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Review article

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Molecular mechanisms of antibiotic resistance revisited

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Abstract

Antibiotic resistance is a global health emergency, with resistance detected to all antibiotics currently in clinical use and only a few novel drugs in the pipeline. Understanding the molecular mechanisms that bacteria use to resist the action of antimicrobials is critical to recognize global patterns of resistance and to improve the use of current drugs, as well as for the design of new drugs less susceptible to resistance development and novel strategies to combat resistance. In this Review, we explore recent advances in understanding how resistance genes contribute to the biology of the host, new structural details of relevant molecular events underpinning resistance, the identification of new resistance gene families and the interactions between different resistance mechanisms. Finally, we discuss how we can use this information to develop the next generation of antimicrobial therapies.

Sections

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Introduction

Antimicrobial resistance (AMR) is a major global health challenge, causing substantial morbidity and death globally. Understanding the molecular mechanisms that underlie resistance can aid in the design of novel strategies to treat infectious diseases. Bacteria use various mechanisms of resistance[1], some are 'intrinsic', whereby the cell can use genes it already possesses to survive antibiotic exposure, and some are 'acquired', whereby gain of new genetic material provides new capacities that mediate survival (Table 1). There has been substantial progress in our understanding of how antibiotics work and the major mechanisms by which bacteria can resist the inhibitory or killing effects of antibiotics (Fig. 1), including the importance of context for many resistance mechanisms in determining their effects (Box 1). For example, the expression of resistance genes and targets can substantially change between diverse growth conditions[2]. New technological advances have revealed the structural details of many resistance mechanisms, including complex, multi-component structures of efflux systems that might point towards possible routes for inhibitor development[3,4]

Table 1 | Major classes of antibiotics, primary targets and mechanisms of resistance

Antibiotic class (examples)	Mechanism of action	Mechanism of resistance
Aminoglycosides (gentamicin, streptomycin, kanamycin)	Interact with the 30S ribosomal subunit of 16S rRNA causing misreading and/or truncated proteins and cell death[75]; positively charged, attach to outer membrane causing pores to increase accumulation[169]	Aminoglycoside-modifying enzymes, for example, acetyltransferases, phosphotransferases and nucleotidyltransferases[131]; 16S ribosomal methylases[170]; mutations in the 16S rRNA gene[171]; decreased influx and/or increased efflux[172]
β-Lactams (penicillins, cephalosporins, cephamycins, carbapenems, monobactams)	Target peptidoglycan crosslinking by inhibiting penicillin-binding proteins, which crosslink the peptide chain in the cell wall[107], leading to lysis of the cell[75]	Production of β-lactamases[173]; modification of penicillin-binding proteins[174]; reduced permeability and increased efflux[174]
Cationic peptides (colistin)	Bind to lipid A in lipopolysaccharide[175]; permeabilizing the outer membrane causing cell death[175]	Modification or removal of lipid A[176,177]
Glycopeptides (vancomycin)	Inhibit crosslinking and therefore synthesis of peptidoglycan by binding to D-alanyl-D-alanine in the peptide chain[178]	Intrinsic resistance in Gram-negative cells by impermeable outer membrane[178]; in Gram-positive cells, enzymes can modify and hydrolyse peptidoglycan precursors[179]; intermediate susceptibility phenotype conferred by mutations leading to thickened membrane and low permeability[180]
Lincosamides (clindamycin)	Target the translation of proteins, specifically 23S rRNA of the 50S ribosomal subunit, causing truncated peptide chains[181]	Methyltransferases that modify 23S rRNA[182]; expression of proteins that inactivate lincosamides and efflux[183]
Lipopeptides (daptomycin)	Insert in the cell membrane and cause depolarization, reducing the ability to create ATP and cell death[184]	Thickening of and increasing the positive charge in the cell wall[185]; reducing the depolarization of membranes induced by lipopeptides[185]
Macrolides (azithromycin, erythromycin)	Inhibit the translation of proteins by targeting 23S rRNA of the 50S ribosomal subunit, causing truncated peptide chains[186]	rRNA methyltransferases, which methylate 23S rRNA[187]; mutations in the ribosome[188]; efflux[188]; macrolide phosphotransferases and esterases[135]; ribosomal protection by ATP-binding cassette F (ABC-F) proteins[189]
Oxazolidinones (linezolid)	Limit translation by binding to 23S rRNA of the 50S subunit and preventing the formation of a functional 70S subunit[190]	Modifications of 23S rRNA, for example, by methyltransferases[191]; protection of the ribosome via ABC-F proteins[191]
Phenicols (chloramphenicol)	Inhibit translation by binding to the A site of the 50S ribosomal subunit, inhibiting protein synthesis[192]	Mutations within 23S rRNA of the 50S ribosomal subunit[192]; enzymatic inactivation via acetyltransferases and efflux[192]
Pyrimidines (trimethoprim)	Affect C1 metabolism and folate synthesis by inhibiting dihydrofolate reductase, blocking production of tetrahydrofolate[193]	The modification or acquisition of novel dihydrofolate reductase genes and efflux of trimethoprim[194]
Quinolones and fluoroquinolones (ciprofloxacin)	Inhibit DNA replication by DNA gyrase and topoisomerase IV, which are involved in DNA supercoiling, strand cutting and ligating[195]	Mutations in DNA gyrase or topoisomerase IV[195]; the efflux of quinolones or proteins that protect DNA gyrase and topoisomerase IV[195]
Rifamycins (rifampicin)	Inhibit transcription, specifically DNA-dependent RNA synthesis, by binding to RNA polymerase[196]	Mutations in the drug target *rpoB*[196]; enzymatic ribosylation or inactivation of rifampicin[144]
Streptogramins (dalfopristin)	Target protein translation by binding to 23S rRNA of the 50S ribosomal subunit at the peptidyl-transferase domain causing truncated peptides[197]	Mutations in 23S rRNA[192]; modification of streptogramins by acetyltransferases[192]; efflux out of the cell[192]
Sulfonamides (sulfamethizole)	Stop dihydrofolate acid synthesis by inhibiting dihydropteroate synthase and arresting cell growth[198]	Mutations in the dihydropteroate synthase gene and *sul1/2* genes, which encode distinct dihydropteroate synthases that are less susceptible to sulfonamides[198]
Tetracyclines (tigecycline, tetracycline)	Inhibit translation by binding to 16S rRNA of the 30S ribosomal subunit, preventing tRNA binding to 30S at the A site[199]	Efflux[199]; protein-mediated ribosome protection[199]; ribosomal mutations[199]; enzymatic inactivation of the drug[199]

