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Cite this: DOI: 10.1039/c0xx00000x

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REVIEW

# Mechanisms of Resistance to Aminoglycoside Antibiotics: Overview and Perspectives

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Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

DOI: 10.1039/b000000x

Aminoglycoside (AG) antibiotics are used to treat many Gram-negative and some Gram-positive infections and, importantly, multidrug-resistant tuberculosis. Among various bacterial species, resistance to AGs arises through a variety of intrinsic and acquired mechanisms. The bacterial cell wall serves as a natural barrier for small molecules such as AGs and may be further fortified *via* acquired mutations. Efflux pumps work to expel AGs from bacterial cells, and modifications here too may cause further resistance to AGs. Mutations in the ribosomal target of AGs, while rare, also contribute to resistance. Of growing clinical prominence is resistance caused by ribosome methyltransferases. By far the most widespread mechanism of resistance to AGs is the inactivation of these antibiotics by AG-modifying enzymes. We provide here an overview of these mechanisms by which bacteria become resistant to AGs and discuss their prevalence and potential for clinical relevance.

## Introduction

This review presents an overview and perspective of the mechanisms of resistance to aminoglycoside (AG) antibiotics. AGs are broad-spectrum antibiotics effective against both Gram-negative and some Gram-positive bacteria. AG structure consists of a 2-deoxystreptamine (2-DOS) ring to which two or more amino-modified sugars are attached *via* glycosidic bonds (Fig. 1).<sup>1</sup> AGs have long been known to exert their antibacterial action by binding to the bacterial ribosome and interfering with bacterial protein translation.

Recently, AGs have been examined as potential treatments for fungal infections, Leishmaniasis parasitic infections, and for genetic diseases arising from premature termination codons, such as cystic fibrosis, Rett syndrome and Duchenne muscular dystrophy.<sup>2</sup> Currently, however, AGs are typically used to treat Gram-negative infections (*Acinetobacter baumannii*, *Enterobacteriaceae spp.*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*) and as second-line of defence treatment for multidrug-resistant (MDR) tuberculosis (*Mycobacterium tuberculosis*).<sup>3</sup> Therefore, our discussion of mechanisms of resistance to AGs will focus on these two classes of bacteria.

Antibiotic resistance can be classified into three main categories: intrinsic, adaptive, and acquired resistance.<sup>4, 5</sup> An example of intrinsic antibiotic resistance is the naturally low permeability of the bacterial cell wall, which limits uptake of many antibiotics including AGs. Adaptive antibiotic resistance occurs as a result of an environmental trigger (*e.g.*, nutrient concentration changes or sub-inhibitory levels of antibiotics) that causes temporary changes in gene and/or protein expression levels contributing to the tolerance of antibiotics.<sup>5, 6</sup> Bacteria

growing on surfaces as biofilms maintain an adaptive resistance (often referred to as tolerance) to antibiotics.<sup>7</sup> Finally, antibiotic resistance may be acquired by either the incorporation of exogenous genetic material, often a plasmid carrying multiple resistance genes, or *via* mutation of existing genes.<sup>8</sup> While intrinsic and acquired resistance elements are passed on vertically during bacterial reproduction, adaptive resistance is transient and typically reverts upon removal of the environmental trigger. Furthermore, resistance genes on plasmids may be transferred horizontally from one bacterium to another. This is the major cause of the dissemination of antibiotic resistance genes among various bacterial species. Additionally resistance mechanisms may be non-specific (*e.g.*, the cell membrane is impermeable to many toxic small molecules) or specific (*e.g.*, AG-modifying enzymes (AMEs) regioselectively modify only particular AG substrates).

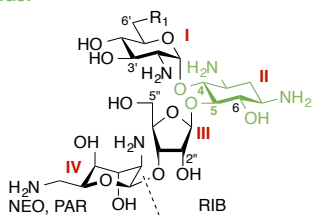
Mechanisms of bacterial resistance to AGs are diverse (Fig. 2). The most common mechanism is inactivation of AGs by a family of enzymes named AMEs. Also, AG resistance can be achieved by mutations of the ribosome target and, increasingly commonly, by modification of the ribosome by a family of ribosomal methyltransferase enzymes.<sup>9</sup> The bacterial cell wall serves as an intrinsic barrier, and its impermeability may be increased by acquired lipid modifications that cause repulsion of AGs. Furthermore, even if AGs do enter the bacterial cell, intercellular concentrations may remain low due to the active expulsion of AGs out of the cell by efflux pumps.<sup>10</sup>

Bacterial resistance to all classes of antibiotics, including AGs, is becoming a global public health crisis.<sup>11, 12</sup> It is crucial to understand the mode of action of AGs as well as mechanisms of AG resistance to be able to fight resistance. This review will focus on recent (2010 onward) research towards the

understanding of mechanisms of AG resistance, except in the case of cell membrane permeability, an area in which little new literature exists.

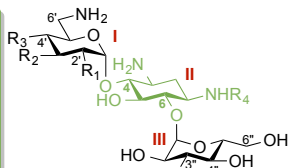
#### 4,5-Disubstituted 2-DOS AGs:

	R <sub>1</sub>
Neomycin B (NEO)	NH <sub>2</sub>
Paromomycin (PAR)	OH
Ribostamycin (RIB)	NH <sub>2</sub>

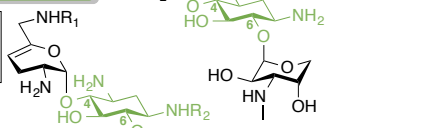
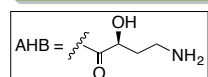
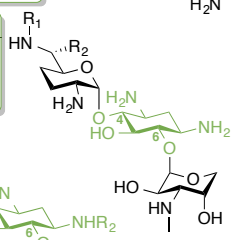


#### 4,6-Disubstituted 2-DOS AGs:

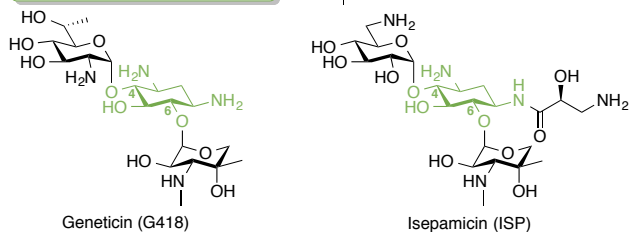
	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>
Amikacin (AMK)	OH	OH	OH	AHB
Arbekacin (ABK)	NH <sub>2</sub>	H	H	AHB
Dibekacin (DBK)	NH <sub>2</sub>	H	H	H
Kanamycin A (KANA)	OH	OH	OH	H
Kanamycin B (KANB)	NH <sub>2</sub>	OH	OH	H
Tobramycin (TOB)	NH <sub>2</sub>	H	OH	H



	R <sub>1</sub>	R <sub>2</sub>
Gentamicin C <sub>1</sub> (GEN C <sub>1</sub> )	CH <sub>3</sub>	CH <sub>3</sub>
Gentamicin C <sub>2</sub> (GEN C <sub>2</sub> )	H	CH <sub>3</sub>
Gentamicin C <sub>1a</sub> (GEN C <sub>1a</sub> )	H	H



	R <sub>1</sub>	R <sub>2</sub>
Sisomicin (SIS)	H	H
Plazomicin (PLZ)	(CH <sub>2</sub> ) <sub>2</sub> OH	AHB



#### Other AG scaffolds:

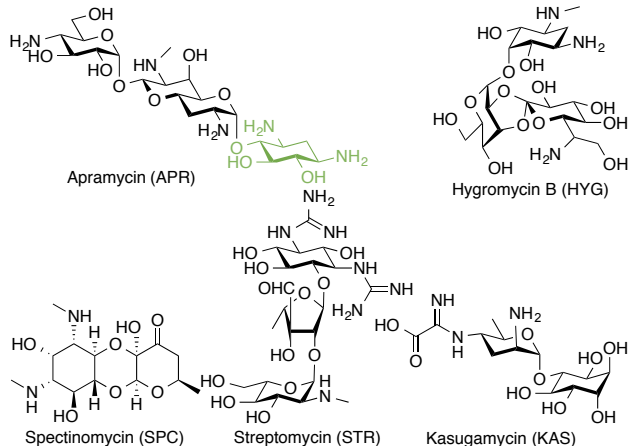


Fig. 1. Structures of AGs presented in this review.

## Ribosomal Mutations and Modifications

The classical mechanism of resistance to an antibiotic is bacterial modification of the antibiotic's target. AGs target the A-site of the bacterial ribosome; to evade inhibition by AGs there are two

potential, acquired mechanisms of resistance: mutations of the ribosome or enzymatic modifications of the ribosome.

