



Structure, mechanism and cooperation of bacterial multidrug transporters

Dijun Du¹, Hendrik W van Veen², Satoshi Murakami³,
Klaas M Pos⁴ and Ben F Luisi¹

Cells from all domains of life encode energy-dependent transmembrane transporters that can expel harmful substances including clinically applied therapeutic agents. As a collective body, these transporters perform as a super-system that confers tolerance to an enormous range of harmful compounds and consequently aid survival in hazardous environments. In the Gram-negative bacteria, some of these transporters serve as energy-transducing components of tripartite assemblies that actively efflux drugs and other harmful compounds, as well as deliver virulence agents across the entire cell envelope. We draw together recent structural and functional data to present the current models for the transport mechanisms for the main classes of multi-drug transporters and their higher-order assemblies.

Addresses

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

² Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1PD, UK

³ Division of Structure and Function of Biomolecules, Department of Life Science, Tokyo Institute of Technology, Yokohama 226-8503, Japan

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

Corresponding author: Luisi, Ben F (bfl20@cam.ac.uk)

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Introduction

What biological purpose do multidrug transporters have?

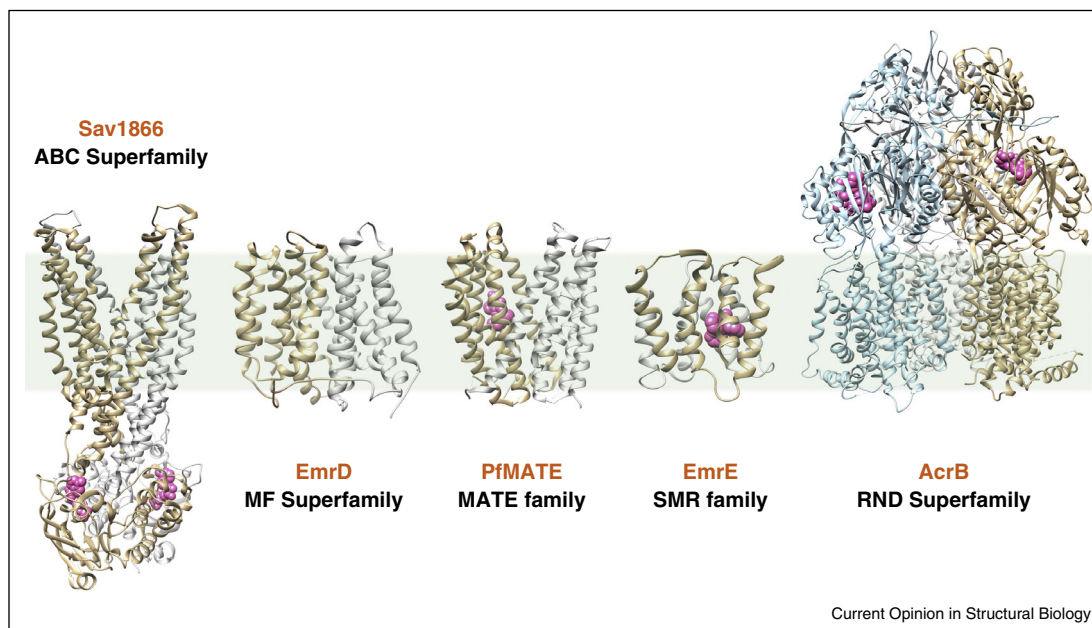
Bacteria encode numerous multidrug transporters, and while some are expressed constitutively, synthesis of others is triggered by environmental factors, including man-made drugs [1,2]. In the Gram-negative bacteria, some of the transporters form large multi-protein ‘pump’ assemblies (to which we will return below), and reflecting upon their considerable size, it seems that the production of such molecular machines requires substantial investment of energy and material. The

question naturally arises why such assemblies should be expressed constitutively, or triggered by artificial compounds such as drugs that might never have been encountered in the wild. Perhaps the answer might lie in part with the similarity of artificial drugs with abundant, naturally occurring compounds for which the transporters do have intrinsic preference. For example, it was shown that *Bacillus subtilis* Blt multidrug transporter, which can expel cationic amphiphiles, has a defined function in the efflux of the natural polyamine spermidine [3]. Some transporters are expressed to protect against nitrosative damage during anaerobic respiration [4], or to efflux free fatty acids [5] or gastrointestinal bile salts [6]. There is growing appreciation that drug transporters are actually required for growth and survival of pathogenic bacteria in their intracellular hosts. In *Mycobacterium tuberculosis*, the causative agent of clinical TB, efflux transporter genes are induced during infection inside macrophages [7,8], where they are likely to be involved into the efflux of host protective molecules. Recent studies have identified a mycobacterial transporter, Rv1258c, to be responsible for macrophage-induced tolerance of the pathogen towards the antibiotic rifampicin and for mycobacterial virulence [7–9]. In addition to supporting survival in the presence of toxic compounds, some of the transporters have been found to export virulence factors and adhesion factors that enable bacterial colonies to aggregate into protective ‘biofilm’ communities [10]. Thus, microbial multidrug transporters can have specific physiological functions, some of which might only be revealed when the organism is in its natural habitat, rather than the nutrient-rich media used in the laboratory [11]. Through their capacity to expel diverse toxic compounds, efflux transporters contribute to environmental fitness, and their ancient functions have likely aided the capacity of microorganisms to survive in competitive and hazardous niches. Understanding how such transporters work and how they might be inhibited may be invaluable for treatment of many threatening bacterial infections.

The main transporter families

Five families of trans-membrane transporters have been well characterized thus far that are involved in active efflux of antimicrobial agents. The family members are widely distributed throughout all domains of life, highlighting their biological importance (Figure 1) [12]. One such family comprises the ATP-binding cassette (ABC) proteins, which are ‘primary-active’ in that they

Figure 1



Schematic of structures for five representatives of multidrug transporters. The molecules are aligned so that the cytoplasmic facing side is on the bottom. The protomers (Sav1866 [13^{*}] and EmrE [14^{**}]) or symmetry related domains (EmrD [16] and PfMATE [67^{**}]) are colored light brown and light grey; the protomers of AcrB trimer [100] are colored light blue, light brown and light grey. The substrates (with the exception of the EmrD apo-structure) are shown as pink sphere. PDB codes: PfMATE: 3VVP; EmrD: 2GFP; EmrE: 3B5D; Sav1866: 2HYD; AcrB: 3AOD.

use the free energy of ATP binding and/or hydrolysis to catalyze drug extrusion [13^{*}]. The ‘secondary-active’ multidrug transporters are antiporters that couple drug efflux to the influx of protons or sodium ions down electrochemical gradients; these define four distinct groups, namely the major facilitator superfamily (MFS), small multidrug resistance (SMR), resistance/nodulation/cell division (RND), and multidrug and toxic compounds extrusion (MATE) families (Table 1) [14^{**},15,16,17^{**},18^{**}]. Whether energized by electrochemical gradients or by the binding and hydrolysis of nucleoside triphosphates, the transporters bear trans-membrane domains that switch conformations as they cycle through different substrate transport states, as we will describe below.