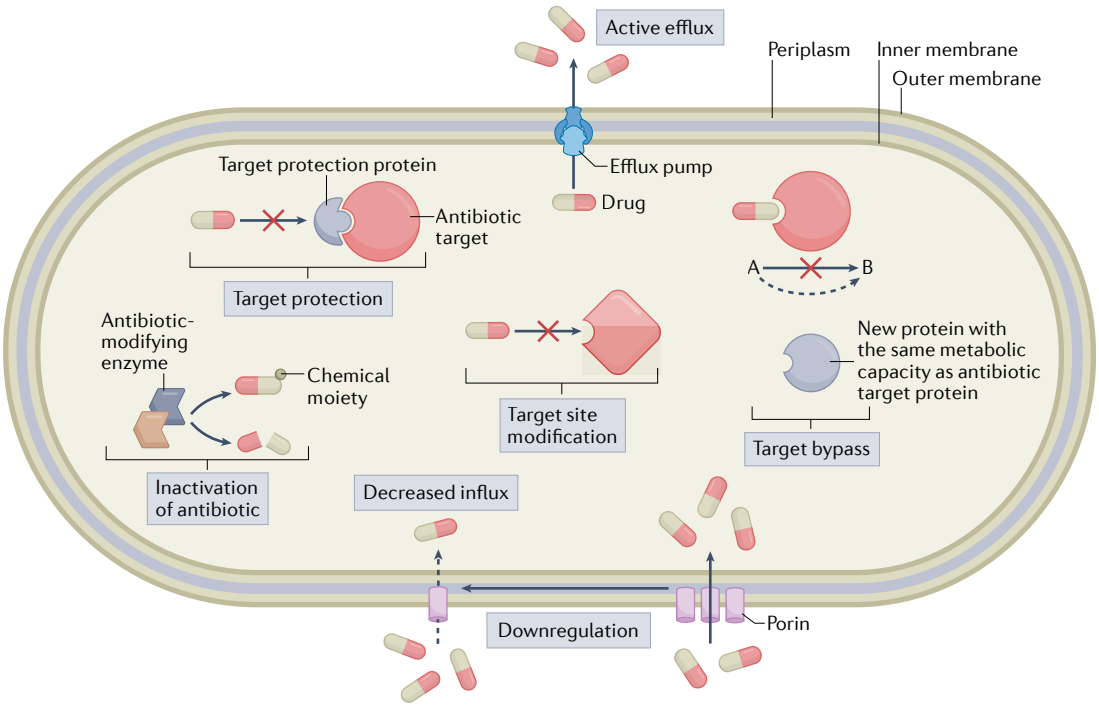


Fig. 1 | Overview of the molecular mechanisms of antibiotic resistance. Inactivation of antibiotics is mediated by enzymes that either degrade or modify the antibiotic molecule. Enzymatic degradation involves hydrolysis of the functional group of the antibiotic, thereby rendering it ineffective[200]. Antibiotic-modifying enzymes transfer various chemical groups to the antibiotic, which prevent binding of the antibiotic to its target[201]. Target site alteration involves alteration of the antibiotic target to reduce binding of the antibiotic. This can involve mutations in the gene encoding the protein target of the antibiotic molecule or enzymatic alteration of the binding site[102,202]. During target bypass, the function of the antibiotic target is accomplished by a new protein that is not inhibited by the antibiotic, making the original target redundant and the antibiotic ineffective[203]. Decreased influx is mediated by changes to membrane structure, for example, the downregulation of porins, which are transmembrane proteins that allow the passive transport of various compounds, such as antibiotics, into the bacterial cell[13]. Active efflux is facilitated by transmembrane efflux pumps, which export antibiotics out of bacterial cells to reduce their intracellular concentration[204]. Target protection generally involves the physical association of a target protection protein with the antibiotic target, thereby relieving it from antibiotic-mediated inhibition[205].

and a proposed mechanism of action for the newly identified ABC-F ribosome rescue proteins. More is also known about the importance and hierarchy of multiple molecular mechanisms working together to generate high-level resistance (for example, the underpinning role of efflux to support all other resistance mechanisms; Box 2).

In addition, the costs and benefits associated with acquiring resistance are now better understood. In particular, the interplay between plasmids, hosts and AMR genes is important in determining the expansion of genes, vectors and strains[5–7]. Examples of successful acquisition of specific resistance genes by globally dominant clones of a species have been identified, for example, acquisition of the extended-spectrum β-lactamase CTX-M-15 by *Escherichia coli* ST131, or the carbapenemase KPC by *Klebsiella pneumoniae* ST258, both of which spread globally[8,9].

In this Review, we explore the mechanisms of resistance and outline the clinical relevance of different mechanisms. Specifically, we discuss the most recent progress in understanding antibiotic resistance via reduction in intracellular drug accumulation (reduced permeability and antibiotic efflux), modification or alteration of the bacterial antibiotic target, modification or destruction of the drug itself, and bypass of whole metabolic pathways. Finally, we also discuss how genomics has revolutionized the study of AMR (Box 3) and how together this information can aid in developing the next generation of antimicrobial therapies.

Reduced permeability

For many antimicrobials to exert their activity, they need to cross the bacterial cell envelope to reach their target. This is particularly important in Gram-negative bacteria, which have a double-membrane structure that makes the cellular envelope relatively impermeable, providing intrinsic resistance to many antibiotics that work against Gram-positive pathogens and presenting a major challenge to the development of novel antimicrobials that can penetrate the cell envelope. In addition, alterations to envelope structure, such as porin loss or changes to phospholipid and fatty acid content of the cytoplasmic membrane, can affect the ability of a drug to penetrate the cell and can contribute to the emergence of AMR. Gram-positive bacteria lack the outer membrane, which makes them naturally more permeable to many antibiotics; however, changes in composition of the cytoplasmic membrane, which affect fluidity, have been shown to be important in reducing permeability to antibiotics[10]. Mycobacteria produce an extensive outer lipid layer and a polysaccharide capsule coat, thereby preventing the entry of hydrophilic molecules into the cell[11].

Box 1

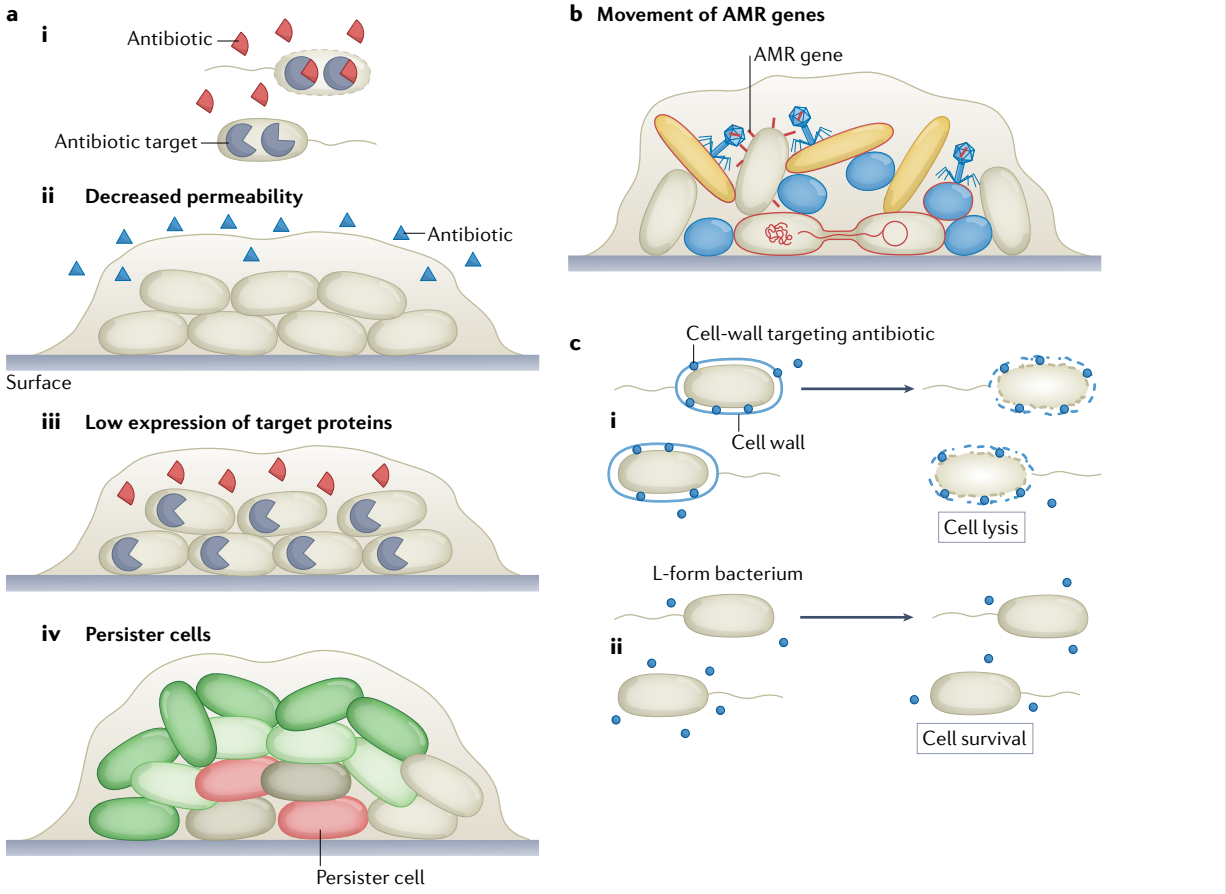
The role of the environment and lifestyle on survival

Bacteria live in varied conditions, where different genes are required for survival[212]. One major common lifestyle for bacteria is growth in biofilms, which are aggregated communities of cells producing a protective extracellular matrix. Biofilms are inherently tolerant to the action of antibiotics and some exhibit resistance due to multiple factors[213]. During the planktonic lifestyle, bacteria are inhibited by the drug (see the figure, part **a**,i). However, cells within a biofilm exhibit a large degree of heterogeneity of metabolic states and gene expression, which results in individual cells being resistant within a biofilm due to reduced permeability (part **a**,ii), low metabolic activity resulting in reduced target expression (part **a**,iii) and production of large numbers of persister cells[213] (part **a**,iv). Biofilms can also experience high rates of genetic exchange, allowing movement of antimicrobial resistance (AMR) genes (figure, part **b**)[214].

A striking example of how a lifestyle change can result in resistance is seen in 'L-form' bacteria, which are cells that lose their cell wall after stress exposure[215]. L-form bacteria are consequently resistant to cell-wall targeting agents (for example, β-lactams) and exposure to these drugs rapidly selects for cell wall-deficient bacteria (figure, part **c**)[216].

A lifestyle change that can be induced by environmental conditions is the formation of persisters, which are dormant cells that are often growth arrested but viability is maintained. Persister cells are highly resistant to the killing action of bactericidal antibiotics but regain normal sensitivity once replication resumes[217]. Various mechanisms for persister cell formation have been suggested, including toxin–antitoxin systems and production of (p)ppGpp, although there remains much debate about the importance of each[218]. Recent data have shown that cells with low levels of ATP are likely to become persisters[219,220].

