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MINIREVIEW

Molecular architecture of the bacterial tripartite multidrug efflux pump focusing on the adaptor bridging model

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Gram-negative bacteria expel a wide range of toxic substances through tripartite drug efflux pumps consisting of an inner membrane transporter, an outer membrane channel protein, and a periplasmic adaptor protein. These pumps form tripartite assemblies which can span the entire cell envelope, including the inner and outer membranes. There have been controversial findings regarding the assembly of the individual components in tripartite drug efflux pumps. Recent structural and functional studies have advanced our understanding of the assembly and working mechanisms of the pumps. Here, we re-evaluate the assembly models based on recent structural and functional studies. In particular, this study focuses on the 'adaptor bridging model', highlighting the intermeshing cogwheel-like interactions between the tip regions of the outer membrane channel protein and the periplasmic adaptor protein in the hexameric assembly.

Keywords: multidrug resistance, Gram-negative bacteria, multidrug efflux pump, structure, TolC

Introduction

Microorganisms can tolerate various toxic chemicals and antibiotics. To cope with these environmental stresses competing bacteria have developed sophisticated export mechanisms and thus acquire resistance against the noxious compounds. Gram-negative bacteria are known to possess numerous resistance mechanisms, including antibiotic inactivation, modification of the antibiotic target site, reduced permeability of the outer membrane, and use of multidrug efflux pumps (Walsh, 2000; Coates *et al.*, 2002). In particular, multidrug efflux pumps located in the membranes rapidly expel a broad range of substances including antibiotics, dyes, detergents, toxic compounds, and organic solvents, conferring a high level of intrinsic antibiotic resistance in many organisms (Putman *et al.*, 2000; Paulsen, 2003). Based on features of the amino acid sequence and structure, active bacterial efflux systems are typically classified into five categories (Marquez, 2005): the major facilitator superfamily (MFS), the ATP-binding cassette family (ABC), the resistance-nodulation-division family (RND), the multidrug and toxic compound extrusion family (MATE), and the small multidrug resistance family (SMR). Since the envelope of Gram-negative bacteria has an outer membrane associated with lipopolysaccharides (Silhavy *et al.*, 2010), RND, ABC, and MFS transporters in Gram-negative bacteria require other two essential components to form tripartite systems (Du *et al.*, 2015).

Tripartite efflux systems comprise the inner membrane transporter (IMT), an outer membrane protein (OMP), and a periplasmic 'membrane fusion protein' (MFP) or adaptor protein (Du *et al.*, 2015). The IMT transports various substrates by means of a proton gradient (for RND and MFS transporters) or ATP hydrolysis (for ABC transporters), and the OMP provides a continuous conduit through the outer membrane (Eswaran *et al.*, 2004; Hinchliffe *et al.*, 2013). The MFP was initially designated as a low sequence homology to viral membrane fusion proteins responsible for the fusion of viral and cellular membranes (Thanabalu *et al.*, 1998). However, periplasmic 'adaptor protein' is a more appropriate term to define the function of these proteins in the tripartite pumps, because it functions to connect the IMT and OMP in the periplasm. Recently, significant advances in the structural biology of tripartite efflux pumps have been achieved, which expand our understanding of the assembly of the pumps. Nowadays, the assembly of the tripartite pumps is still a topic in active debate (Du *et al.*, 2015; Yamaguchi *et al.*, 2015). In this study, the structural organization of tripartite drug efflux pumps is plausibly described by the 'adaptor bridging model'.

Outer-membrane protein: an exit channel spanning the outer membrane

OMPs in the tripartite efflux pumps play a role in the final exit of substrates through the outer membrane of Gram-negative bacteria (Gerken and Misra, 2004). These proteins are also involved in the export of small molecules, heavy

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metals, and protein toxins, in addition to toxic compounds (Koronakis *et al.*, 2004). Enteric bacteria such as *E. coli* and *Salmonella* species contain *tolC* as a global OMP, which functions with several multidrug efflux pumps, and *Pseudomonas* species contain several copies of TolC homologs (OprM, OprJ, and OprN). These TolC homologs work together to provide broad and overlapping substrate specificities and affect pathogenicity of Gram-negative bacteria (Hinchliffe *et al.*, 2013). Based on the defined substrates, function of OMP families are generally determined: multidrug efflux (e.g., TolC, OprM), cation efflux (e.g., CusC) and protein export (e.g., type I secretion system) (Hinchliffe *et al.*, 2013).

