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# MECHANISM OF ANTIBIOTIC EFFLUX IN GRAM-NEGATIVE BACTERIA

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## 1. ABSTRACT

Active efflux of antibiotics mediated by multidrug transporters is a mechanistic basis of multidrug resistance in bacteria. The most versatile multidrug transporters are those found in Gram-negative bacteria. They have a high level of constitutive expression and provide an immediate response to structurally diverse antimicrobial agents including clinically important antibiotics. The versatility and efficiency of multidrug transporters in Gram-negative bacteria heavily depend on coupling of drug efflux with the transport across the outer membrane. The coupling is achieved through the assembly of multi-component protein complexes that span both the inner and the outer membranes of Gram-negative bacteria. In this review we discuss the mechanistic and structural features of multidrug efflux complexes with the major focus on the tight coupling of drug efflux with transport across the outer membrane.

## 2. INTRODUCTION

Antibiotic efflux as a mechanism of bacterial resistance is known to clinicians and scientists for a long time. Tet transporters carried by plasmids or transposons provide clinically significant resistance to tetracyclines in many bacterial species (1). Recently, it became evident that antibiotic efflux affects not only tetracyclines, but almost all classes of antimicrobials. Multidrug efflux transporters encoded on bacterial chromosomes are easily overexpressed during exposure to antibiotics and provide efficient protection against broad spectrum of structurally unrelated drugs (2-5).

Bacterial genomes invariably contain genes encoding established and putative multidrug transporters (MDRs). MDRs are structurally diverse and belong to six families of transporters. The ATP-dependent MDRs are members of the ATP-Binding Cassette (ABC) superfamily of proteins. All other MDRs are powered by the transmembrane ion gradients. Usually bacteria constitutively express at least one multidrug transporter characteristic of bacterial species. The majority of Gram-negative bacteria are protected by the action of multidrug efflux transporters, which belong to Resistance-Nodulation-cell Division (RND) superfamily of proteins (4, 5). The MFS-type (Major Facilitator Superfamily) and the SMR-type (Small Multidrug Resistance) transporters reportedly confer multidrug resistance in Gram-positive bacteria (6, 7).

Interestingly, among RND-type proteins found in Gram-positive bacteria, transporters conferring multidrug resistance have not been identified so far (8). The reason for this selectivity possibly resides in structural differences of bacterial envelopes. Gram-negative bacteria enveloped into two lipid bilayers employ unique mechanisms to achieve transport across two membranes. The MDRs in Gram-negative bacteria often function in complexes with another two proteins, which are indispensable for the MDRs' function. One of them is located in the periplasm and belongs to the Membrane Fusion Protein (MFP) family (9). The biochemical function of MFPs is still unclear. However, structural studies suggested that these proteins could span the periplasm and provide connection with the second, outer membrane. The third component of MDRs from Gram-negative bacteria is the outer membrane channel, which provides the route for expelled drug to cross the outer membrane (10).

Some bacteria simultaneously express several MDRs. For example, *E. coli* cells simultaneously express at least four MDRs: EmrAB, AcrAB, MdfA and EmrE (11). *P. aeruginosa* cells are protected by at least two MDRs: MexAB-OprM and MexXY (4). The simultaneous expression of structurally different MDRs was shown to result in additive or even synergistic effects, which could further boost antibiotic resistance (12). The mechanistic

particularities of MDRs appear to be responsible for synergistic effect in multidrug resistance.

The constitutive expression of MDRs provoked discussion of physiological roles, which these transporters might play in the normal physiology of bacteria. Enterobacteria colonizing gut of mammals are exposed to high concentrations of bile salts. Bile salts are excellent substrates of constitutive multidrug transporter AcrAB-TolC from *E. coli* and its homologs in other Gram-negative bacteria (13). Perhaps protection from toxic substances in the environment, such as detergent-like action of bile salts, is the natural function of these proteins. Such protection is absolutely required for bacteria to establish themselves in particular niche and successfully proliferate there. Consistent with this notion is finding that *P. aeruginosa* mutant cells lacking the major MDRs are defective in killing mice and worms (14). On the other hand, such bacteria were also unable to colonize the cell cultures, which are generally free of many antimicrobial substances, suggesting that these transporters could be involved in transport of virulence factors (15).

What putative virulence factors depend on the activity of MDRs remains unclear. Recent studies suggested that these possible factors might be signaling molecules involved in quorum sensing. Quorum sensing (QS) is a phenomenon of high-density cell populations, which regulates virulence factor expression and biofilm formation. *P. aeruginosa* lacking constitutive MDR pump MexAB-OprM is deficient in production of pyoverdine pigment (16). The expression of this pigment is regulated by QS. The analysis of mutants with defects in genes encoding proteins of QS suggested that MexAB-OprM might be responsible for establishing the correct concentration of autoinducer inside the cells. Similarly AcrAB-TolC was proposed to contribute to efflux of autoinducers in *E. coli* cells (17).