Tolerance refers to populations of cells temporarily able to survive antibiotic exposure at concentrations that exceed the minimum inhibitory concentration[221]. Tolerant cells exhibit slow growth (but not complete arrest) due to mutation within genes that impact growth. This slower growth allows cells to survive antibiotic exposure and favours the accumulation of resistance mutations when exposed to drugs[222].



The outer membrane of Gram-negative bacteria is a complex organelle that has evolved to provide protection and has a barrier function, while still allowing the uptake of nutrients. Recent evidence from *Enterobacterales* shows how permeability of the outer membrane dynamically changes during bacterial growth, which, in turn, affects how much drug can penetrate the membrane[2]. The outer membrane contains porins, which are β-barrel protein channels categorized, based on their function and architecture, into families: general nonspecific channels (for example, OmpF and OmpC); substrate-specific channels (for example, PhoE and LamB) and small β-barrel channels (for example, OmpA and OmpX)[12]. Generally, porins allow the influx of hydrophilic compounds <600 Da into the cell, including many antibiotics[13]. OmpF and OmpC found in *E. coli* and related organisms are trimeric β-barrel structures through which different antibiotic classes are known to be able to pass. OmpF has a larger pore size than OmpC (6.5–7 Å versus 5.5–6 Å, respectively), which makes it generally easier for substrates to pass through OmpF compared with OmpC[14]. Despite earlier beliefs that porins exhibited selectivity towards certain substrates, recent studies suggest that diffusion occurs in a spontaneous, passive way, without an active interaction observed[15].

Recently, alterations to porin structure have also been identified as important contributors to antibiotic resistance, demonstrating that these are not evolutionarily static structures, and our knowledge of the structure–function relationship of porins has improved. For example, carbapenem resistance in *Klebsiella pneumoniae* is partially mediated by modification of the non-selective porins OmpK35 and OmpK36; a selected insertion (Gly115-Asp116) into loop 3 of OmpK36 causes significant constriction of the pore and, thus, increased tolerance to carbapenems[16]. Multidrug-resistant *E. coli* isolates from patients were recently found to carry multiple mutations within OmpC that alter the electric charge within the pore and, in turn, affect the permeability of antibiotics such as cefotaxime, gentamicin or imipenem[17].

Porin expression in *Enterobacterales* is actively regulated in response to environmental stimuli (Fig. 1). Perhaps the best-studied examples are, again, OmpC and OmpF in *E. coli*, the expression of which is under the control of the EnvZ–OmpR two-component system. EnvZ is a periplasmic sensor protein that senses changes in the environment and controls the phosphorylation state of OmpR, its cognate response regulator[18]. High levels of phosphorylated OmpR in the cell lead to reduced *ompF* and increased *ompC* transcription. This differential regulation of the two porins allows appropriate porin expression: in high-osmolarity environments, where nutrients are abundant, the small pore is predominant. Mutants that only produce the smaller pore survive antibiotic exposure better. It has been shown that carbapenems select for multiple first-step mutations within OmpR, conferring reduced porin expression[19]. Small regulatory RNAs (sRNAs) can also control outer membrane structure at a post-transcriptional level. The sRNA *micF* is encoded by a sequence divergent to *ompC* and, when transcribed, it inhibits expression of *ompF* by direct base pairing to the ribosome-binding site and start codon of the *ompF* mRNA, thus preventing translation[20]. Expression of this sRNA is conditional and subject to various environmental stimuli such as high osmolarity conditions[21]. More recently, the *micC* sRNA has been identified and observed to repress OmpC translation by directly binding to the 5′ untranslated region of the *ompC* mRNA[22]. Transcription of these sRNAs is co-regulated in response to antimicrobial stress, and β-lactams actively induce the transcription of *micC*[23].

Nonspecific porins, such as OmpF and OmpC, are characteristic of *Enterobacterales*. However, other important pathogens, such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, are instead

Box 2

Underpinning role of efflux and synergies with other resistance mechanisms

High-level multidrug resistance (MDR) is often conferred by a combination of multiple interacting mechanisms. In particular, there is a growing body of evidence indicating that many mechanisms of antibiotic resistance rely on or interact with the intrinsic resistance provided by efflux pumps.

First, efflux activity can cause altered expression of other genes involved in intrinsic antibiotic resistance. For example, in *Enterobacterales*, the deletion or inhibition of *acrAB* can lead to decreased expression of the outer membrane porin OmpF, thereby reducing membrane permeability and limiting intracellular drug accumulation[223].

Second, the efflux status of cells affects the rate of evolution of resistance in a population. Lack of efflux function has been shown to decrease the frequency with which antibiotic-resistant mutants are selected[41,224,225]. Furthermore, there is single-cell level heterogeneity in *acrAB* expression, and cells with higher levels of expression of *acrAB* had lower levels of expression of the DNA mismatch repair gene *mutS* and subsequently an increased mutation rate, which enables the rapid evolution of high-level resistance via the accumulation of point mutations[63,226]. Similarly, in *Staphylococcus aureus*, amplification of the NorA efflux pump led to more rapid evolution, whereas inhibition of the pump prevented resistance evolution. Interestingly, positive epistasis was also detected, whereby increased NorA expression interacted positively with mutations conferring ciprofloxacin resistance in *S. aureus* to further increase resistance[225].

Finally, there is also evidence that resistance–nodulation–division efflux affects horizontal gene transfer. It was shown, in *Escherichia coli*, that, in the presence of the translation-inhibiting antibiotic tetracycline, the AcrAB–TolC efflux pump was required for the acquisition of plasmids carrying TetA, which confers high-level tetracycline resistance. This is because the efflux pump reduces intracellular levels of the drug, thus allowing time to translate proteins encoded on the newly acquired plasmid[227]. Furthermore, the acquisition of an MDR plasmid in *Klebsiella pneumoniae* has been shown to cause increased transcription of efflux genes, thus further strengthening the link between MDR plasmids and efflux[228].

Box 3

Genomics and the study of antimicrobial resistance

The study of bacteria has moved from the first reported genomes of pathogenic species, to comparisons of the first hundred, to today where for some species, hundreds of thousands of individual strains have been sequenced[229]. Analysis of large numbers of genomes of a species now allows epidemiology of antimicrobial resistance to be studied and can show how the acquisition of resistance genes and elements interplay with host fitness in globally dominant clones. For example, the acquisition of carbapenem-resistant plasmids in dominant clones of *Escherichia coli* has been facilitated by mutation in core metabolic genes[230].

Studying large isolate panels has been used to predict novel mechanisms of resistance. An analysis of 95 *Staphylococcus aureus* isolates was used to identify genes involved in daptomycin resistance, which was previously not well understood[185], and a genome-wide association study suggested that convergent evolution has selected for daptomycin-resistant *mprF* mutants on multiple occasions[231].

Genomic sequencing following laboratory evolution experiments is a simple and accessible tool to identify routes to resistance. For example, parallel selection of mutations within *fusA1* and *ptsP* in response to tobramycin in *Acinetobacter baumannii* grown in different conditions demonstrated the primary importance of these mutations in resistance[232].

Functional genomics approaches have also become highly advanced, CRISPR methods now allow defined mutants to be generated in species previously not tractable for manipulation, and extremely high-density transposon mutant libraries have been used to screen whole genomes for changes related to antibiotic susceptibility at almost base pair resolution[233]. This allows the identification of many genes that have small contributions to resistance but together form the 'secondary resistome'. Using this approach, a study of genes involved in colistin sensitivity in *Klebsiella pneumoniae* showed that inactivation of a non-essential gene (*dedA*) reversed antimicrobial resistance in isolates with high colistin minimum inhibitory concentrations[234].

equipped with multiple, specific porins that allow the entry of molecules no larger than 200 Da (ref.[24]). This lack of larger, general porins results in highly impermeable membranes, particularly towards hydrophilic molecules[25]. In the case of *P. aeruginosa*, loss of OprD porins is very commonly reported as a mechanism of clinically important, high-level carbapenem resistance[26]. This is often seen in conjunction with other mechanisms, and a recent analysis of the impacts of inactivating all porins of *P. aeruginosa* showed that the loss of no single porin could completely abolish drug entry, demonstrating the importance of synergy between porin loss and other mechanisms[27].