ABC transporters

ABC transporters share a similar overall architecture, consisting of two core units, namely, an intracellular nucleotide-binding domain (NBD) dimer, which binds and hydrolyses ATP at the dimer interface, and a cognate membrane-domain (MD) dimer, which is embedded in the plasma membrane and acts as a trans-membrane pathway for substrates [19,20]. ABC transporters are organized as either, homodimers or heterodimers. In *Salmonella* and the NBD-MD subunit(s) may be encoded separately or as a single polypeptide. The domains may be arranged with an N-terminal MD and C-terminal NBD, or vice versa [21,22]. Despite these variations, the family members likely share a common transport mechanism.

Crystal structures of the multidrug ABC exporter Sav1866 have revealed a dimer with the two closely associated NBDs sandwiching the nucleotide (‘closed’), and the two MD ‘wings’ facing towards the cell exterior [13^{*}]. Accordingly, this conformation was termed ‘outward-facing’. Nucleotide-bound crystal structures for MsbA from *Salmonella typhimurium* have been reported in a similar, outward-facing conformation [23]. Moreover, MsbA from *Escherichia coli* was crystallized in an inward-facing ‘open’ (open-apo) conformation in the absence of nucleotides, where the NBDs were distant and the presumed substrate-binding chamber in the MDs was facing the cell interior [23]. The nucleotide-free structures of murine and *Caenorhabditis elegans* multidrug resistance P-glycoprotein ABCB1 were obtained in a similar inward-facing conformation [24,25]. The crystal structures for *Vibrio cholerae* MsbA [23] and the antimicrobial peptide/multidrug transporter McjD from *E. coli* [26] show closed structures that might represent an occluded transition state between the inward-facing and outward-facing conformation. Many of these conformations have also been observed individually using cryo-EM [27,28], and are supported by data from biochemical and biophysical experiments [29–38]. Cysteine cross-linking studies first described the entire conformational cycle for a single ABC exporter at major checkpoints of the ATPase reaction under experimental conditions where the exporter was transport-active in a biological membrane [39–41].

Table 1

Structures of multidrug transporters*						
Family	Protein	PDB code	Substrates	Resolution (Å)	Refs	
ABC	Sav1866	2HYD	ADP	3.00	[13*]	
	ABCB1 (mouse)	3G5U	None	3.80	[24]	
	ABCB1 (<i>C. elegans</i>)	4F4C	None	3.40	[25]	
	McjD	4PL0	AMP-PNP	2.70	[26]	
MFS	EmrD	2GFP	None	3.50	[16]	
	YajR	3WDO	None	3.15	[51]	
MATE	PfMATE	3VVN	None	2.40	[67**]	
		3VVO	None	2.50		
		3VVR	MaD5	3.00		
	NorM-VC	3MKT	None	3.65	[15]	
		4HUL	Cs ⁺	3.81	[66]	
	NorM-NG	4HUM	Ethidium	3.49		
		4HUN	Rhodamine 6G	3.59		
		4LZ9	Rhodamine 6G	3.70	[65]	
		DinF-BH				
SMR	EmrE	3B5D	TPP ⁺	3.80	[14**]	
RND	AcrB	1IWG	None	3.50	[94]	
		2DHH	None	2.80	[17**]	
		2DRD	Minocycline	3.10		
		2DR6	Doxorubicin	3.30		
		2GIF	None	2.90	[18**]	
		4DX5	Minocycline	1.90	[147]	
		4DX7	Doxorubicin	2.25		
		3AOC	Erythromycin	3.34	[100]	
		3AOD	Rifampicin, Minocycline	3.30		
		3W9H	ABI-PP inhibitor	3.05	[144]	
		AcrB/AcrZ	4C48	None	3.30	[113**]
		MexB	3W9J	ABI-PP inhibitor	3.15	[144]
			2V50	None	3.00	[145]
		MtrD	4MT1	None	3.54	[146]

* <http://blanco.biomol.uci.edu/mpstruc/>.

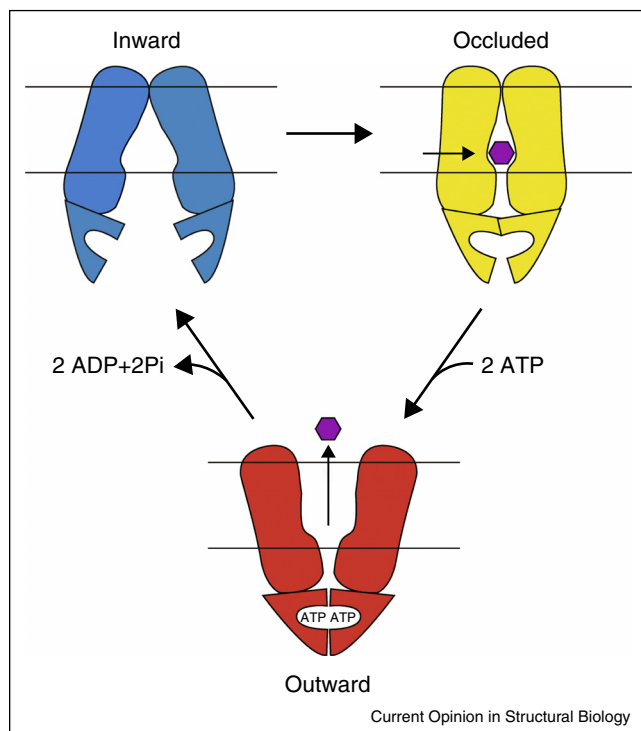
Taken together, the available data support an ‘alternating access’ model in which the substrate-binding pocket in the MD [42*] is exposed to the interior and exterior of the cell as the transporter alternates between inward-facing and outward-facing conformational states. The transition between states occurs via an intermediate with a binding pocket that is inaccessible to either side, and is governed by ATP binding-associated NBD dimerization. ATP hydrolysis and ADP/Pi release-associated NBD dissociation would finally reset the transporters to the inward-facing conformation (Figure 2). The question whether the two ATP sites in homodimeric ABC exporters hydrolyse the nucleotide simultaneously, sequentially or in an alternating fashion has not yet been resolved in detail. It is also unclear whether the inward-facing conformation with disengaged NBDs is a generic state for all ABC exporters, or whether in some ABC exporters the NBDs remain in close contact throughout the catalytic cycle [43]. Furthermore, one of two ATP sites is only active in ATP binding but not in ATP hydrolysis in heterodimeric ABC exporters [44]. However, regardless of these variations in the detailed mechanisms of ATP-dependent transport, the basic principles of alternating access in ABC exporters are most likely quite similar to those proposed for other classes of membrane transporters, to which we now turn.

