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Du, Dijun; Wang-Kan, Xuan; Neuberger, Arthur; van Veen, Hendrik W.; Pos, Klaas M.; Piddock, Laura J.V.; Luisi, Ben F.

DOI:
10.1038/s41579-018-0048-6

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Document Version
Peer reviewed version

Citation for published version (Harvard):
Du, D, Wang-Kan, X, Neuberger, A, van Veen, HW, Pos, KM, Piddock, LJV & Luisi, BF 2018, 'Multidrug efflux pumps: structure, function and regulation', *Nature Reviews Microbiology*, vol. 16, no. 9, pp. 523-539. https://doi.org/10.1038/s41579-018-0048-6

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Publisher Rights Statement:
Checked for eligibility 14/09/2018

Dijun Du, Xuan Wang-Kan, Arthur Neuberger, Hendrik W. van Veen, Klaas M. Pos, Laura J. V. Piddock & Ben F. Luisi Multidrug efflux pumps: structure, function and regulation Nature Reviews Microbiologyvolume 16, pages 523–539 (2018) https://doi.org/10.1038/s41579-018-0048-6

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Multi-drug efflux pumps: structure, function and regulation

Dijun Du[1], Xuan Wang-Kan[2], Arthur Neuberger[1,3], Hendrik W. van Veen[3], Klaas M. Pos[4], Laura Piddock[2] and Ben F. Luisi[1]

1. Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1GA, UK

2. Institute of Microbiology and Infection, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

3. Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1PD, UK

4. Institute of Biochemistry, Goethe Universität Frankfurt, Max-von-Laue-Straße 9, D-60438, Frankfurt, Germany

Email: B.F. Luisi bfl20@cam.ac.uk

Summary

Infections arising from multidrug-resistant pathogenic bacteria are spreading rapidly throughout the world and threaten to become untreatable. The origins of resistance are numerous and complex, but one underlying factor is the capacity of bacteria to rapidly export drugs through the intrinsic activity of efflux pumps. In this Review, we describe recent advances that have increased our understanding of the structures and molecular mechanisms of multidrug efflux pumps in bacteria. Clinical and laboratory data indicate that efflux pumps function not only in the drug extrusion process, but also in virulence and the adaptive responses that contribute to antimicrobial resistance during infection. The emerging picture of the structure, function and regulation of efflux pumps suggests opportunities for countering their activities.

[H1] Introduction

Bacterial antibiotic resistance has numerous origins, and several general mechanisms of adaptive responses have been described that give rise to resistant behavior in bacterial populations [1]. Resistant phenotypes can arise from the boost of intrinsic efflux activity through the overexpression, asymmetric accumulation during division or mutation of genes encoding energy-dependent transporters [2-4]. Depending on the antibiotic or toxin challenge, efflux can be the fastest acting and most effective resistance mechanism in the bacterial repertoire of stress responses. Exposure to antibiotics and other drugs often triggers very complex bacterial reactions that involve changes in the level of expression of numerous transporter genes, as seen by

phenotypic profiling of *Escherichia coli* [5]. These transporters provide several antibiotic efflux pathways that can work in a cooperative manner or provide redundant functionality [6,7]. Currently, six families of bacterial drug efflux pumps have been identified that contribute to the efflux pathways. One of these, the ATP-binding cassette family (ABC) directly utilizes ATP as the energy source to drive transport. The other five groups are secondary-active transporters that are powered by electrochemical energy captured in transmembrane ion gradients; they are the major facilitator superfamily (MFS), the multidrug and toxin extrusion (MATE) family, the small multidrug resistance (SMR) family, the resistance-nodulation-cell division (RND) superfamily and the proteobacterial antimicrobial compound efflux (PACE) family [8,9]. Most of the efflux families have an early origin and have been sustained during the course of evolution, as seen for example in the ubiquity of the MFS, MATE and RND families or superfamilies among all domains of life. The efflux machinery is well-tuned to cope with hazardous compounds and harmful metabolic waste products of immensely diverse chemical character.

Currently, about 80% of all severe bacterial infections observed clinically are attributed to multi-drug resistant Gram-negative species [10,11]. These bacteria are characterized by a cell envelope comprising two membranes that function as a barrier to the entry of drugs and other compounds. Tripartite efflux pumps span this envelope to drive the efflux of compounds across this barrier (Figure 1). Not all transporters form such assemblies, but the pumps can cooperate as part of a system that moves the efflux substrates first into the periplasm and then outward through a tripartite machine [6,7].

Advances in structural analyses of the different classes of transporters have recently provided unprecedented insight into their detailed function. Investigations of pump expression patterns reveal complex regulatory networks at the transcriptional and post-transcriptional level with connections to many cellular processes, including central metabolism. Accumulating evidence suggest that efflux pumps have much broader functional roles during infection, beyond transport of noxious compounds (Box 1). For example, pumps can contribute to bacterial pathogenicity through transport of proteinaceous toxins and other virulence factors, as well as having roles in cell-to-cell communication and formation of protective biofilms. In addition, efflux pumps play a part in lipid transport (in *Mycobacteria*) and possibly persistence in the presence of antibiotics [12]. In this Review, we summarize our current understanding of the structures and molecular mechanisms of multidrug efflux pumps in bacteria. We discuss their regulation by two component systems as well as by transcription and post-transcription factors and explore efflux-mediated resistance to antibiotics.

[H1] Molecular mechanisms of multidrug efflux

[H2] ATP-binding cassette transporters.

The ABC transporters are functionally diverse and mediate ATP-dependent import or export of solutes. Some function as modulators of ion channels [13]. The structures of ABC transporters reveal transmembrane domains (TMDs) that contain substrate-binding pockets and nucleotide-binding domains (NBDs) that bind and hydrolyse ATP to drive the transport cycle [14-19].

The ABC exporters can be divided into homo- and heterodimeric groups, with the latter being particularly relevant for intrinsic and acquired antibiotic resistance in the Gram-positive bacteria (for example, PatAB, LmrCD, BmrCD, EfrCD). Although most homodimeric ABC exporters are thought to have two equivalent nucleotide-binding sites, heterodimeric ABC exporters contain a degenerate binding site that does not support ATP hydrolysis [20]. ATP binding at the degenerate site establishes additional contacts across the NBD-NBD dimer interface and prevents the NBD dimers from fully separating, a molecular feature that might distinguish the hetero- and homodimeric ABC transporters. For the heterodimeric ABC exporter BmrCD, the nucleotide-binding domains have been shown to be non-equivalent, conferring an intrinsic asymmetry in the transporter [21].

The current structural and functional data for both importers and exporters support an 'alternating access' mechanism, whereby the conformation switches states between inward-open, occluded and outward-open to translocate substrates across the membrane bilayer (Figure 2A). The conformational changes are linked with NBD dimerization and dissociation mediated by ATP binding and hydrolysis [22-24].

The bacterial homodimeric MsbA uses its ATPase activity to move the lipopolysaccharide precursor lipid A from the cytoplasmic leaflet of the inner membrane to the periplasmic leaflet. This catalytic action confers the enzyme with the descriptive title of 'flippase'. The structures of MsbA in defined functional states have been elucidated by cryo-electron microscopy (cryo-EM) and enabled the visualization of the transport process [25]. In the absence of ATP, the lipopolysaccharide can be identified in the transmembrane region of MsbA near the periplasm. In the presence of a transition state analogue that mimics ATP hydrolysis (ADP-vanadate), MsbA assumes a closed state conformation, but when ADP is present, an inward-facing conformation is observed. These structures can be considered as snapshots of the transport process and are consistent with the alternative access model. The bacterial ABC transporter McjD exports antibacterial peptides, and structural and spectroscopic data support a mechanism whereby a binding cavity is transiently opened in the outward-facing conformation causing release of the peptide [26]. In addition to the conformational changes in ABC exporters facilitated by ATP binding and hydrolysis, a role of electrochemical ion gradients in transport has been indicated by studies of the bacterial homodimeric ABC exporters LmrA [27] and MsbA [28]. By analogy with the chemiosmotic coupling of secondary-active transporters, the coupling to electrochemical ion gradients most likely imposes directionality on steps in the transport cycle of ABC transporters that are not regulated by nucleotide. Thus, although bacterial ABC multidrug exporters share proteins motifs and common features, the detailed structural differences might translate into a diversity in molecular mechanisms [29].