Active transport of antibiotics

As well as preventing drugs from entering the cell, bacteria can actively export them in a process known as efflux. Efflux pumps are transmembrane proteins that can transport a wide variety of toxic compounds, including antibiotics, across bacterial membranes in an energy-dependent manner. While all bacteria contain multiple efflux pumps, they are particularly important mediators of AMR in Gram-negative bacteria. They work synergistically with the impermeable double membrane to make these pathogens intrinsically resistant to many antibiotics. The impact of different efflux systems on specific drugs varies, with some providing high levels and others low levels of resistance; however, efflux acts as a crucial 'platform' mechanism that enables most other resistance mechanisms to have an impact[28]. Efflux transporters are categorized into six families, with members of the resistance–nodulation–division (RND) family conferring the most clinically relevant levels of resistance in Gram-negative bacteria[3]. As inner membrane proteins, RND transporters associate with both periplasmic adaptor proteins (PAPs) and an outer membrane factor (OMF) in a 3:6:3 protomer stoichiometry to form tripartite complexes that span the entire Gram-negative cell envelope[29] (Fig. 2). RND pumps can export a broad range of structurally and chemically dissimilar antibiotics, and the overexpression of these pumps contributes to multidrug resistance (MDR) in clinical isolates[30,31].

Understanding of the structure and assembly of RND tripartite systems has been revolutionized by technological improvements, particularly in cryo-electron microscopy (cryo-EM)[3]. Cryo-EM has allowed us to visualize efflux transporters in bound and unbound states, in addition to providing completely assembled tripartite efflux complexes in situ[32,33].

RND transporters are homotrimeric proteins that recognize ligands and transduce electrochemical energy from the proton (H^+) gradient across the inner membrane[3,30]. PAPs are elongated periplasmic proteins that mediate the assembly and stabilization of the tripartite complex via six PAP monomers directly interacting with both the inner membrane protein and OMF components. The assembled complex constitutes a continuous tunnel through which substrates are exported[3]. As the exterior portion of the channel, OMFs are outer membrane-bound, homotrimeric structures that protrude deep into the periplasmic space and serve as the final conduit of the tripartite complex from which drugs exit the channel and are released into the

Glossary

β-Lactamase
Enzymes produced by bacteria that degrade β-lactam antibiotics.

Epistasis
Where a mutation can exert a phenotypic effect but only in concert with other genes, making the impact conditional on the genetic background of where it occurs.

Horizontal gene transfer
The movement of genetic information between bacterial cells.

Insertion sequences
Small pieces of DNA that encode their own recombination machinery and can move within or between genomes.

Minimum inhibitory concentration
(MIC). The lowest concentration of antibiotic that prevents growth of bacteria.

Topoisomerases
Essential enzymes involved in DNA replication.

Two-component system
A system that allows bacteria to respond to specific environmental stimuli. Usually, it consists of a membrane-bound histidine kinase that senses the stimuli and activates a response regulator that alters the expression of relevant genes.

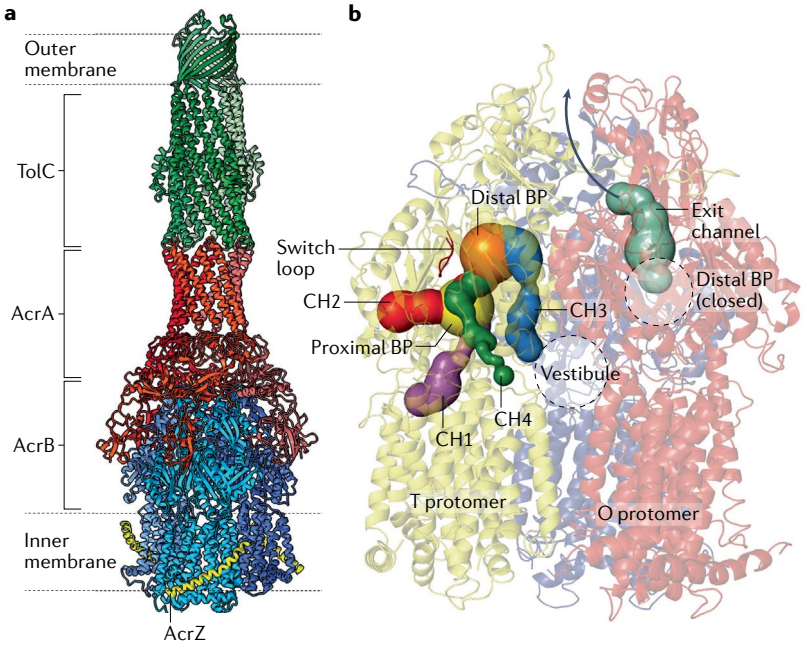


Fig. 2 | Structure and entry channels of RND efflux systems. a, Shown is the crystal structure of the tripartite AcrAB(Z)–TolC efflux complex (Protein Data Bank (PDB) ID: 5O66)[34]. The trimeric inner membrane resistance–nodulation–division (RND) transporter AcrB has a crucial role in substrate recognition and energy transduction[206]. The AcrB trimer interacts with six copies of the periplasmic adaptor protein AcrA, which form a sealed tubular structure that links AcrB to the outer membrane factor TolC[37]. The AcrA hexameric ring interacts with the TolC trimer in a tip-to-tip fashion[207]. The TolC trimer is a cannon-shaped channel, of which one end is inserted in the outer membrane with the other end extending into the periplasmic space and interacting with AcrA[37]. The small protein AcrZ interacts with AcrB and is proposed to play a role in allosterically modulating the activity of AcrB[51]. **b**, The AcrB trimer consists of multiple substrate entry channels (L protomer not shown for clarity). The entrance of channel 1 (CH1) is open to the outer leaflet of the inner membrane. Substrates entering CH1 are guided to the proximal binding pocket (proximal BP). The entrance of CH2 is situated at the cleft formed by the PC1 and PC2 subdomains of AcrB (not indicated) and is open to the periplasmic space[208]. High-molecular-mass drugs (for example, erythromycin or rifampicin) preferentially enter through CH2 and bind to the proximal BP[49]. The switch loop separates the proximal BP and the distal binding pocket (distal BP) and is important for the translocation of high-molecular-mass drugs from the proximal BP to the distal BP[209]. Low-molecular-mass drugs (for example, chloramphenicol and linezolid) preferentially enter through CH1 and bypass the proximal BP and the switch loop and bind directly to the distal BP[48]. CH3 is open to the central cavity formed by the vestibules between the protomer interfaces and is preferentially used by planar aromatic cations (for example, berberine or ethidium bromide) for transport directly to the distal BP[47]. The entrance of CH4 is situated at the groove formed by TM1 and TM2 and leads directly to the distal BP. CH4 is preferentially accessed by carboxylated drugs (for example, β-lactams or fusidic acid)[50,210]. During the transition of the AcrB protomers from tight to open (T-to-O), all substrate channels are closed, and an exit channel is created in the O protomer. The exit channel is connected to the closed distal BP, allowing substrates to be exported[211].

extracellular space, thereby completing substrate extrusion[3,34]. Cryo-EM structures of AcrAB–TolC from *E. coli*, at near-atomic resolution, have shown that a closed-state complex is formed upon tripartite assembly when substrates are absent[34,35]. In the presence of substrates, such as puromycin, the periplasmic tip of the OMF TolC is dilated, while the three protomers of the RND transporter AcrB assume an asymmetric conformation — loose, tight and open — presumably in preparation for drug efflux[34] (for a graphical representation see ref.[30]). The quaternary structural changes involved with the apo-to-active state transition of AcrB are reflected onto TolC via the PAP AcrA[34], which also seals the pump channel to prevent leakage during substrate export[34,36]. Based on the most recent cryo-EM structures of tripartite pumps, successful opening of the OMF conduit has been shown to depend on the disruption of 'primary gates', formed by predominantly ionic interactions, at its periplasmic tip by the α-helical hairpin domain of PAPs in a 'tip-to-tip' cogwheel manner[3,34,37–39]. PAPs have also been proposed to enforce the directionality of cycling between RND transition states based on functional and cryo-EM structural analysis of MexAB–OprM of *P. aeruginosa*[3,39]. Far from the simple bridging role they were initially assigned, it is now clear that the PAPs have a crucial and active role in both pump assembly and substrate expulsion[3,40]. This importance has led to PAPs being considered as effective targets to combat efflux-mediated antibiotic resistance[41,42].

RND efflux pumps contribute to the MDR phenotype of all clinically relevant Gram-negative pathogens, including *E. coli*, *P. aeruginosa*, *Neisseria gonorrhoeae* and *A. baumannii*, partly due to their extraordinarily large substrate range (see, for example, refs.[43,44]). The structural basis for the poly-specificity of RND transporters is not fully understood, although they do contain both proximal binding pockets and distal binding pockets (distal BPs), which can accommodate different substrates (Fig. 2). In AcrB, the surface of its distal BP allows for multisite binding due to the presence of weakly hydrophobic and weakly polar residues[45,46]. In addition, multiple entry pathways to AcrB provide different access routes to the distal BP for substrates with different chemical properties[47,48]. Entrances at the membrane–periplasm interface (channel 1) and at the periplasm (channel 2) permit entry to drugs with low and high molecular mass, respectively[30,49], whereas an opening between the periplasm and central cavity of the AcrB trimer (channel 3)

is favoured by planar, aromatic cations such as ethidium bromide[47,50]. Most recently, a fourth channel located between transmembrane helices 1 and 2 of AcrB has been proposed as an entry point for carboxylated substrates such as fusidic acid and hydrophobic β-lactams[50].