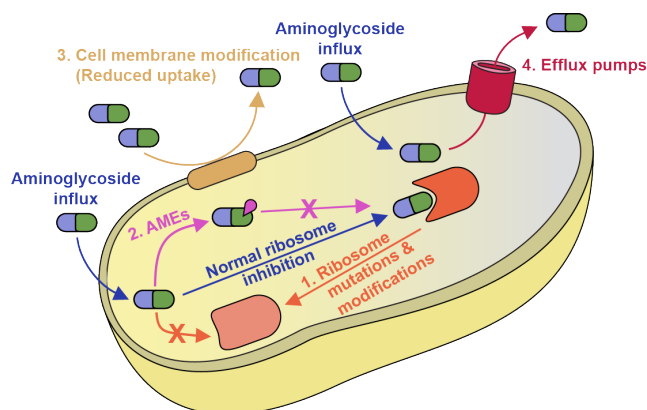


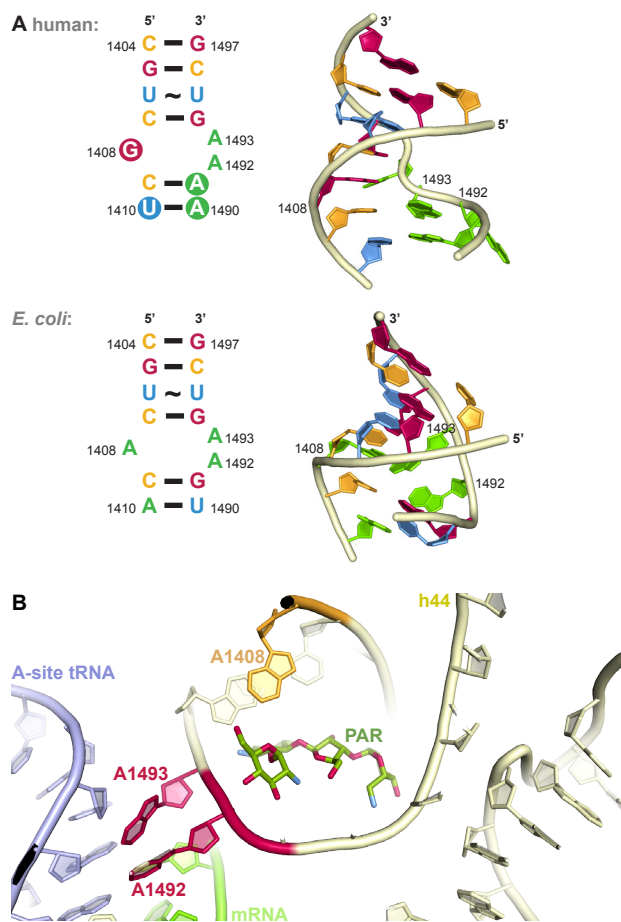
Fig. 2. Schematic overview of mechanisms of resistance to AGs discussed in this review.

## Ribosomal Mutation

In both eukaryotes and bacteria, protein translation occurs at the ribosome. The bacterial ribosome consists of a large subunit (50S) comprised of 5S and 23S rRNA and 34 proteins and a small subunit (30S) comprised of 16S rRNA and 20 proteins.<sup>13</sup> The steps of protein translation occur in three sites at the ribosome, the E-, P-, and A-sites. At the A-site, addition occurs of the amino acyl tRNA complementary to the three base codon of the mRNA. Selectivity for AGs to bind to the bacterial, but not human ribosome, comes from structural differences. Eukaryotic ribosomes contain nucleotide substitutions at two key AG-binding residues (prokaryotic A1408 and G1491 are replaced with guanine and adenine, respectively, Fig. 3A).<sup>14</sup> However, human mitochondrial ribosomes contain the prokaryotic residues, therefore are susceptible to inhibition by AGs. This is likely responsible for ototoxicity side effects.<sup>15</sup> The recent crystal structures of the human mitochondrial ribosome will aid in the design of AGs that avoid this toxicity.<sup>16, 17</sup>

The bacterial A-site, to which AGs bind, is located on the 16S RNA of the 30S bacterial ribosomal subunit.<sup>9</sup> In addition to the interactions at the codon, the 16S RNA makes many contacts with the tRNA confirming the correct codon-anticodon match and induces a closed conformation when a match is achieved. Recent crystal structures have elucidated the details of contacts made between various AGs and highly conserved nucleotide bases (A1408, A1492 and A1493 by *E. coli* numbering) of helix 44 (h44) of the 16S RNA (Fig. 3B). AGs of the 2-DOS scaffold (Fig. 1) may each bind slightly differently to the 16S RNA, but the binding mode of the 2-DOS core is conserved and binding leads to inhibition of tRNA translocation and consequently protein synthesis.<sup>9, 18</sup> Additionally, AGs have been thought to induce conformational change to mimic the closed, or active, ribosomal conformation.<sup>9, 13</sup> Specifically, RNA bases A1492 and A1493 flip from intra-helical to extra-helical to accommodate the AG (Fig. 3B). This signals the continuation of translation despite incorrect mRNA-tRNA pairing, resulting in mistranslated proteins. Secondary effects of these mistranslated proteins, such as incorporation into and subsequent disruption of the cell membrane, have been hypothesized to be the true mechanism of

AG lethality. Recent work demonstrates that the energetic changes induced by AG binding may be more complex than originally thought.<sup>19</sup>



**Fig. 3.** A. Comparison of internal loop of h44 of human and bacterial (*E. coli*) A-site. B. Structure of PAR bound to h44.

Some 2-DOS AGs (neomycin B (NEO), gentamicin (GEN), and paromomycin (PAR)) have been reported to also bind to a secondary site – the major groove of helix 69 (H69) of the 23S RNA of the 50S subunit.<sup>20-22</sup> Binding at this allosteric site has been demonstrated to affect the mobility of ribosomal subunits, which interferes with translation and ribosome recycling. Details regarding ribosomal binding of non-2-DOS AGs are beyond the scope of this review and can be found in other recent reviews.<sup>1, 2, 9, 13</sup>

AG resistance may arise from mutations in the *rrs* gene, which codes for 16S rRNA, that hinder AG-binding. These mutations, however, are not very common, as changes to this vital cellular machinery are often lethal. One viable mutant is A1408G. This mutation disrupts a key hydrogen bonding interaction between 2-DOS AGs and the h44 nucleotide A1408 (Fig. 3B). This mutation, which corresponds to A1401G in *M. tuberculosis*, as well as C1402T and G1484T have been found in clinically isolated strains of resistant *M. tuberculosis*.<sup>23</sup> Other less clinically prevalent ribosomal mutations have recently been analysed and summarized.<sup>24</sup> *In vitro*-selected mutations in *Mycobacterium abscessus* nucleotides 1406 and 1408 were found to be viable and confer resistance to 2-DOS AGs.<sup>25</sup> Recent structural analysis

reveals that AGs bearing a 6'-OH group (geneticin (G418), PAR) may evade resistance typically caused by A1408G.<sup>26</sup>

In addition to contacts made with h44 nucleotides, the non-2-DOS AG streptomycin (STR) interacts with ribosomal protein S12. Mutations in *rspL*, the gene encoding the S12 protein, lead to high-level STR resistance in *M. tuberculosis*.<sup>27, 28</sup> Similarly, spectinomycin (SPC)-resistant *Neisseria gonorrhoeae* has been determined to contain a mutation in ribosomal protein S5.<sup>29</sup>

Another ribosomal mechanism of resistance, demonstrated *in vitro*, is the overexpression of a 16S rRNA fragment resembling helix 34, which sequesters SPC and decreases its binding to the ribosome.<sup>30</sup>

### Ribosomal Modification by Methyltransferases

In addition to mutations, the AG binding site may be modified enzymatically by 16S ribosomal RNA methyltransferases (RMTases, also commonly referred to as 16S rRNA methylases in the literature).<sup>31</sup> RMTases naturally occur in *actinomycetes*, the bacterial group from which AGs were originally isolated. To protect their ribosomes from inhibition by the AGs they produce, *actinomycetes* produce RMTases to methylate their own 16S rRNA.

RMTases are acquired by other bacterial species most commonly by uptake of a plasmid containing the RMTase gene, and potentially other resistance genes (Table 1). RMTases were only recently discovered; an AG-resistant *P. aeruginosa* strain isolated in 1997 in Japan was found to contain a plasmid carrying the RMTase RmtA.<sup>32</sup> A resistance plasmid isolated from a *Citrobacter freundii* strain in Poland in 2002 and a multidrug-resistant strain of *K. pneumoniae* in France in 2003 were found to contain ArmA (aminoglycoside resistance methyltransferase A).<sup>33, 34</sup> This isolated RMTase displayed from 37 to 47% sequence similarity to intrinsic RMTases from various *actinomycetes*.<sup>34</sup> Low identity between the intrinsic and acquired RMTase genes (less than 30%) suggests that this gene transfer did not occur recently.