MFS transporters

The MFS group is one of the largest families of secondary-active transporters. Most members are 400–600 amino acid residues in length and possess 12 or 14 trans-membrane (TM) helices. Crystal structures of prokaryotic and eukaryotic MFS symporters, antiporters and uniporters have been determined in different conformational states. These transporters exhibit a high degree of structural conservation, despite the limited sequence similarity, distinct substrate specificities and coupling mechanisms. The common structural architecture comprises two domains, each composed of bundles of six helices. The domains are related by a pseudo-twofold symmetry axis in the membrane plane and are linked by a long cytoplasmic loop or two TM helices [45–50].

The structures of representatives of the Drug:H⁺ Antiporter-1 (DHA1) group of MFS transporters have been determined in occluded (EmrD) and outward open (YajR) conformations, and comparing these structures suggests a general mechanism for the conformational change between the inward and outward states [16,51]. Crystal structures of a single protein captured in different conformational states have provided a direct view of conformational switch [45–48,52]. These observations

Figure 2



Schematic representations of the drug transport mechanism of ABC efflux pumps. The transport cycle has been simplified to show only three states: inward-facing, occluded state and outward-facing.

and biochemical evidence suggest that MFS transporters operate via a single binding site, alternating-access mechanism, with the translocation pathway of the transport substrate following the interface between the two six-helices domains, and the substrate-binding cavity lies halfway into the membrane. The rocker-switch type movement comprises consecutive and concerted conformational changes of the two halves of the protein between inward open, occluded and outward open states. The transport coupling of MFS proteins involves sequential binding/releasing of substrate/counterion, and this varies between family members. The DHA1 transporter MdfA shows an indirect competition mechanism between H^+ and substrates (Figure 3) [53], whereas both direct and indirect mechanisms of competition might be relevant in LmrP [54,55].

Although MFS transporters generally function as monomers, a recent crystal structure of plant NRT1.1 reveals an unexpected homodimer with different affinity modes [49]. In Gram-negative bacteria, some MFS transporters form tri-partite pumps with partner proteins, which directly transport substrate from the inner membrane and cytoplasm to the cell exterior [56]. The MFS transporter EmrB, which belongs to the DHA2 group and is a component of a tripartite pump, was observed to form a dimer in complex with another assembly component, EmrA [57].

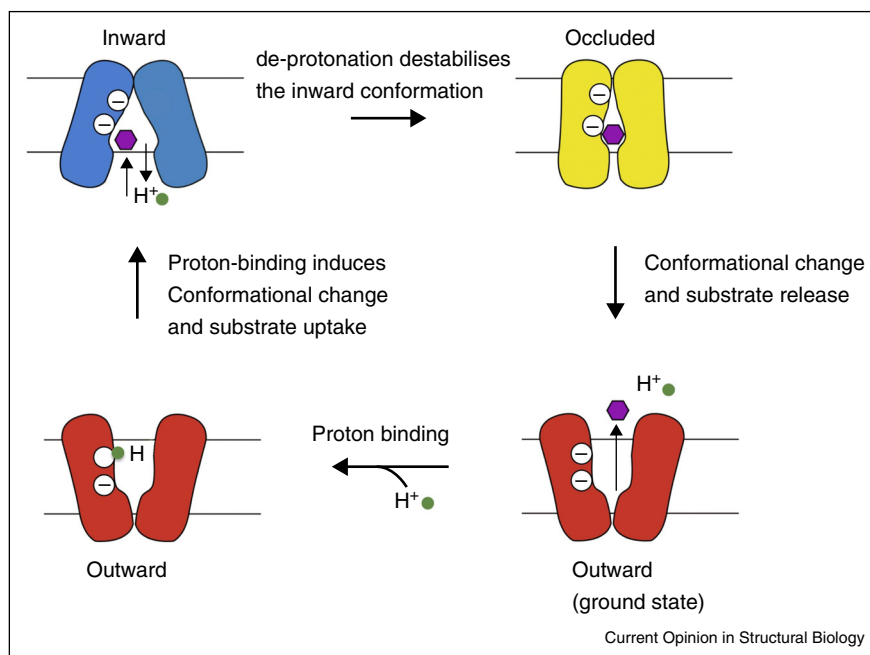
MATE transporters

MATE transporters function in the efflux of endogenous cationic, lipophilic substances and xenobiotics [58–60], and confer multidrug resistance on bacterial pathogens and cancer cells [61,62]. Members of the MATE family are thought to couple transport with influx of either Na^+ or H^+ , but ion coupling in this family is most likely more complex [63]. The SLC47 family of MATE proteins are involved in the efflux of important medications, and disruption of MATE transporter activities can cause severe changes in drug bioavailability and pharmacokinetics [64]. The MATE proteins range from 400 to 700 amino acids in length and have 12 putative transmembrane helices. Although they do not bear any apparent conserved sequence motif, all MATE proteins share ~40% sequence similarity, suggesting an overall conserved structure and transport mechanism [15,58]. Structural information for several MATE transporters has advanced our understanding of the mechanism of members in this family, and suggests a rocker-switch mechanism similar to that used by the MFS transporters described in the previous section [15,65,66,67].

The structure of the PfMATE protein, which is an H^+ -coupled MATE transporter, reveals an internal repeat that is likely to have arisen from an ancient gene duplication event and comprises two bundles of six transmembrane helices (N-lobe TM1–TM6 and C-lobe TM7–TM12) [67]. The bundles are related by pseudo-two-fold symmetry along an axis normal to the membrane plane and form a large internal cleft. In an outward-open state, the cleft is open towards the extracellular side and presents two non-equivalent portals within the lipid bilayer. The hydrophobic central cleft can be divided into two chambers, referred to as the N-lobe and C-lobe cavities. A drug-binding pocket has been identified in the N-lobe cavity located halfway into the membrane. Thioether-macrocyclic peptide inhibitors of PfMATE bind in this pocket, but are not transported possibly by sterically preventing the rocker-switch motion of the N-lobe and C-lobe. The question of how the movement of protons and substrates is coupled is addressed by the finding that protonation of Asp41 induces bending of TM1 at Pro26, resulting in the collapse of the N-lobe cavity that may displace the bound substrate into the extracellular space [67]. A similar mechanism of (de)protonation-induced helix movement is seen in the AcrB structure [68], described in the RND subsection below.