OMPs commonly exist as homotrimers reminiscent of a long cylinder with a central channel. The cylinder is formed by a 12-stranded β-barrel in the outer membrane-traversing region and a long α-barrel largely composed of 12-α-helices in the periplasmic region (Akama *et al.*, 2004a; Koronakis *et al.*, 2004). The mixed α/β extended equatorial domain forms an outer belt around the periplasmic α-barrel domain (Fig. 1). However, no significant sequence homology was found in the equatorial domain (Kim *et al.*, 2008).

TolC is composed of 471 amino acids excluding the signal peptide, and the crystal structure revealed a homotrimeric assembly forming a long cylinder of 140 Å in length (Koronakis *et al.*, 2000). The TolC trimer contains a 12-stranded right-twisted β-barrel (~40 Å) and an α-barrel with 12 α-helices or 6 coiled-coil α-hairpins (~100 Å) (Koronakis *et al.*, 2000). Each α-hairpin is composed of a long helix (67 residues) and a pair of short helices (23 and 34 residues) that stack to produce a pseudo-continuous helix. The two short helices are connected by the equatorial domain, which provides the structural flexibility of the α-hairpin structures. This cylinder is open to the outside of the cell at the β-barrel end region. Although the α-barrel forms a cylindrical channel with an internal diameter of 35 Å, it is closed at the periplasmic α-barrel end by an approximately 3.9-Å diameter coiled-coil α-hairpin structure composed of highly conserved motifs (Koronakis *et al.*, 2000).

Highlighting a channel-closing feature at the periplasmic end region of the TolC cylinder, the inwardly curving pattern of pairs of coiled-coil α-hairpins is mediated by salt bridges between the residues in the inner helix tip regions, which results in closing of the channel (Bavro *et al.*, 2008). Channel opening of OMPs is induced by binding to the other components and may be coupled to binding to the adaptor proteins (Tikhonova *et al.*, 2009; Xu *et al.*, 2012). To date, only the partially open structure of TolC and a pseudoatomic structure of the fully open structure in the ternary complex are available (Bavro *et al.*, 2008; Trepout *et al.*, 2010; Du *et al.*, 2015; Kim *et al.*, 2015).

Inner membrane transporters

RND transporters

RND transporters are driven by a proton motive force that occurs across the inner membrane, and their crystal structures revealed a mushroom-like homotrimeric assembly composed of a transmembrane domain, a porter domain, and a docking domain (Murakami *et al.*, 2002, 2006; Murakami