The substrate preference of AcrB is also modulated by the effects of a fourth transmembrane component, AcrZ[51]. Located within the inner membrane, AcrZ is a small α-helical protein that binds to AcrB (Fig. 2) and promotes the preferential extrusion of antibiotics, such as chloramphenicol, tetracycline and puromycin, by AcrAB–TolC[34,51]. Recent cryo-EM data have suggested that lipids work with AcrZ in a synergistic manner to allosterically modulate AcrB activity[52]. Understanding the mechanisms that support the activity of RND transporters would be beneficial for the rational design of efflux pump inhibitors to inhibit drug sequestration and restore antibiotic susceptibility[46,53,54].

Efflux pump production is carefully regulated, and most systems are controlled by a repressor encoded adjacent to the structural efflux system genes. Mutations in these transcriptional regulator genes are frequently observed to promote pump overexpression in clinical isolates (see, for example, refs.[43,44,55]). For example, MtrR is responsible for the repression of *mtrCDE*, the only RND pump of *N. gonorrhoeae*[56]. Point mutations within the MtrR-binding site or its DNA-binding domain are common and lead to increased *mtrCDE* expression and resistance against structurally diverse antimicrobial agents, including penicillin, tetracycline, azithromycin and third-generation cephalosporins (see, for example, refs.[56–58]). The *mtrCDE–mtrR* locus was recently reported to be a hotspot for genetic recombination between multiple *Neisseria* species, with epistatic interactions at the *mtr* region conferring resistance against azithromycin, polymyxin B and crystal violet[59,60]. Indeed, overexpression of MtrR has been shown to increase gonococcal susceptibility towards penicillin and ceftriaxone[57]. The relationship between impaired regulatory factors and efflux-mediated resistance has also been observed in other clinically relevant pathogens, including *P. aeruginosa*[61], *Campylobacter jejuni*[62] and *Salmonella enterica* subsp. *enterica* serovar Typhimurium[63].

The induction of expression of each RND component involves both local and global transcription regulators responding to various environmental signals. For instance, *acrAB–tolC* expression in *S.* Typhimurium is regulated by RamA which, in turn, is under the control of RamR. Recently, RamR was co-crystallized with bile components cholic acid and chenodeoxycholic acid, compounds typically found in the liver and further metabolized in the intestinal tract; ligand binding to RamR reduced its DNA-binding affinity, resulting in increased *acrAB–tolC* expression through the upregulation of *ramA*[64]. RamR was also shown to interact with other substrates, although in a different manner, suggesting that different recognition mechanisms permit transcriptional regulators to respond to several inducing signals.

Additionally, the activities of these regulators are not simply constrained to efflux regulation. Indeed, MarA, SoxS and Rob — global regulators of *acrAB–tolC* expression — have important roles, including in membrane integrity, DNA repair, biofilm formation, quorum sensing and virulence[65–68]. The multifactorial activities of these regulators therefore indicate that efflux pump expression is part of a wider network of genes that promote bacterial survival in diverse stressful conditions[65,67]. The identification of key regulatory features for efflux pump expression would serve as useful therapeutic targets to combat MDR in clinically relevant pathogens[69].

The evident contributions of RND efflux pumps towards MDR, particularly in clinically relevant Gram-negative pathogens, present a clear opportunity to reinstate drug susceptibility by targeting the structure, function and regulation of these transporters[3,30,70,71]. Indeed, this would also be applicable to members of other efflux families, such as NorA of *Staphylococcus aureus* and P55 of *Mycobacterium tuberculosis*, from the major facilitator superfamily[72–74].

Target alteration, modification and protection

Central to the selective toxicity of most antibiotics against bacteria is their high specificity for important bacterial cellular targets. Many antibiotics bind a primary target with high affinity, which generally inhibits an essential cellular function and leads to inhibition of growth or death[75]. If the structure of the primary target is altered or protected by decoration with other chemical moieties, then antibiotic binding can become inefficient, conferring resistance to the antibiotic (Fig. 3a). For example, the quinolone antibiotics inhibit essential topoisomerase enzymes by binding near the active site. Amino acid substitutions in the target proteins, which result in lower binding efficiency while still allowing the enzyme to function, confer resistance[76]. Similarly, decreased susceptibility to β-lactam antibiotics is conferred by mutations in genes coding for penicillin-binding proteins (PBPs). For example, mutation of PBP3 in *E. coli* has been identified to be almost ubiquitous in clinical isolates from India resistant to aztreonam and avibactam[77]. Altered target sites can be generated by random point mutations that accumulate during growth and can expand to dominance under drug pressure. Alternatively, mutant alleles of target genes can be generated at high frequency by recombination between alleles if multiple copies exist within the cell (for example, mutation and recombination between homologues of the 23S ribosomal RNA (rRNA) gene can rapidly confer linezolid resistance in Gram-positive species[78]) or by transformation, whereby alternative alleles can be gained from related species and mosaic genes are generated by recombination, an approach common in competent species such as members of the *Neisseria* genus[79].

Mycobacteria are unusual in that carriage of plasmids seems to be rare[80] and of minor importance as a mechanism of AMR. Treatment of tuberculosis requires combination therapy, with isoniazid, rifampin, pyrazinamide and ethambutol being common first-line treatments. For each drug, a target-site mutation can cause resistance[81]; for example, a recent study documented the structural basis for resistance to pyrazinamide, whereby mutation of residues near the active site of PanD impair affinity and residence time of the active prodrug, resulting in resistance[82].

Moreover, the addition of moieties to the drug target can prevent antibiotic access and thus protect the target. This is a well-known mechanism of resistance to macrolides, whereby the rRNA target can be methylated by ribosomal methyltransferases, thereby preventing binding of macrolides (as well as of lincosamines and streptogramins)[83]. Methylation of the 16S rRNA is an emerging mechanism of high-level resistance to aminoglycoside antibiotics[84]. Resistance to the polymyxin colistin has also been recognized to result from target modification. As resistance to traditional first-line therapies for Gram-negative pathogens has increased in prevalence, colistin, an old drug, is often used, being one of the few efficacious agents remaining. Colistin has a complex mode of action, but central to its efficacy is its ability to target lipopolysaccharide (LPS), which results in membrane damage and cell death[85,86]. Resistance can occur by decorating LPS with moieties that alter the charge of the overall molecule and inhibit interaction between the drug and its target. Transfer of phosphoethanolamine (pEtN) by pEtN transferase enzymes from phosphatidylethanolamine to LPS results in a modified LPS conferring resistance. The action of pEtN transferases is controlled by the regulatory systems PmrAB or PhoPQ[87],