The NorM proteins are well-studied representatives of MATE transporters. Recognizing a broad range of transport substrates, NorM proteins confer resistance to dyes, fluoroquinolones and aminoglycosides [69]. The structures of NorM-VC and the related NorM-NG reveal similar overall conformation to that of the H^+ -driven PfMATE described above [15,66]. The structures of NorM-NG with bound-substrates point to a multidrug-binding cavity that

Figure 3



The indirect competition mechanism of MFS multidrug/proton antiport [53]. The substrate and proton bind to different residues in the transporter. This mechanism involves a sequential process in which the substrate is bound after releasing of proton at the inside surface of the membrane, and substrate is released before the binding of the proton at the outside surface of the membrane. Some MFS multidrug transporters show both direct and indirect mechanisms of competition [54,55].

is enriched in negative charges but rather limited hydrophobic moieties, implying reduced affinity for hydrophobic substrates [66]. Since the substrate binding sites are located near the membrane-periplasm interface, these structures may represent intermediate states of transport. Substrates might first bind to a cavity that is alternatively accessible from both sides of the membrane bilayer, as shown in PfMATE, and then move to a binding site near the membrane-periplasm interface before being released, as suggested for NorM-NG. Na⁺ and H⁺ play different roles in the substrate transport by NorM-VC: Na⁺ was found to stimulate ethidium binding, whereas ethidium binding was found to drive H⁺ release [63^{*}]. Similar to PfMATE, coupling ions might therefore play a role in the collapse of the substrate-binding cavity to displace bound substrates. In addition, coupling ions might directly drive conformational transitions required for progression of the transport cycle.

All the current MATE transporter structures have been captured in an outward-open state, however, the binding/releasing of H⁺/cation/substrates cause substantial rearrangement of the trans-membrane helices, such as TM7 and TM8, which may be important for the conformational switch between outward-open and inward-open states [15,65,66,67^{**}]. The elucidation of the structures capturing the occluded and inward-open states and detailed

biochemical analysis of transport mechanisms will be critical for the understanding of the H⁺ and/or Na⁺/drug antiport cycle of MATE transporters.

SMR transporters

The SMR family belongs to the drug/metabolite transporter (DMT) superfamily, whose members are typically composed of around 100–120 amino acid residues with four predicted trans-membrane helices, and function in general as homodimers. Some SMR family transporters are composed of two dissimilar but homologous subunits [70]. EmrE from *E. coli* is a H⁺:drug antiporter conferring resistance to cations such as ethidium, proflavine, pyronin Y, safranin O, and methyl viologen as well as to erythromycin, sulfadiazine and tetracycline [71,72].

The structure of EmrE bound to tetraphenylphosphonium (TPP⁺), determined by X-ray crystallography and cryo-EM, reveals an asymmetric, antiparallel homodimer consisting of eight α -helix bundles, which are arranged pairwise across the dimer interface [14^{**},73^{*}]. This antiparallel topology within the dimer was confirmed by single-molecule fluorescence-energy transfer experiments [74^{**}]. TPP⁺ binds in a chamber formed by TM1–3 from each protomer. The dimer is favoured by inter-helical contacts between TM4, which is nearly perpendicular to the membrane [75–77]. The loops between TM3 and TM4

become structured upon substrate binding to form a β -strand, which holds TM4 close to the substrate-binding chamber [77,78].

A single-site alternating access model suggests that EmrE converts between inward-facing and outward-facing states to move substrates across a membrane barrier. Trapping EmrE in a single state by cross-link abrogates pump activity, suggesting the importance of conformational switching for the transport process [79]. The coupling of protons and cationic substrates occurs through competition for common binding to a conserved glutamate in the TM1 (Glu14), with two protons exchanging one polyaromatic cation [71,80–83]. The details of the conformational exchange process were directly observed in recent solution NMR studies, which show that the conversion is achieved by conformational exchange of protomers in the asymmetric EmrE dimer, so that the two states are identical except that they have opposite orientation in the membrane [74**].

The structure and topology of SMR transporters have long been controversial [70,84]. Since it has been shown that a genetically-fused parallel dimer is active [85], as well as fused anti-parallel dimers [86], both topoforms can be given credibility, albeit that antiparallel dimers appear to be more stable [87]. The topology of SMR proteins is extremely sensitive towards modifications in the primary sequence [88], making it difficult to attain the native form of these proteins.

RND transporters

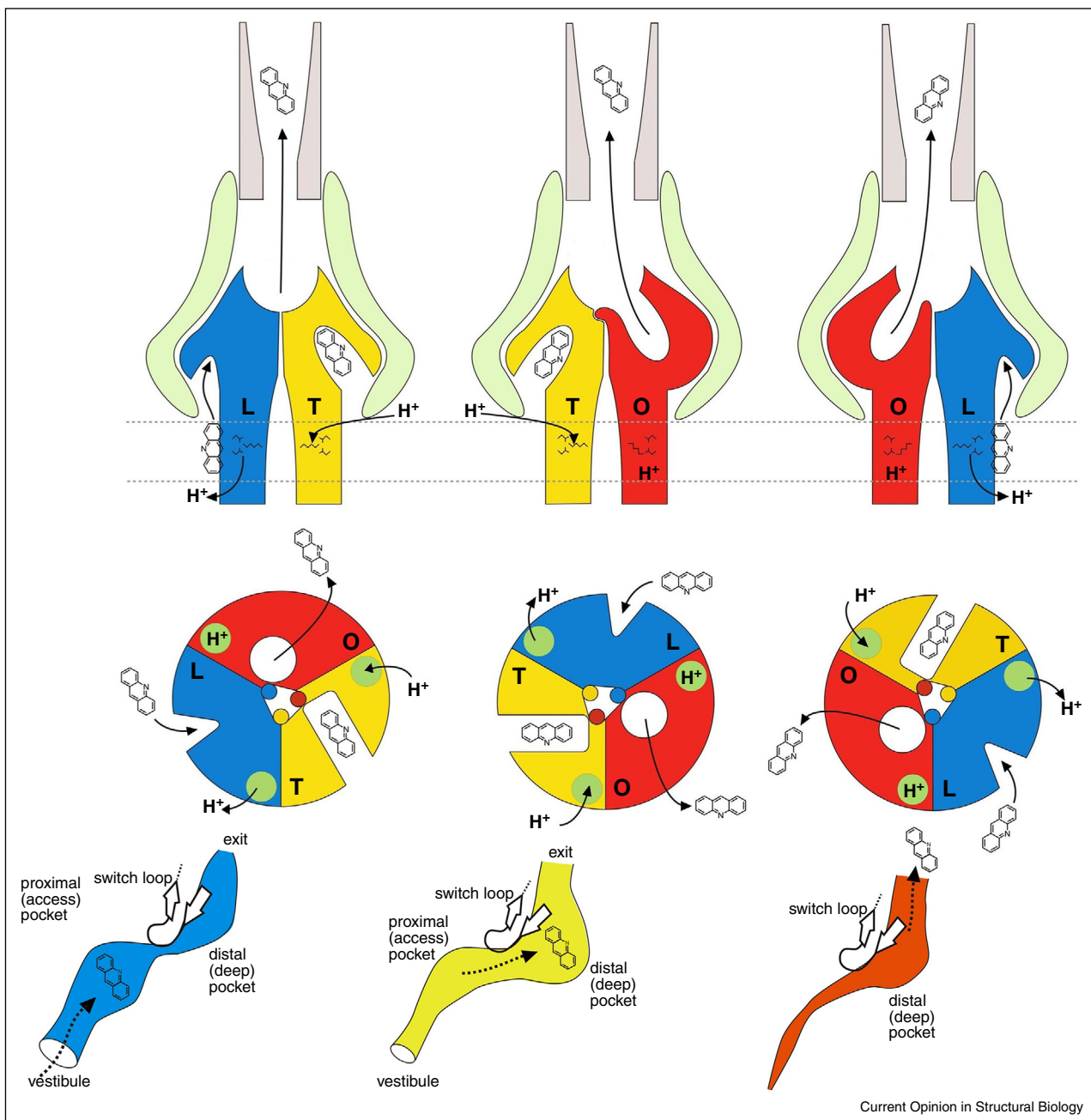
AcrB from *E. coli* is a well-studied representative of the RND family of multidrug transporters. Recognizing a broad range of transport substrates, it confers resistance to numerous anti-microbial compounds including tetracycline, chloramphenicol, β -lactams, novobiocin, fusidic acid, nalidixic acid, and fluoroquinolones [89]; moreover, it also transports sodium dodecylsulfate, Triton X-100, detergent-like bile salts, cationic dyes, disinfectants [90], and even nonpolar solvents [91,92]. The capacity of AcrB to transport such a broad range of compounds raises the intriguing structural puzzle of how this and other general transporters recognize so many chemically distinct transport substrates [93]. AcrB is a homotrimer and its protomers bear a structural repeat as a result of an ancient gene duplication event. AcrB has a 12-helix trans-membrane domain and an extensive periplasmic portion comprising porter and funnel domains [94,95]. The funnel domain is composed of two subdomains, DN and DC. The porter domain, located nearest to the inner membrane, can be divided into four subdomains, PN1 and PN2, situated in the N-terminal half between trans-membrane helices TMH1 and TMH2, and the repeat-related subdomains PC1 and PC2, which are located in the C-terminal half between TMH7 and TMH8 (these domains are depicted schematically in Figure 5b). The four subdomains pack to