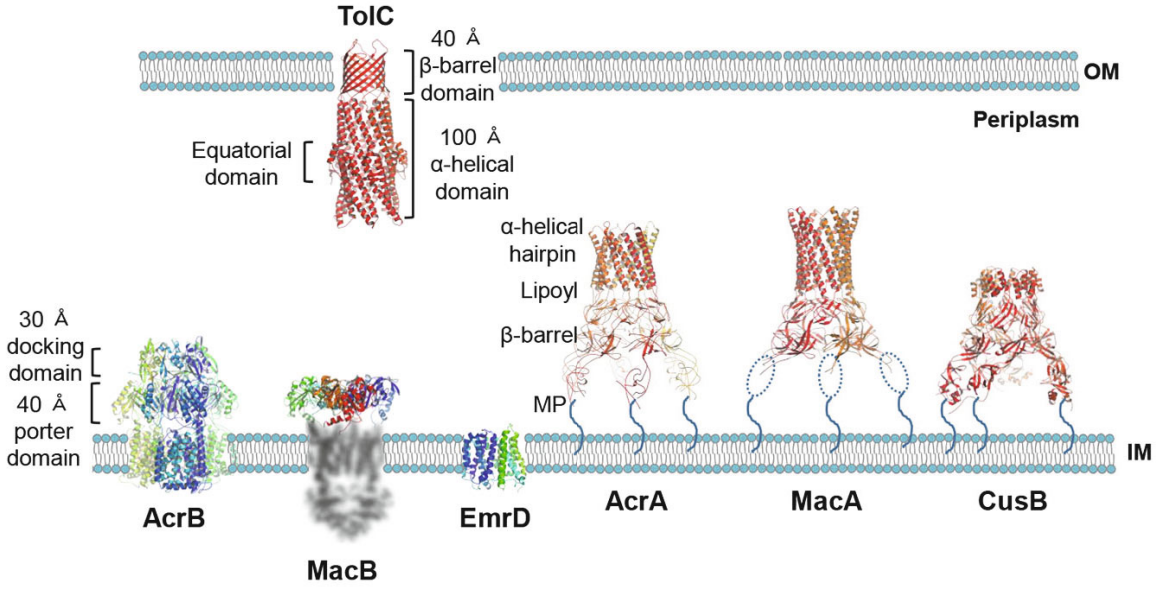

Fig. 1. Structural gallery of the individual components of tripartite drug efflux pumps. The outer membrane (OM) and inner membrane (IM) are shown schematically, and the proteins are drawn as ribbon representations: transporters; AcrB (PDB ID: 3AOA) from the RND drug efflux transporter of *E. coli*, the periplasmic region of MacB (PDB ID: 3FTJ) from the ABC transporter of *E. coli*, EmrD (PDB ID: 2GFP) from the MFS transporter of *E. coli*. adaptors; AcrA (PDB ID: 2F1M) from the AcrAB-TolC of *E. coli*, MacA (PDB ID: 4DK0) from the macrolide efflux pump MacAB-TdeA of *Aggregatibacter actinomycetemcomitans*, CusB (PDB ID: 3H94) from the heavy metal efflux pump CusBAC of *E. coli*. TolC (PDB ID: 1EK9) is the outer membrane protein of *E. coli* which is in a putative open conformation in this figure. In AcrB, the porter domain and the docking domain are indicated. The names of the domains of the adaptor proteins between AcrA and MacA are labeled.

and Yamaguchi, 2003; Nakashima *et al.*, 2011) (Fig. 1). The best-studied RND transporter is *E. coli* AcrB in the AcrAB-TolC multidrug efflux pump. The broad substrate specificity of AcrB is responsible for both intrinsic and acquired multidrug resistance, which is most commonly found in bacteria with these characteristics. In the AcrB trimer, the transmembrane (TM) domain comprises 36 α-helices (12 α-helices per each monomer) and has a central cavity filled with lipid. The porter domain is divided into four subdomains, which form two substrate-binding pockets enriched in aromatic, polar, and charged amino acids that can interact with diverse substrates (Murakami *et al.*, 2002). The docking domain, which was previously known as the 'TolC'-docking domain, is located at the top region of the periplasmic region of AcrB. A protomer of the docking domain is composed of two subdomains and displays a funnel-like shape with an internal diameter of ~30 Å (Murakami *et al.*, 2002). The substrate enters the hydrophobic binding pocket in the porter domain and exits from the center of the funnel in the docking domain (Murakami *et al.*, 2002; Murakami and Yamaguchi, 2003).

The AcrB trimer has an intrinsic asymmetry among the protomers, adopting distinct conformations–'access', 'binding', and 'extrusion'–depending on the status of the substrate-binding pocket (Murakami *et al.*, 2006; Nakashima *et al.*, 2011). The AcrB trimer can bind to only one drug molecule at a time. The proton motive force coming from the inner membrane drives the conformational change of the asymmetrical protomers during translocation of the drug molecule. Structural homologs corresponding to AcrB include MexB of *P. aeruginosa* and CusA in the CusABC heavy metal efflux pump of *E.* coli and *Salmonella* (Sennhauser *et al.*, 2009; Long *et al.*, 2010). Compared with AcrB and MexB, the CusA structure has a similar structural architecture and has multiple Cu^{+} binding sites for the efflux of the metal ions (Long *et al.*, 2010).