[H2] Tripartite assemblies involving ABC transporters

In *E. coli* and other Gram-negative bacteria, the ABC exporter MacB contributes to drug resistance and virulence. In those organisms, MacB forms a tripartite assembly with the outer membrane protein TolC and the periplasmic partner protein MacA. These pumps drive not only the efflux of macrolide antibiotics, but also the transport of outer membrane lipopeptides, protoporphyrin, polypeptide virulence factors and lipopolysaccharides. The transport processes are coupled to ATP hydrolysis by MacB.

Cryo-EM structures have been obtained for the MacAB-TolC assembly, which consists of a TolC homotrimer, six protomers of MacA and a MacB homodimer (Figure 1) [30]. The 3:6:2 stoichiometry of the assembly is consistent with results from biophysical experiments [31], and the dimer seen in the crystal structure of MacB homologues [32,33]. The assembly may also accept some transport substrates from the periplasm. For example, the precursor of one of its substrates, the heat-stable enterotoxin II [34], is transported by the Sec machinery into the periplasm, where it undergoes maturation and might subsequently enter the MacAB–TolC pump through an opening observed in the cryo-EM structure [30,33]. For substrates that gain access from the periplasmic side, MacB possibly uses an 'outward-only' transport mechanism proposed for some ABC transporters, whereby the substrate-binding pocket remains in an outward-facing state. The transporters may intercept their substrates from in an outward-facing binding pocket, then undergo a conformational change that is coupled to ATP hydrolysis that decreases affinity for the ligand so that it is displaced into the exterior compartment [35,36].

[H2] Transporters of the major facilitator superfamily.

The MFS group is found in all domains of life and is the largest and most diverse family of transporters. The group encompasses 'uniporters' (that move substrates across the lipid bilayer without any coupling ions), 'symporters' (that couple transport with ion-transport in the same direction as substrate) and 'antiporters' (that facilitate the movement of ion and substrates in opposite directions). Most members of this superfamily function as single, monomeric units. They range from 400–600 amino acid residues in length and possess 12 or 14 transmembrane helices (TMH) organized as two domains, each of which is composed of bundles of six helices (referred to as the MFS fold) [37]. The available structural data support an 'alternating access' mechanism for the MFS proteins, whereby the two domains undergo a conformational switch between inward-open and outward-open states during a transport cycle, similar to that described above for ABC transporters. Data for the lactose permease symporter LacY show that the conformational switch for alternating access is triggered by induced fit from ligand binding, and that the electrochemical proton gradients controls the rate of transport [38]. The key element of the model is that the binding of proton and substrate are ordered, and that a ternary complex is formed (Figure 2b). For various MFS transporters, the sequence of binding (that is, proton binding followed by substrate binding, or substrate binding followed by proton binding) is different.

Multidrug efflux is conferred by members of the drug:H^+ antiporters-1 (DHA1) and DHA2 families. The crystal structures of DHA1 proteins from *E. coli* have been determined in outward-open (YajR), occluded (EmrD) and inward-open (MdfA) conformations [39-41]. The structural gallery of three different conformational states resembles closely the states captured for the symporters lactose permease LacY and XylE [38,42,43]. The common structural architecture of the DHA1 group comprises amino-terminal- and carboxy-terminal domains that are linked by a long cytoplasmic amphipathic helix-containing loop. In the substrate-bound structures of MdfA, drugs and inhibitors bind in a central cavity (formed by TMH1, TMH4, TMH7 and TMH10) (Figure 4c). DHA2 family homologues, such as YbgG, have similar domain organization to DHA1 proteins, except that the domains are connected by two V-shaped TMHs [44]. A motif (termed

motif C) within the TMH5 situated in the interface between the two N-terminal and C-terminal bundles could participate with other helices to prevent proton leakage. Two conserved acidic residues, E26 and D34, have been identified in motif D of MdfA and those residues were shown to be involved in proton transfer [45], although only D34 participates in substrate-binding [40]. Spectroscopic data suggest that protonation could drive the conformational switch between alternating access states in the antiporters MdfA and LmrP [46]. Moreover, lipid-protein interactions are reported to affect the proton-dependent and substrate-dependent conformational dynamics of MFS transporters (and other classes of pumps) [47-49].

The transport cycle of MFS proteins seems to involve ordered binding and release of proton and substrate, but the stoichiometry of drug-proton exchange varies between family members[50,51]. Whereas the DHA2 family transporter exchanges two protons with one substrate, regardless of whether the transported drug is cationic with one or two charges, MdfA mostly catalyzes efflux of electroneutral or monovalent cationic drugs by exchanging a single proton with a single drug molecule. MdfA also transports divalent cations with two charged moieties separated by a long linker; however, the export of such a cation drug molecule needs two consecutive transport cycles [50]. MdfA shows an indirect competition mechanism between H^+ and substrates [45], whereas both direct and indirect mechanisms of competition might be relevant to the case of LmrP [51].

Although MFS transporters generally function as monomers, some family members are fusions of two homologous but distinct MFS permeases. A recent crystal structure of the plant NRT1.1 reveals a homodimer and suggests that the protomers might have different affinity modes [52]. In Gram-negative bacteria, some DHA2 family proteins can form tripartite assemblies with partner proteins to directly transport drugs across the entire cell envelope [53].

[H2] Multidrug and toxic compound extrusion transporters.

The multidrug and toxic compound extrusion (MATE) transporters can be categorized into the NorM, DinF (DNA-damage-inducible protein F) and eukaryotic subfamilies based on their amino-acid sequence similarity. Bacterial MATE transporters use transmembrane H^+ and/or Na^+ gradients to drive the efflux of poly-aromatic and cationic drugs. The structures of NorM and DinF subfamily members reveal two bundles of six transmembrane helices [54-57] in which the topology is distinct from that seen for the MFS transporters [58]. There are N-terminal and C-terminal domains related by pseudo-twofold symmetry that form a V-shaped central cavity open to the extracellular space. The structures apparently adopt an outward-open state with the lower portion of the central cavity situated about halfway through the membrane bilayer. NorM may use an alternative access transport mechanism, as indicated by the proximity of residue pairs *in vivo* [59].

In the structure of *Neisseria gonorrhoeae* NorM, a drug-binding site has been identified in a central cavity situated near the membrane-periplasm interface (Figure 2c; Figure 4d). The interaction with the substrate is largely mediated by ionic and H-bonding [56,60]. Three key residues that are involved in Na^+-coordination (corresponding to *N. gonorrhoeae* NorM Y294, E261 and D377) are highly conserved in the NorM and eukaryotic subfamilies and probably

participate in the transport process [60]. *Vibrio cholerae* NorM can use both H^+ and Na^+ gradients to transport substrates, and an aspartate (residue D371 in *V. cholera* NorM) has been identified to be involved in proton coupling [61]. The substrate and cation interact with distinct subsets of amino acids and can bind to the protein simultaneously [62]; therefore, the coupling between the fluxes of Na^+ and drug is likely to be indirect and mediated by protein conformational changes. As the drug-binding pocket is proximal to the periplasmic side and is inaccessible in a presumptive inward-open state, the protein must adopt a different conformation to recognize substrates from the cytoplasmic side.

For the DinF subfamily member DinF-BH, substrates can be deeply bound within the central cavity, where the interactions are mostly mediated by hydrophobic amino acids [56,63] (Figure 2c). Residue D40 in DinF-BH makes charge-charge interactions with cationic substrates, and evidence indicates that protons directly compete with this interaction [63]. DinF-BH was also captured in an asymmetric outward-open state, in which the pseudo-twofold symmetry is broken by the kinking of TMH7 and TMH8 [63]. For another DinF subfamily member, PfMATE, the drug-binding site is formed exclusively within the N-terminal domain, and is dominated by polar amino acids (Figure 2c). The TMH1 of PfMATE might undergo protonation-dependent bending, but its function in the transport cycle and generality to other family members is not clear [56,57].