form two substrate-binding pockets, namely a proximal (or access) and a distal (or deep) binding pocket separated by a 'switch-loop' (Figure 4, lower panel). The pockets are enriched in aromatic, polar and charged amino-acid residues that form favourable interactions with the transport substrates. The access pocket appears to favour interactions with large substrates (or those with a large minimal projection area [96]), like erythromycin, rifampicin or doxorubicin dimers. The deep binding pocket has been shown by X-ray crystallography to harbour minocycline or doxorubicin (as a monomer), and mutational as well as molecular dynamics analyses predict binding of other substrates [68**,97–100,101*]. These pockets might therefore each contribute to the broad substrate 'polyspecificity' of AcrB. Details of the pathways by which drugs enter AcrB from different cellular compartments remain to be established experimentally. One proposed pathway for drugs is through the leaflet of the outer cytoplasmic membrane, with entry through a TM8/TM9 groove entrance [95]. It has also been proposed that substrate can enter from the periplasm, and this might occur through the PC1/PC2 cleft that is open from the periplasm and reaching in the access pocket [95,102].

One fascinating aspect of AcrB is its loss of molecular symmetry with drug binding, whereupon the three protomers each adopt a distinct conformation, designated Loose (L), Tight (T) and Open (O) (Figure 4). Compelling structural and biochemical evidence indicates that these conformations are consecutive steps within the transport cycle, and correspond to 'access' (L), 'binding' (T), and 'extrusion' (O) states, in accord with the status of the substrate-binding pocket [17**,18**,103]. During the transport cycle, the PN1/PC2 repeat is rather rigid, while the PN2/PC1 repeat undergoes cyclic changes internally due to its function to bind substrates within the hydrophobic core of the tandem. However, both PN1/PC2 and PN2/PC1 tandems undergo large conformational changes during cycling through the three different states. The DN and DC subdomains of each protomer form a funnel with an internal diameter of about 30 Å, and its conformation is largely unchanged throughout the transport cycle of the pump.

The mechanism for drug transport involves a proton translocation process, and evidence supports a proton pathway involving residues D407, D408, and K940; these are located in the middle of trans-membrane helices TMH4 and TMH10 [104–106]. Recent crystallographic and computational analyses show that the structural repeats in the trans-membrane portion undergo a relative rotation with respect to each other during the transport cycle (rocking motion from the 'access' (L) to the 'binding' (T) state and a shearing motion from the 'binding' (T) to the 'extrusion' (O) state), which is linked with a piston-like and tilting movements of TMH2 and TMH8 into the porter domain (Figure 5) [68**]. Interestingly, TMH2 connects to the flexible PN2/PC1 repeat, where

