2 mg/ml) for 14- and 16-member macrolides and also for the new ketolide class of macrolides (Ref. [65]; V. D'Costa and G. Wright, unpublished results). While these genes are not widespread at his time, the fact that they confer resistance to the ketolides suggests a selection mechanism for wider distribution in the future.

The tuberactinomycins are basic cyclic pentapeptide inhibitors of bacterial translation with potent antitubercular activity. The prototypes of the class are viomycin and capreomycin, both produced by species of *Streptomyces* [66,67]. Self-resistance to these antibiotics occurs via kinases (VPHs) that modify the antibiotics on the Ser residue (Fig. 8) and share sequence homology in the ATP-binding region with the aminoglycoside, macrolide, and protein kinases, specifically in the signature sequences HGDL and GXV(I)D where the invariant Asp in the first sequence is involved in phosphate transfer and in the second sequence is a Mg^{2+} ligand [68].

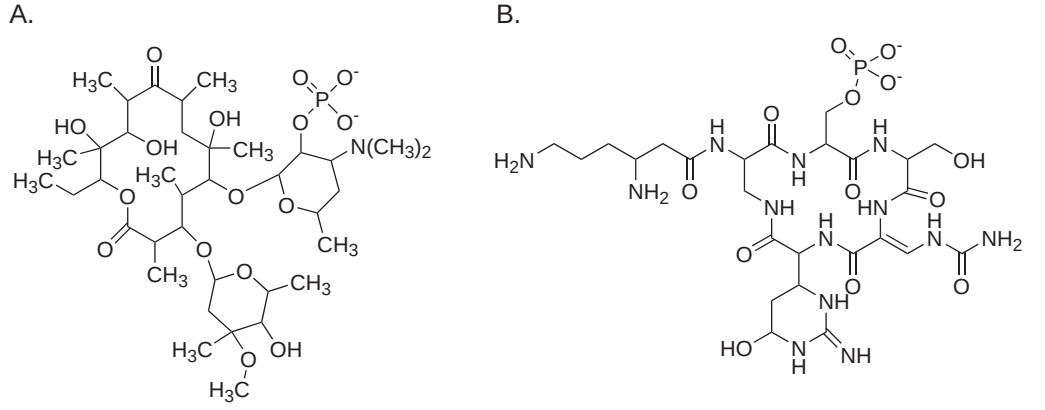

Fig. 8. Macrolide (MPH) and tuberactinomycin (VPH) kinase reaction products. (A) 2′-Phosphoerythromycin; (B) phosphoviomycin.

Modification of rifampin by an uncharacterized kinase in pathogenic bacteria of the genera *Nocardia* [69,70] and *Rhodococcus* [71] has been reported. Modification occurs on the hydroxyl group at position 23 and presumably interferes with binding to RNA polymerase (Fig. 9).

2.2.3. Thioltransferases

As noted above, the reactive epoxide of fosfomycin makes this antibiotic vulnerable to nucleophilic ring-opening reactions. The fosfomycin resistance enzyme FosA, encoded on resistance plasmids found in Gram-negative bacteria [72] and on the chromosome of *P. aeruginosa* [24], is a metallo-enzyme that catalyzes fosfomycin epoxide ring opening using the thiolate of glutathione as the reactive nucleophile (Fig. 3). An equivalent gene, *fosB*, is found on resistance plasmids in Gram-positive bacteria such as staphylococci [73,74] and on the chromosome of *Bacillus subtilis* [75]. The absence of glutathione in these Gram-positive bacteria [76] has necessitated a retooling of the inactivation strategy and FosB has evolved to be an efficient fosfomycin Cys transferase (Fig. 3).

2.2.4. Nucleotidyltransferases

There are two major classes of nucleotidyltransferases: the ANTs that modify aminoglycosides, and the Lin proteins that inactivate the lincosaminide antibiotics that include lincomycin and its semi-synthetic derivative, clindamycin. These enzymes transfer the NMP portion of NTPs to an accepting hydroxyl group on the antibiotic. While all antibiotic *O*-nucleotidyltransferases studied at the protein level have a broad ability to utilize a variety of NTPs, given the high relative concentration of ATP in the bacterial cell, it is likely that these enzymes are exclusive AMP transferases in vivo.