ABC transporters

MacB was first identified in 2001 as a macrolide-specific ABC transporter (Kobayashi *et al.*, 2001), and subsequent studies have revealed the roles of MacB in the efflux of macrolide antibiotics and the secretion of protein enterotoxin II, lipopeptides, and glycolipid (Yamanaka *et al.*, 2008; Vallet-Gely *et al.*, 2010). A recent study suggested that MacB in *E. coli* and *Salmonella enterica* expels the endogenous protoporphyrin when bacterial heme homeostasis is disrupted by iron shortage, as it happens when bacteria invade the mammalian host (Turlin *et al.*, 2014). Another study showed that MacB expels unknown compounds that can decompose reactive oxygen species (ROS), and is required for survival of *Salmonella* in macrophages (Bogomolnaya *et al.*, 2013).

Typical ABC transporters function as homodimers and operate using ATP energy. Each protomer of a typical ABC transporter has a cytoplasmic ATP binding domain, six transmembrane α-helices, and a very small periplasmic domain (Dawson and Locher, 2006). However, MacB exhibits an atypical and novel architecture including four transmembrane helices, an N-terminal nucleotide binding domain (NBD), and a large periplasmic domain, which is distinct from typical ABC transporters (Kobayashi *et al.*, 2001, 2003). According to the crystal structure of the periplasmic region of *Actinobacillus actinomycetemcomitans* MacB, the periplasmic core domain (PCD) of MacB consists of two subdomains comprising one elongated domain with mixed α and β folds (Lin *et al.*, 2009; Xu *et al.*, 2009a). However, to date there is no structural model of full-length MacB, so further studies will be necessary to explain how MacB assembles and operates with the other pump components (Xu *et al.*, 2009a). Like other ABC transporters, MacB depends on ATP substrate for the function using the free energy from ATP binding and hydrolysis in NBD at the cytoplasm (Dawson and Locher, 2006). Kinetic analysis of ATP hydrolysis and functional studies showed that MacA promotes formation of the ternary complex MacAB-TolC and stabilizes the ATP-binding form of MacB (Lin *et al.*, 2009; Modali and Zgurskaya, 2011).

MFS transporters

MFS transporters are the largest group of secondary active membrane transporters present in all phyla from bacterial to mammals and have multiple physiological functions (Pao *et al.*, 1998). MFS proteins function primarily in the uptake of sugars, although some drug efflux systems belong to this family (Pao *et al.*, 1998). Like oligosaccharide:H^{+} symport permeases, the MFS transporter in drug efflux pumps uses a proton gradient in the membrane (Pao *et al.*, 1998). However, the proton motive stoichiometry of MFS transporters is not yet completely known. MFS transporters contain 12 or 14 transmembrane helices, and usually function as a monomer with two pseudosymmetric domains that form a large central cavity (Abramson *et al.*, 2003; Huang *et al.*, 2003; Dang *et al.*, 2010). The cavity is flip-flopped between periplasm and cytosol, thereby resulting in outward-facing conformation or inward-facing conformation, respectively. Similar to the ABC transporters, the flip-flops between two conformations are considered to mediate substrate transport across the membrane. Crystal structures of three MFS antiporters showed that they possess a single binding site and an alternating access mechanism with a rocker-switch type movement of the protein (Abramson *et al.*, 2003; Huang *et al.*, 2003; Dang *et al.*, 2010). In *E. coli*, the MFS transporter EmrD contains 14 transmembrane helices and contributes to drug resistance when complexed with the adaptor protein EmrA and the OMP TolC (Fig. 1) (Pao *et al.*, 1998; Borges-Walmsley *et al.*, 2003).