RMTases contribute to resistance by methylating a nucleotide in the AG-binding site of the 16S rRNA (the A-site) using *S*-adenyosyl-L-methionine (SAM) as a cosubstrate. The AG resistance RMTases are divided into two families: those which methylate at the N7 position of nucleotide G1405 (ArmA, RmtA, RmtB, RmtC, RmtD1, RmtD2 RmtE, RmtF, RmtG and RmtH) and those which methylate at the N1 position of A1408 (NpmA). N7-G1405 RMTases confer resistance to 4,6-disubstituted 2-DOS AGs such as amikacin (AMK), GEN, tobramycin (TOB), and kanamycin A (KAN), but not to 4,5-disubstituted 2-DOS AGs (e.g., NEO) or apramycin (APR). The N1-A1408 RMTase NmpA confers panAG resistance to both 4,5- and 4,6-disubstituted DOS AGs and to APR. This can be rationalized structurally due to the lack of N7-G1405 hydrogen bond interaction between the ribosome and 4,5-disubstituted DOS AGs or APR; methylation at N7-G1405 will not affect binding for these AGs therefore does not cause resistance to them (Fig. 4A).<sup>31</sup> Neither family of RMTases confer resistance to non-A-site binding AGs such STR or SPC.<sup>35</sup>

The structures of several RMTases have been elucidated using X-ray crystallography (Fig. 4B, Table 2). The three-dimensional structures of intrinsic RMTases when compared with acquired, resistant bacterial RMTases are similar, despite low (~30%)

Cite this: DOI: 10.1039/c0xx00000x

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## REVIEW

**Table 1.** Exogenously acquired 16S RMTases, bacterial species, and coexisting resistance genes.

16S RMTase	Bacterial species	Observed coexisting genes <sup>a</sup>	References
NpmA	<i>E. coli</i>	<i>bla</i> <sub>CTX-M-14</sub> , <i>bla</i> <sub>SHV-12</sub> , <i>bla</i> <sub>TEM-1</sub>	35, 36
ArmA	<i>A. baumannii</i> , <i>C. freundii</i> , <i>K. pneumoniae</i> , <i>E. cloacae</i> , <i>E. coli</i> , <i>S. enterica</i> , <i>S. flexneri</i> , <i>S. marcescens</i> , <i>Providencia</i> sp., <i>P. aeruginosa</i> , <i>P. mirabilis</i> , <i>P. stuartii</i> , <i>Providencia rettgeri</i>	<i>aac(3)-Ia</i> , <i>aac(3)-II</i> , <i>aac(6)-Ib-cr</i> , <i>aacA4cr</i> , <i>aacC2</i> , <i>aadA1</i> , <i>aadA2</i> , <i>ant3''9</i> , <i>aphA1</i> , <i>aph(3)''I</i> , <i>la</i> , <i>aph(3)''-Ib</i> , <i>arr-1</i> , <i>bla</i> <sub>ADC-30</sub> , <i>bla</i> <sub>ADC-67</sub> , <i>bla</i> <sub>CMY-2</sub> , <i>bla</i> <sub>CMY-16</sub> , <i>bla</i> <sub>CMY-30</sub> , <i>bla</i> <sub>CTX-M-3</sub> , <i>bla</i> <sub>CTX-M-14</sub> , <i>bla</i> <sub>CTX-M-15</sub> , <i>bla</i> <sub>IMP-1</sub> , <i>bla</i> <sub>KPC-2</sub> , <i>bla</i> <sub>NDM-1</sub> , <i>bla</i> <sub>OXA-1</sub> , <i>bla</i> <sub>OXA-9</sub> , <i>bla</i> <sub>OXA-10</sub> , <i>bla</i> <sub>OXA-23</sub> , <i>bla</i> <sub>OXA-30</sub> , <i>bla</i> <sub>OXA-48</sub> , <i>bla</i> <sub>OXA-51</sub> , <i>bla</i> <sub>OXA-66</sub> , <i>bla</i> <sub>OXA-72</sub> , <i>bla</i> <sub>OXA-82</sub> , <i>bla</i> <sub>OXA-202</sub> , <i>bla</i> <sub>PER-1</sub> , <i>bla</i> <sub>SHV</sub> , <i>bla</i> <sub>SHV-2</sub> , <i>bla</i> <sub>SHV-11</sub> , <i>bla</i> <sub>SHV-12</sub> , <i>bla</i> <sub>SHV-28</sub> , <i>bla</i> <sub>SHV-32</sub> , <i>bla</i> <sub>SHV-33</sub> , <i>bla</i> <sub>SHV-130</sub> , <i>bla</i> <sub>SHV-133</sub> , <i>bla</i> <sub>TEM-1</sub> , <i>bla</i> <sub>TEM-16</sub> , <i>bla</i> <sub>VEB-1</sub> , <i>bla</i> <sub>VIM-1</sub> , <i>cmlA1</i> , <i>dfrA12</i> , <i>dfrA14</i> , <i>dfrXII</i> , <i>florR</i> , <i>linF</i> , <i>mel</i> , <i>mph2</i> , <i>qnrA1</i> , <i>qnrB2</i> , <i>qnrS</i> , <i>sul1</i> , <i>sul2</i> , <i>tet(A)</i>	33, 37-61
RmtA	<i>P. aeruginosa</i>	$\kappa\gamma$ (mercury resistance mobile element) <sup>31</sup>	32
RmtB <sup>b</sup>	<i>A. baumannii</i> , <i>C. freundii</i> , <i>E. aerogenes</i> , <i>E. amnigenus</i> , <i>E. coli</i> , <i>K. pneumoniae</i> , <i>L. adecarboxylata</i> , <i>M. morgani</i> , <i>P. aeruginosa</i> , <i>P. mirabilis</i> , <i>S. marcescens</i>	<i>armA</i> , <i>aac(3)-II</i> , <i>aac(6)-Ib-cr</i> , <i>aadA2</i> , <i>aadA4</i> , <i>aadA5</i> , <i>aphA1-IAB</i> , <i>bla</i> <sub>CMY-58</sub> , <i>bla</i> <sub>CTX-M-3</sub> , <i>bla</i> <sub>CTX-M-12</sub> , <i>bla</i> <sub>CTX-M-14</sub> , <i>bla</i> <sub>CTX-M-15</sub> , <i>bla</i> <sub>CTX-M-24</sub> , <i>bla</i> <sub>CTX-M-65</sub> , <i>bla</i> <sub>DHA-1</sub> , <i>bla</i> <sub>KPC</sub> , <i>bla</i> <sub>LAP-1</sub> , <i>bla</i> <sub>NDM-1</sub> , <i>bla</i> <sub>NDM-8</sub> , <i>bla</i> <sub>OXA-1</sub> , <i>bla</i> <sub>OXA-10</sub> , <i>bla</i> <sub>OXA-23</sub> , <i>bla</i> <sub>OXA-51</sub> , <i>bla</i> <sub>SHV</sub> , <i>bla</i> <sub>TEM-1</sub> , <i>bla</i> <sub>VIM-1</sub> , <i>catA1</i> , <i>catB4</i>	48, 62-78
RmtC	<i>K. pneumoniae</i> , <i>P. mirabilis</i> , <i>S. enterica</i>	<i>aac(3)-II</i> , <i>aac(6)-Ib</i> , <i>aacA4</i> , <i>aadA1</i> , <i>aadB</i> , <i>aphA1</i> , <i>arr2</i> , <i>bla</i> <sub>NDM-1</sub> , <i>bla</i> <sub>CMY-6</sub> , <i>bla</i> <sub>CTX-M-15</sub> , <i>bla</i> <sub>OXA-1</sub> , <i>bla</i> <sub>OXA-9</sub> , <i>bla</i> <sub>TEM</sub> , <i>bla</i> <sub>VEB-6</sub> , <i>cmlA7</i> , <i>ereC</i> , <i>sul1</i>	57, 79-83
RmtD1	<i>K. pneumoniae</i> , <i>P. aeruginosa</i>	<i>bla</i> <sub>CTX-M-15</sub> , <i>bla</i> <sub>KPC-2</sub> , <i>bla</i> <sub>SPM-1</sub>	84, 85
RmtD2	<i>E. aerogenes</i> , <i>K. pneumoniae</i>	<i>aadA2</i> , <i>cat</i> , <i>dfrA12</i> , <i>sul1</i> , <i>bla</i> <sub>KPC-2</sub>	85, 86
RmtE	<i>E. coli</i>	<i>aph(3)''-Ia</i> , <i>aphA7</i> , <i>strA</i> , <i>strB</i>	87
RmtF	<i>C. freundii</i> , <i>E. cloacae</i> , <i>E. coli</i> , <i>K. pneumoniae</i>	<i>aac(6)-Ib</i> , <i>armA</i> , <i>bla</i> <sub>CIT</sub> , <i>bla</i> <sub>CTX-M</sub> , <i>bla</i> <sub>DHA</sub> , <i>bla</i> <sub>NDM-1</sub> , <i>bla</i> <sub>OXA-1</sub> , <i>insEA</i> , <i>rmtB</i> , <i>rmtC</i>	88, 89
RmtG	<i>K. pneumoniae</i> , <i>P. aeruginosa</i>	<i>bla</i> <sub>CTX-M-2</sub> , <i>bla</i> <sub>CTX-M-15</sub> , <i>bla</i> <sub>CTX-M-59</sub> , <i>bla</i> <sub>KPC-2</sub> , <i>bla</i> <sub>SHV-1a</sub> , <i>bla</i> <sub>TEM-1</sub>	85, 90, 91
RmtH	<i>K. pneumoniae</i>	<i>bla</i> <sub>CTX-M-15</sub> , <i>bla</i> <sub>SHV-1</sub> , <i>bla</i> <sub>OXA-15</sub> , "ISCR2, an IS91-like transposable element containing resistance genes for sulfonamide, trimethoprim, and florfenicol, and tetracycline and cephalosporin"	92