Even if the efflux of antibiotics by MDRs is only accidental, these transporters confer very impressive levels of antibiotic resistance in both Gram-positive and Gram-negative bacteria. One of the biggest surprises is finding that antibiotics generally believed to be effective only against Gram-positive bacteria, such as macrolides, are highly potent against Gram-negative bacteria when constitutive MDRs are inactivated by mutations or inhibitors (18). Similarly *Enterococcus faecalis* mutants deficient in MDRs are highly susceptible to a number of commonly used antibiotics (19). These drugs appeared to be excellent substrates of MDRs and the resistance is mainly maintained through the active efflux.

Multidrug efflux-based antibiotic resistance is a problem in clinical settings when constitutive or otherwise silent MDRs are overproduced due to mutations. Depending on the class of antibiotics, the level of resistance can range from that which is still therapeutic to extreme resistance, which must be treated vigorously, often with more than one antibiotic. Several excellent review articles addressed the problem of MDR in clinical settings (6, 20, 21).

In this review we discuss the mechanistic particularities of MDR transporters. In particular, we focus on the constitutive MDRs from Gram-negative bacteria belonging to the RND superfamily of transporters. Recent advances in structural and functional studies illuminated features that distinguish RND-type multidrug transporters from other classes of MDRs.

## 3. MULTIDRUG RECOGNITION AND TRANSPORT

### 3.1. Biochemical studies

The mechanistic signature of all MDRs is the ability to bind multiple structurally unrelated compounds. Majority of MDRs interact predominantly with hydrophobic cations. The RND-type transporters are the most versatile among MDRs. Besides hydrophobic cations they also provide protection against neutral, zwitterionic and negatively charged compounds. The broad substrate specificity of the RND-type transporters is further highlighted by finding that some of these transporters along with hydrophobic substrates expel from the cell such highly hydrophilic antibiotics as aminoglycosides (21).

The common feature of the majority of substrates appears to be large hydrophobic domains, which ensure the partition of drug molecules into phospholipid bilayer. This observation prompted a model that binding of substrates occurs in the membrane environment (22). The direct evidence for binding within the membrane was obtained in reconstitution studies of purified MDRs. Structurally divergent transporters LmrA (ABC-type) and AcrB (RND-type) both transport the fluorescent analogs of phospholipids (23, 24). This transport was dependent on energy of ATP hydrolysis or proton-motive force, respectively. This feature of bacterial transporters is similar to eukaryotic Mdr2, which was also found to transfer fluorescent phospholipids (25). Among other compounds used to demonstrate substrate binding within phospholipid bilayers are lipophilic fluorescent probes such as Hoechst 33342 and TMA-DPH. A strong decrease in fluorescence upon the efflux of Hoechst 33342 from the membrane to the aqueous phase enabled the studies of the drug specificities of such transporters as P-glycoprotein, LmrA and LmrP (26-28). The transport of fluorescent phospholipids and probes by MDRs were inhibited by other substrates including antibiotics. This finding implied that transport of fluorescent probes or lipids and drugs occurs by the same mechanism. These studies also showed that several substrate molecules can simultaneously bind to the protein. Competitive, noncompetitive, or uncompetitive inhibition was observed with various substrates of the MFS-type MDR transporters such as QacA (29), LmrP (30) and MdfA (31) and the ABC-type transporters P-glycoprotein and LmrA (32). These studies suggested existence of at least two distinct drug interaction sites. These drug interaction sites may represent distinct drug binding sites on the proteins or alternatively, may represent drug-binding regions within a common hydrophobic binding pocket.

The approach for mapping drug-binding sites is based on the labeling of transporters with photoreactive radioactive or fluorescent substrates. Based on these studies several regions directly interacting with drugs were identified in eukaryotic MDRs including human P-

glycoprotein and MRP1 (33, 34). The exact amino acid sequence of binding sites is presently unknown; however, several reports support the role of transmembrane helices in drug binding. These studies reiterated the phospholipid bilayer as a site of multidrug binding. Importantly, different photoreactive substrates labeled the same regions within P-glycoprotein suggesting that the drug-binding regions in transporters are organized within a common binding pocket. The presence of other substrates caused a significant decrease in the extent of MDRs photoaffinity labeling indicating the possible competition for the same binding site (34).

Substrate capture within phospholipid bilayer by MDRs must prevent drugs from reaching their targets located in the cytoplasm. Two possible mechanisms could result in a decrease in drug concentration within the cell. First, the substrate is captured within the inner or outer leaflet of the cytoplasmic membrane and then released into the external medium. Alternatively, the substrate is captured within the inner leaflet and then drugs are moved from the inner to the outer leaflet, followed by equilibration with the aqueous phase (35, 36). Accumulated experimental data indicate that both mechanisms are utilized by various MDRs.

Bolhius *et al.* showed that LmrA expels the fluorescent substrate TMA-DPH from the cytoplasmic leaflet of the plasma membrane into the extracellular aqueous medium (37). Similar conclusion was reached for the transport of Hoechst 33342 by the human multidrug transporter P-glycoprotein (28) and lactococcal multidrug transporter LmrP (27) as well as for staphylococcal QacA transporter (29). However, whether these transporters release their substrates in the outer leaflet of the cytoplasmic membrane or pump them directly into the extracellular medium remains unclear.