The ANTs represent the smallest class of aminoglycoside-inactivating enzymes, although they have a major impact on clinical resistance. The clinically

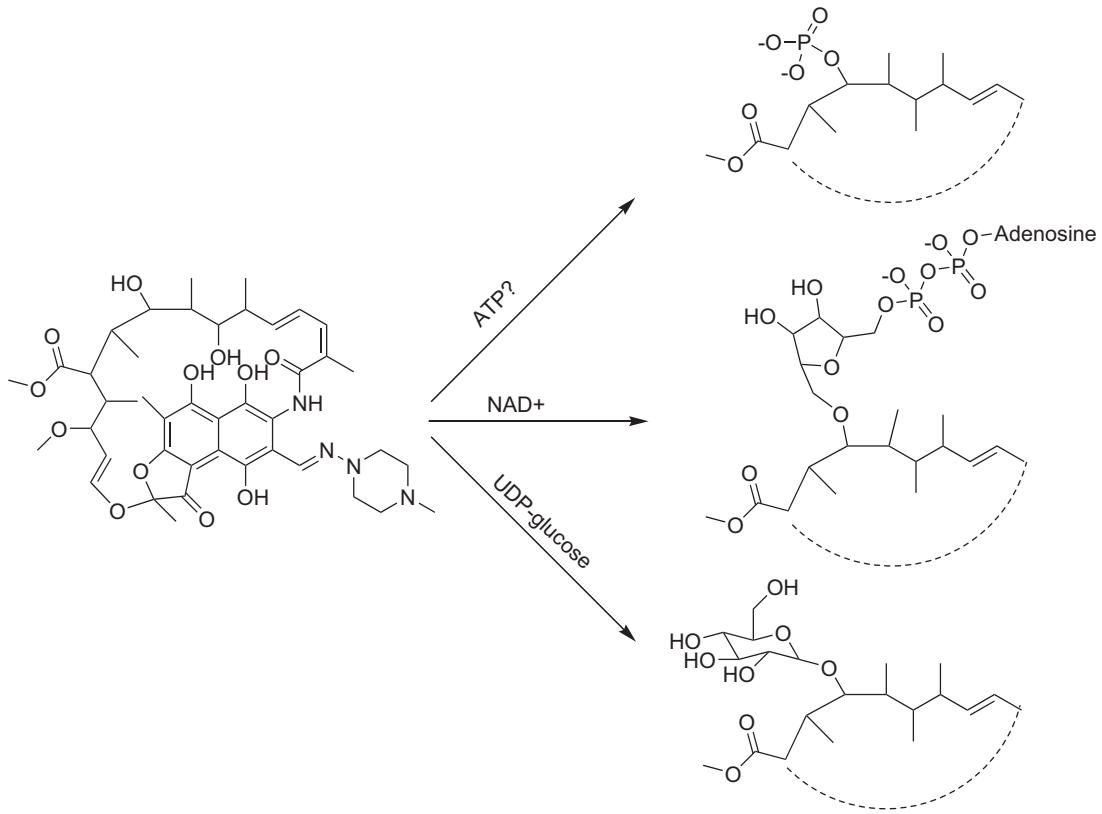

Fig. 9. Chemical modifications of rifampin resulting in antibiotic resistance.

important, gentamicin and tobramycin, are both modified by ANT(2″) (Fig. 10). The gene encoding this enzyme is widely distributed among pathogenic bacteria and its local prevalence is clearly selected by aminoglycoside usage in different clinical environments [77]. The enzyme has been purified and its substrate specificity and kinetic mechanism characterized [78–80]. The structure of aminoglycoside substrates bound to the enzyme has been characterized by NMR methods [81], although a three-dimensional structure of the entire enzyme remains elusive.