<sup>a</sup> Only subsets of these coexisting genes exist in various strains of the specified bacterial species. <sup>b</sup> In ref. <sup>92</sup>, RmtB1 and RmtB2 are reported.

**Table 2.** Crystal structures of 16S RMTases.

16S RMTase	Role	PDB codes	References
<b>N7-G1405</b>			
Sgm	Intrinsic	3LCU, 3LCV	93
ArmA	Resistance	3FZG	94
RmtB	Resistance	3FRH, 3FRI, 3B89	94
<b>N1-A1408</b>			
Kmr	Intrinsic/Resistance	4RWZ, 4RX1	95
KamB	Intrinsic	3MQ2	96
NpmA	Resistance	3MTE, 3P2E, 3P2I, 3P2K, 3PB3, 4OX9	96-98

sequence conservation. Furthermore, residues required for enzymatic activity are highly conserved.<sup>31</sup>

The clinical prevalence of RMTases, while still low, is increasing. This poses a considerable potential threat because RMTases confer resistance to many clinically relevant AGs, including AMK. A noted exception is the structurally rigid APR, which remains resistant to N7-G1405 RMTases, but the N1-A1408 RMTase NmpA can render APR inactive. Also, STR and SPC retain efficacy in the presence of resistance RMTases. Plazomicin (PLZ), an AG currently in clinical trials, was, unfortunately, found to be inactive against *Enterobacteriaceae* strains containing the RMTase genes *armA* and *rmtC*.<sup>99, 100</sup>

RmtB and ArmA are currently the predominant 16S RMTases, and their genes have spread around the world to resistant bacterial strains isolated from humans as well as livestock.<sup>31, 78</sup> Recently, RMTase resistance genes have been found in food products,

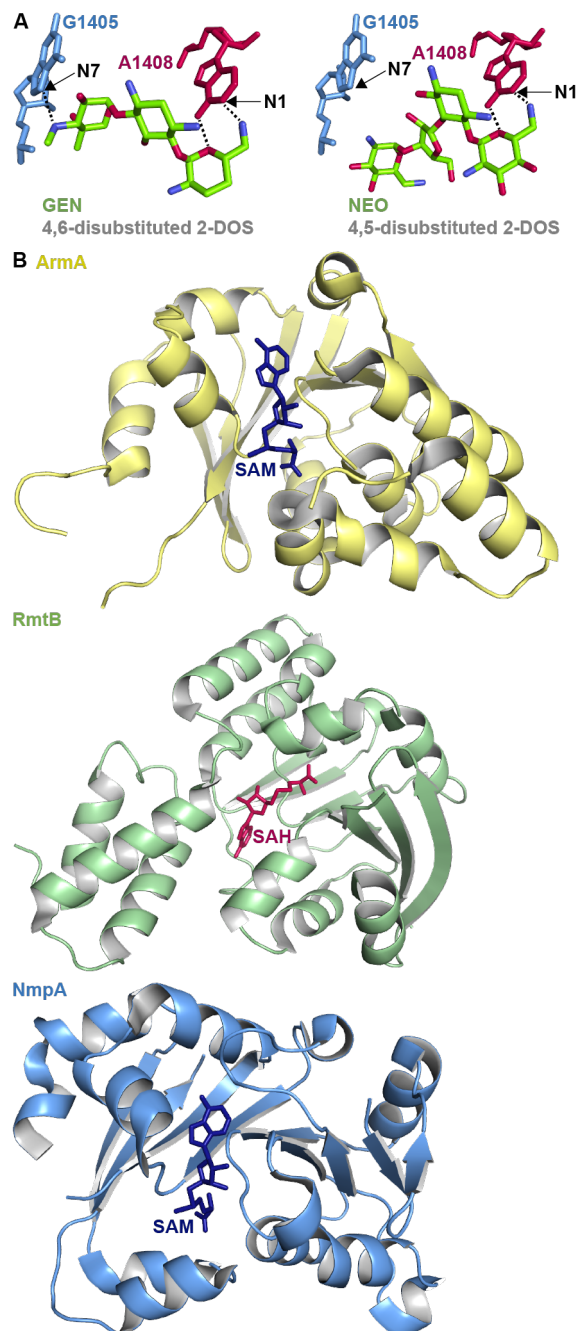
suggesting that food may be a possible vehicle for the spread of AG-resistant bacteria.<sup>41</sup>

Acquired resistance 16S RMTases will co-exist with endogenous ribosomal methyltransferases, for example RsmH and RsmI, which methylate C1402 in *E. coli* for proper ribosome function. Recently, it was found that the presence of resistance-causing N7-G1405 methyltransferase RmtD impedes C1402 methylation.<sup>101</sup> ArmA's methylation at G1405 slowed the growth rate of *E. coli* by blocking methylation at C1402, while NpmA had seemingly little effect on *E. coli* fitness. Both ArmA and NpmA methylations affected translation fidelity.<sup>102</sup> This work will lend insight on predictions of rates of dissemination of 16S RMTases based on fitness. These studies are also crucial for understanding the endogenous methylation at bacterial AG binding sites for the design of improved AGs to evade resistance.

Continued development of improved monitoring of RMTases, as well as other resistance genes, will be crucial to the slowing the spread of resistance. These methods include RT-PCR,<sup>103</sup> loop-mediated assays,<sup>104</sup> and bioinformatics tools<sup>105</sup> to detect both previously identified and novel resistance genes. Recent studies have identified putative resistance RMTases in *Pyrococcus furiosus*<sup>106</sup> and four other diverse bacterial species.<sup>107</sup>

One approach to overcome resistance due to RMTases would be the design small molecule inhibitors. Working towards this, more structural information is needed for the design of selective RMTase inhibitors. While RMTases unfortunately contribute to resistance to many AGs, the structural similarities among

RMTases would lend to the possibility of developing inhibitors that would target multiple RMTases.



**Fig. 4.** A. Structure of the 4,6-disubstituted 2-DOS AG GEN and 4,5-disubstituted 2-DOS AG NEO in close proximity to ribosome (PDB code 4V53 and 4V52, respectively) with positions methylated by RMTases. B. Representative example of crystal structures of the RMTases ArmA as yellow cartoon with SAM as navy stick (PDB code 3FZG),<sup>94</sup> RmtB as green cartoon with SAH as red stick (PDB code 3FRH),<sup>94</sup> and NmpA as pale blue cartoon with SAM as navy stick (PDB code 3P2K).<sup>97</sup>

## AG-Modifying Enzymes

The most common mechanism of AG resistance is chemical modification by aminoglycoside-modifying enzymes (AMEs). This large family of enzymes contains three subclasses, divided based on the type of chemical modification they apply to their

AG substrates: AG *N*-acetyltransferases (AACs), AG *O*-nucleotidyltransferases (ANTs), and AG *O*-phosphotransferases (APHs).<sup>108</sup> Each AME modifies an AG at a specific position, and this information is included in the enzyme name (Fig. 5A). Also, bifunctional enzymes, such as AAC(6')-Ie/APH(2'')-Ia from *Staphylococcus aureus*,<sup>109-112</sup> AAC(3)-Ib/AAC(6')-Ib' from *P. aeruginosa*,<sup>113, 114</sup> ANT(3'')-Ii/AAC(6')-IId from *Serratia marcescens*,<sup>115-117</sup> and AAC(6')-30/AAC(6')-Ib from *P. aeruginosa*<sup>118, 119</sup> exist and are capable of multiple types of AG modification. AAC(6')-Ib is the most prevalent and clinically relevant AME; approximately fifty variants of AAC(6')-Ib exist in numerous Gram-negative species.<sup>120</sup> In *Mycobacterium* and other bacterial species, upregulation of the enhanced intercellular survival (Eis) protein<sup>121</sup> is responsible for resistance to AGs via their multi-acetylation.<sup>122-130</sup> In addition to AGs, Eis is also capable of acetylating other anti-tubercular drugs, such as capreomycin.<sup>131</sup>