The currently available structures of MATE transporters are all captured in an outward-facing state. The intracellular-facing state may resemble the inward-facing conformation of the structurally related MurJ [64], a member of the newly defined supergroup that includes the MATE proteins (referred to as the multidrug oligosaccharidyl-lipid polysaccharide (MOP) transporter superfamily in the transporter classification database). Having the structure of an intracellular-facing state with substrate bound would substantially advance our understanding of MATE-mediated multidrug extrusion.

Comment [Editor1]: CE: please can you hyperlink transporter classification database to http://www.tcdb.org

[H2] RND transporters.

The well-characterized Gram-negative members of the RND family include *E. coli* AcrB, *Pseudomonas aeruginosa* MexB, *Neisseria gonorrhoeae* MtrD and *Campylobacter jejuni* CmeB, for which high-resolution structures are available [65-70]. The RND proteins are relatively large in comparison to other bacterial membrane proteins, and the members of this superfamily form mainly homotrimers (Figure 3a). The ubiquitous RND superfamily also include members with different quaternary structures, such as the monomeric eukaryotic NPC1 [71], which is involved in cholesterol homeostasis, heterodimeric SecDF, which is part of the protein export complex [72], and homodimeric HpnN that shuttles hopanoids to the outer membrane [73]. All RND transporters have a rather atypical structure for the secondary transporter group and display a conserved transmembrane region structure, as well as a periplasmic and luminal domain which comprise up to 60% of the mass of the protein. The RND transmembrane domain contains an internal structural repeat that originated through a gene duplication event, whereas the fold of the periplasmic luminal domain can differ substantially amongst the RND homologs.

Aside from their physiological relevance to the conferment of antibiotic resistance, the molecular mechanism of drug poly-specificity, the movement of the drug through the protein, the coupling

of proton and drug antiport (that is, energy transduction), as well as the dynamics of the assembly and disassembly of the Gram-negative tripartite systems are the most prominent questions. The bacterial RND protomer is central to these questions and comprises three larger domains: the transmembrane domain, the pore domain and the docking domain that engages a periplasmic partner in cases when the RND protein is part of a tripartite assembly (Figure 1). The transmembrane domain consists of 12 α-helices, and is the conduit for proton movement, which is coupled to drug efflux in the pore domain [67,74-76]. The pore domain is the main location of drug entry and consists of the subdomains PN1 and PN2, which extend into the periplasm (between TMH1 and TMH2), and PC1 and PC2 (which lie between TMH 7 and TMH8) [67,74] (Figure 3a; sup info fig 1). These four subdomains form the two main drug-binding cavities: the proximal (or access) and distal (or deep binding) pockets. Each is enriched with a variety of aromatic, charged and polar residues thought to confer each pocket with slightly different substrate preferences (see for example Figure 4a,b). The docking domain consists of the DN and DC subdomains, and its interaction with the periplasmic protein will be discussed further in the subsection on the tripartite assemblies.

AcrB undergoes marked conformational change upon drug binding. Although the protomers of the AcrB trimer are symmetric in the apo state, they have different conformations when actively engaged with substrate: the loose (L), tight (T), and open (O) states [74,77]. It is thought that these conformations represent three states in drug efflux, and that each protomer cycles through these states consecutively, with a high degree of cooperativity [4] (Figure 3a, sup info Fig 1). The exact details of the process remain to be elucidated, but in brief the model holds that drugs are introduced in the L state, move further into the drug binding pocket in the T state, and then are extruded into a funnel-shaped ‘canyon’ in the docking domain in the O state [77]. The conformational changes occur in an apparently strict order, with drugs moving further into the protein but unable to leak back into the periplasm, and has been called a ‘peristaltic’ mechanism in analogy to the physical process [77]. The movements between the PN2 and PC1 subdomains in the L to T transition constitute the opening and closing of the distal binding pocket, whereas the PN1 and PC2 subdomains seem to move as a rigid body [76]. The relative movement of the PN2 and PC1 and PN1 and PC2 subdomains in the T to O transition closes the periplasmic entry pathway and as such prevents the reverse flow of drugs to the periplasm. The intrinsic and asymmetric inter-protomer interaction of the PN1 and PN2 subdomains supports the consecutive and cooperative drug transport model which is also found consistent with functional dependencies of the protomers in the heterotrimeric *E. coli* MdtBC pump [78]. It is therefore a surprising finding that the three protomers of the *C. jejuni* homologue CmeB seem to undergo conformational switches independently during the efflux process in microsecond to second time range, as determined by fluorescence energy-transfer measurements [70].

Structures of AcrB bound to different substrates illustrate the surprisingly diverse range of compounds that the transporter can efflux [54,66,68,74,76,79-82]. These structures show slightly different substrate preferences between the proximal and distal binding pockets, possibly also based on the substrate size or minimal projection areas [81,83,84], and are thought to represent, at least for the high molecular weight substrates, a progression of drug entry into the proximal pocket during the L state, and movement of drug into the distal pocket during the L to T transition (Figure 3b). A short switch loop is located between the two binding pockets of AcrB that seems to regulate drug entry into the distal pocket [66,68].

The distal pocket seems to be a universal binding site for the known AcrB drugs. Its binding site consists of mainly Phe, but also Ile, Val, Ala and Asn side chains (Figure 4a). The pocket represents a hydrophobic pit and seems to accommodate all the known substrates. Although the co-structures show binding of single drugs to this pocket, it is not known whether the pocket might bind different (smaller) drugs concomitantly. If so, it might provide one of the explanations for the strong cooperativity of drug transport observed [85]. Water molecules, also found to stabilize the binding of substrates and inhibitors [66,79], have been postulated to be involved in drug transport as well by assisting substrate-protein interactions in a way compatible with the poly-specificity of AcrB [86].

Apart from the periplasmic entry pathway which includes the proximal pocket in the L protomer and further transport towards the distal binding pocket, other pathways have been proposed using channels or tunnels located at the membrane-periplasmic boundary (Figure 3b). One channel leading to the distal binding pocket (-tunnel 1) is preceded by a major groove comprising TMH7, TMH8 and TMH9, and in most structures, a dodecylmaltoside molecule is deeply bound inside [68,87,88 89]. Channel 2 is defined by the PC1 and PC2 subdomains in the pore domain and directly guides substrates from the periplasm to the proximal pocket. This channel seems to be present in both L and T protomers, but the PC1-PC2 cleft seems more closed in the latter protomer. Drugs originating from the periplasm are thought to enter AcrB through this channel [50,52,68] . A third channel has its entry from within the central cavity formed by the three protomers within the trimer [67,68,90]. This channel was shown to have a preference for planar, low molecular mass, cationic aromatic compounds such as ethidium [90]. These substrates are suggested to be directly transported to the distal binding pocket, bypassing the proximal binding pocket and the switch loop. Other substrates, such as the high molecular weight macrolides, are preferentially taken up via channel 2. More channels might be present, as carboxylated drugs (such as β-lactams) bind to the TMH1-TMH2 groove [53,69], which does not show a defined channel entrance in any of the conformations, but which is close to the entrance of channel 3 [90]. One hypothesis holds that the drug-bound TMH2 moves in different conformational sub-states as a type of ‘elevator mechanism’ to deliver the transport substrate to the periplasmic pore domain [69]. It is interesting to note that an analogous channel for substrate transport has been proposed to occur for the HpnN transporter, which shuttles hopanoid molecules to the outer membrane of the Gram-negative species *Burkholderia* sp., modifying the fluidity of the outer membrane [73].