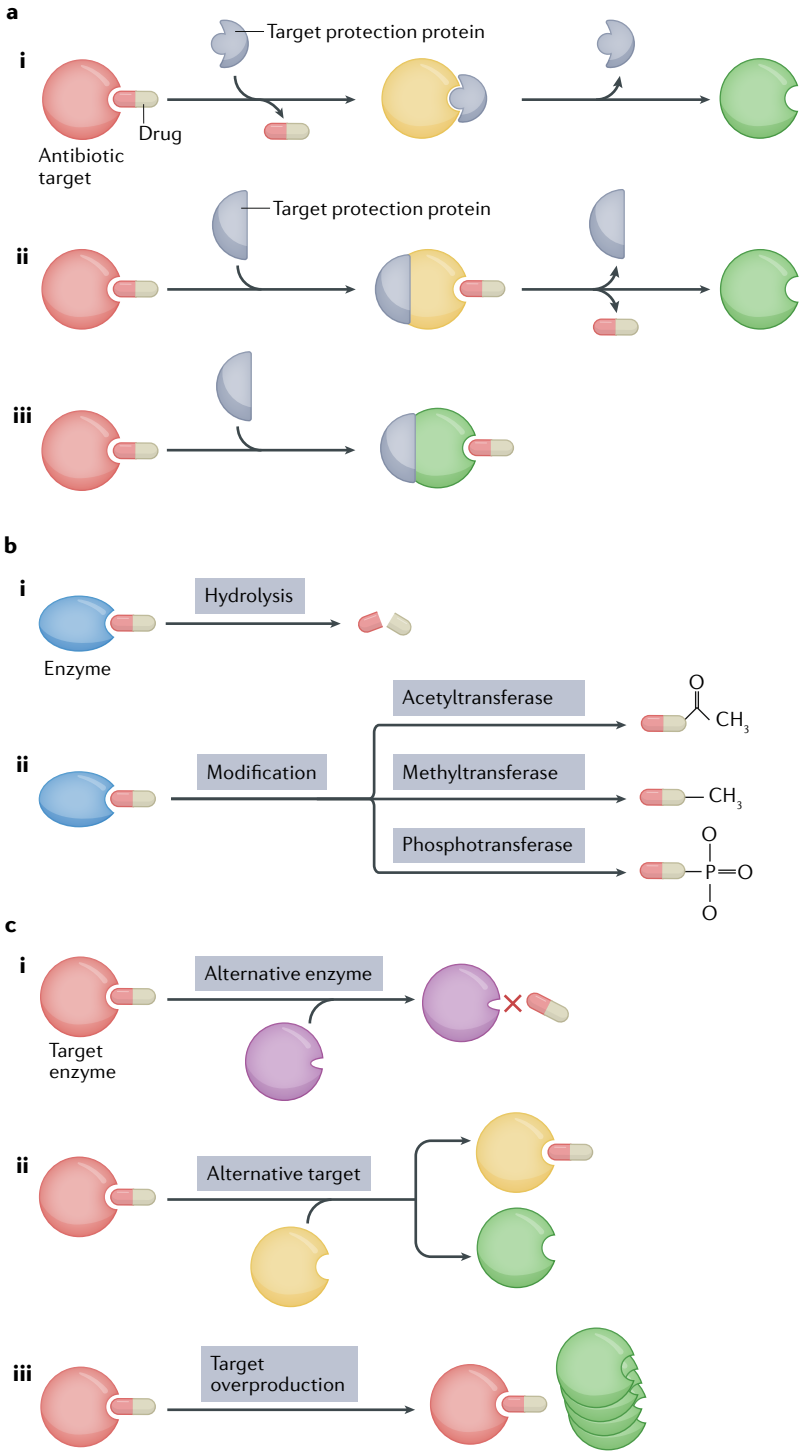


Fig. 3 | Antibiotic resistance via target protection, drug inactivation and target bypass. Schematic diagram of antibiotic resistance mechanisms mediated by target protection, drug inactivation and target bypass. **a**, Mechanisms of target protection. Target protection proteins can bind to the drug target and sterically remove the drug from the target (part **a**,i). Target protection proteins can bind to the drug target and mediate allosteric dissociation of the drug from its target (part **a**,ii). Target protection proteins can bind to the drug target and cause conformational changes to allow the target protein to function even in the presence of the drug (part **a**,iii). **b**, Mechanisms of drug alteration. Enzymes can hydrolyse the functional group of the drug, thereby destroying its antibacterial activity (part **b**,i). Enzymes (such as acetyltransferases, methyltransferases or phosphotransferases) can modify the drug by covalent transfer of various chemical groups to prevent it from binding its target (part **b**,ii). **c**, Mechanisms of target bypass. The drug target (such as an enzyme) becomes redundant due to acquisition of a gene that encodes an alternative enzyme that fulfils the function of the drug target (part **c**,i). The drug target can be replaced by an alternative target that sequesters the drug, thereby allowing the drug target to resume its function (part **c**,ii). The drug target can be overproduced and thus there is insufficient amount of drug to inhibit the increased available target (part **c**,iii). Part **a** adapted from ref.[205], Springer Nature Limited.

which are chromosomally encoded in many Gram-negative species and resistant mutants are vertically inherited but not shared between strains. However, in 2015, a novel mobilizable pEtN transferase family was identified and named *mcr* (mobile colistin resistance). This was an extremely concerning finding given the importance of colistin and the potential for rapid spread of mobile colistin resistance. Subsequent analysis highlighted a family of *mcr* genes that have now been identified in a wide range of species around the world[88]. MCR enzymes act in a similar manner to chromosomal systems[89]. In isolation, the acquisition of an *mcr* gene may confer a limited increase in the minimum inhibitory concentration (MIC) of colistin and therefore the clinical importance of *mcr* has been debated. There is a high prevalence of carriage of *mcr* in

colistin-resistant isolates[90] and strains carrying *mcr* have been shown to be protected from low-level colistin exposure, although the same study found that carriage of *mcr* actually inhibited the selection of mutants with very high MICs of colistin[91]. Expression of pEtN transferases results in the production of diacylglycerol (DAG) as a by-product. DAG is a dead-end metabolite that can become toxic; to arrest this, the cell relies on diacylglycerol kinase A (DgkA) to recycle DAG into useful precursor molecules. DgkA has recently been shown to be required for colistin resistance to prevent toxic by-products inhibiting growth[92]. Together, these data support previous observations of a balance needed for pEtN transferase expression that affects resistance and fitness[93].

Protection of a target may confer a relatively mild increase in the MIC of the relevant antibiotic; however, in combination with mutation of the target site, very high MICs can be achieved. This is commonly seen with the Qnr proteins — a family encoded by genes carried on plasmids commonly seen in Gram-negative species which protect topoisomerases from inhibition by quinolones. Acquisition of a *qnr* gene (there are now seven families recognized) alone has a mild impact but quinolone-resistant isolates will often carry a *qnr* gene in concert with chromosomal mutations in *gyrA*, which can together result in high-level resistance[94]. The variety of different quinolone-resistance mechanisms, each with different impacts on MICs and fitness, offers many possible evolutionary routes to high-level resistance[94].

Target protection can also occur without prevention of drug binding, in which case the drug reaches the target but the impact can then be alleviated, resulting in protection. For example, fusidic acid-resistant *S. aureus* often express FusB-type proteins. Fusidic acid inhibits translation by binding to elongation factor G (EF-G), the ribosome translocase that is involved in processing mRNA and tRNAs and in preventing dissociation of the complex once RNA translocation is complete. FusB proteins contain a zinc finger domain that rescues translation by promoting dissociation of the stalled complex, even though drug binding has occurred[95].

Another recent example of target rescue also involves the ribosome. As mentioned earlier, macrolides, lincosamides, streptogramins and pleuromutilins target translation and act by binding to the peptidyl-transferase centre crucial in the elongation phase of protein synthesis. Proteins belonging to the ATP-binding cassette F (ABC-F) family can confer resistance to these drugs. In contrast to ABC transporter proteins, ABC-F proteins are not membrane bound, and members of this group provide resistance to antibiotics binding to the peptidyl transferase centre of the ribosome by binding to the ribosome–antibiotic complex[96]. Recent evidence from structural and genetic studies suggests that the ABC-F proteins contain 'antibiotic-resistance domains' that interact with the drug-binding domains of the target and cause release of the drugs[97,98]. There are diverse ABC-F families of proteins, and it is now known that they may have evolved on multiple occasions in many species. These have varying structures thought to provide differential affinities for different drug classes[99]. A model for how Vga and Lsa ABC-F proteins can protect a stalled ribosome complex has recently been proposed based on structural and genetic data[96]. The model suggests that ABC-F proteins recognize a stalled ribosome complex and bind to the E-site of the ribosome, which then induces a shift in the P-site tRNA conformation within the ribosome. This allows access of the antibiotic-resistance domain of the ABC-F to the peptidyl transferase centre of the ribosome, which results in expulsion of the antibiotic. Further work has investigated the structural basis for how the Sal ABC-F proteins in staphylococci confer resistance to lefamulin, the first pleuromutilin used for systemic disease in humans[100]. This study confirmed that the Sal proteins are responsible for resistance to pleuromutilins, group A streptogramins and lincosamides in a panel of staphylococci isolates. The structure of the SalB–ribosome complex was analysed and, thus, an allosteric mechanism of resistance was proposed, whereby SalB binding alters the structure of 23S rRNA and results in the release of bound pleuromutilins[100]. While pleuromutilin resistance currently seems to be rare, ABC-F proteins are often found on plasmids, making potential spread possible, and may likely result in clinically problematic rates of resistance in the future[100].

Inactivation and modification of the drug

A widespread mechanism of resistance in many pathogenic bacteria is the modification or inactivation of the antimicrobial drug itself[101] (Fig. 3b). This is typically achieved by the action of enzymes and, therefore, often does not involve changes to any core components of the bacterial cell, which can give an advantage in that there are less likely to be associated fitness costs than for other mechanisms such as mutation or alteration of the drug target. The modification of antibiotics can be broadly split into two mechanisms: inactivation of the antibiotic by degradation or modification by the transfer of a chemical group. Both of these mechanisms are widespread among bacteria due to the mobility of the encoding genes.