Periplasmic adaptor proteins

Periplasmic adaptor proteins are absolutely necessary for assembly of the functional tripartite efflux pumps (Zgurskaya and Nikaido, 1999). For example, AcrA, the adaptor protein of the AcrAB-TolC efflux pump, is required to mediate the interaction between the two other components (Touze *et al.*, 2004). The function of the adaptor protein is to connect the IMT to the OMP in the periplasmic space. The crystal structures of *E. coli* AcrA and *P. aeruginosa* MexA in the MexAB-OprM pump show that these adaptor proteins are composed of four linearly arranged domains: membrane proximal (MP), β-barrel, lipoyl, and α-hairpin domains

(Akama *et al.*, 2004b; Higgins *et al.*, 2004; Mikolosko *et al.*, 2006). The α-hairpin domains of AcrA and MexA resemble the coiled-coil α-hairpin regions of TolC and OprM, and the connecting loop of the α-hairpin structure of AcrA plays a crucial role in binding to TolC (Kim *et al.*, 2010; Xu *et al.*, 2010, 2011a). The lipoyl domain is composed of a β-sandwich of two interlocking motifs of four β-strands (Higgins *et al.*, 2004). The β-barrel domain consists of six antiparallel β-strands capped by a single α-helix and the MP domain at the N-terminus forms a β-roll structure that is essential for anchoring into the inner membrane (Symmons *et al.*, 2009). However, the crystal structures of AcrA and MexA do not seem to directly reflect the oligomeric state of the adaptor proteins in the functional state, so it is important to reveal the assembly of the tripartite efflux pumps (Akama *et al.*, 2004b; Mikolosko *et al.*, 2006).

The first crystal structure reflecting the functional oligomer of the periplasmic adaptor proteins was that of MacA protein in the MacAB-TolC pump. MacA forms a hexameric funnel-like structure, in which six protomers are arranged by side-by-side packing (Yum *et al.*, 2009). In the MacA hexamer, the coiled-coil α-hairpins pack into a cylinder like a funnel stem, while the β-barrel and lipoyl domains form a cone like the funnel mouth. Except for the disordered MP domain, the overall structure of MacA resembles that of AcrA and MexA. It is noteworthy that the α-barrels of the MacA hexamer and the TolC trimer have the same number of α-hairpins, and thus exhibit the same dimensions of the central channel and the same inclination of the helices along the central axis of the channel (Fig. 1) (Yum *et al.*, 2009).

It was noted that the connecting loop (or hairpin tip region) between the two helices of α-hairpin of MacA has high sequence conservation with that of AcrA and MexA (Yum *et al.*, 2009) although the coiled-coil α-hairpin of MacA protein is 11 Å long with six heptad repeats per helix, which is longer than that of AcrA (9 Å long with five heptads) and MexA (7 Å long with four heptads) (Fig. 1). Mutational and biochemical analyses proved that the conserved residues (Arg131, Leu135, and Ser142) of the hairpin tip of *E. coli* MacA play a crucial role in the binding of TolC (Xu *et al.*, 2010, 2011b). This suggested that the hairpin tip of the adaptor protein plays an important role in binding to the OMPs (Xu *et al.*, 2009a, 2010, 2011b).

EmrA, the adaptor protein of the MFS efflux pump EmrAB-TolC, contains an α-helical hairpin that is 127 Å long with 11 heptad repeats per helix, a lipoyl domain, and a β-barrel domain, but lacks a MP domain compared to all identified RND and ABC adaptors (Hinchliffe *et al.*, 2014). It has been reported that EmrA can oligomerize to form dimers or trimers *in vitro* (Tanabe *et al.*, 2009). However, its stoichiometry was predicted to form a hexameric structure by in silico modeling when in the complex with IMT EmrB and trimeric TolC (Hinchliffe *et al.*, 2014).