A growing body of evidence indicates that in contrast to MDRs described above, the RND-type bacterial transporters such as AcrB bind their substrates in the outer leaflet of the cytoplasmic membrane. AcrB and its homologs provide the cell protection on both the cytosolic and the periplasmic sides of the inner membrane. Several lines of experimental evidence support this notion. First, these MDRs efficiently expel some beta-lactam antibiotics whose target is located in the periplasmic space rather than in cytoplasm (22, 38). In addition, some transported drugs were shown not to traverse the cytoplasmic membrane. Secondly, mutagenesis and chimeric protein studies showed that the periplasmic domains of the RND-type MDRs define the substrate specificity of these transporters (39-41). Taken together these data suggest that RND-type multidrug transporters establish the gradient of substrate concentration not across the cytoplasmic but across the outer membrane (see also below).

Recently the mechanistic diversity of MDRs has further expanded. Identification of bacterial MDR transporters capable of extruding aminoglycosides, such as AcrD from *E. coli* (42) and MexY from *P. aeruginosa* (43) suggested that the drug capture within the phospholipid bilayer is not the only drug-binding mechanism. Aminoglycosides are highly hydrophilic antibiotics carrying multiple positive charges. In this case the substrates do not partition into the membrane and possibly they are captured directly from the periplasm or cytoplasm. A similar observation was also made with human MRP1 and related multidrug transporters, which have specificity for strictly hydrophilic drugs, e.g. anionic carboxyfluorescein derivatives (44). Interestingly, these transporters along with hydrophilic drugs are also capable of expelling lipophilic compounds. Perhaps these transporters have multiple drug-binding sites to capture drugs from different locations within the cell (43). Or alternatively, the topology of these transporters is such that both hydrophilic and lipophilic compounds can gain an access to drug-binding sites. In the latter case, the drug-binding cavity could be located on the water:lipid interface.

### 3.2. Structural studies

The ultimate answer to the question of multidrug binding and recognition awaits the high-resolution structures of MDRs in the substrate-bound state. Membrane proteins are in general very difficult to crystallize. Presently, high-resolution structures are available only for a limited number of transporters. However, MDRs are not the only proteins capable of multidrug recognition. The ability of interaction with multiple structurally unrelated compounds has arisen many times during evolution. Examples include xenobiotic receptors in eukaryotic cells such as the human pregnane X receptor (PXR) (47). Among soluble proteins, a number of transcriptional regulators of the MDRs' expression (45, 46) were also demonstrated to be multidrug specific proteins. The structural determinants of multidrug recognition are hypothesized to be similar in MDR transporters and soluble proteins. The structures of the *E. coli* multiple antibiotic resistance repressor, MarR, the *Bacillus subtilis* BmrR activator and the *Staphylococcus aureus* QacR repressor have been determined (reviewed in 48). All three were crystallized in ligand-bound form. Among these proteins the structure of QacR was solved with six different drugs (45). These structural studies revealed a number of features that will probably be shared by the multidrug transcriptional regulators and by MDR efflux pumps. All these proteins may possess a sizeable drug-binding pocket that is exceptionally rich in aromatic residues and contains one or more charge-neutralizing acidic residues. Structures of QacR-drug complexes also showed a remarkable feature: a large drug-binding pocket contains several partially overlapping binding sites (45). Such structure is ideal for the accommodation of numerous structurally diverse compounds.

The major advances in structural studies of MDRs are highlighted by recently solved crystal structures of two *E. coli* transporters, a lipid A transporter MsbA and a multidrug transporter AcrB (49, 50). Although MsbA is not MDR, this transporter shares a high degree of homology with other ABC-type transporters including MDRs such as human P-glycoprotein and LmrA transporter from lactobacteria. Besides being an MDR transporter, AcrB also is the first proton-motive force-dependent