Figure 4

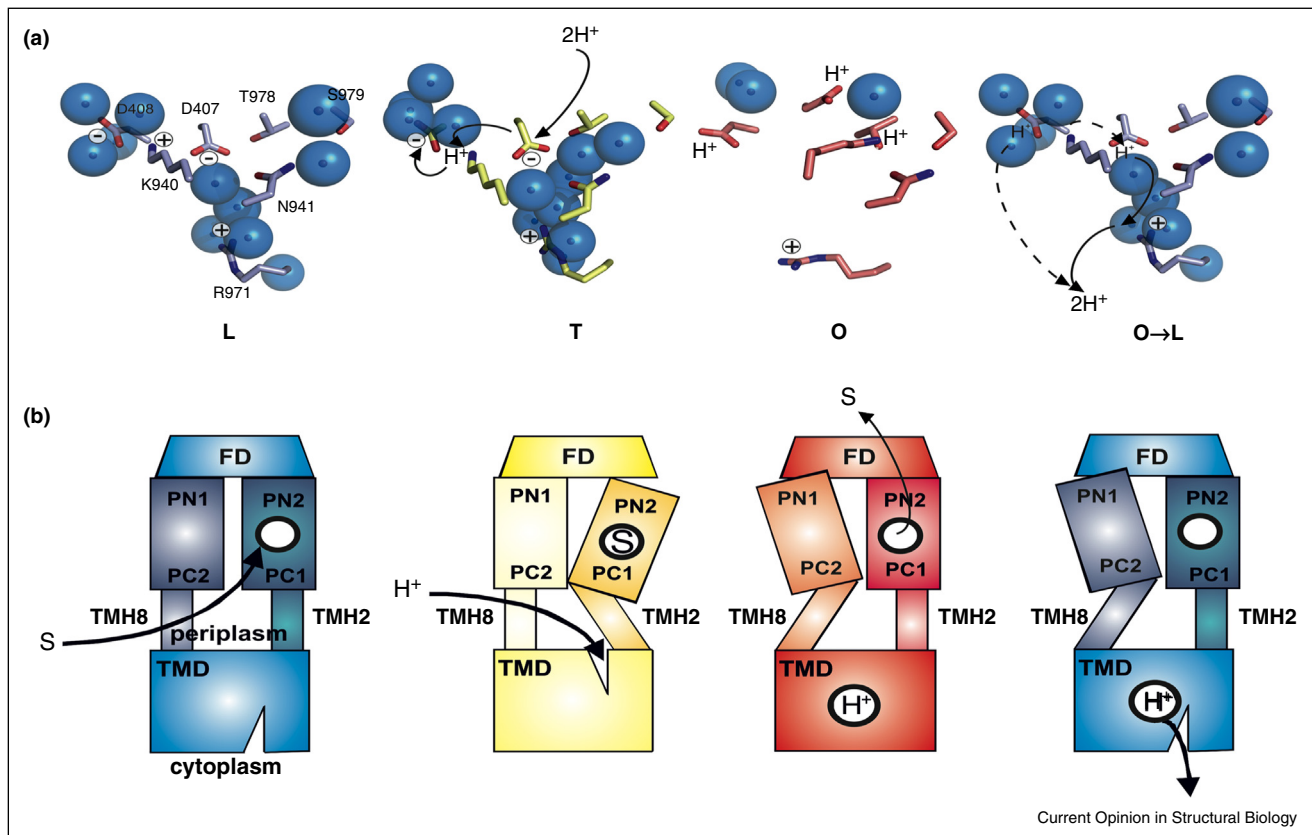


Schematic representation of the 'peristaltic' transport mechanism of the RND transporter AcrB. Through coupling of proton and drug binding, the AcrB trimer cycles through three conformational states with different shapes for the drug binding pocket: the 'access' (or 'loose' (L)), 'binding' (or 'tight' (T)) and 'extrusion' (or 'open' (O)) states. The upper panel depicts a cross section (two protomers of the AcrB/TolC trimers or AcrA hexamer) through the full tripartite assembly, which is shown in more detail in Figure 6. TolC is at the top and AcrA is contacting TolC and AcrB. The middle panel is a view of the AcrB trimer, from the perspective of the porter domain of the pump. The schematic in the lower panel depicts the passage of drugs between the drug-binding pockets in a protomer; these are color coded to correspond to the three states shown in the other panels. In the upper panel, the TolC is proposed to remain in an 'open' state throughout the conformational switches in AcrB. Source: The figure is a modification of an image from Seeger *et al.* [18**]; a similar model was also proposed by Murakami *et al.* [17**].

substrate binding occurs in the 'binding' (T) state, and TMH8 is linked to the more rigid PN1/PC2 repeat. Binding of substrate to the PN2/PC1 unit causes stabilization of the 'binding' (T) state and triggers the

movement of TMH2 and formation of a chain of water molecules leading from the periplasm to the titratable residues D407 and D408 within the trans-membrane domain. Upon protonation of these residues, the transition

Figure 5



Mechanistic detail of the proton coupling mechanism of the RND transporter, AcrB from *E. coli* (pdb entry: 4DX5). (a) Salt bridges form and break in the different conformational states of AcrB. (b) The protonation states are linked to displacements of trans-membrane helices (TMH2 and TMH8), which trigger changes in the pockets shown in Figure 4. The structures are for the *E. coli* AcrB. FD: funnel domain; PN1, PN2, PC1, PC2: subdomains of the AcrB porter domain; TMD: trans-membrane domain.

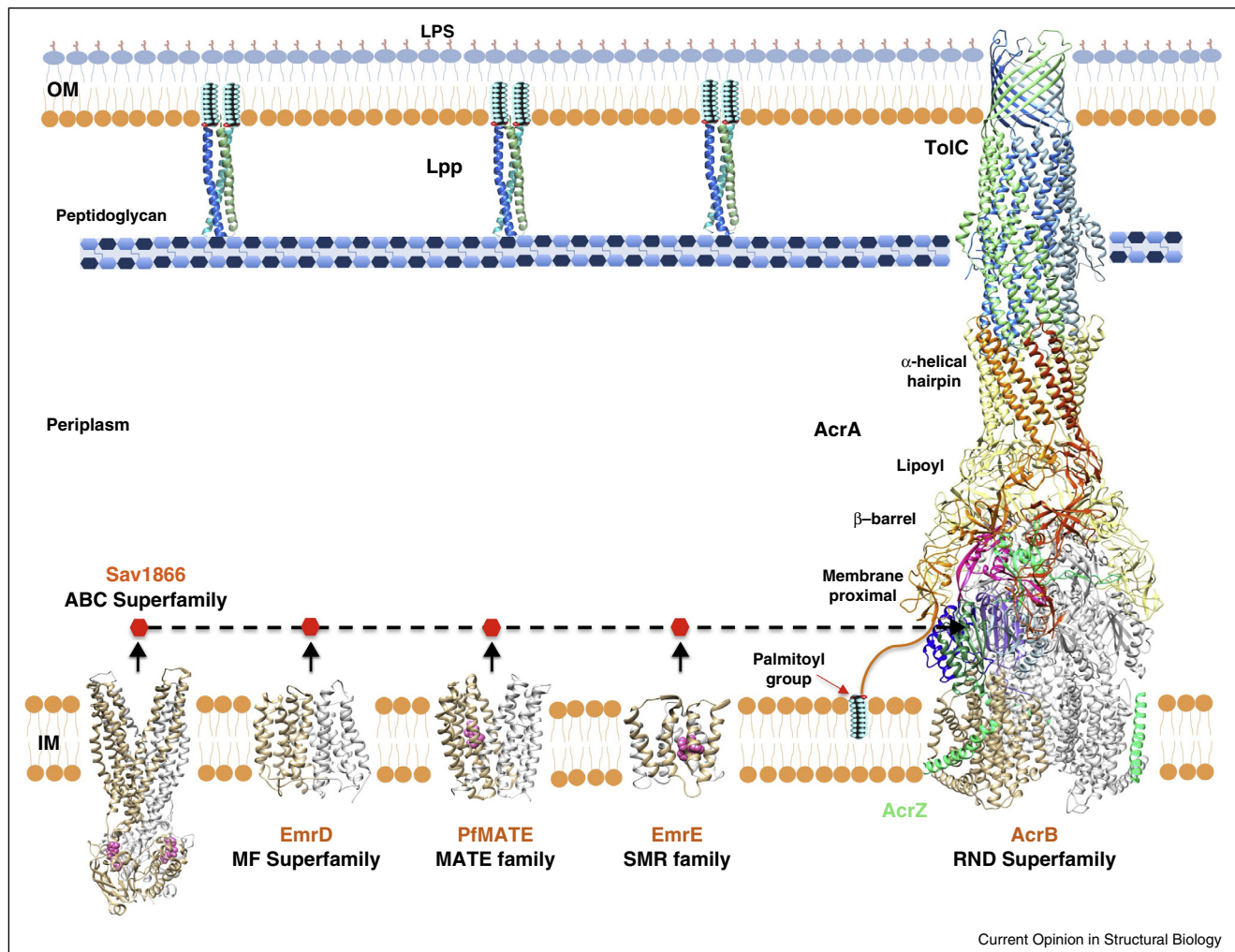
of the 'binding' (T) towards the 'extrusion' (O) state is triggered. Protonation causes the shearing motion of the structural repeats within the trans-membrane domain, which not only disrupts the water chain and precludes return of the protons towards the periplasm, but also induces the movement of TMH8 and TMH2. Movement of TMH8 causes the PN1/PC2 tandem to undergo a large conformational change leading to the closure of the periplasmic PC1/PC2 cleft, therefore sealing off the return path of the drug towards the periplasm. Moreover, it creates a new drug exit tunnel leading towards the funnel domain (and eventually towards the exit duct). Concomitantly, movement of TMH2, linked to the PN2 subdomain, causes the flexible PN2/PC1 to close its deep, hydrophobic substrate-binding pocket, most likely in a peristaltic fashion [107], squeezing the drug out. These changes are concerted, and directly link the directionality of proton influx and substrate efflux. The conformational changes described above depict a rather simple model that only focuses on a single protomer. Since AcrB is only active as a trimer [108,109] and displays a highly cooperative drug transport behaviour [110,111] inter-monomeric