The transport substrates for AcrB, MexB and other RND transporters encompass diverse compounds that have some lipophilic character but can also harbor moieties that are cationic, neutral (chloramphenicol and solvents) or acidic (β-lactams). Can the structural basis for such broad poly-specificity be rationalised? A recent study on the differences between the AcrB and AcrD binding pockets [91], known to accept preferentially lipophilic and hydrophilic molecules, respectively, indicated that besides the higher lipophilicity of the AcrB binding pocket, the distal binding pocket in AcrD has a higher hydration tendency, and these water molecules could facilitate binding of hydrophilic drugs; a similar effect is seen in *Salmonella* Typhimurium AcrB by G288D substitution[2]. Moreover, simulations also indicated that dynamics of the loops found especially in the proximal pocket, are different in both proteins and may infer induced fit-like binding. The binding of drugs to the pocket of RND transporters shares certain features seen in crystallographic studies of MDR pump transcription regulators, such as QacR [92]. For those regulators, activating ligands bind in a large pocket enriched with aromatic side chains, similar to

what is seen for the RND transporters. The distal binding pocket of the RND transporters is comparatively large, and different ligands prefer different areas of the pocket for binding. Transport substrates with hydrophilic groups tend to bind to the region of the pocket that is enriched with hydrophilic and charged residues but hydrophobic ligands such as cyclohexane or chloramphenicol tend to be excluded in these binding pockets. The general principle that might account for poly-specificity is that hydrophobic drugs do not require small, shape-complementary interaction surfaces in the multidrug transporters or their regulators to form favourable interactions, because the ligands are not strongly stabilised in bulk solvent and do not require extensive desolvation. By contrast, in case of the RND transporters, many of the hydrophobic drugs will most likely reside in the outer leaflet of the inner membrane and enter the periplasmic pore domain via the channels (channels 1 and 3). Compounds with hydrophobic moieties that are likely to partition spontaneously into the bilayer seem to be good substrates of the pump, whereas compounds with hydrophilic side chains are apparently not pumped out efficiently [93]. For the amphipathic substrates, side chain interactions might stabilize first contact to the channel entrance, and the hydrophobic moieties can exert favourable binding energies to small regions of large surfaces, similar to that of the distal binding pocket, so that a broad range of ligands are accepted with only small differences in binding free energies [94].

[H2] Tripartite assemblies involving RND transporters.

In *E. coli*, AcrB forms a tripartite assembly, the AcrAB-TolC tripartite efflux pump (Figure 1). Homologous RND-based assemblies occur throughout the group of Gram-negative bacteria, and the AcrAB-TolC pump shares some similarities with the MacAB-TolC pump. The cryo-EM structures of the AcrAB-TolC assembly in the drug transport state reveal that the TolC channel is open and is likely to remain open when the pump cycles through three distinct conformations. In the context of the complete pump assembly, the AcrB trimer adopts asymmetric LLT, LTT or LTO conformations in the presence of transportable ligands [80]. As described above, ligand binding to AcrB is associated with large conformational changes for both the PN1 and PC2 and the PN2 and PC1 structural modules that form the drug-binding pocket [76], but the interfaces between TolC and AcrA, and between AcrA and AcrB do not change considerably in the different transport states.

In contrast to the drug-bound forms of the pump, in the apo-form TolC is sealed closed, and this probably represents a resting state or an early assembly state. A proposed quaternary structural switch couples and synchronizes initial binding of ligand with channel opening by long-distance communication through allostery [81]. Quaternary structural changes in AcrB associated with drug binding leads to repacking of the helical hairpin domains of AcrA into a configuration that can optimally fit to the open state of TolC. The structural change of AcrA is critical to seal the gaps in the pump, which would otherwise provide a route for substrates to leak into the periplasm. These conformational changes are accompanied by a contraction of the pump along the long axis in its transport state by nearly 10 Å that must entail a local compression of the periplasm, and local curvature of the outer membrane and inner membrane near the portal of AcrB. These changes are likely to influence the energetics and kinetics of the transport process.

[H2] Small multidrug resistance and proteobacterial antimicrobial compound efflux family transporters.

The small multidrug resistance (SMR) family proteins confer resistance against quaternary ammonium compounds and other lipophilic cations in archaea and bacteria. They are indeed small, having only four TMHs, but function as homo- or heterodimers [95] (Figure 1). A representative family member is EmrE, and structural analyses of the *E. coli* protein by X-ray crystallography, cryo-EM and NMR reveal an architecture of protomers arranged with antiparallel orientation to form an asymmetric dimer [96-98] with the two possible configurations occur at roughly equal frequencies [99,100]. Four TMHs from each protomer are arranged pairwise across the dimer interface. The first three TMHs form a substrate-binding chamber, whereas the fourth helix is mainly involved in dimerization interactions. The loop connecting TMH3 and TMH4 forms a β-strand structure, which holds TMH4 close to the substrate-binding chamber [96-98]. During the transport process, the pump alternates between inward- and outward-facing states through conformational exchange of the two protomers. Therefore, the two states are identical except that they have opposite orientation in the membrane [98]. Such global conformational motions are modulated in an allosteric fashion by protonation (of E14 in TMH1), which causes extensive rotation and tilt of TMH1, TMH2 and TMH3 in conjunction with repacking of loops [101,102]. Substrate property affects the transport rate of the pump, and single residue substitution in the drug-binding chamber could change the specificity of SMR pumps [103,104].

Although SMR-like, the *Acinetobacter* chlorhexidine efflux protein (AceI) has recently been identified as a member of a novel family of multidrug efflux pumps termed PACE [9]. AceI and its homologues were shown to contribute to resistance against various synthetic bactericidal agents (chlorhexidine, acriflavine, proflavine and benzalkonium). AceI has a similar size and predicted secondary structure to SMR family members and shares the conserved Glu residue in TMH1 [8]. The lack of PACE protein-encoding genes in the *E. coli* chromosome suggests that these genes were lost early after the divergence from other γ-proteobacteria.

[H1] Drug efflux pump regulation

The association of efflux with drug resistance was first reported nearly four decades ago. To confer effective drug resistance, efflux must match or exceed the rates of influx, and this in turn depends on the composition of the cell envelope and the access to conduits for drug entry such as porins or other routes [4]. Indeed, porin loss is one mechanism that can confer resistance and has been observed to develop during the course of therapy [4]. There have also been numerous reported cases of efflux-mediated resistance in clinical isolates (Table 1), some of which are associated with overexpression of pumps [105].

Understanding the regulation of efflux is important as alterations in the regulatory system, such as mutations in the local and global transcriptional regulators, lead to overexpression of efflux pumps. Efflux regulation can differ between species, and even within a species depending on cellular physiological status. The regulation is complex and highly interconnected, and inhibition

or deletion of one pump can result in expression of others. For example, the deletion of *acrB* results in potentially compensating overproduction of AcrD and AcrF [105]. Thus, efflux pump networks complicate the interpretation of resistant phenotypes.

[H2] Regulation by two component systems.

Bacteria sense and respond to various environmental stimuli, including stress associated with drug action, using two-component systems (TCSs), which contribute to the adaptive responses that confer drug resistance (Figure 5). The systems comprise a sensor histidine kinase and response regulator, and they can be found in many organisms from all domains of life. The histidine kinase and response regulator together function to link signal detection and the generation of the appropriate cellular response (Figure 5a). A few studies reported that the histidine kinase senses the antibiotic directly through binding , such as VanS, which directly interacts with vancomycin [106], but in most cases sensing seems to be indirect. An interesting example is provided by the mechanism of bacitracin resistance in *Bacillus subtilis*: the bacitracin efflux ABC transporter BceAB itself functions as the sensor by communicating its transport activity to the histidine kinase BceS through protein-protein interaction [107,108].

In terms of molecular organization, the membrane-associated sensor histidine kinase functions typically as a homodimer and consists of a sensor module, a transmembrane domain (TMD), and one or several intracellular signal transduction domains (such as HAMP, PAS or GAF), as well as an intracellular catalytic core, which comprises a dimerization and histidine-phosphotransfer (DHp) domain and a catalytic ATP-binding (CA) domain [109,110]. The response regulator protein generally contains a conserved N-terminal receiver (REC) domain and a highly diverse C-terminal effector domain. The response regulator proteins can be divided into five classes according to how the effector domain exerts its response, namely DNA-binding, RNA binding, enzymatically active, protein-binding and single-domain effector [110].
Some two component systems, such as CpxAR in *Enterobacteriaceae*, AdeSR in *Acinetobacter baumannii* and AmgRS in *Pseudomonas aeruginosa* [111] regulate the expression of multidrug resistance efflux pumps. Mutations in *adeSR* were found in numerous clinical isolates of *A. baumannii* [112] and are associated with overexpression of the RND pump AdeAB [112,113]. The response regulator component AdeR activates *adeABC* expression by binding to a direct-repeat motif in the intercistronic region [114], and mutations in AdeR lead to enhanced efflux activity of AdeB [115]. AdeRS not only regulates the expression of the tripartite pump system encoded by *adeABC*, but also genes for biofilm formation and virulence [116]. Interestingly, TCSs can regulate more than one type of pump, such is the case of BaeSR in *A. baumannii*, which is proposed to indirectly regulate AdeAB and AdeIJK RND efflux pumps and also the MacAB ABC pump, and which has been suggested to ~~influence~~ increase resistance to tigecycline. Similarly, in *Salmonella enterica* subsp. *enterica* serovar Typhimurium BaeSR regulates the expression of AcrD and MdtABC RND pumps [117]. In *E. coli*, fifteen TCSs have been identified that contribute to drug resistance, five of which modulate the expression of drug efflux pump genes. The *E. coli* BaeSR and CpxAR systems activate the expression of *mdtABC* and *acrD* in response to indole and envelope stress, respectively. The two-component EvgAS system controls the expression of *mdtEF, emrKY*, *acrAB*, *tolC* and *mdfA*, but the triggering stimuli are currently unknown [4,118]. Expression of the TolC outer membrane channel is also affected by another two-component system, PhoPQ.