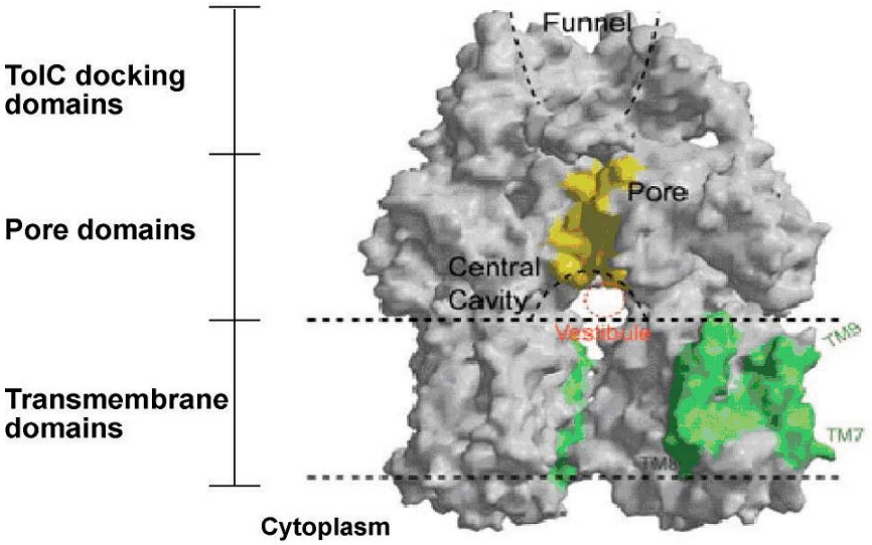


**Figure 1.** A cutaway view displaying the solvent accessible surface of AcrB (reproduced with permission from Dr. A. Yamaguchi). The transmembrane domain of AcrB trimer and the periplasmic domain (TolC docking domains and pore domains (shown in yellow)) are indicated. Possible proton translocation pathway is colored in green (transmembrane helices TM7 and TM9). The large central cavity is proposed to be multidrug binding site. Once collected in the central cavity, drugs could be actively transported through the pore into the outer membrane channel.

secondary transporter, for which the crystal structure is available. Importantly, these two structures represent transporters that bind their substrates on different sides of the cytoplasmic membrane: MsbA binds lipid A in the inner leaflet of the cytoplasmic membrane and then presumably flips it into the outer leaflet, whereas AcrB binds the substrates in the outer leaflet of the membrane.

There are some similarities in MsbA and AcrB structures. Both are oligomers: AcrB is a trimer, MsbA is a dimer. The dimeric structure of MsbA is supported by the finding that functional unit of its homolog LmrA is a dimer (37). The trimeric structure of AcrB awaits further verification. It is possible that the functional unit of this protein is a monomer or a dimer. Recently discovered cluster of transporters MdtABCD from *E. coli* contains two RND-type proteins, MdtB and MdtC. These transporters appear to function as heteromultimers since resistance to novobiocin is achieved only when both transporters are present in the cells (51).

In both AcrB and MsbA the transmembrane domains are not tightly packed. MsbA contains a large cone-shaped chamber in the interior of the molecule. This chamber is formed between two angled blocks of six alpha-helices that correspond to the two transmembrane domains and is closed at the extracellular face of the membrane (and, hence, does not appear to form a pathway across the entire bilayer). However, the chamber has two substantial ~25 Å openings on either side facing the lipid bilayer. These openings provide free access of substrate from the cytoplasmic leaflet of the lipid bilayer while excluding molecules from the outer leaflet. The chamber is proposed to be the site for substrate binding. In agreement with this notion are the cysteine accessibility studies of LmrA (52). The transmembrane domains of the LmrA transporter appear to form, under nonenergized conditions, an aqueous chamber within the membrane, which is open to the cytoplasm.

A large hole in the transmembrane portion of AcrB is open to both the cytoplasmic and the periplasmic sides of the membrane (Figure 1). The drug molecules could easily diffuse in both directions. The distinctive feature of AcrB structure is the presence of two large hydrophilic domains protruding 7 nm deep into the periplasm. These periplasmic domains of AcrB trimer form a central cavity on the lipid:water interface of the membrane, which could be the substrate-binding pocket. There is also a pore spanning the periplasmic domain of AcrB, which is proposed to be a route for the drug to reach the outer membrane channel. Two putative pathways for substrate entry into AcrB pore are seen. Murakami *et al.* (2002) proposed that substrates located in the cytoplasm or inner leaflet of the membrane might be transported across the membrane through the transmembrane groove at the periphery of each transmembrane domain. Then they might be collected in the central cavity. Substrates located on the outer surface of the membrane could gain access to the cavity through the vestibules open into the periplasm. They would then be actively transported through the pore into the outer membrane channel (49).

The structural features of AcrB are in agreement with the previous studies and point onto ability of AcrB to protect the periplasm from the action of such antibiotics as beta-lactams. Recent mutagenesis studies are consistent with the hypothesis that some drugs can gain access to the drug binding site from the periplasm. Spontaneous mutations in the periplasmic loops of MexD from *P.*

*aeruginosa* have enabled this transporter to expel the normally non-transported by MexD beta-lactam antibiotic carbenicillin (41). Furthermore, these mutations also had a substantial impact on the transport of other numerous substrates of MexD. In agreement are results of the chimeric analysis of AcrB and its homolog from *P. aeruginosa* MexB (39). We found that the first 60 N-terminal amino acid residues of AcrB/MexB are important for transport of numerous substrates of these transporters. The periplasmic portion of this region lines the central cavity in AcrB structure (Figure 1). The second region containing structural determinants of substrate recognition is entirely located in the C-terminal periplasmic loop of AcrB/MexB. The role of periplasmic loops in drug recognition was specifically targeted in the recent study by Elkins and Nikaido (40). Researchers constructed AcrD chimeras in which the periplasmic loops were replaced with the corresponding loops of AcrB. Such constructs provided resistance to AcrB substrates at levels similar to native AcrB. Conversely, AcrB chimeras containing both loops of AcrD conferred resistance only to the typical substrates of AcrD. Researchers came to the conclusion that substrate recognition (and presumably binding) is determined largely by the two periplasmic loops. Taken together all these data imply that RND-type multidrug transporters do not transfer substrates across the inner membrane but bind their substrates on the periplasmic side of the inner membrane.