The three-dimensional structure of only one ANT has been reported—that of ANT(4′,4″) from *S. aureus* [82,83]. The enzyme functions as a dimer, with the active site at the interface and with both monomers contributing residues to stabilize the substrates [82]. The positioning of the substrates supports independent mechanistic evidence for direct attack of the nucleophilic hydroxyl on the α-phosphate of ATP [84,85].

There are three characterized lincosaminide nucleotidyltransferase genes, *linA* from *Staphylococcus haemoliticus* [86], the very similar *linA′* from *S. aureus* [87], and *linB* from *Enterococcus faecium* [88]. All the Lin proteins modify lincomycin at position 3, and LinB also adenylates clindamycin at this site (Fig. 11), although both LinA and LinA′ are reported to modify clindamycin at position 4 [88,89].

2.2.5. ADP-ribosyltransferases

ADP-ribosyl transfer is a common mechanism of protein modification in both eukaryotes and prokaryotes. These modifications occur on amino acid residues and require nicotinamide adenine dinucleotide (NAD) as the ADP-ribosyl donor. For example, in higher eukaryotes, poly(ADP-ribose) polymerases (PARPs) are well known to modify nuclear proteins in response to DNA damage and are markers of programmed cell death [90]. The bacterial cholera, pertussis, and botulinum toxins are members of the A:B toxin family where a delivery protein, B, recognizes a binding partner on eukaryotic cells and shuttles the ADP-ribosyltransferase component (A) into the cell where it is involved in protein modification, typically on His (or diphthamide), Arg, or Cys residues [91]. ADP ribosylation of rifampin is thus far the only well-documented example of this modification in antibiotic resistance. Rifampin is an inhibitor of bacterial RNA polymerase that finds primary use in the treatment of infections caused by *Mycobacterium tuberculosis*. In other mycobacteria such as *Mycobacterium smegmatis*, a unique rifampin ADP-ribosyltransferase (ARR) blocks the antimicrobial activity of this drug [92]. Another enzyme with similar activity, ARR-2, is associated with multidrug resistance integrons in Gram-negative bacteria [93]. These enzymes show approximately 55% identity to each other and are unique among ADP-ribosyltransferases in that they are quite small (~150 amino acids) and do not show sequence similarity to the protein ADP-ribosyltransferases. Analysis of the product of enzymatic modification reveals that the hydroxyl at position 23 is the site of ADP ribosylation and that this compound may lose ADP to generate the ribosyl derivative [94–96] (Fig. 9).

2.2.6. Glycosyltransferases

Glycosyltransfer is so far not a widespread mechanism of antibiotic resistance, although it does play a

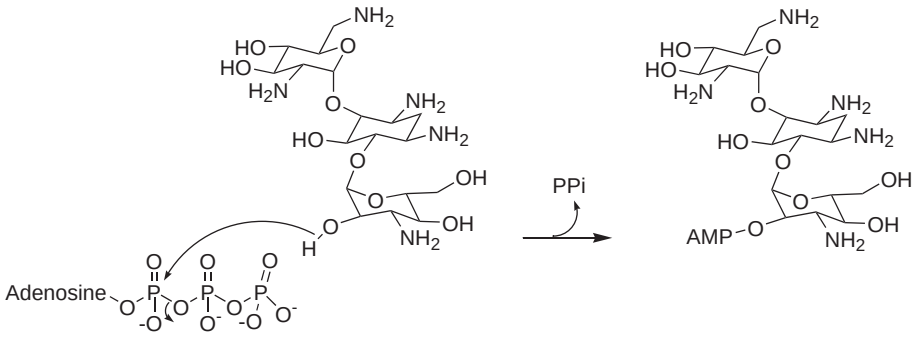

Fig. 10. Modification of kanamycin by ANT(2″).