Structural and functional data have advanced the mechanistic understanding of TCS systems. The crystal structures of *E. coli* nitrate and/or nitrite sensor histidine kinase NarQ provide a direct demonstration of signal transduction through the TMD and HAMP domains [109]. Ligand binding to the sensor domain causes piston-like shifts of TMHs, which is accompanied by extensive rearrangements. The HAMP domain protomers undergo lever-like motions and convert the piston-like movements of the TMHs into helical rotations. It is presently unclear how this conformational change transduces downstream of the HAMP domain to activate the catalytic core. The structure of HK853-RR468 complex from *Thermotoga maritima* reveals the communication between the histidine kinase and response regulator, during which a phosphoryl group is transferred from phosphorylated H260 in the HK853 catalytic core to D53 of the RR468, and thereby activates RR468 [119] (Figure 5a). The signal-activated HK853 auto-phosphorylates the conserved H260 in the DHp domain. The RR468 dimer docks onto the DHp domain and catalyzes its own phosphorylation at D53, using the phosphorylated histidine as a substrate. For the DNA-binding response regulator subfamilies, the activated response regulator can form a homodimer that binds to a specific DNA-binding motif and activates the transcription of target drug transporter gene (Figure 5b).

[H2] Regulation by transcription and post-transcription factors.

Clinical isolates of drug-resistant bacteria have been found to contain genomic mutations in transcriptional repressors and in the promoters of the genes encoding RND and ABC transporters. Mutations in genes encoding local regulators, such as the TetR family of transcriptional [repressors (for example, *emrR*, *acrR*, *mtrR*) [120-123], or overexpression of global transcriptional regulators, including AraC/XylS transcription factors (for example, MarA, SoxS, RamA), results in overexpression of efflux pumps and increased transport activity. Although rare, 4% of *Neisseria gonorrhoeae* isolates carry mutations in the *mtrA* global regulator gene, resulting in lack of overexpression of the MtrCDE RND efflux pump [124].

The AcrB RND transporter is encoded in the *acrRAB* operon, and AcrR functions as the local repressor of the operon (Figure 5C). Mutations in *acrR* lead to overexpression of AcrAB and have been described in clinical and veterinary isolates resistant to the AcrB substrate fluoroquinolone [4]. AcrR is one of many regulators of the operon, examples of other regulators are Mar, RamA, Sox and Rob (see below). AcrB overproduction in *Klebsiella* sp. and *Salmonella* sp. is caused mainly by mutations in *ramR* and in one case in *soxR* [4]. In the same way in which the expression of one pump is controlled by multiple regulators, it has also been shown that one regulator can control the expression of multiple pumps. For example, MarA in *E. coli* regulates the expression of AcrAB-TolC and is also involved in regulation of the ABC transporter encoded by the *mlaFEDCB* operon [125]. The transcription of *acrAB* and *tolC* genes encoding the tripartite assembly is controlled by many regulators, including MarA, RamA, SoxS and Rob. These are transcription factors that bind to the marbox, a degenerate sequence of approximately 20 base-pairs found in multiple promoter sequences. These factors can activate the transcription of multiple genes, including those encoding efflux pumps and porins. Together, the different regulatory mechanisms can give rise to multi-drug resistance by decreasing influx through suppression of the expression of the OmpF porin, while increasing efflux mediated by AcrAB-TolC.

The ArmZ-MexZ system functionally links the activation of the pump with ribosome activity, and at least two signal transduction regulatory systems interconnect MexXY-OprM or MexXY-OprA with other cellular processes. Transcriptional attenuation is another mechanism that is involved in regulating some antibiotic efflux pumps [4].

Post-transcriptional processes are involved in fine-tuning regulation of gene expression and are likely to be involved in controlling antibiotic susceptibility and efflux pump expression [126,127]. The recent discovery of antibiotic-responsive riboswitches from studying changes in RNA levels in bacterial communities has highlighted key regulatory role of RNA in the emergence of resistance [128]. Expression of efflux pumps can be regulated by attenuation through *cis*-encoded RNA elements as primary regulatory mechanism. In these cases, the antibiotics target the ribosome and cause stalling when translating a short leader peptide upstream of the resistance gene. As a result, RNA secondary structure is changed and the resistance gene can be translated or transcribed (depending whether translation or transcription termination is regulated by attenuation) [128].

Trans-acting small regulatory RNAs (sRNA) (Figure 5d) are also likely to have important roles in efflux regulation, as suggested by the phenotype of strains that are defective for expressing the Hfq protein that is a chaperone for sRNAs. Such mutants are not only attenuated in virulence but also more susceptible to many antibiotics [123]. There are at least three known mechanisms of efflux pump regulation by sRNA. In one such case, sRNA can repress translation by blocking the ribosome-binding site (RBS) through binding to the latter. sRNA can also promote mRNA degradation through its specific binding to a cognate site in the transcript and via recruitment of ribonucleases. In addition to its repressor function, sRNsA are able to promote translation (activator function) by preventing the formation of inhibitory secondary structure in the target mRNA **[Au: can you please add a brief description of the regulatory mechanism shown in the figure: blocking the ribosome binding site; promoting mRNA degradation through specific binding to a cognate site in the transcript; promotion of translation in an activator function through prevention of inhibitory secondary structure. OUR REPLY: THE NEW TEXT IS HIGHLIGHTED IN YELLOW ABOVE]** Some notable examples are the sRNA SdsR, which binds and represses the *tolC* mRNA in *E. coli* ~~and *Salmonella*~~ ~~**[Au: a specific species?]**~~ [129] and DsrA which regulates expression of MdtEF, albeit indirectly [130]. In *Neisseria gonorrhoeae*, **[Au: a specific species? – yes, added]** expression of MtrF (a component of MtrCDE) is regulated by sRNA NrrF [131]. sRNAs can contribute to 'rewiring' TCSs and linking these with other processes, and can thereby affect the expression of efflux pumps [132]. These regulatory RNAs can also regulate numerous processes related to virulence [129,130]. In principle, compounds that target such riboregulators could affect antibiotic resistance and could be useful leads for drug development.

[H2] Regulation of AcrB by a small peptide.

Small proteins have been discovered in various organisms that can have important biological roles. Often overlooked in genome studies, these small proteins affect spore formation and cell division, regulate transport, membrane-bound enzymes, protein kinases and signal transduction,

and function as chaperones, among other functions [133]. There are approximately 60 confirmed small proteins in *E. coli* [134]. These proteins are often expressed under specific conditions, and many are thought to modify the activity of large membrane proteins [134].

E. coli AcrZ is a 49 amino acid small protein that interacts with and regulates AcrB [134]. Deletion of *acrZ* results in sensitivity to some but not all of the antibiotics for which AcrB provides resistance [134]. This indicates that AcrZ has a role in substrate recognition or AcrB regulation. Indeed, AcrB and AcrZ are co-regulated by the transcription factors MarA, Rob and SoxS, supporting a key functional role for the small protein [134]. Structural studies of the AcrBZ complex by X-ray crystallography and in the context of the full pump assembly (by cryoEM) indicate that AcrZ forms a helix that interacts with AcrB in the transmembrane portion [80,135]. Perhaps AcrZ and other related peptides might exercise allosteric effects on their RND partners to modulate efflux activities.