The native functions of AMEs remain unclear; they likely had roles in normal cellular metabolism, but have since evolved from their original “proto-resistance genes” to modify AGs upon selective pressure from exposure to these antibiotics.<sup>132</sup> AMEs are highly mobile; their genes are transferred on plasmids, integrons, transposons, and other transposable gene elements, often along with other resistance genes (such as RMTases or  $\beta$ -lactamases, “*bla*” genes, Table 1). Most pathogenic bacteria acquire resistance AMEs through horizontal gene transfer. Eis, however, is natively expressed in *M. tuberculosis* and mutations in the promoter region or a translational activator cause its upregulation, leading to resistance.<sup>121, 133</sup> Also, there is good evidence that another pathogenic mycobacteria, *M. abscessus*, contains a native AAC(2'') responsible for its intrinsic resistance to AGs.<sup>134, 135</sup>

Over 100 AMEs have been reported, here we highlight those identified and characterized since the publication of a comprehensive list of AMEs in 2010.<sup>108</sup> Newly discovered and substrate scope-characterized AACs include several AAC(6')s: AAC(6')-Iag from *P. aeruginosa*,<sup>136</sup> AAC(6')-Iaj from *P. aeruginosa*,<sup>137</sup> AAC(6')-Iak from *Stenotrophomonas maltophilia*,<sup>138</sup> and AAC(6')-Ian from *S. marcescens*.<sup>139</sup> A novel AAC(2''), AAC(2'')-IIa confers resistance to the agricultural AG kasugamycin (KAS) in rice pathogenic bacteria *Burkholderia glumae* and *Acidovorax avenae* subsp. *avenae*.<sup>140</sup> The Gram-positive *Corynebacterium striatum* BM4687 bacterial strain has a unique resistance profile (resistant to GEN and TOB, but susceptible to KAN and AMK), due to the presence of what was named AAC(3)-XI.<sup>141</sup>

A new APH, APH(2'')-If from *Campylobacter jejuni*, was found to have the same AG substrate scope as and to have high sequence identity to the APH(2'') of the bifunctional AAC(6')-Ie/APH(2'')-Ia.<sup>142</sup>

Because of their clinical prominence as mechanism of AG resistance, an active area of research has been obtaining structural information regarding AMEs. A comprehensive list of resolved crystal structures of AMEs can be found in a recent review.<sup>143</sup> Since that publication, new AME crystal structures have been solved and are listed in Table 3 and representative structures shown in Fig. 5B.

Noteworthy is the first structure of an ANT other than

Cite this: DOI: 10.1039/c0xx00000x

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## REVIEW

**Table 3.** List of AMEs for which crystal structures were recently determined.

Enzyme	PDB code	Substrate	Cosubstrate	Bacterial species	Oligomeric state	References
AAC(3)-Ib	4YFJ	-	-	<i>P. aeruginosa</i>	Structural dimer <sup>a</sup>	-
AAC(6')-Ie	4QC6	KANA	CoA	<i>Staphylococcus warneri</i>	From bifunctional AAC(6')-Ie/APH(2'')-Ia	144
EisC204A	4JD6	TOB	CoA	<i>M. tuberculosis</i>	Hexamer	127
ANT(2'')-Ia	4WQL (4WQK)	KAN (-)	-	<i>K. pneumoniae</i>	Monomer	145
APH(4)-Ia <sup>b</sup>	3W0O	HYG	ADP	<i>E. coli</i>	Monomer	146
APH(3')-VIII	4H05	-	-	<i>Streptomyces rimosus</i>	Structural dimer <sup>a</sup>	-
APH(2'')-Ia	4ORK	-	GDP	<i>S. aureus</i>	From bifunctional AAC(6')-Ie/APH(2'')-Ia	144
APH(2'')-IVa	4N57	-	ADP	<i>Enterococcus casseliflavus</i>	Structural dimer <sup>a</sup>	-

<sup>a</sup> Indicates that there are no reference to confirm the dimeric state, but this is what is observed in the PDB. <sup>b</sup> Indicates thermostable mutant. Please also see PDB codes 3W0M, 3W0N, 3W0P, 3W0Q, 3W0R, and 3W0S for structures of other thermostable mutants of APH(4)-Ia with hygromycin B (HYG) and various cosubstrates.

ANT(4'),<sup>147</sup> the structure of ANT(2'')-Ia reveals that this enzyme shares molecular features with other nucleotidyltransferases including lincosamide nucleotidyltransferases and DNA polymerase  $\beta$ .<sup>145</sup> Augmenting previous studies that suggest that the bifunctional enzyme AAC(6')-Ie/APH(2'')-Ia adopts a rigid conformation in solution,<sup>112</sup> Vakulenko and co-workers recently constructed an updated homology model of this bifunctional enzyme using small angle X-ray scattering data and their independently resolved structures of each AAC(6')-Ie and APH(2'')-Ia.<sup>148</sup> During studies of this bifunctional enzyme, the first structure of APH(2'') variant Ia was determined, revealing that like APH(2'')-IIIa, APH(2'')-Ia uses exclusively GTP as a cosubstrate, unlike APH(2'')-IIa and APH(2'')-Iva, which both can use either ATP or GTP.<sup>144</sup> Also worth mentioning is the first resolved structure of the multiacetylating AME Eis with an AG (TOB), providing structural rationale for the diacetylation of this AG substrate.<sup>127</sup>

Several strategies are being investigated to overcome resistance caused by AMEs including attempts to regulate AME expression, the design of new AGs that evade AMEs, and the design of AME inhibitors. Recent review articles detail these strategies, but noteworthy advances from the past two years are described here.<sup>120, 143, 149-151</sup> Interestingly, metal cations have been demonstrated to inhibit AAC activity, increasing AG efficacy in resistant strains.<sup>152, 153</sup> This approach has yet to be explained mechanistically, but provides a potential combined therapy approach to treat AG-resistant strains. Newly designed AGs showing promise as antibacterials that evade AMEs include thioether, alkylated, and acylated AG variants,<sup>154-160</sup> AG homo- and hetero-dimers,<sup>161-163</sup> and NEO analogues with a fluorinated (S)-4-amino-2-hydroxybutyrate (AHB) side chain that evade resistance of many AMEs as well as ArmA.<sup>164</sup>

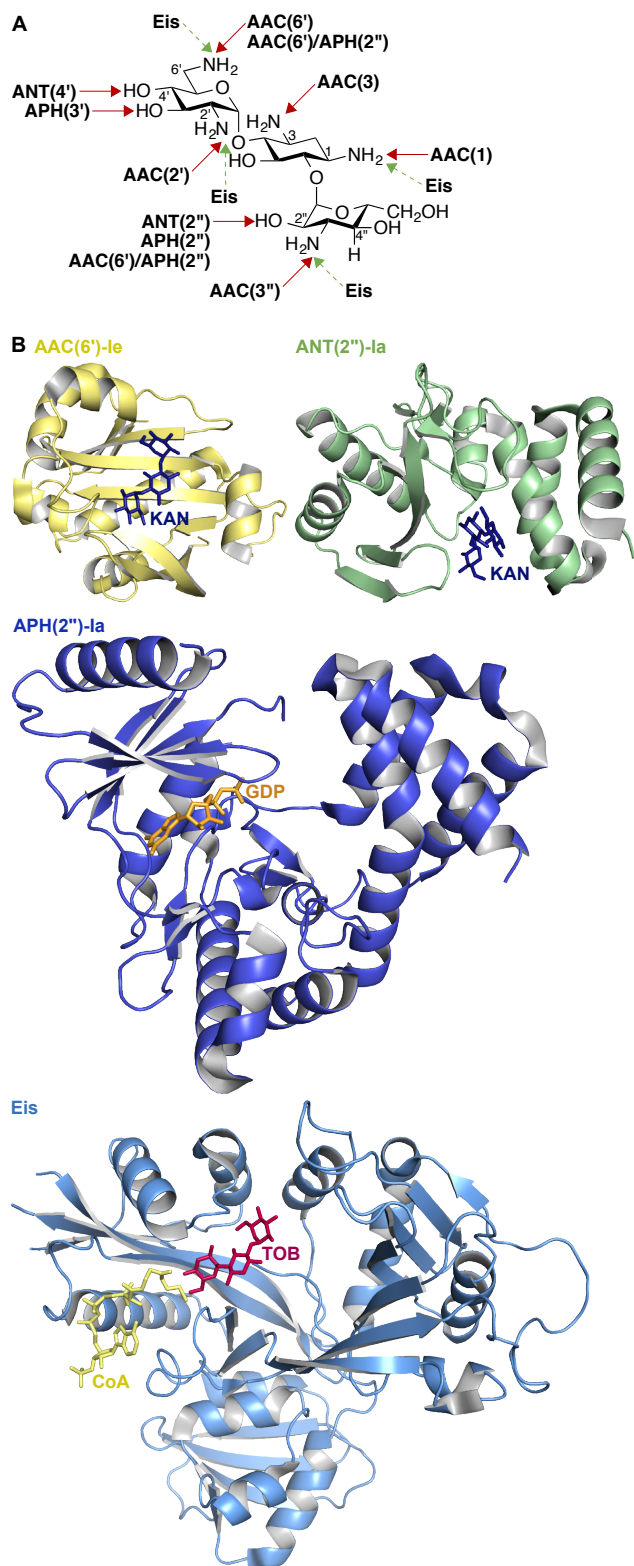
### Cell Membrane Modification

To reach their bacterial target, AGs must traverse the bacterial cell wall. In Gram-negative bacteria the cell envelope consists of an inner cellular membrane, followed by periplasm containing