Inactivation of antibiotics

The inactivation of antibiotics is a major mechanism of drug resistance, whereby the structure of the drug is damaged or degraded rendering it less effective; therefore, it can contribute to reduced treatment success in the clinic[102]. Examples of the inactivation of antibiotics include the hydrolysis of β-lactam antibiotics by β-lactamases and the binding of tetracycline hydroxylases to inactivate tetracyclines. β-Lactamases are enzymes that provide resistance to β-lactam drugs by hydrolysing the amide bond of the β-lactam ring, thus degrading the drug[103]. β-Lactamases have evolved in nature in response to the production of β-lactam antibiotics by microorganisms and have been studied since the 1940s. The list of characterized β-lactamases continues to increase; at the time of writing, the Beta Lactamase DataBase records over 7,000 distinct β-lactamases[104]. Two main schemes for β-lactamase classification have been developed, based either on sequence in the Ambler class[105] or by function[103,106]. Functionally, there are four classes of β-lactamases (A–D); classes A, C and D are serine β-lactamases and members of class B are zinc-dependent metallo-β-lactamases[103].

Carbapenem resistance is of particular concern as carbapenems are among the most potent antibiotics in use, and carbapenem resistance in combination with resistance to other β-lactams can exclude the use of an entire class of drugs[107]. Extended-spectrum β-lactamases provide resistance to extended-spectrum cephalosporins and monobactams[108]. Carbapenem resistance can be mediated by carbapenemases or by production of an extended-spectrum β-lactamase in combination with porin loss. In 2017, WHO listed three 'critical' priority pathogens, all of which were carbapenem resistant[109]. Carbapenemases, such as KPC (class A), NDM (class B) and OXA (class D) types, have the capacity to hydrolyse penicillins, cephalosporins and carbapenems, greatly reducing the number of drug treatment options for a given infection[103,110]. Novel variants of carbapenemase enzymes are continually being discovered, for example, KPC-55, which is particularly efficient at catalysing aztreonam and meropenem[111], NDM-19, which can hydrolyse β-lactams even in low-zinc conditions[112], and OXA-679 in *Acinetobacter calcoaceticus*, conferring high-level resistance to carbapenems[113]. Of the carbapenemases, NDM enzymes have had a huge impact on

resistance in the past decade. First discovered in 2009 in India[114], they are now found globally across several different species due to their presence on multiple different plasmids, and they can confer resistance to all β-lactams except aztreonam[115–118]. It was shown that different species within the same hospital intensive care unit frequently shared antibiotic resistance genes and, of these, β-lactam resistance genes were most common, with >40% having predicted carbapenemase activity[119]. This, and other work[120,121], shows how frequently β-lactamases can spread between bacteria. Multidrug-resistant plasmids are often characterized by the presence of β-lactamases, enabling these genes to spread more easily between different bacteria[122]. In addition to plasmids, β-lactamases are often found near insertion sequences. This can both mobilize the resistance gene and affect its expression levels[123,124]. For example, in *A. baumannii*, the insertion sequence IS*Aba1* is found upstream of the bla_{ampC} gene (which encodes AmpC β-lactamase) increasing its expression and, in turn, providing resistance to extended-spectrum cephalosporins[124].

Another important example of the inactivation of antibiotics is exemplified by tetracycline-inactivating enzymes that catalyse the oxidation of tetracyclines. The best known is the Tet(X) family, which has been characterized in many different classes of bacteria and can move horizontally on transposable elements, conferring high-level tetracycline resistance[101,125]. The *tet(X/X2)* genes are widely spread and commonly found in multidrug-resistant bacteria from various environments, including isolates from patients, and the presence of them in an environment is positively correlated with tetracycline use[125,126]. Tet(X3/X4/X5) enzymes can confer resistance to tigecycline and have been found in both *Enterobacterales* and *Acinetobacter* isolates in China[127–129]. While Tet(X) enzymes have the capacity to confer resistance, their ability to degrade tetracyclines varies. Structural analysis compared the ability of different enzymes in the family to degrade substrates and suggested that defined substitutions in the most effective enzymes reduce turnover time, allowing faster processing of substrates[126].

Modification of antibiotics by the transfer of a chemical group

Antibiotics can also be rendered ineffective by the transfer of a chemical group. Drug-modifying enzymes have been identified for several antibiotics, including aminoglycosides, macrolides, rifamycins, streptogramins, lincosamides and phenicols.

Aminoglycosides can be modified by acetyltransferases, phosphotransferases or nucleotidyltransferases, modifying the hydroxyl or amino groups of the drug, which in turn substantially reduces the affinity of the drug to the target[130,131]. Many aminoglycoside-modifying enzymes are encoded on mobile genetic elements, in addition to chromosomes, and they are found in both Gram-positive and Gram-negative species. A recent example of a novel aminoglycoside-modifying enzyme is ApmA, which is an acetyltransferase capable of inactivating apramycin, an antibiotic that can currently evade other mechanisms of aminoglycoside resistance[132].

Lincosamide antibiotics can be modified by nucleotidyltransferases, which add phosphate-containing groups to the antibiotic. Nucleotidyltransferases are encoded by *lnu* genes, for example, *lnu(A)* in *Staphylococcus* species[133]. Novel *lnu* genes are still being characterized, and while their clinical impact remains uncertain, it is concerning that they can often be found on mobile genetic elements with the capacity to disseminate across a number of different bacteria[134].

Phosphotransferases and esterases can modify and confer resistance to macrolides because the modified macrolides cannot bind as efficiently to the 50S ribosome. The structure for macrolide phosphotransferases in complex with macrolides has been elucidated, showing that they are members of the same protein superfamily as aminoglycoside phosphotransferases[135]. Esterases are a diverse set of modifying enzymes and there is yet to be a resolved structure for the esterase–macrolide complex[135].

Phenicol and streptogramin antibiotics are both commonly modified by acetyltransferases, which are widespread. The chloramphenicol acetyltransferase (CAT) enzyme transfers an acetyl group from coenzyme A, preventing chloramphenicol from binding to its target on the ribosome[136]. Streptogramin antibiotics can be classified into two groups based on their structure: group A, which binds to the peptidyl transferase centre, and group B, which binds to the peptide exit tunnel. Streptogramins are enzymatically modified by virginiamycin acetyltransferases (Vats), which acetylate the alcohol, changing the conformation of the drug and thereby reducing the activity of the antibiotic[137]. A recent study has developed a pipeline specifically to search for novel streptogramin antibiotics that are less affected by Vats, which could be important in developing more efficacious drugs in this class[137].

Finally, rifamycins can be inactivated and modified by ADP-ribosyltransferases, glycosyltransferases, phosphotransferases and monooxygenases. Rifamycins are often the first line of defence against *M. tuberculosis* infections[138]. ADP-ribosyltransferases in *Mycobacterium smegmatis* were first characterized in the late 1990s but, recently, ADP-ribosyltransferases have also been found, conferring high levels of rifamycin resistance in *Mycobacterium abscessus*[139]. At the time of writing, there has yet to be an ADP-ribosyltransferase characterized in *M. tuberculosis*. ADP-ribosyltransferases catalyse the transfer of ADP ribose to hydroxyl-linked C_{23} on the antibiotic, blocking its interaction with RNA polymerase[140]. Rifamycin resistance by glycosyltransferases[141] is similar to the action of ADP-ribosyltransferases, whereby the enzymes glycosylate the hydroxyl position at C_{23} (ref.[140]). Phosphotransferases convert rifamycins to inactive phospho-rifamycins[142] at C_{21} (ref.[140]). The expression of such enzymes has the potential to reduce the susceptibility profile of the organism if the phosphotransferases are found on mobile genetic elements, such as plasmids, where expression can be high[143]. More recently, a novel mechanism of rifamycin inactivation conferred by rifamycin monooxygenases (Rox) enzymes has been described. Rox enzymes have been shown to linearize the rifamycin structure after oxygenation of a naphthyl group, which abolishes the binding of the drug to RpoB[144].

Target bypass

Target bypass is a strategy that consists of producing an alternative pathway that bypasses the antibiotic by making the original target redundant. This can occur via the acquisition of an alternative gene that can confer the required properties to the cell but is not efficiently inhibited by the original antibiotic[145] (Fig. 3c).

The best-known example of target bypass is probably the development of methicillin-resistant *S. aureus* (MRSA). β-Lactam antibiotics, such as methicillin, bind to PBPs and inhibit the transpeptidase domain, causing disruption of the cell wall synthesis. *S. aureus* can acquire an exogenous PBP (PBP2a) that is homologous to the original target but with lower affinity for β-lactam antibiotics[145,146]. This protein is encoded by the *mecA* gene (methicillin-resistant gene), located in the staphylococcal cassette chromosome *mec* (SCC*mec*), a mobile genetic element that confers methicillin resistance[146].

When methicillin binds to this alternative target site, inhibition of cell wall synthesis is prevented as the transpeptidase activity of PBP2a is maintained[145]. Native PBPs are also required as PBP2a does

not possess a transglycosylase domain. With this mechanism, *S. aureus* can bypass the action of methicillin to ensure cell survival[146]. Recent evidence has suggested that common lineages of MRSA often seen in human infection first appeared in European hedgehogs in response to β-lactams produced by endogenous dermatophytes. This was well before human antibiotic use and illustrates the broad diversity of selective pressure operating in different contexts, which can have implications for human health[147].