communication, or even communication with the AcrA adaptor protein (described below) is likely involved in the coupling of the three states leading to efficient drug efflux.

Modulators of RND transporters

In screens of the interacting partners of small proteins, a 49-residue protein was identified that interacts with the AcrB protein [112]. Although not required for activity of AcrB, this 49-residue protein, known as AcrZ (formerly named YbhT) was found to impact on the sensitivity to certain antibiotics, such as tetracycline, puromycin, and chloramphenicol that are known substrates for AcrB. Crystallographic analysis shows that AcrZ traverses the TM domain of AcrB (Figure 6, bottom right) [113**]. It is not clear yet how AcrZ modulates the activity of the transporter for a subgroup of antibiotics, but potential mechanisms could be based on alterations in drug binding or access to drug binding pockets. The interaction surfaces of AcrB and AcrZ are conserved in homologues, and we speculate that other RND family members are likely to have modulating partners that bind in the transmembrane groove in a similar fashion to AcrZ.

Figure 6



The cell envelope of Gram-negative bacteria and cooperative organization of transporters. The lower panel depicts the conformational switch for the different classes of drug transporters during the transport process, as well as the pathway for transport substrates into the periplasm and then exiting through the tripartite assembly as a 'super-system'. The outer membrane (OM) is an asymmetric bilayer, with the lipids on the inner and outer leaflets composed of phospholipids and glycolipids (principally lipopolysaccharides (LPS)), respectively. For *Escherichia coli*, the outer membrane is anchored through abundant lipoproteins to the underlying peptidoglycan, which is composed of repeating units of the disaccharide N-acetyl glucosamine-N-acetyl muramic acid, conferring mechanical robustness to the dual membrane system. The inner membrane layer (IM) is a phospholipid bilayer, consisting largely of phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin. The two membrane layers delimit an aqueous cellular compartment densely packed with proteins, the periplasm, with a width estimated to be roughly 230 Å, judging from electron cryo tomography images.

Tripartite multidrug efflux pumps in Gram-negative bacteria span the cell envelope

The cell envelope of Gram-negative bacteria, a formidable protective barrier, has three principal layers: the outer membrane with an associated lipopolysaccharide (LPS) coat on the cell exterior, the inner membrane that adjoins the cytoplasm, and the peptidoglycan cell wall situated in the space between the two membranes—the periplasm—and which contributes to mechanical rigidity (Figure 6) [114]. Moving molecules through this barrier requires specialised machinery that helps to guide the

transport substrates through the different layers [115–119]. Cytotoxic compounds, including clinical drugs, can be driven from the cell by tripartite assemblies that span the width of the envelope. Such assemblies include an outer membrane protein, an inner membrane transporter, and a periplasmic 'membrane fusion' protein that connects the two trans-membrane components. The inner membrane transporters typically belong to the MFS, RND, or ABC families described above [57,113,120]. For the RND type tripartite assemblies, transport substrates enter the pump from the periplasm or from the periplasm-facing

outer leaflet of the cytoplasmic membrane. The drugs are moved into these locations by the action of ‘stand alone’ transporters that are not components of tripartite assemblies [121]. As a collective body, the tripartite and solitary transporters can be considered as a super-system that confers tolerance to an enormous range of harmful compounds, including clinically applied antibiotics (Figure 6).

Structural information on the individual components of the tripartite pump has advanced our understanding of how the individual pump components might work in the assembly. Recent electron microscopy structures of an RND-type tripartite multidrug efflux pump have shown how the components interact to form an operating machine [113^{••},122]. We will discuss each of the components in the following sections before describing the full assembly.

Outer membrane proteins (OMP)

A representative outer membrane component of the tripartite multidrug efflux pump is TolC from *E. coli* (Figure 6, right). TolC assembles as a homotrimer with three distinct structural regions: trans-membrane β -barrel, periplasmic α -helical barrel, and a mixed α/β -fold also in the periplasm and referred to as the equatorial domain [123]. Like its partner RND transporter AcrB, the TolC protomer bears a structural repeat, originating from an ancient gene duplication. As a consequence, the tube-like architecture of TolC has approximate six-fold symmetry. The TolC tube has a large interior cavity that is mostly solvent-filled with an average accessible interior diameter of roughly 20 Å and is well shaped to form an exit duct, except that it is tightly constricted at the end distal to the outer membrane. Here, the effective diameter is only ~ 3.9 Å, which is much too small for the passage of transport substrates. This constriction arises from the inwardly curving trajectory of pairs of coiled-coil helices, which meet at an apex and associate through salt bridges near the constriction point. Carboxylates from conserved side chains decorate the interior surface near this constriction and provide a ‘gating ring’ with selectivity for cations [124,125]. Insight into how the channel opens has been provided from crystal structures of partially opened states of TolC [126,127] and the homologous *Neisseria gonorrhoeae* MtrE (a component of the MtrC–MtrD–MtrE tripartite pump) [128]; these reveal changes in the super-helical trajectory of the coiled coils and breaking of the gating salt bridges that favour the closed state. The energy costs associated with these conformational switches must be provided through interactions with the other components of the efflux pump.