If all substrates are bound by AcrB and its homologs on the periplasmic side of the membrane, these proteins represent a new class of transporters, which create the gradient of substrate concentration across the outer membrane (see below). Does this mechanism apply to all members of RND superfamily of proteins? Heavy metal transporters belonging to RND superfamily presumably transport cations across the inner membrane (53). But these transporters also require for their activity the periplasmic membrane fusion proteins (MFPs). Perhaps all RND-type transporters, which function in cooperation with MFPs, reduce the concentration of substrates in the periplasm. RND transporters from Gram-positive and eukaryotic cells are poorly characterized, in many cases their biochemical activities are unknown. However, some RND-type transporters appear to be involved in transport of proteins (8). Examples include SecDF associated with the general secretory protein complexes of bacteria (54) and eukaryotic proteins involved in sterol homeostasis such as Patched and Dispatched (55). One could imagine that these transporters are responsible for the release of proteins from the outer leaflet of the cytoplasmic membranes.

The possible substrate binding in the periplasmic domains of RND-type transporters illuminates the question how substrate translocation is coupled to the proton transfer across the membrane. Proton transfer activity has been confirmed for heavy metal transporter CzcB and AcrB (24, 53). In latter case, the proton transport activity was stimulated by the presence of antibiotics. The possible amino acid candidates for the proton-translocating pathways were identified by site-directed mutagenesis of CzcB, AcrB and MexF (49, 53, 56). The amino acid residues Asp 407, Asp 408 and Lys 940 are located in the middle of TM 4 and TM10, and form ion pairs. Mutations in these amino acid residues lead to complete loss of protein functionality. Reconstitution studies of CzcA showed that D407A mutant although incapable of transporting substrate against the gradient still was able to promote passive diffusion of substrate across the membrane (53). These studies suggested that Asp407 is a part of proton-translocating channel and that the substrate channel is independent of proton. One possible mechanism how proton translocation is coupled to efflux of drugs is that the protonation/deprotonation of the transmembrane amino acid residues results in the conformational change leading to closing of the vestibules in the periplasmic domains of RND transporters and opening the pore. Then drug molecules trapped insight the central cavity could diffuse through the pore into the outer membrane channel. Further studies are needed to understand this mechanism of coupling.

## 4. DRUG TRANSPORT ACROSS THE OUTER MEMBRANE

### 4.1. Intermembrane complex assembly

The ability of RND-type MDRs to reduce periplasmic concentration of some drugs strictly depends on the accessory proteins. If RND-type MDRs do not transfer the substrates across the cytoplasmic membrane, the function of accessory proteins is crucial to ensure that none of the captured drug molecules escape back into the periplasm. Indeed, genetic studies showed that mutations in the periplasmic MFPs and the outer membrane channels lead to complete loss of multidrug resistance in Gram-negative bacteria (5).

The best characterized examples of these proteins are the periplasmic AcrA and MexA and the OM channels TolC and OprM. These proteins form complexes AcrAB-TolC and MexAB-OprM in *E. coli* and *P. aeruginosa*, respectively. Whereas all three genes encoding MexAB-OprM are arranged in a single operon and probably expressed from a single mRNA, gene encoding TolC is located apart from the *acrAB* operon. Thus, the *tolC* gene is regulated and expressed independently from the other two components of the complex.

The interactions within AcrAB-TolC and MexAB-OprM complexes are highly specific. Despite 70% identity shared by AcrB and MexB transporters the corresponding accessory proteins cannot be interchanged between these two complexes (39). It is particularly surprising for the OM channels (Figure 2). TolC is found to interact with a myriad of transporters in *E. coli*. Among its counterparts are protein secretion complex HlyBD and colicin V exporter CvaAB (57, 58). Furthermore, TolC supports the function of MexCD and MexXY, the MDRs from *P. aeruginosa,* when they are expressed in *E. coli* (59, 60). Although, MexCD is produced by *P. aeruginosa* in the complexes with the specific OM channel OprJ, this pump can also function with OprM (61). The latter is also required for the activity of MexXY in *P. aeruginosa* cells (43). In contrast, when MexAB is expressed in *E. coli* without OprM, it remains non-functional indicating that