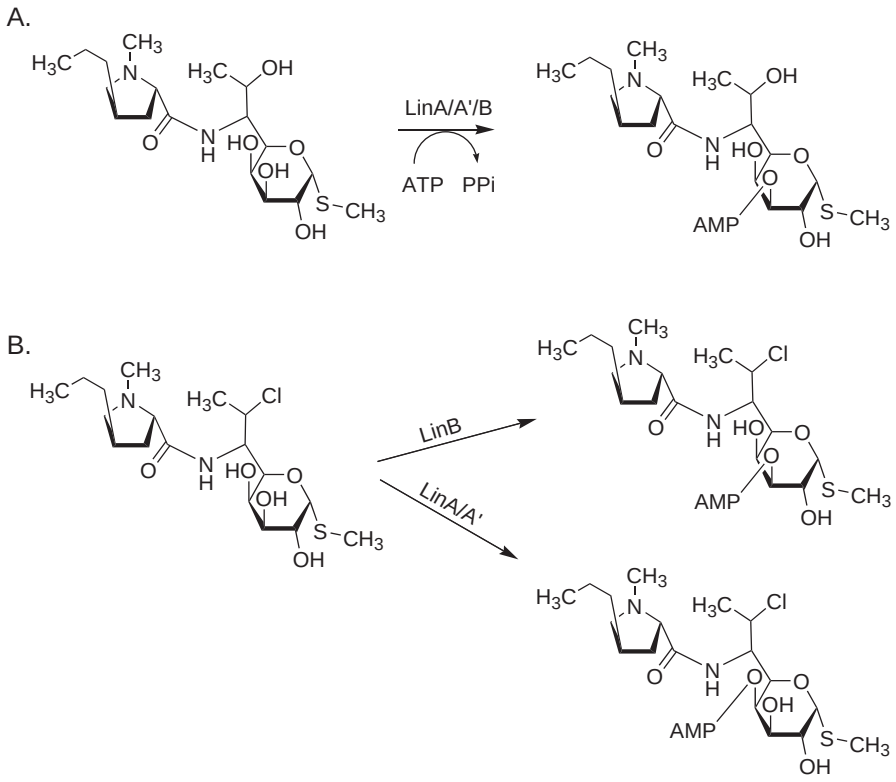

Fig. 11. Inactivation of lincosamides by the Lin nucleotidyltransferases. (A) Lincomycin; (B) clindamycin.

role in self-protection in antibiotic-producing organisms. Macrolide glycosylation by the product of the *mtg* gene in *Streptomyces lividans* is the prototype of this class [97,98]. The enzyme catalyzes glucosylation of erythromycin and other macrolides at position 2′ of the desosamine sugar using UDP glucose as the glucose donor (Fig. 12). Glycosylation of rifampin at position 23 by species of *Nocardia* has also been reported, although the enzymes have not been characterized (Fig. 9) [70,99].

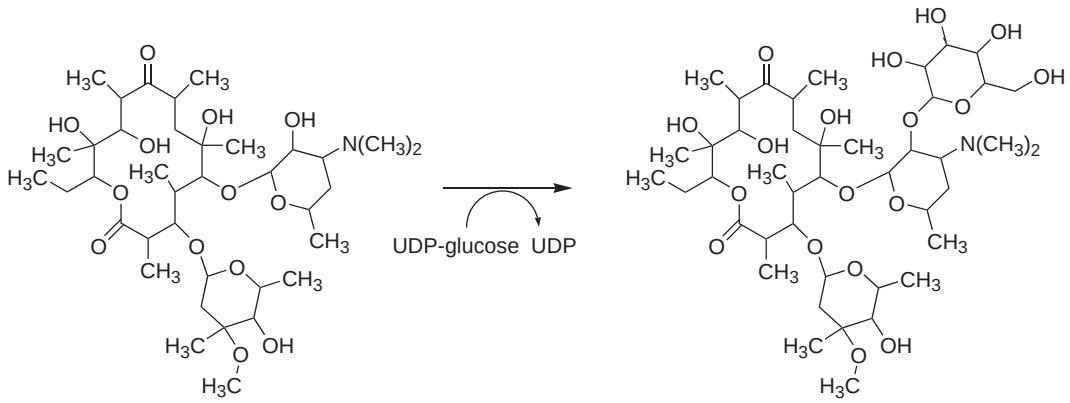

Fig. 12. Glycosylation of macrolides antibiotics by *mtg*.