***[H1]* Efflux-mediated resistance to antibiotics.**

Whole-genome sequencing case studies suggest that adaptive evolution within the host has an important role in bacterial infections [136]. Sub-lethal doses of antibiotics can trigger numerous processes that enable bacteria to survive antibiotic exposure. Epigenetic effects have been proposed to play a part in upregulating efflux transporters. The observed phenomenon of persistence, whereby a subpopulation of bacteria that may be metabolically quiescent survives initial exposure to antibiotic even though they may be genetically identical compared to the drug-susceptible group, may complicate strategies for treatment [137,138]. It is interesting to note that in *E. coli*, persister cells have been proposed to have enhanced efflux activity and lower intracellular antibiotic concentrations [12]. The persistent subpopulation might be greater in a biofilm [139]. Efflux pumps also have a role in the establishment of hetero-resistance, which is characterized by subpopulations of bacteria that have a resistant phenotype, conferring clinical levels of resistance to the isolate.

In many cases, MDR clinical isolates of bacteria exhibit multiple mechanisms of resistance [4]. For example, fluoroquinolone-resistant MDR *E. coli* were found to have increased efflux, decreased permeability and a mutation that decreases gyrase sensitivity to the drug (GyrA S83L) [140]. Similar findings were made for fluoroquinolone-resistant *S. enterica* serovars [141] and strains of *M. tuberculosis* [142] resistant to quinolones and other drugs. For example, mutations in *inhA* or *katG* confer resistance to isoniazid in *M. tuberculosis* [142].

Overexpression of efflux pumps is one mechanism that can contribute to multidrug resistance. Redirecting protein synthesis to make efflux pumps is demanding metabolically, so perhaps it not surprising that a metabolic cost can be associated with drug resistance [143]. Changes in various metabolic processes have been observed with overexpression of RND-based pumps in mutants of *N. gonorrhoeae* MtrCDE [144], MexAB and *P. aeruginosa* MexXY-OprM [145], MexEF-OprN and MexCD-OprJ [146]. Overexpression of the AcrB RND pump, in response to high concentration of chloramphenicol, drives central carbon metabolism from an oxidative state to a fermentative one [143] and may compensate the stress induced by overexpression [145]. In this regard, it is important to note that efflux pump gene-deletion mutants may not be the best model to study the physiological role of pumps, as there are notable transcriptome differences between a *S.*

Typhimurium Δ*acrB* and an AcrB D408A mutant [147]. The AcrB D408A mutant expresses the inactive protein that lacks efflux function (and was found to be attenuated in multiple infection models). Importantly there was no increased expression of homologous RND efflux pumps (in comparison to the Δ*acrB* mutant strain). This suggests that the compensatory role of these proteins is fulfilling a structural requirement rather than an efflux function.

Although overexpression of pumps might confer drug tolerance, too much overexpression can in some cases be detrimental to the fitness and virulence of the bacterium, as seen for instance in a *S.* Typhimurium mutant overexpressing AcrAB [148]. Similarly, a *P. aeruginosa* mutant overexpressing MexEF was shown to be less virulent in a *Caernorhabditis elegans* infection model. However, there is no simple rule relating expression and fitness, as shown by the finding that a *Klebsiella pneumoniae* mutant overexpressing AcrAB, via deletion of the transcriptional regulator *ramR*, had increased virulence in a mouse infection model [149]. Furthermore, overexpression of the MtrCDE RND-based efflux pump in *N. gonorrhoeae* led to increased fitness in a mouse model [144].

Mutations in pumps is a potential mechanism to alter activity, and mutated binding pockets in AcrB have been observed to confer clinically relevant MDR that evolved in a patient during treatment of an infection by *S. Typhimurium*. A G288D substitution changed the substrate specificity of the pump, conferring ciprofloxacin resistance [2]. In drug-resistant clinical isolates of *Campylobacter jejuni*, mutations in the drug-binding pocket of the RND transporter CmeB have been identified [150], which map to the corresponding drug binding pocket of AcrB [68,74].

[H1] Conclusions and perspectives

Structural studies of transporters and their assemblies have provided insights into the molecular origins of MDR. The mechanisms of the transporters – how they transduce energy to drive translocation of substrates – have advanced for the main classes of these proteins. In Gram-negative species, the transporters function as a coordinated network, in which the final step of efflux is achieved by a tripartite assembly. Similar systems are likely to contribute to resistance and survival in Mycobacterial pathogens. These advances are complemented by improved understanding of the numerous biological roles of pumps beyond simple transport, such as the contribution of bacterial efflux pumps towards virulence and community behavior. Future efforts to explore the dynamics of pump assembly and the interplay between transporters and transporter components, including the regulation of expression of the different components controlled by specific regulators, and the identification of antimicrobial targets. Targeting the protein-to-protein interfaces of the tripartite assemblies might be an efficacious means to counter drug tolerance, hence the dynamic process of efflux pump assembly will be an important field to study in future (supplementary box 1). Regulators that control the stress response, including RNA-mediated regulatory networks, may also represent targets to address in future for therapeutic intervention.

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On reflection, the detailed analysis of efflux systems, from the level of stereochemistry to the intricate interconnections with physiology and host interactions, illustrates the immense complexity of the hidden layers of those biological systems.

Acknowledgements

The authors thank S. Murakami, B. Görke, J. Blaza, M. Welch, A. Vargiu, P. Ruggerone and M. Osman for helpful discussions and the reviewers for helpful comments. B.F.L. and D.D. are supported by the Wellcome Trust and the ERC (742210). K.M.P. is supported by the German Research Foundation (DFG-SFB 807, Transport and Communication across Biological Membranes, and DFG-FOR2251, Adaptation and persistence of the emerging pathogen *Acinetobacter baumannii*), the DFG-EXC115 (Cluster of Excellence Frankfurt—Macromolecular Complexes), the Innovative Medicines Joint Undertaking (IMI-Translocation) under Grant Agreement no.115525, and the National Institute of Allergy and Infectious Diseases (Grant R44 AI100332). H.W.v.V., K.M.P. and B.F.L. are supported by a grant from the HFSP (RGP0034/2013). A.N. is recipient of a Herchel Smith Scholarship. X.W.K. is supported by Consejo Nacional de Ciencia y Tecnología (CONACyT), Mexico. . L.J.V.P. is supported by the BBSRC (Grant BB/N014200/1) and the MRC (MR/022596/1).

Further information
Transporter classification database: http://www.tcdb.org

Contributions
D.D., X. W.-K., A.N., H.W.v.V., K.M.P., L.P. and B.F.L. researched data for the article and wrote the article. D.D., X.W.-K., A.N., H.W.v.V., K.M.P., L.P. and B.F.L. made substantial contributions to discussions of the content and reviewed and edited the manuscript before submission.

Competing interests
There is NO Competing Interest.

Publisher's note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Reviewer information
Nature Reviews Microbiology thanks K. Beis, H. Schweizer and the other anonymous reviewer(s) for their contribution to the peer review of this work.

Table of context
One factor contributing to the emergence of antimicrobial resistance is the capacity of bacteria to rapidly export drugs through the intrinsic activity of efflux pumps. This Review-describes recent insights into the structure, function and regulation of efflux pumps are described.

TEXT BOX 1

Efflux pumps have diverse biological roles

Molecular genetic studies support the concept that efflux pumps provide an ancient mechanism to meet the challenge of surviving in environments laden with noxious compounds. For example, *Rhizobium etli*, a nitrogen-fixating bacterium that forms nodules in roots of *Leguminosae*, uses the RmrAB MFS-based pump to withstand the toxic effects of plant flavonoids [151]. Likewise, AcrAB, the paradigm RND class pump of the Gram-negative *Enterobacteriaceae* confers resistance to bile salts and so contributes to survival in the mammalian gut [152]. Some species, such as *Pseudomonas* sp., have up to a dozen RND pumps, suggesting that it is beneficial for the bacterium to have a repertoire of these pumps [153].