peptidoglycans, and finally a second phospholipid bilayer, the outer membrane (OM) (Fig. 6). Mycobacteria have a unique cellular envelope consisting of an inner membrane followed by a peptidoglycan layer linked to a layer of arabinogalactan, which is linked to high-molecular weight mycolic acids and coated with an outer monolayer of phospholipids (Fig. 7). These multi-layered cell walls act as a barrier and provide an innate mechanism of resistance to AGs for Gram-negative bacteria and mycobacteria. Modifications to cell wall compositions can cause them to be even less permeable.

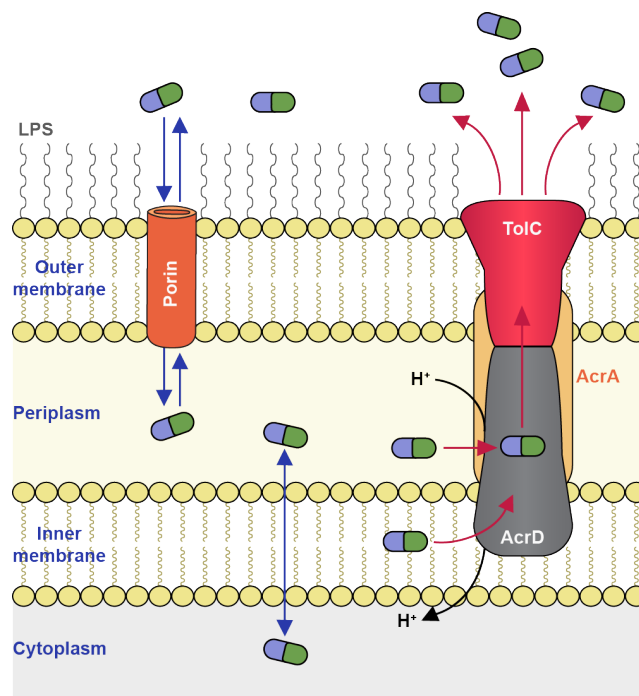
Due to their cationic, hydrophilic structures, AGs have been hypothesized to penetrate bacterial cell walls through porin channels rather than direct diffusion through the phospholipid bilayer.<sup>165, 166</sup> However, some experiments have demonstrated that porin-deficient mutants are still capable of AG-uptake.<sup>167</sup> Additionally, it has been hypothesized that AGs undergo "self-promoted uptake" in which they interact with and disrupt the OM, allowing their penetration into the bacterial cell.<sup>108, 168-170</sup> Upon binding to the ribosome AGs interfere with translation, and the aberrant polypeptides synthesized interrupt the cell membrane, further allowing AGs to penetrate the cell wall. AG uptake has been demonstrated to be an energy-consuming process, but details remain unclear.<sup>108, 168</sup> Bacteria may become resistant to AGs by modification of their OM permeability *via* alteration of their outermost lipopolysaccharides (LPSs) or down regulation of porins (Fig. 6).

The OM serves as an intrinsic first-line of defence offering Gram-negative bacteria protection from foreign molecules such as AGs. The outward facing half of the OM consists of sugar-functionalized phospholipids (LPSs), which bear a net negative charge, attracting cationic AGs. The most common LPS modification leading to reduced AG uptake is the incorporation of the positively charged 4-amino-4-deoxy-L-arabinose sugar, which effectively reduces the net negative charge of the LPS layer, decreasing affinity for AGs.<sup>171-173</sup> Among many Gram-negative bacteria, several two-component systems have been identified (PhoPQ, PmrAB, ParRS, CprRS) to respond to the presence of cationic metals or cationic antimicrobial peptides, or



**Fig. 5.** A. Chemical modifications and positions affected by various AMEs on KANB. B. Representative example of recent crystal structures for AACs (AAC(6')-Ie as yellow cartoon with KANA as navy stick; PDB code 4QC6;<sup>148</sup> from the bifunctional enzyme AAC(6')-Ie/APH(2'')-Ia), APHs (APH(2'')-Ia as dark blue cartoon with GDP as orange stick; PDB code 4ORK;<sup>144</sup> from the bifunctional enzyme AAC(6')-Ie/APH(2'')-Ia), ANT(2'')-Ia as green cartoon with KANA as navy stick; PDB code 4WQL;<sup>145</sup> normally a monomer), and Eis as pale blue cartoon with CoA as yellow stick and TOB as red stick (PDB code 4JD6;<sup>127</sup> normally an

hexamer) resistance enzymes. *Note:* Only the monomer of each of these enzymes is shown.



**Fig. 6.** AG transport in Gram-negative bacteria. Influx of AGs occurs through hydrophilic porin protein channels. Efflux of AGs may occur through active transport pumps such as the RND-type efflux pump (e.g., AcrAD-TolC, as shown.)

low pH, by attachment of this arabinose onto the phospholipid of the LPS.<sup>174, 175</sup> Additionally, phosphoethanolamine was reported to be incorporated into the LPS in response to the presence of cationic molecules, under the regulation of the two-component system ColRS (*P. aeruginosa*) or PmrAB (*E. coli*, *Salmonella enterica*).<sup>176</sup>

The complex, lipid-rich cell wall of mycobacteria is also a very effective barrier for the entry of AGs. The *Mycobacterium smegmatis* cell membrane lipid profile was recently examined and it was found that the innermost layer of the inner membrane lipid composition consists of an unusual lipid, diacyl phosphatidylinositol dimannoside (Fig. 7).<sup>177</sup> This lipid has four hydrocarbon chains, and is proposed to lead to low membrane fluidity and therefore poor drug permeability.

Porin proteins are large water-filled channels allowing the passive diffusion of hydrophilic small molecules. Several types of porins have been identified and studied in Gram-negative bacteria. In *E. coli*, OmpF is the classical porin -a trimer of  $\beta$ -barrels that quickly transports small, hydrophilic molecules,<sup>10</sup> but there is currently no conclusive evidence that AGs are transported through this porin. The relatively narrow size restrictions of porins are hypothesized to limit AG transport. While the transport of beta-lactam antibiotics through porins has been investigated,<sup>178</sup> much remains unknown about AG transport through porins in Gram-negative bacteria. OmpF has been hypothesized to be involved in kanamycin resistance, but experiments remain inconclusive.<sup>179</sup>



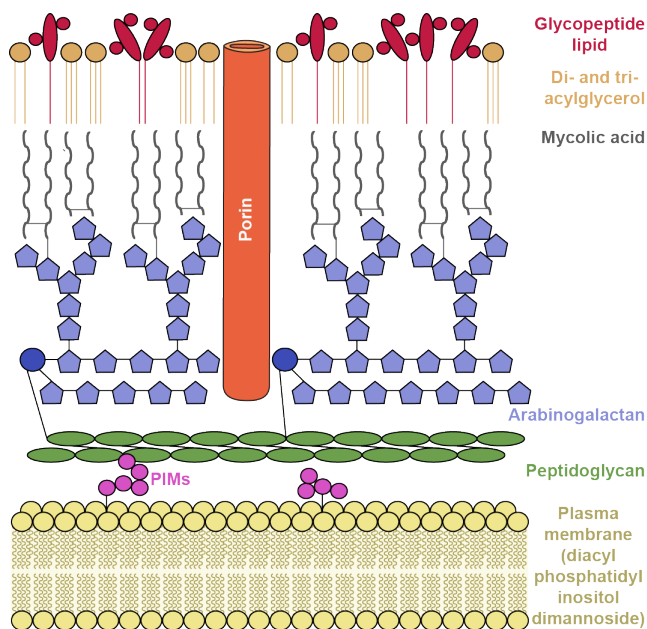


Fig. 7. Mycobacterium cell wall.