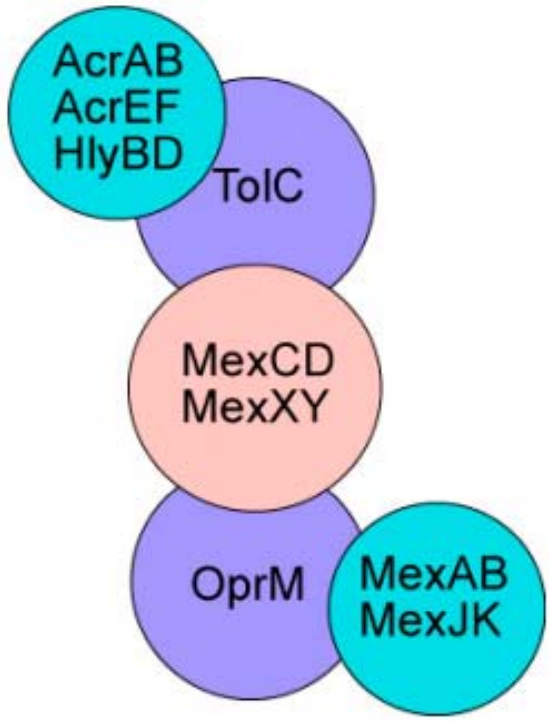


**Figure 2.** Overlap of TolC and OprM in interactions with the IM bi-partite complexes. TolC from *E. coli* and OprM from *P. aeruginosa* are multifunctional outer membrane channels. Both can support the activities of multiple transporters with various substrate specificities. In contrast, TolC and OprM cannot be interchanged in constitutively expressed multidrug AcrAB and MexAB complexes, respectively.

TolC cannot complement OprM (39). Similarly, the TolC-deficient *E. coli* cells transformed with OprM-containing plasmid remain highly susceptible to antibiotics. Among all named above transporters only AcrAB-TolC and MexAB-OprM are always expressed in cells. The constitutive expression of these MDRs might be the reason of highly demanding specificity of protein interactions within the complexes.

Although for many transporters periplasmic MFPs cannot be interchanged between different complexes recent studies suggest that in *E. coli* the AcrA protein can function with several RND pumps. Elkins and Nikaido provided evidence that AcrA forms complex with AcrD transporter (40). Interestingly, AcrA-AcrD interaction is required to expel hydrophobic molecules pointing onto involvement of TolC in this process. On the other hand, AcrA was not required for transport of hydrophilic aminoglycosides by AcrD. AcrA was also found to complement the function of AcrE protein, which is the MFP component of AcrEF-TolC pump in *E. coli* (62). In contrast, highly homologous MexA and MexC proteins from *P. aeruginosa* cannot replace each other in the corresponding complexes (63).

The architecture of tri-partite MDR complexes from Gram-negative bacteria remains unknown. Complexes containing the IM components AcrA-AcrB and MexA-MexB were detected in the membrane fractions of *E. coli* cells treated with amine reactive cross-linkers (39, 64). These complexes seem to assemble independently from the outer membrane channels TolC or OprM. The MDR complexes containing all three components were not detected using *in vivo* cross-linking approach. Similarly, the IM components of the hemolysin secretion machinery HlyD and HlyB were shown to exist in a pre-formed complex in the IM independent of the translocating substrate hemolysin and the OM channel TolC (58). But in contrast to the MDRs, engagement of TolC was demonstrated for both HlyBD-TolC (58) and colicin V exporter CvaAB-TolC (65). Thanabalu *et al.* showed that the assembly of the HlyBD-TolC complex requires active transport event. These studies suggested that this tri-partite complex is stabilized by the amino acid chain of the translocating hemolysin and as a result the complex exists only transiently. Studies of the protein secretion complexes also suggested that periplasmic MFPs are responsible for the recruitment of OM protein (see also below).

The substrate-dependent complex formation might also take place in three-component MDRs. However, small drug molecules are unlikely to play the critical role in complex stabilization. Although MFPs mediating protein secretion and drug efflux are homologous proteins, the IM transporters in these assemblies belong to different superfamilies of proteins. The RND-type MDRs, unlike the ABC transporters such as HlyB and CvaB, possess large periplasmic domains, which protrude into periplasm and might directly interact with OM channels. The periplasmic headpiece of AcrB trimer was proposed to constitute the TolC docking domain with the funnel-like structure (Figure 1) (49). The top of AcrB and the bottom of TolC fit well with each other by manual docking. Six vertical hairpins at the top of AcrB trimer are proposed to contact with the six alpha-helix-turn-alpha-helix structures at the bottom of TolC (Figure 3) to form a tight seal. But experiments to demonstrate such direct interaction by co-precipitation or co-purification approaches have failed so far (unpublished data).

The direct interactions between AcrB and the periplasmic AcrA are confirmed by co-immunoprecipitation approach (unpublished data). Furthermore, using chimeric analysis we identified the region of RND transporters responsible for the specificity of interaction with the accessory proteins (39). Amino acid residues from both periplasmic loops contribute to the recognition of MFPs. In particular, the short stretch of 22 amino acids V590-V612 located in the C-terminal periplasmic loop appears to be important in complex formation. On the crystal structure of AcrB these residues are part of a deep cleft along to the outer surface of the AcrB periplasmic headpiece (49). This cleft is proposed to be a binding site for AcrA.