Membrane fusion proteins (MFP)

Crystal structures of MFP proteins from diverse bacterial species reveal that many are comprised of four domains: membrane-proximal, β -barrel, lipoyl, and a coiled-coil α -helical hairpin (Figure 6, right) [129–131]. Some MFP proteins lack the membrane proximal domain [132], while

others have longer or shorter α -helical hairpin domains or none at all [133], so there must be some diversity in the way that these proteins assemble into active efflux pumps. One feature that does seem to be general to the MFPs, however, is that the inter-domain regions are flexible, enabling the proteins to mould to accommodate their partners in the efflux pumps. Another interesting aspect of MFP proteins is that they can often self-associate into oligomers, and these may mimic their organisation in the full pump assembly.

Assembly of tripartite multidrug efflux pump AcrAB–TolC

AcrAB–TolC is a paradigm of RND-type tripartite multidrug efflux pump. Du *et al.* developed a strategy to stabilize the AcrABZ–TolC complex by fusion of the pump components, and purified the full pump for single particle cryo-EM assay [113^{••}]. A cryo-EM structure at roughly 16 Å resolution shows that the AcrABZ–TolC assembly comprises an AcrA hexamer, a TolC trimer, three AcrZs, and an AcrB trimer (Figure 6). The reconstructed shape and pseudoatomic model is in agreement with a recently reported negative stain EM structure of the pump prepared with a different engineering procedure and without AcrZ [122].

The helical hairpins of AcrA pack to form bundles with the ends of the helical portion of TolC [134], which adopts a fully open state in the assembly. The importance of this region of TolC is suggested by the observation that, with a few residue mutations at the periplasmic tip, outer membrane components could be exchanged between different tripartite pumps to make active assemblies [135,136]. The membrane proximal domain and β -barrel domain of AcrA are involved in defined interactions with AcrB. One protomer of AcrA bridges the upper regions of subdomains PC1, PC2, and DC of AcrB, while the adjacent protomer interacts with the upper regions of PN2 and DN subdomains. The modeled docking of the membrane-proximal domain onto the surface of AcrB is consistent with the disruptive behaviour of mutants at that interface [137].

The structure of CusBA complex, a homologue of AcrAB, shows that the β -hairpin residues in the DN and DC subdomains are involved in an interaction with the β -barrel domains of the membrane-fusion protein [138^{••}]. These interactions were shown to be important for the proper assembly of a hexameric AcrA suitable for presentation to TolC [139]. The lipoyl domains principally interact with each other and make no predicted interactions with either AcrB or TolC.

The side-by-side arrangement of the β -barrel, lipoyl and helical hairpin domains of the AcrA hexamer generates a funnel-like structure with a sealed central channel along the long molecular axis [113^{••},140,141]. The bottom of

the channel opens into a chamber in the funnel domain of AcrB. The channel is partially constrained near the middle of lipoyl domains and then runs from the helical hairpin domains of the AcrA through the periplasmic domain and transmembrane β -barrel of TolC to the cell exterior. This conduit forms an exit pathway for efflux substrates, which will be delivered into the channel through successive opening and closing of ligand-binding pockets in AcrB, adjacent to the apex of the funnel-shaped periplasmic canyon.

Due to the sequential rotation mechanism of AcrB, described above, three protomers adopt an extrusion state in turn, which is ready to release a substrate molecule into the funnel of the AcrB. Accordingly, TolC must adopt an open state throughout the transport cycle, so that the channel opening of TolC should be independent on the conformational change of AcrB. Indeed, the cryo-EM model, which represents the pump in a relaxed state, shows that the interaction between AcrA and TolC can directly open the channel. Although the surface area contacted between the individual AcrA and TolC protomers is not extensive, having six such surfaces pre-organised in an optimal orientation for interaction must significantly boost the overall binding free energy. AcrB provides a platform for the proper assembly of hexameric AcrA suitable for presentation to TolC and opening of the channel. The interaction between TolC and AcrA, in turn, stabilizes the assembly of hexameric AcrA, accounting for the change in the proteolytic sensitivity of the membrane-proximal domains of AcrA and MacA by TolC [137,142]. The AcrAB–TolC pump is likely to be dynamic, and the pump might accommodate the asymmetrical cycling of AcrB during the transport through the inter-domain flexibility of AcrA, allowing the TolC–AcrA interaction to be maintained during the conformational changes of AcrB throughout the substrate translocation process.

Conclusions

Similar to other drug resistance mechanisms, efflux pumps have likely existed from a very early stage in the evolutionary history of bacteria [143], and they have enabled this domain of life to occupy hazardous niches and outwit competitors. We described here the RND-based tripartite assembly, but there are also ABC and MFS based assemblies that are currently not structurally characterised; the interactions of the MFP and inner membrane components are likely to differ markedly for the different energy transducing classes, but all are likely to share some architectural similarities in the organisation of the MFP and outer membrane components. Structural data for the RND based pumps has provided some insight into how the periplasmic portions of the ABC and MFS based pumps may be organised. Related tripartite assemblies that are energised by an ABC transporter drive virulence determinants of pathogenic bacteria across

the cell envelope, such as adhesins, toxins, or other proteins that are important for the colonization and infection of human and animal cells [2]. These protein translocators, known as type I secretion systems, also use TolC as an exit duct.

In Gram-negative bacteria, RND family drug transporters mostly assemble with their partner proteins to form tripartite pumps, which acquire substrates from the outer leaflet of inner membrane and periplasm and efflux to the cell exterior. In contrast, members of the other four 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. In this scenario, it is envisaged that the drugs are transported by the ‘unitary’ transporters into the periplasm or outer leaflet of the plasma membrane, where they are intercepted and expelled by the RND tripartite assemblies (Figure 6) [121].

Multidrug transporters have many properties that are unexplained at present. For example, the capacity of RND and ABC exporters to transport a broad range of compounds raises the question whether there might be some trade-off between substrate recognition and transport efficiency. The answer awaits accurate experimental measurements of the amount of metabolic energy required to transport numerous types of substrates. The growing structural information on these systems may also guide rational approaches to design compounds that interfere with the function of the transporters [144] or the assembly of the pumps. The recently discovered PACE family transporters are also involved in multidrug efflux and are likely to be structurally distinct [148,149]. Characterizing the structure and function is another key research question in the field. There is clearly much remaining to be discovered regarding these fascinating nanomachines.

Conflict of interest statement

Nothing declared.

Note added in proof

A recent study reports the crystal structure of *E. coli* MdfA in complexes with chloramphenicol, deoxycholate and LDAO. The structures reveal how multi-drug resistance MFS transporters engage ligands in the inward-facing conformational state [150]. Also reported are crystal structures of the polypeptide processing and secretion transporter from *Clostridium thermocellum*; this is an ABC transporter related to the type I secretion system of Gram negative bacteria. The structures capture two different conformations and illustrate the proposed transport cycle for another ABC exporter [151].

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