3. Other enzymatic mechanisms of resistance

While group transfer and hydrolysis represent the lion's share of characterized enzymatic resistance mechanisms, there are a growing number of alternative strategies that have been exploited by bacteria to detoxify antibiotics.

3.1. Redox enzymes

Oxidation is a frontline mechanism of mammalian detoxification of xenobiotics. This molecular strategy occurs primarily through a cadre of membrane-bound cytochrome *P*-450s with broad substrate specificity that hydroxylate xenobiotics to facilitate their excretion [100]. In contrast, the oxidation or reduction of antibiotics has not been frequently exploited by pathogenic bacteria. Nonetheless, there are a few of examples of this strategy in the literature. The best studied of these is the oxidation of tetracycline antibiotics by the enzyme, TetX [101–104]. The gene-encoding TetX was found on conjugative transposons in the obligate anaerobe *Bacteroides fragilis* [102,103]. Paradoxically, the TetX protein is an oxygen-requiring flavoprotein [104] and, as such, the tetracycline resistance phenotype was only uncovered when the gene was cloned into *E. coli* [101,102]. TetX catalyzes the monohydroxylation of tetracycline antibiotics at position 11a, which disrupts the Mg^{2+}-binding site of the antibiotic that is required for antibacterial activity (Fig. 13). Subsequent to TetX-catalyzed hydroxylation, the antibiotic undergoes non-enzymatic rearrangement into unstable products that polymerize into a black product after several hours [104,105].

Another predicted monooxygenase with antibiotic inactivation properties was cloned from rifampin-resistant *Rhodococcus equi* [106]. Expression of the gene in *E. coli* resulted in rifampin resistance by an uncharacterized degradative mechanism.

In yet another example of redox-mediated resistance, *Streptomyces virginiae*, the producer of the type A streptogramin antibiotic virginiamycin M_1, protects itself from its own antibiotic by reducing a critical ketone group at position 16 to an alcohol, thereby generating an inactive compound (Fig. 14) [107]. Reduction was NADPH-dependent and regiospecific, generating the (14*S*,16*R*)-dihydrovirginiamycin M_1 isomer exclusively.

Resistance to the anti-mycobacterial drug, isoniazid, presents a variation on the theme of redox-associated resistance. Isoniazid is a prodrug that is activated to a reactive product through the action of the catalase peroxidase KatG, a non-essential enzyme [108]. Subsequent to KatG activation, the antibiotic can covalent modify targets such as NAD bound to InhA, an enoyl-ACP reductase that is an essential fatty acid biosynthetic enzyme required for cell wall mycolic acid synthesis [109]. Resistance to isoniazid can, therefore, occur in strains with impaired KatG proteins [110] and so a lack of redox activity is associated with resistance in this example [111].

3.2. Lyases

Lyases are enzymes that cleave carbon–carbon, carbon–oxygen, carbon–nitrogen, and carbon–sulfur bonds by non-hydrolytic or non-oxidative routes. These reactions also frequently result in double bond formation or ring closure. There is one well-characterized antibiotic resistance lyase, Vgb, which is responsible for type B streptogramin resistance [112]. Type B streptogramins are cyclic hexadepsipeptides or heptadepsipeptides that are cyclized via an ester bond between a Thr side chain hydroxyl and the carboxyl of the invariant C-terminal phenylglycine (Fig. 15). Vgb was cloned from streptogramin B-resistant staphylococci [38,113] and initially reported to be a hydrolytic lactonase associated with ring opening of the antibiotic at the susceptible ester

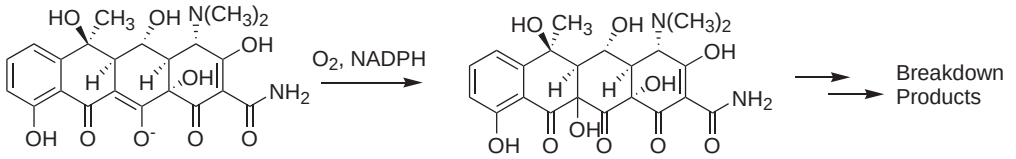

Fig. 13. Inactivation of oxytetracycline by TetX.