### 4.2. Outer membrane channels

These proteins form a distinct family of proteins specific for Gram-negative bacteria, the Outer Membrane Factor (OMF) family (10). All members of this family are

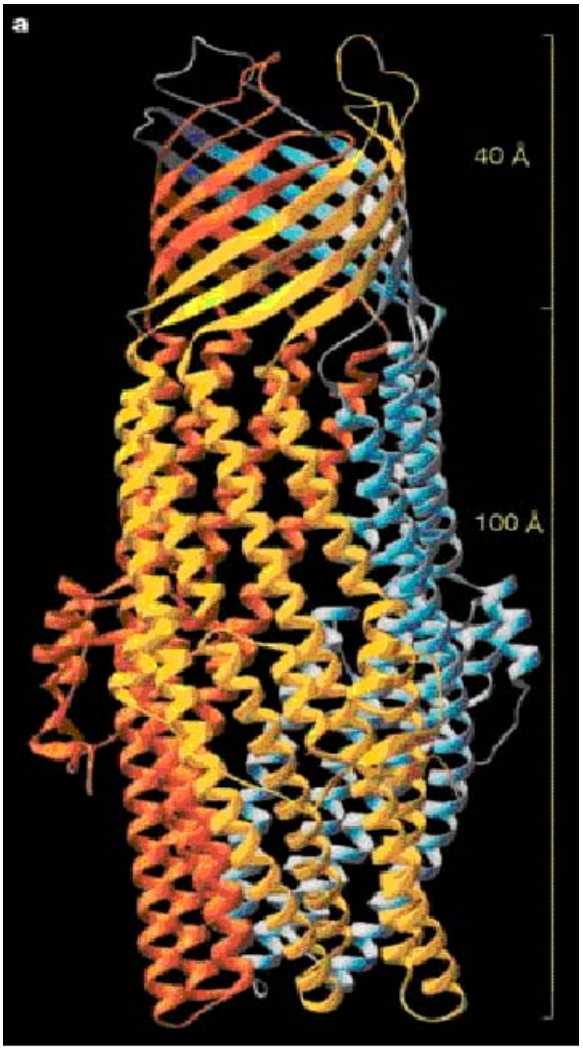


**Figure 3.** The overall architecture of TolC (reproduced with permission from Dr. V. Koronakis). The protomers are individually colored. The embedded into OM beta-barrel is at the top end. The large periplasmic domain is at the bottom end. The diameter of TolC bottom end is about the same as that of the TolC docking domain of AcrB (Figure 1 and (49)). The periplasmic entrance of TolC is constricted by helical two-stranded coiled coils.

believed to function as channels that facilitate translocation of diverse substrates across the OM. They also serve as receptors for bacteriophages and are required for penetration of bacteriocins into cells (66-68). The crystal structure of TolC, the best studied member of this family, has been solved (69). The structure of trimeric TolC represents a new class of protein fold (Figure 3). This protein contains two domains: the embedded into the OM beta-barrel and the large periplasmic alpha–helical domain. The large periplasmic domain is unique among characterized OM proteins. Furthermore, in contrast to other OM channels such as general porin OmpF or ligand-gated channel FepA (67), in which beta-barrel is formed by amino acid residues from a single protomer, in TolC structure all three protomers contribute residues to a beta-barrel. How the TolC-like proteins are assembled in the OM remains unknown. Judging by relatively efficient extraction of TolC from OM by non-ionic detergents, this protein is not linked to the peptidoglycan layer underlying the OM of the Gram-negative bacteria.

The average accessible interior diameter of the single central pore of TolC is 19.8 Å (30 Å measured from backbone to backbone) (69). The extracellular side of channel is constitutively open. The periplasmic entrance of TolC is constricted by helical two-stranded coiled coils and has an effective diameter of only ≈3.9 Å. This small opening is consistent with electrophysiological characterization of TolC assembled in planar lipid bilayers, which has shown that TolC generates only a small conductance of about 80 pS in 1 M KCl (70). The mechanism of TolC opening remains unclear. Conductivity of TolC is strongly affected by charged amino acid residues, which form bonds on the distant end of TolC entrance. Mutagenesis studies combined with the electrophysiological characterization of mutant TolC protein showed that weakening these bonds relaxes the helical arrangement, increasing the flexibility of the entrance and opening the constriction (71-73). Authors propose that *in vivo* the opening of TolC is triggered by interaction with the periplasmic MFPs. This interaction could initiate destabilization of the TolC entrance network by disturbing one or more critical links.

As discussed above, in the MDR complexes IM transporters might be in direct contact with the OM channels and be responsible for opening of channel. Interestingly, TolC mutants with the periplasmic entrance wide open remain functionally competent (72). Furthermore, *E. coli* cells expressing the constitutively open TolC remain drug resistant. This finding suggests that in the cells TolC is engaged into some sort of stable interactions, which efficiently block this channel. Otherwise, the presence of such open channels in the OM would substantially compromise the permeability properties of the OM and thus lead to increased drug sensitivity independently of AcrAB-mediated drug efflux. Indeed, *E. coli* cells expressing the components of the hemolysin secretion machinery HlyBD displayed higher susceptibility to antibiotic vancomycin (74). This increased susceptibility was proposed to be a result of a pore formed by TolC in the OM, which is extended through the periplasm by the periplasmic MFP HlyD. Through this pore in the OM hemolysin was proposed to be released and vancomycin taken up into cells. Vancomycin attacks the process of peptidoglycan synthesis. Thus, the HlyD-TolC channel appears to leak into periplasm.