Efflux pumps are required to colonize and disseminate during host infection by many pathogenic bacteria and can help bacteria extrude innate host defenses. This is particularly clear in *Neisseria*, which uses RND efflux pumps to extrude host antimicrobial fatty acids and antimicrobial peptides. The role of RND pumps in *Neisseria sp., Vibrio* sp. and *Klebsiella pneumoniae* in export of antimicrobial peptides has been established. For *Neisseria gonorrhoeae*, the MFS efflux pump FarAB is implicated in facilitating rectal infection in mice by ridding the bacteria of anti-gonococcal long-chain fatty acids such as linoleic and palmitic acids [154]. *Salmonella enterica* mutants of the tripartite assembly AcrAB-TolC have diminished virulence in mice and poultry [147,155,156], and deletion of *macAB,* encoding components of an ABC-type tripartite pump, also results in loss of virulence for *S.* Typhimurium in mice [157].

In some species, efflux pumps can also actively contribute to infection processes, for example in the export of virulence factors, such as siderophores used for the acquisition of iron and other required metals. The catechol siderophore vibriobactin is exported by the *Vibrio cholerae* RND pump VexGH [158]. Enterobactin, the strongest binding siderophore known, is exported by the *E. coli* MFS transporter EntS, with 'back-up' transport function provided by AcrB and other RND pumps [159]. Group A and B Streptococci internalize heme for chelation of iron [160], but the heme itself may then be exported [160]. Efflux of heme and protoporphyrins also occur in *N. gonorrohoeae* [161] and *E. coli* [162]. The MATE family member ClbM transports the precursor of the toxin colibactin from the cytoplasm to the periplasmic space in *E. coli* [54]. Efflux pump activity may in some cases be required for host invasion. Subpopulations of *Mycobacterium* spp. infecting human macrophages showed overexpressed efflux pumps and as a consequence increased tolerance to a range of antibiotics [163]. Although not identified, the efflux substrate might be a host defensive molecule.

In some bacterial species, efflux pumps can export signaling molecules involved in quorum sensing, which is a mechanism of cell-to-cell communication that responds to bacterial population density. Quorum sensing controls gene expression at a population level and thereby affects colony behavior including virulence and biofilm formation. In the opportunistic pathogen *P. aeruginosa*, the expression of roughly one-tenth of the genome is controlled through quorum sensing [164]. The sensing signaling molecules acyl-homoserine lactones are exported by the RND efflux pump MexAB-OprM [165], and the efflux systems MexGHI and MexEF-OprN export a precursor of the *Pseudomonas* quinolone signaling system [166]. Similarly, quorum sensing inhibitors based on non-native N-acylated l-homoserine lactones and derivatives are known to be exported by MexAB-OprM [167].

The MexGHI system also exports signaling molecules for biofilm formation (namely, phenazine precursors) [168]. Inactivation of RND efflux pumps in *P. aeruginosa* leads to attenuation of virulence, presumably due to lack of signaling molecules. Conversely, efflux pump overexpression can also lead to an attenuated phenotype because the signaling molecules cannot achieve the intracellular concentration threshold required to activate the quorum response as well as the potential consequences for cellular energy use [146].
The AcrB pump has a role in the phenomenon of contact-dependent growth inhibition in *E. coli* that is characterized by the secretion of the toxin CdiA and growth arrest upon inter-cellular contact [169,170]. Deletion of *acrB* confers resistance to CdiA [169], and while it is unclear how AcrB contributes to this phenomenon, the transporter is likely to have some role in CdiA entry.

FIGURE 1. **Schematic of representative structures of multidrug transporters and tripartite assemblies**. The envelope of Gram-negative bacteria has three principal layers: the outer membrane, the inner membrane and the peptidoglycan cell wall in the interstitial periplasm between the two membranes. In Gram-negative bacteria, all the drug transporters locate in the inner membrane. Structures of representatives of each of the transporter families is presented: ABC, MFS, MATE, SMR and RND. The ABC transporters use ATP to drive transport processes, and the other families depicted use electrochemical gradients for energy source. The groups differ in architectural features, as described in more detail in the main text. The ligands for the MFS, MATE and SMR representatives are indicated. The RND family drug transporters mostly assemble with their partner proteins to form tripartite pumps, and these bind substrates at the outer leaflet of the inner membrane and periplasm, and efflux them to the cell exterior. By contrast, members of the other families of drug transporters usually function as independent units in the inner membrane to translocate substrates across the membrane bilayer. It is likely that these transporter systems cooperate with RND-type tripartite efflux pumps to deliver substrates across the entire cell envelope as part of a larger drug-efflux super-organization. The structure of the PACE class of transporters have not yet been experimentally elucidated and are therefore not included here. Some ABC family and 14-TMH MFS family transporters could also form tripartite pumps and directly transport substrate from the inner leaflet of inner membrane and cytoplasm to the exterior. AcrAB-TolC and MacAB-TolC are RND-based and ABC-based tripartite multidrug efflux pumps, respectively.

FIGURE 2. **Transport mechanisms of efflux pumps**.

Figure 2A. Cartoon schematics of the transport mechanism for ABC transporters. Shown is the alternating access model with clothes-peg-like movement of protomers, which alternate between an inward-facing and outward-facing conformation to translocate substrates across the membrane bilayer. The transmembrane domains of ABC transporters comprise the substrate-binding pocket and nucleotide-binding domains (NBDs), which bind and hydrolyse ATP to drive the transport cycle. The conformational changes are linked to the dimerization and dissociation of the NBDs, which is mediated by ATP binding and hydrolysis.

Figure 2B. A rocker-switch model for the galactoside and H+ symport, exchange and counterflow reaction of the MFS group. During the symport cycle, the lactose permease symporter LacY binds lactose with high affinity as a result of its protonation (see step 1 for influx reaction; step 6 for efflux reaction). The binding of galactoside (see steps 2 or 5) triggers a conformational switch from an outward-open (influx) or inward-open (efflux) state to an occluded conformation of the dimer (see steps 3 or 4). This is followed by opening up of the dimer towards the respective opposite side inward-open (influx) or outward-open (efflux), the release of the substrate and deprotonation of LacY (1 or 6). At this state, LacY adapts an apo-occluded conformation (see steps 7 or 8). In case of an exchange or counterflow cycle, LacY only undergoes steps 2-5 (yellow area). An analogous process may occur for the drug-exporting group of the MFS family. Figure 2C. Cartoon schematics of the transport mechanism of MATE transporters. The amino-terminal and carboxy-terminal domains of MATE transporters form a V-shaped central cavity. The structures apparently adopt an outward-open state with the lower portion of the central cavity situated about halfway through the membrane bilayer. In the *Neisseria gonorrhoeae* NorM, a drug-binding site has been identified in a central cavity situated near the membrane-periplasm interface (top panel). The substrate and cation (Na^+) interact with distinct subsets of amino acids (the three key residues that are involved in Na^+-coordination (corresponding to *N. gonorrhoeae* NorM Y294, E261 and D377) are indicated), and can bind to the protein simultaneously. Therefore, the coupling between the fluxes of Na^+ and drug is likely to be indirect and mediated by protein conformational changes. The location of the transport substrates rhodamine 6G and ethidium bromide are indicated.

Substrates bind more deeply within the central cavity of the DinF subfamily member DinF-BH, where the interactions are mostly mediated by hydrophobic amino acids. For another DinF subfamily member, PfMATE, the drug-binding site is formed exclusively within the N domain and is dominated by polar amino acids. The substrate and cation (proton) interact with the same amino acids, and compete each other to bind. The coupling between the fluxes of H^+ and drug is likely direct (not indicated) The location of transport substrates verapamil and Br-NRF are indicated.

Figure 3: **Transport mechanism and drug pathways for RND transporters**.

Figure 3A. Cartoon schematics of the transport mechanism for RND transporters. The representative RND transporter AcrB cycles through three non-equivalent structure states with high cooperativity, as depicted in the top panel. The model holds that drugs gain entrance to the binding pocket in the periplasmic domain (indicated by dashed lines) in the access (or L) state.

These can move further into the drug binding pocket in the binding (or T) state, and then are extruded into a funnel-shaped 'canyon' in the docking domain in the extrusion (or O) state. The red lines depict the opening and closing of the entrances to the pockets on the exterior face and canyon face. The switch between states is associated with changes in protonation of side chains in the transmembrane domain, as depicted in the bottom panel.