Two types of porins have been identified and characterized in mycobacteria: the MspA-like porins from *M. smegmatis* and 5 OmpA-like porins from *M. tuberculosis*.<sup>180</sup> Staining and microscopic analysis revealed that the number of MspA pores in *M. smegmatis* is approximately 50-fold less than pores counted in the outer membranes of Gram-negative bacteria.<sup>181</sup> This likely contributes to mycobacteria's characteristically low drug- 10 permeability.<sup>182</sup>

Numerous studies of the MspA-like porins (MspA, MspB, MspC and MspD) have demonstrated that porin deletion causes a reduction of drug uptake and an increase in MIC.<sup>180</sup> However, the MIC value for KANA did not increase significantly in Msp 15 mutants, suggesting that these porins do not transport AGs.<sup>183</sup> The crystal structure of MspA has been resolved and while MspA is not directly involved in AG resistance in a pathogenic bacterial species, this structure provides valuable insight of bacterial cell membrane proteins.<sup>184</sup> *M. tuberculosis* porin-like molecule 20 "OmpATb", named as a homologue of *E. coli* porin OmpA, plays a role in adapting to low pH environments, but does not appear to function as a transport channel.<sup>180, 181</sup> Furthermore, OmpATb mutants do not show increased drug resistance.<sup>185</sup> *M. tuberculosis* protein Rv1698 was thought to function like MspA; initial 25 experiments demonstrated it is capable of uptake of ampicillin, cephaloridine, and chloramphenicol (AGs not examined),<sup>186</sup> but this result was later deemed an artifact of overexpression.<sup>187</sup> Recently, Rv1698 (now named MctB) was demonstrated to protect *M. tuberculosis* from high concentrations of copper.<sup>188</sup> 30 The newly discovered *M. tuberculosis* outermembrane protein CpnT plays a role in nutrient uptake and susceptibility of hydrophilic drug molecules.<sup>189</sup> The contribution of CpnT to *in vitro* resistance of large, hydrophilic drugs including STR was only moderate.<sup>190</sup>

The difference in porin systems, especially that *M. tuberculosis* does not contain the well-studied MspA-like porins from *M. smegmatis*, highlights the need for better characterization of AG uptake in the pathogenic species. Unfortunately *M. smegmatis* may not be accurately serving as a non-pathogenic

40 model organism of *M. tuberculosis* when it comes to studies of AG influx and resistance. Clearly, much remains to be examined regarding AG-specific uptake by in mycobacteria.

While no documented cases of clinical AG resistance due to porin changes have been reported to our knowledge to date, 45 porin-related resistance to other classes of antibiotics has been reported.<sup>10</sup> Possibilities of mutational resistance associated with porins include decreased expression, no expression, or structural mutations that, for example, result in narrowing of the channel size to exclude relatively large AGs. Porin mutations or 50 expression changes, however, may be a limited mechanism of resistance, as they could also lead to decreased uptake of nutrients. Changes in membrane lipids and porin expression are only minor mechanisms of AG resistance – AMEs, RMTases, and efflux pumps are more clinically prominent mechanisms of 55 resistance.

## Efflux Pumps

An additional bacterial mechanism of resistance to AGs is the active transport of AGs out of cells through efflux pumps. Due to the polycationic structures of AGs, only a few efflux pumps have 60 been demonstrated to remove AGs.<sup>191, 192</sup>

The main AG efflux pump in Gram-negative bacteria, AcrAD, is a multidrug transporter and a member of the resistance-nodulation-division (RND) family of efflux pumps. The name AcrAD describes a three-component system that spans the cell 65 envelope; AcrD spans the innermost cellular membrane and functions as a drug-proton antiporter, AcrA is a membrane fusion protein found in the periplasm, and TolC is the outer membrane component of the pump (Fig. 6).<sup>193</sup> Intrinsic AcrAD-TolC-type efflux pumps have been identified in many Gram-negative 70 bacterial species (see Li *et al.* 2015 for a comprehensive review)<sup>166</sup> including *E. coli*,<sup>194, 195</sup> *S. enterica*,<sup>196</sup> *A. baumannii* (AdeABC and AdeDE),<sup>197</sup> *P. aeruginosa* (MexXY-OprM),<sup>198</sup> and *Burkholderia pseudomallei* (AmrAB-OprA and BpeAB-OprB).<sup>199</sup> AcrAD-TolC homologues also exist in numerous 75 *Enterobacteriaceae*<sup>200</sup> and even in *Erwinia amylovora*, a plant pathogenic bacteria responsible for causing fire blight in apple, pear and rose species.<sup>201</sup> In *Stenotrophomonas maltophilia*, AGs have also been identified as substrates for RND family efflux pumps SmeIJK and SmeYZ.<sup>202, 203</sup> Other efflux pumps of which 80 AGs may be (poor) substrates include MexAB-OprM and EmrE (a small multidrug resistance, "SMR" transporter) in *P. aeruginosa*,<sup>204</sup> LmrA (a multidrug ABC transporter) in *Lactobacillus lactis*,<sup>205</sup> and MdfA (a major facilitator superfamily "MFS" transporter) in *E. coli*, though these are pumps are not 85 likely involved in resistance.<sup>206</sup> VcmB and VcmH efflux pumps from in *Vibrio cholerae* of the multidrug and toxic compound extrusion (MATE) family have also been demonstrated to transport AGs.<sup>207</sup>

Crystal structures have been solved of AcrB and MexB, RND 90 multidrug transporters whose substrate scope includes mostly hydrophobic molecules and not hydrophilic AGs.<sup>208, 209</sup> The structures revealed two distinct substrate binding regions – a large proximal substrate channel that accommodates high molecular weight substrates as well as a distal, phenylalanine- 95 containing pocket that accommodates low molecular weight substrates.<sup>210</sup> Recently structures of AcrB with an inhibitor

present have been solved showing that the inhibitor binds to the hydrophobic phenylalanine trap.<sup>209</sup> A homology model of *P. aeruginosa* MexY was constructed from these structures of AcrB suggesting that the position of a tryptophan residue in MexY, which is a phenylalanine in AcrB, likely prevents the inhibitor from binding MexY and subsequently serving as a universal inhibitor of multidrug efflux transporters.<sup>209</sup> In a related study, mutagenesis of residues in the larger, proximal substrate-binding pocket of MexY demonstrated their involvement in AG binding.<sup>211</sup> Hopefully soon resolved crystal structures of AcrD or MexY will reveal details of how these pumps accommodate the cationic AG structures and will aid in future work to evade resistance caused by these AG efflux pumps.

The role of MexXY-OprM from *P. aeruginosa* in AG resistance has been well studied.<sup>198</sup> MexXY-OprM accommodates many antibiotic classes, but only AGs, erythromycin, tetracyclines, and glycolcyclines have been demonstrated to induce *mexXY* expression, and consequently overexpression of MexXY-OprM has been implicated in resistance to these antibiotics.<sup>191</sup> The expression of the MexXY-OprM efflux pump has also been shown to be inducible by exposure to reactive oxygen species, contributing to pan-AG resistance.<sup>212</sup> The contribution of efflux to AG resistance is currently low. One exception is that the overexpression of MexXY due to a mutation in the repressor gene *mexZ* is the most common mechanism of AG resistance in lung isolates from cystic fibrosis patients with chronic *P. aeruginosa* infections.<sup>213</sup> In addition to the repressor MexZ, in-depth studies of MexXY have found the two-component system AmgRS to be involved in the regulation pathway of *mexXY*. AmgRS also protects the cell membrane from damage caused by aberrantly synthesized polypeptides due to AG-ribosomal inhibition.<sup>214, 215</sup> Despite currently low clinical prominence, efflux pump expression may be used to monitor resistance of other classes of antibiotics; AcrAB (AcrAD homologue) overexpression may be a biomarker for determining the mechanism by which bacteria become MDR.<sup>216</sup>

AG efflux pumps can also be found in mycobacteria. *M. tuberculosis* encodes many putative efflux pumps, many of which have yet to be identified and their substrate scopes characterized.<sup>187, 217</sup> AGs are substrates for mycobacterial Rv1410c (P55) efflux pump.<sup>218</sup> *M. tuberculosis* knockout of efflux pump Rv1258c ("Tap", an MFS transporter) was reported to have decreased MIC values for AGs, suggesting that AGs are substrates for this pumps but not for Rv0849 (MFS transporter) or Rv1218c (an ABC transporter).<sup>219</sup>

Approaches to combat AG resistance by efflux pumps include devising AGs to evade pumps or adjuvant inhibitors to block them. Spectinamides, semi-synthetic SPC derivatives were found to have increased efficacy against *M. tuberculosis*, hypothesized to be due to their ability to evade the Rv1218c efflux pump.<sup>220</sup> *In vitro* studies show efflux inhibitors to be successful against AMK-resistant *M. tuberculosis*.<sup>221</sup> Other recent work includes a competitive, fluorescent dye assay for studying the efficacy of efflux pump inhibitors.<sup>222</sup>