The mutagenesis studies of TolC and its homolog from *P. aeruginosa* OprM confirmed that the *in vivo* structure of these proteins similar to that in crystals (75, 76). Several functionally important regions have been identified in these studies. The C-terminal domain appears to be crucial for TolC functionality. Indeed, a single substitution of Leu412 on polar amino acid residue completely abolished the function. The role of this residue remains unclear but mutated protein is still targeted into

OM and appears to form normal trimers (75). On the TolC crystal structure this residue is located in alpha-helix H9. The H9 helix is a part of the equatorial domain of TolC, which form a "strap" around the mid-section of the periplasmic helical barrel (Figure 3). This domain was proposed to be a site of interaction with the periplasmic MFPs of complex. However, deletion of this H9 helix as well as sizeable insertions in this region of OprM does not affect the function of the protein (76). These results indicate that this region of the equatorial domain of OprM/TolC is unlikely to participate in protein-protein interactions.

The structural determinants of TolC important for the export of proteins but not for the translocation of small molecules were identified by genetic screens. A number of residues located in the beta-barrel OM domain and the entrance of TolC on the periplasmic side appears to be essential for the export of functional hemolysin (77). The mutations in these residues, however, had no effect on the drug resistance of *E. coli* strains producing mutated TolC. This result suggests that drugs and protein substrates do not share the sites of interaction within the TolC channel.

### 4.3. Periplasmic membrane fusion proteins

The crystal structure of MFPs is not currently available. The hydrodynamic studies showed that purified AcrA is a monomeric, highly asymmetric molecule in solution (78). The direct visualization of AcrA by lipid-layer crystallization techniques confirmed this asymmetric shape of AcrA (79). This study also showed that AcrA retains its asymmetric shape even when bound to lipids.

The hypothetical mechanisms of MFPs' function postulate the interaction with both, the inner and the outer membranes. The elongated shape of AcrA (about 21 nm) is consistent with the possibility that AcrA spans the periplasm and forms a connection between the IM and OM (78). There are little doubts that MFPs interact with the IM through their N-termini. AcrA and its homologs have the N-terminal lipid modification, which anchors these proteins into IM. Furthermore, members of MFP family that interact with ABC transporters, such as HlyD do not undergo processing in the periplasm and possess a single N-terminal transmembrane domain (80). These transmembrane domains play important role in complex assembly and function (see below). On the other hand, besides lacking the TMSs, AcrA or MexA retain their functions even without lipid modification (78, 81). These studies demonstrate that anchoring into bilayer through the lipid moiety is not the only mode of AcrA/MexA association with IM. Additionally, the specific interactions with MDR transporters keep these proteins in the IM.

The association with the OM is more controversial. A highly conserved approximately 100-residue domain near the C-terminus is suggested to be the region of interaction with OM (9). Site-directed mutagenesis of amino acid residues of HlyD and CvaA located in this domain confirmed its functional importance (65, 82). However, the question whether these mutations affect association of MFPs with OM was not addressed in these studies. HlyD was found to co-precipitate with both the IM and OM, after membrane separation, whereas a truncated HlyD lacking the last 10 amino acid residues was exclusively associated with the IM (83). Similarly, AcrA was found to associate with both membranes, with the distribution between the OM and IM being 30% and 40% correspondingly (unpublished data). The truncated unlipidated AcrA protein lacking the C-terminal 100 amino acid residues was completely recovered in the periplasmic fraction indicating that C-terminal deletions in this protein severe the ability to associate with either membrane. AcrA association with OM does not depend on the presence of TolC protein since in mutants lacking this protein AcrA still co-precipitates with the OM. However, in the absence of the IM transporter AcrB, AcrA was predominantly associated with the IM. This result suggested that interaction with AcrB might affect the conformation of AcrA in a way to promote binding of this protein to the OM.

The mechanism of intermembrane complex assembly of multidrug efflux pumps remains unclear. The accumulated data indicate that this mechanism is different for the protein exporters and transporters of the small molecules. The HlyBD-TolC secretion complex is short-lived and assembled during the process of substrate translocation (58). The translocating hemolysin at some point during secretion spans both the inner and the outer membranes and thus could stabilize the protein-protein interactions in the secretion complex. In contrast, small drug molecules are unlikely to play a role in complex assembly. Interestingly, mutagenesis studies suggested that substrate engagement by the HlyB transporter alone is insufficient to trigger TolC recruitment, and that substrate binding to the HlyD cytosolic domain is essential (84). Such recruitment mechanism, however, cannot be realized, for example, by AcrAB-TolC. AcrA and its close homologs involved in transport of small molecules do not have cytosolic domains, which could be involved in recruitment of TolC channel. These differences in complex formation might be attributed to structural features of the IM transporters. Large periplasmic loops of RND-transporters might be in direct contact with OM channels and be responsible for complex specificity and assembly. In such scenario MFPs could play a role in the stabilization of interactions between IM transporter and OM channel.

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