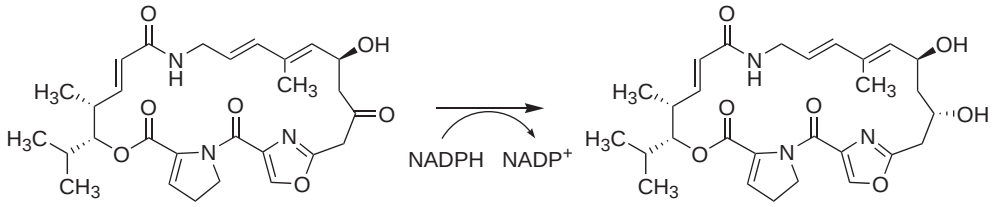

Fig. 14. Redox-based resistance to type A streptogramins.

bond [114]. Purification and characterization of the linear product of *S. aureus* Vgb catalysis, however, demonstrated that the reaction does not require water as a co-substrate [112] analogous to the inactivation of the streptogramin etamycin by extracts of *S. lividans* [115]. The inactive product also contained a double bond at the site of cleavage, confirming that the resistance enzyme Vgb is a lyase (Fig. 15).

4. Evolution of enzymatic resistance

Antibiotic inactivation mechanisms share many similarities with well-characterized enzymatic reactions. Hydrolysis, group transfer, and redox enzymes are all involved in primary and intermediary microbial metabolism and, thus, likely serve as the origins of resistance. As noted several times above, primary sequence analysis of resistance proteins, and in particular determination of their molecular mechanisms and three-dimensional structures, has revealed homologies to known metabolic and signaling enzymes with no antibiotic resistance activity. Therefore, one can speculate that these are the original sources of resistance. A reasonable scenario, then, would have such housekeeping genes encoding enzymes with modest and fortuitous resistance properties evolving as a result of exposure to the antibiotic into a bone fide and efficient resistance enzymes (Fig. 16). The antibiotic-producing organisms are possible originators for many resistance enzymes, as here resistance *and* biosynthesis must co-evolve and, thus, the evolutionary pressure would be chronic. Furthermore, the soil environment where many of these organisms reside is home to neighboring species that produce their own antibiotics, thereby adding to the evolutionary pressure to develop resistance enzymes. The presence of resistance elements in antibiotic-producing bacteria that have orthologues in clinical isolates supports this hypothesis [116,117].

Antibiotic-producing bacteria are not the only source of resistance genes. The sequencing of whole bacterial genomes has revealed an unexpected number of chromosomal genes encoding putative resistance enzymes in organisms not previously characterized as antibiotic-resistant. Verification of annotation, however, requires characterization of the isolated enzymes and only this rigorous approach

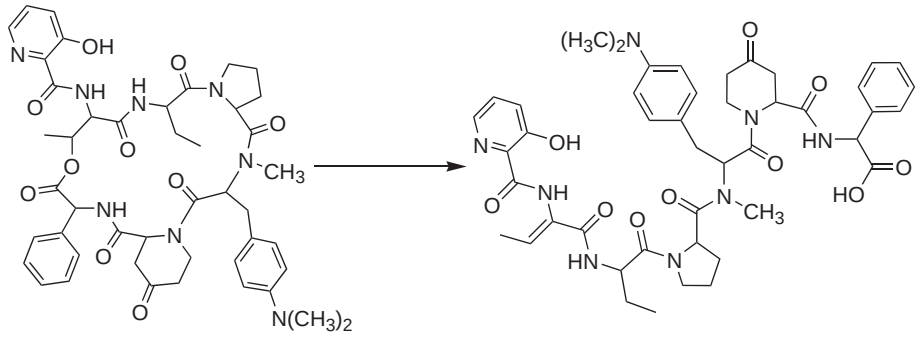

Fig. 15. Action of Vgb on type B streptogramins.