At their intrinsic level of expression, efflux pumps contribute little to resistance. It is the overexpression of efflux pumps (usually due to mutations in regulatory genes), the acquisition of

mutations within efflux pumps that improve substrate affinity, and synergy with other resistance mechanisms that leads to high levels of antibiotic resistance. The contribution of efflux pumps to resistance also depends on the rate of influx of the drug; in organisms such as *P. aeruginosa* or *M. tuberculosis* in which AG influx is slow, increased efflux will be a large contributor to AG resistance. Furthermore, a danger of AG efflux pumps as a mechanism of resistance is that they are multi-drug transporters and will also confer resistance to other classes of antibiotics. They have even been shown to remove other biocides such as disinfectants.<sup>206, 223, 224</sup> Further details of work towards the design of AGs to evade efflux pumps and efflux pump inhibitors to block these resistance-causing pumps is well covered in recent reviews.<sup>225-230</sup>

## Other Mechanisms of Resistance

Though not traditionally considered a mechanism of resistance, membrane proteases may offer protection from AGs. Membrane proteases are part of an intrinsic system of protein biosynthesis quality control that recognizes and degrades misfolded and mistranslated proteins. Because one of the effects of AGs is synthesis of aberrant proteins, which accumulate and perturb cell membrane integrity, any protection from this would provide tolerance to AGs. Mutations in FtsH, a membrane protease in *P. aeruginosa* lead to increased sensitivity to TOB, demonstrating that FtsH plays a role in intrinsic AG resistance.<sup>231, 232</sup> Deletion mutations in genes associated with lipid biosynthesis or metabolism (*lptA*, *faoA*), phosphate uptake (*pstB*), and the two-component regulator (*amgRS*) also result in increased TOB sensitivity.<sup>233</sup> In another study of *P. aeruginosa*, upon exposure to TOB, the expression of *asrA*, which encodes a Lon-type protease, as well as heat shock genes, was increased.<sup>234</sup> This is an example of adaptive resistance, common to chronic *P. aeruginosa* infections of cystic fibrosis patients.<sup>213</sup>

Adaptive resistance to AGs in these chronic *P. aeruginosa* infections is associated with the bacteria existing as a biofilm. Biofilms are characterized as bacteria growing aggregately on a surface, surrounded by a matrix of proteins, DNA and carbohydrates. This biofilm matrix has been hypothesized to bind cationic molecules, such as AGs, decreasing their efficacy.<sup>213, 235</sup> Co-delivery of an AG with a cationic steroid antibiotic, CSA-13, has been demonstrated to overcome this resistance.<sup>236</sup> More recently, adaptive resistance in biofilms has been associated with the previously described gene expression changes and the upregulation of MexXY efflux pump expression described in an earlier section of this review.<sup>192</sup> Meta-analysis revealed that a variety of genes and proteins undergo expression changes in biofilm when compared to non-biofilm cells, yet, as expected, no smoking gun was revealed.<sup>237</sup> Much remains to be understood about adaptive response to AGs.<sup>6, 238</sup>

## Perspective and Conclusions

The mechanisms discussed here differ in their degree of contribution to bacterial AG resistance. These mechanisms also vary in mobility; some spread quickly to other species via horizontal gene transfer, others are less transferable. Mechanisms also vary in their substrate scope; for example AMEs often have

only a few specific AG substrates (e.g., GEN/TOB or AMK), while RMTases work on many AG substrates. In this sense, RMTases are a worrisome mechanism of resistance due to their high mobility and broad substrate scope. The bacterial cell wall and efflux pumps contribute to resistance more broadly. They often work against all AGs and other entire classes of antibiotics too. The less common mechanism of ribosomal mutations can offer either broad resistance to an entire sub-class of AGs (e.g., 2-DOS) or specific resistance to one AG.

The most threatening bacterial species harbour multiple mechanisms of resistance, commonly from a single plasmid bearing multiple resistance genes. The effects of multiple mechanisms are additive. For example, the intrinsic nature of the outer membrane as a barrier to restrict AG uptake can act synergistically with other mechanisms of resistance; the combination of minor changes that individually result in only small MIC increases can collectively result in a highly AG-resistant phenotype.<sup>239</sup>

To combat multi-faceted bacterial resistance, clinicians will need to be able to execute a breadth of therapies. Additionally rapid detection and identification of resistance genes will allow for tailored therapies. This is not only more effective at fighting each uniquely resistant bacterial infection, but also prevents unnecessary use of irrelevant antibiotics. Individualized diagnostics may not be as daunting as it sounds; a recent study demonstrates that in *E. coli* and *K. pneumoniae* cultures, resistance to GEN can be pinpointed to just three genes in 97% of the 267 geographically localized isolates.<sup>58</sup> Executed efficiently, a combination of periodic complete resistance gene scans and regular screens for resistance genes of known prevalence may be more effective in the long run.

Work to design new AGs that evade resistance continues. Second generation AGs include semi-synthetic derivatives of dibekacin (DBK) such as arbekacin (ABK), bearing an N1-AHB side chain, and isepamicin (ISP) bearing the 1-C shorter (S)-3-amino-2-hydroxypropate N1 side chain (Fig. 1). Both of these compounds were originally synthesized in the 1970s and they were clinically approved in 1990 and 1988, respectively. Despite their structural modifications, which seem to decrease toxicity side effects, ABK and ISP are still susceptible to resistance caused by RMTases and modification from AMEs, though surprisingly sometimes not to modification by AAC(6)'s.<sup>116, 240, 241</sup> The high prominence of AAC(6') enzymes is likely why ISP is often still quite clinically effective.<sup>242</sup>

The only new AG currently in the pipeline is plazomicin (PLZ, formerly ACHN-490), a sisomicin (SIS) derivative bearing the N1-AHB side chain (Fig. 1). It has been shown to be effective against methicillin-resistance *S. aureus* (MRSA) and carbapenem-resistant *Enterobacteriaceae* (CRE) and to overcome the action of many AMEs, including AAC(6)'s.<sup>243, 244</sup> PLZ was recently shown to be effective against several strains of *Brucella*, a zoonotic pathogen,<sup>245</sup> and carbapenem-resistant *A. baumannii*.<sup>246</sup> PLZ is currently in Phase 3 clinical trials for patients with bloodstream infections or nosocomial pneumonia due to CRE. PLZ has a few weaknesses; it is not effective against *Enterobacteriaceae* carrying RMTases ArmA or RmtC, or *Providencia stuartii* AAC(2')-I.<sup>247</sup> Despite these deficiencies, PLZ is an important new weapon in the pipeline to fight

antibiotic resistance.

With better understanding of AG resistance mechanisms, follow novel potential strategies to combat resistance. The discovery of a secondary ribosomal AG-binding site at H69, launches the potential for exploiting this as a bactericidal method.<sup>21</sup> Recently it was shown that the binding of NEO induced different ribosomal conformation changes when compared to the structurally similar AG PAR, highlighting the subtleties of AG-ribosome binding.<sup>22</sup>

Due to their dramatic clinical prevalence, much current research is aimed at blocking AMEs via novel AG derivatives or small molecule AME inhibitors as discussed earlier in this review (*AG Modifying Enzymes*). Additionally, as resistance due to dissemination of the RMTases increases, inhibitors of these enzymes may also serve as valuable AG adjuvants.

Because multiple resistance genes may coexist within a mobile gene element and therefore are often acquired together, to prevent the spread of resistance, the transfer of these resistance genes must be prevented. Selective pressures, which occur in hospital environments and regions in which antibiotic use is unregulated, must be limited. Ongoing surveillance of resistance genes in humans, animals, and foods will be crucial in delaying the spread of resistance to AGs.

Many of these mechanisms of resistance to AG antibiotics were discovered relatively recently; RMTases in the late 1990s, the first RND pump was characterized in the early 1990s. Due to recent technological advances and increased efforts in scientific discovery, just a few years later we have acquired an in-depth understanding of some of these molecular mechanisms of antibiotic resistance. If our scientific work continues at this pace and is combined with better management of antibiotic use, hopefully bacterial antibiotic resistance will be classified as a manageable medical issue, rather than a global health crisis.

## Acknowledgements

We thank all of those working in the field of aminoglycoside drug discovery and apologize if their work is not cited due to the scope of the review presenting examples from 2010-2015. Our work on aminoglycosides and bacterial resistance is supported by the National Institutes of Health (NIH) Grant AI090048 (S.G.-T.) and by startup funds from the College of Pharmacy at the University of Kentucky (S.G.-T.).

## Notes and references

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