Another recently characterized example of target bypass is seen in *E. coli*, whereby the production of an alternative crosslinking mechanism, which includes the L,D-transpeptidase YcbB, can bypass the D,D-transpeptidase activity of PBPs and lead to β-lactam resistance[148]. In this example, besides the L,D-transpeptidase activity of YcbB, additional factors, such as high-level synthesis of the alarmone (p)ppGpp, class C monofunctional PBP5 and the glycosyltransferase activity of class A PBP1b, are also needed[149]. Although YcbB is not inhibited by β-lactam antibiotics, it remains susceptible to carbapenem antibiotics such as meropenem and imipenem[149].

Vancomycin is a glycopeptide antibiotic widely used for the treatment of enterococcal and MRSA infections[150]. This drug also inhibits cell wall synthesis but, instead of directly binding to PBPs as do β-lactams, vancomycin binds to the terminal D-alanine-D-alanine from the pentapeptide precursors. This inhibits peptidoglycan crosslinking, which is essential for the synthesis of the bacterial cell wall. Vancomycin resistance in enterococci is mediated by the acquisition of the *van* cluster, with the *vanA* gene cluster being the most prevalent in clinical vancomycin-resistant strains. Expression of the genes in the *vanA* gene cluster, located on a transposon (Tn*1546*), leads to the abnormal synthesis of peptidoglycan precursors, which is the target site. Consequently, instead of binding to D-alanine-D-alanine, vancomycin now binds, with reduced affinity, to terminal D-alanine-D-lactate or D-alanine-D-serine[151,152].

This bypass strategy confers resistance to vancomycin in *Enterococcus* species and other Gram-positive bacteria. Some MRSA strains can also carry the Tn*1546* transposon acquired from vancomycin-resistant *Enterococcus faecalis*[150]. This led to the appearance of MRSA with vancomycin resistance conferred by the *vanA* gene cluster, with the first case being reported in 2002 in the USA[153]. Although vancomycin-resistant *S. aureus* infections are rare, a methicillin-resistant and vancomycin-resistant *S. aureus* strain was isolated in Europe[154].

The combination of the two antibiotics trimethoprim and sulfamethoxazole (known as trimethoprim–sulfamethoxazole (TMP–SMX) or co-trimoxazole) is commonly used to treat urinary tract infections and prophylaxis for pneumonia caused by *Pneumocystis carinii*, which occurs in individuals with HIV[155]. The two active agents block the biosynthesis of bacterial folic acid, which is essential for nucleic acid and protein synthesis. TMP acts as a dihydrofolate reductase (DHFR) inhibitor and SMX is a sulfonamide that inhibits dihydropteroate synthase (DHPS)[156,157]. In general, resistance emerges more slowly to combination therapy, making it an attractive strategy. However, resistance to TMP–SMX can occur when additional novel alleles of DHFR and/or DHPS are acquired and/or overproduced. This causes an increase in target availability and a decrease in the binding activity of TMP–SMX, maintaining folic acid production and ensuring cell survival[145,157].

Bacteria possess different ways to recycle components of their own cell wall. In *E. coli*, the enzyme MurNAc-6P etherase (MurQ) is used to recover uridine diphosphate (UDP) N-acetylmuramic acid (UDP-MurNAc), the first precursor of peptidoglycan de novo biosynthesis. However, the MurQ enzyme, which is also required for amino sugar recycling and catabolism, is absent in many Gram-negative bacteria[158]. An 'anabolic recycling pathway', which bypasses the first steps in peptidoglycan biosynthesis, has been reported[159,160] in most Gram-negative bacteria (absent in *E. coli*)[160]. This recycling pathway channels MurNAc-6P directly into peptidoglycan biosynthesis in the form of UDP-MurNAc[160]. As the UDP-MurNAc pool is affected, the target of fosfomycin (MurA), which is present in the first steps of peptidoglycan biosynthesis, is also altered. Therefore, this pathway also provides intrinsic resistance to fosfomycin[159,161].

The promise of resistance breakers

An important reason to understand the molecular mechanisms of resistance is to use this information to design novel strategies that interfere with or inhibit the resistance mechanism. So-called antibiotic resistance breakers are compounds that can disrupt or inhibit a specific mechanism of antibiotic resistance to restore the clinical efficacy of a specific antibiotic. This is an attractive strategy because it could be used to potentiate the use of existing antibiotics, and there is extensive proof-of-principle that this strategy works clinically as inhibitors of β-lactamase enzymes have been used successfully since the introduction of clavulanic acid in 1981. Generally, β-lactamase inhibitors work by modifying β-lactamase enzymes, rendering them inactive. Many, including clavulanic acid, sulbactam and tazobactam, are themselves β-lactam compounds with minimal antibacterial effect but with the ability to modify the β-lactamase enzyme, creating an inactive inhibitor–enzyme complex. Most recently, new β-lactamase inhibitors belonging to the diazabicyclooctane (for example, avibactam, relebactam and durlobactam) or boronic acid (vaborbactam) classes have been developed with regulatory approvals for meropenem–vaborbactam and imipenem–relebactam showing viable clinical potential[162]. Although β-lactamase inhibitors have been hugely successful, there is now a growing problem with resistance; just as there has been resistance reported to all clinically available antibiotics, there has now also been resistance reported to all β-lactam–β-lactamase inhibitor combinations available. Resistance is often mediated by high-level expression of the targeted enzymes or mutations in the enzymes. Although they have not entered clinical use, promising aminoglycoside-modifying enzyme inhibitors have also been developed[163].

Our increased understanding of how resistance mechanisms interact with core physiology also offers opportunities, for example, the observation that colistin resistance conferred by pEtN transferases results in toxic DAG production as a by-product, which is alleviated by DkgA. Loss of this control mechanism results in repression of pEtN transferases and loss of resistance; therefore, targeting DkgA could potentiate colistin activity[92].

An alternative resistance breaker strategy is to target the impermeable Gram-negative outer membrane to increase drug uptake. However, compounds that do this are often themselves bactericidal while also potentiating the activity of other antibiotics. For example, polymyxins including colistin are potent antimicrobials and work by permeabilizing the membrane but there is evidence that, along with other antimicrobial peptides, they could be used in combination with other drugs to increase activity[164].

Finally, the concept of inhibiting efflux pump activity is particularly attractive, not only because efflux underpins many other mechanisms of resistance (Box 2) but also because many efflux pumps are also required for virulence and biofilm formation. The idea is that, by blocking or otherwise inhibiting major efflux pumps, susceptibility to antimicrobials would increase because the extrusion of antibiotics

would be prevented, causing them to accumulate to high levels intracellularly. Several competitive inhibitors of RND efflux pumps have been discovered or developed. However, none have reached the clinic, largely due to host toxicity. Competitive inhibition is also complicated by complexity of the systems and, therefore, it is possible that this approach will ultimately be unsuccessful. In fact, it seems likely that alternative strategies to inhibit efflux may be needed; other strategies being explored include the prevention of efflux complex assembly, targeting either the outer membrane[165] or periplasmic components[166], decoupling the energy source, or inhibition of efflux pump expression[167,168] (recently reviewed in detail in ref.[3]). Additionally, recent work has shown that the impact of efflux on drug accumulation is most apparent in actively growing cells, which informs which types of infection should be targeted for efflux inhibitor development[2].

The promise of resistance breakers has not been clinically exploited beyond the extensive deployment of β-lactamase inhibitors. The examples outlined above show how understanding the biology of resistance may aid in the development of future combinations; however, this is complex and the development of clinical products will require significant effort.

Conclusions and future perspectives

The epidemiology of AMR continues to paint a worrying picture and its threat is accelerating. However, efforts of the many researchers studying different aspects of the problem give some cause for hope. We now understand much more about the biochemistry of how different antibiotics work and the corresponding mechanisms of resistance. Allied with a greater understanding of the selection of resistance and impacts on host fitness we are better placed to develop dosing regimens for any new agents in a way that will minimize the emergence of resistance. Understanding the genetic basis of resistance is also a foundation for various rapid diagnostic methods being developed, which offer the promise of guiding initial antibiotic selection to minimize use of ineffective antibiotics. Ultimately, new antibiotics or novel synergistic therapeutic combinations are urgently required and understanding resistance is an essential prerequisite to these efforts.

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J.M.A.B., E.M.D., E.T., P.S., M.S.G., I.A. and M.A.W. researched data for the article. J.M.A.B. and M.A.W. contributed substantially to discussion of the content. J.M.A.B., E.M.D., E.T., P.S., M.S.G., I.A. and M.A.W. wrote the article. J.M.A.B., E.M.D., E.T., P.S. and M.A.W. reviewed and/or edited the manuscript before submission.

Competing interests

The authors declare no competing interests.

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