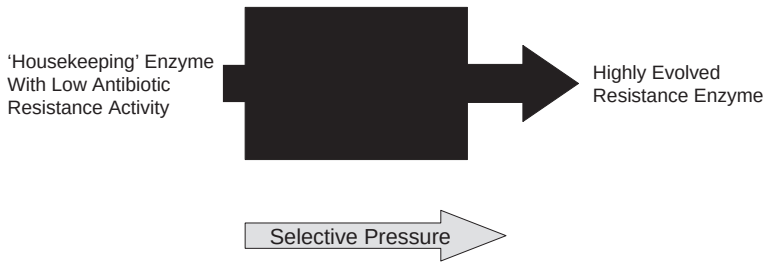

Fig. 16. Evolution of resistance enzymes. External pressure of antibiotic presence selects for more efficient resistance elements.

can support or refute the predicted resistance phenotypes [112,118,119].

5. Strategies to overcome resistance

Knowledge of the molecular mechanism and structure of antibiotic degrading enzymes is essential to develop approaches to ultimately prevail over resistance. For example, understanding of the 3′-regiospecificity of aminoglycoside resistance by the resistance kinases that emerged in the 1960s enabled the development of aminoglycosides such as tobramycin and gentamicin that lacked sites of inactivation [120]. Similarly, florfenicol (Fig. 5) was developed to overcome CAT-mediated resistance by acetylation at the hydroxyl linked to C3 [31]. This approach has also driven the development of the numerous generations of semi-synthetic β-lactam antibiotics, which were designed to keep one step ahead of evolving β-lactamases. The penems and carbapenems, for example, are among the more recent β-lactam scaffolds developed to circumvent resistance. These antibiotics show broad-spectrum activity against bacterial pathogens and enhanced stability vs. β-lactamases [121]. The first antibiotic in this class, imipenem, was unstable to a renal dehydropeptidase and required co-administration of cilastatin, an inhibitor of this enzyme, although the newer agents such as ertapenem and faropenem are single agent drugs with improved spectrum and stability [122–124].

Another application of a thorough understanding of resistance mechanisms is the development of inhibitors of resistance enzymes. These inhibitors can be administered as co-drugs with the antibiotics, thereby blocking resistance and rescuing the antimicrobial activity of the drugs. This approach has been highly successful as a strategy to overcome resistance to the penicillinases (type 2 class of β-lactamases) where the mechanism-based inactivators of β-lactamases clavulanic acid, sulbactam, and tazobactam have been brought to the clinic [125]. There have been considerable efforts to discover other inhibitors of β-lactamases and, in particular, molecules that target the emerging metallo-β-lactamases [126–128], although this highly successful approach has not yet seen clinical candidates emerge for these other classes. Given the success of the type 2 class β-lactamase inhibitors, however, expanding this strategy to other antibiotics is a possibility that should be explored with vigor to maintain the effectiveness of our current arsenal of antibiotics.

Another strategy to overcome resistance is to improve the delivery or otherwise enhance the accessibility of antibiotics to their sites of action. For example, liposomal preparations of hydrophobic antibiotics such as ethambutol for treatment of mycobacterial infections have been reported [129,130]. Another approach that has been explored is the linking of two different classes of antibiotics (e.g., β-lactams and quinolones [131], or β-lactams and oxazolidinones [132]). These examples used stable or labile linking bonds to tether the antibiotics, although a clever alternative strategy that harnesses the enzymatic action of β-lactamases to release tethered antibiotics has been reported. For example, a cephalosporin–β-chloro-Ala adduct released the Ala racemase inhibitor β-chloro-Ala upon β-lactamase action [133], and cephalosporin–triclosan hybrids have been designed to release triclosan upon cleavage of the β-lactam bond by β-lactamases [134].

All of these alternative strategies to overcome resistance require expanded knowledge of the molecular mechanism of antibiotic resistance, their origins and

evolution, and their distribution throughout bacterial populations and genomes. Given the fact that the antibiotic drug discovery pipeline is going dry [135,136], creative leverage of the understanding of the details of enzyme-based resistance has a significant impact on the treatment of infectious diseases.

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