Figure 3B. Drug pathways in AcrB. Drug-binding pockets have been identified in AcrB from X-ray crystallography and cryoEM studies. The different drug pathways are depicted. The periplasmic entry pathway includes the proximal pocket in the L protomer and further transport towards the distal binding pocket. Another pathway proposed involves channel 2, defined by the PC1 and PC2 subdomains in the pore domain and directly guides substrates from the periplasm to the proximal pocket. Drugs with higher molecular mass, such as rifampicin and erythromycin, favour an access pocket in the L monomer, whereas smaller drugs, such as minocycline and doxorubicin, tend to locate to a deep binding pocket within the T monomer. Pump inhibitors, such as ABI-PP and MBX3132 (not shown), ~~were shown to~~ penetrate even more deeply into the T monomer and insert into a hydrophobic chamber that branches from the deep binding pocket. The pockets have multiple sites of interactions and can contact different drugs in very different ways, as seen in the overlay of the minocycline and doxorubicin complexes (not shown). In an entirely distinct mode of interaction, fusidic acid and other lipophilic carboxylated drug locate to a groove at the interface between transmembrane helix 1 (TMH1) and TMH2. The poly-specificity of this transporter may be underpinned by the multiplicity of interactions within the pocket and dynamic hydration patterns during the transport process.

Figure 4 **Drug and inhibitor-binding sites of RND, MFS, and MATE transporters**.

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a) Minocycline bound to the periplasmic distal binding pocket of the RND transporter AcrB from *E. coli* (PDB: 4DX5)
b) MBX-3132 inhibitor bound to the hydrophobic trap region of AcrB (PDB: 5ENQ)
c) Chloramphenicol bound to the binding area of the MFS transporter MdfA from *E. coli* (PDB: 4ZOW)
d) Rhodamine 6G bound to the binding pocket of the MATE transporter NorM from *Neisseria gonorrhoeae* (PDB: 4HUN)
The protein main chain is shown in cartoon representation (gray color), drugs or inhibitor are shown as sticks and side chains involved in their binding. Color code: side chain C-atoms: marine blue, drug C-atoms: slate blue, inhibitor MBX3132 C-atoms: orange. O-atoms: red, N-atoms: dark blue, S-atom: yellow, Cl-atoms: green.

FIGURE 5. **Regulation of efflux pumps**.
(A,B) Cartoon schematics of the regulation by two component systems. (A) Drug binding induces a conformational change in the periplasmic sensor domain. This conformational change is transduced downstream to activate the catalytic core, which autophosphorylates at a conserved Histidine residue in the dimerization and histidine-phosphotransfer (DHp) domain. The phosphoryl group is then transferred from the phosphorylated Histidine residue to an aspartic acid residue of the response regulator to activate it. (B) For the DNA-binding response

regulator subfamilies, the activated response regulator generally forms a homodimer, which binds to a specific DNA-binding motifs and activates the transcription of target drug transporter genes.

Figure 5C. Complex transcriptional regulatory network of efflux pumps. Expression of *acrEF* and *acrAB* is repressed by AcrS. *acrAB* is also repressed by the local repressor AcrR. The global regulators, Rob, SoxS, and MarA, activate the expression of *acrAB*, *tolC*, and *micF*. Other regulators include the histone-like nucleoid structuring protein (H-NS) (repressor) and the SdiA global regulator (activator). *acrAB* and *tolC* expression is also controlled by the two-component regulatory systems EvgAS and/or PhoQP. The *micF* transcript inhibits the translation of OmpF mRNA. *ompF* transcription is also regulated by several phosphorylated OmpR proteins, which bind in hierarchical order to discrete positions on the DNA according to the galloping model [171]. SoxS and Rob can also stimulate MarA expression. Regulator inactivation can be achieved by ligand binding (for example, antimicrobial agents and metabolites) or oxidative stress induction (see MarR, SoxR, AcrR). Some regulators can also be activated upon ligand-binding (for example, Rob is activated through binding of bile salts or fatty acids). Expression of *acrZ* is co-regulated with the expression of *acrAB* via MarA, SoxS and Rob [134]. (Figure adapted after [4] and [171]). Red lines symbolise transcription repression by the repressors AcrR, AcrS, H-NS, MarR, and SoxR. Green lines indicate activation of relevant gene expression by the activators MarA, SoxS, SdiA, Rob, EvgS, and PhoP.

Figure 5D. Regulation of efflux pumps by small regulatory RNAs . Small regulatory RNAs (sRNAs) regulate antibiotic resistance genes in numerous ways. Binding of sRNAs to the ribosome-binding site can repress translation. sRNAs can also induce mRNA degradation by binding to a specific cognate site in the transcript and recruit ribonuclease. In addition to repressor functions, sRNAs can promote translation by preventing the formation of inhibitory secondary structures. Regulatory RNA can also occur in *cis*-acting elements [128]. Antibiotic trigged RNA mediated regulation is common in bacteria of human commensal species and pathogens, and includes most classes of resistance genes including several predicted classes of multidrug antibiotics efflux pumps and exporters [172].

Table 1. Examples of clinically relevant MDR efflux pumps.

Microorganism	MDR efflux pump family	Efflux system	Drug resistance phenotype	References

Acinetobacter baumannii	RND	AdeABC	Aminoglycosides Cefotaxime Chloramphenicol Erythromycin Fluoroquinolones Tetracyclines Tigecycline Trimethoprim	[173]
	SMR	AbeS	Chloramphenicol Ciprofloxacin Erythromycin	[174]
Burkholderia sp.	RND	BpeEF-OprC	Trimethoprim	[175]
Enterobacteriaceae	RND	AcrAB-TolC	β-lactams Chloramphenicol Erythromycin Fluoroquinolones Novobiocin Tetracycline Linezolid	[176]

		OqxAB*-TolC	Cetrimide Chloramphenicol Fluoroquinolones Quinolones Nitrofurantoin	[177]
	MFS	EmrAB-TolC	Novobiocin Fluoroquinolones	[155]
	ABC	MacAB-TolC	Macrolides	[162]
Mycobacterium tuberculosis	MFS	EfpA	Isoniazid Fluoroquinolones Rifampicin Tetracycline Clofazimine	[142,178]
		Rv1258c	Quinolones	[142]
	RND	MmpS5-MmpL5	Isoniazid Diarylquinolones Rifampicin	[142,179]
	SMR	Mmr	Ofloxacin Rifampicin	[142,180]
Pseudomonas aeruginosa	RND	MexAB-OprM	Aminoglycosides Amphenicols β-lactams (except imipenem) Fluoroquinolones Macrolides Novobiocin Sulfonamides Tetracyclines Thiolactomycin Tigecycline Trimethoprim	Reviewed in [181]

		MexCD-OprJ	Chloramphenicol Fluoroquinolones Macrolides Zwitterionic cephalosporins Tetracyclines Trimethoprim	
		MexEF-OprN	Chloramphenicol Fluoroquinolones Tetracycline Trimethoprim	
		MexXY-OprM	Aminoglycosides Fluoroquinolones Macrolides Tetracyclines Tigecycline Zwitterionic cephalosporins	
	SMR	EmrE	Aminoglycosides	[182]
Neisseria gonorrhoeae	RND	MtrCDE	Penicillin (β-lactams) Azithromycin (macrolides) Ceftriaxone	Reviewed in [181,183]
		MtrF	Sulphonamides	

	MATE	NorM	Hydrophilic fluoroquinolones	
Staphylococcus aureus	MFS	NorA	Hydrophilic fluoroquinolones (e.g. norfloxacin, ciprofloxacin)	Reviewed in [184]
		QacAB*	Biocides and antiseptics, e.g. quaternary ammonium benzalkonium salts, chlorhexidine, etc.	
	ABC	MsrA	Macrolides Streptogramins	
Streptococcus pneumoniae	MFS	PmrA	Fluoroquinolones	Reviewed in [184]
	ABC	PatAB	Fluoroquinolones	
	MATE	PdrM	Chloramphenicol Erythromycin	

(*) denotes pumps that are encoded in plasmids, except for *Klebsiella pneumoniae*. QacAB have only been described in *S. aureus*.

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Comment [Editor4]: References should be cited sequentially in the following order: main text, boxes, figures and then tables. Currently, the references in the table are out of order, and cited before the boxes

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