Assembly of the tripartite multidrug efflux pumps

Extensive studies have been performed to describe how tripartite efflux pumps operate and function, and three possible models are most appealing to explain the assembly of the pumps (Yum *et al.*, 2009; Du *et al.*, 2014; Kim *et al.*, 2015). The major difference between these models is the binding mode of components and the stoichiometry of the tripartite efflux pumps. The first model is termed the 'adaptor wrapping model' in this review, because three periplasmic adaptor protomers wrap around the outside of the IMT and OMP complex that is formed by direct tip-to-tip interactions between these proteins. The second model is called the 'adaptor bridging model', in which the hexameric adaptor proteins forms a bridge between the IMT and OMP without allowing direct interaction between them. Another key feature of the adaptor bridging model is the cogwheel-to-cogwheel interaction between the MFP hexamer and the OMP trimer in a tip-to-tip manner. This is based on the same cogwheel features of the α-barrels of the adaptor hexamer and the OMP trimer, which can intermesh with each other. The last model is called the 'adaptor partial wrapping model', and is an intermediate model. The stoichiometry is same as in the adaptor bridging model, but the adaptor hexamer wraps around part of the periplasmic tip region of the OMP trimer (Yum *et al.*, 2009; Du *et al.*, 2014, 2015). However, there are continued debates over how the complex is organized and the transport process operates because of the lack of high-resolution structures of the complexes (Du *et al.*, 2015; Yamaguchi *et al.*, 2015). Therefore, further in-depth structural and functional studies are required to fully understand the assembly mode and opening of tripartite multidrug efflux pumps.

Adaptor wrapping model of AcrAB-TolC pump

The adaptor wrapping model has dominated opinions on tripartite assembly since 2002 when the AcrB structure was first solved (Murakami *et al.*, 2002). These studies emphasized the structural complementarity between the docking domain of the AcrB trimer and the periplasmic end region of the TolC trimer, and proposed the adaptor wrapping model for the AcrAB-TolC pump (Murakami *et al.*, 2002). The key feature of this model is that TolC makes direct contact with the top region of the AcrB trimer. However, the notion of physical binding between AcrB and TolC is not straightforward because simple incubation did not produce a binary complex of AcrB and TolC; instead chemical cross-linkers had to be added to observe the affinity between the components (Touze *et al.*, 2004; Tamura *et al.*, 2005). The model was further supported by subsequent detailed interaction mapping between the components based on *in vivo* site-specific cross-linking experiments in which numerous cysteine-substituted AcrA, AcrB, or TolC variants were constructed (Lobedanz *et al.*, 2007; Symmons *et al.*, 2009). The substituted cysteine residue in one protein could covalently link to a nearby lysine residue of the other protein via a chemical cross-linker that contains both cysteine and amine-reactive moieties. The interaction map was built by combining information on which residues are in close proximity to each other.

At that time, results from the cysteine-substituted cross-linking experiments looked quite convincing and this approach was considered the most accurate method for investigation of the assembly of the tripartite efflux pumps. Based on these results, the adaptor wrapping model, which

Table 1. Functional impairment of AcrB and AcrA used in *in vivo* cross-linking experiments (Lobedanz *et al*., 2007; Symmons *et al*., 2009).

Background	BW25113*ΔacrAB*				
acrA	-	wt	wt	G58C	Q196C
acrB	-	wt	C493S/C887S	C493S/C887S	C493S/C887S
MIC (μg/ml)[a] Novobiocin	12.5	800	200	100	100
Background	BW25113*ΔacrA*				
acrA	-	wt	R104C	E118C	Q112C
MIC (μg/ml)[b] Novobiocin	6.25	100	6.25	12.5	25

[a] The values were adapted from Xu *et al.* (2011a).
[b] The values were adapted from Kim *et al.* (2010).

proposes a 3:3:3 ratio of AcrB:AcrA:TolC with direct contact between AcrB and TolC, was regarded the standard model (Symmons *et al*., 2009; Yamaguchi *et al*., 2015). Many subsequent *in silico* docking and molecular dynamics studies detailed the model, expanding the OMP channel opening mechanism (Symmons *et al*., 2009; Pei *et al*., 2011; Wang *et al*., 2011; Ruggerone *et al*., 2013). Results from *in vivo* cross-linking experiments have greatly impacted subsequent research, including work on the *E. coli* heavy-metal efflux pump CusABC that appeared to oppose the adaptor wrapping model (Su *et al*., 2011). Functional and structural data favoring a 3:6:3 ratio and denying direct contact between AcrB and TolC have recently accumulated (Narita *et al*., 2003; Stegmeier *et al*., 2006; Mima *et al*., 2007; Xu *et al*., 2009a, 2009b, 2010, 2011a, 2011b, 2012; Yum *et al*., 2009; Kim *et al*., 2010, 2015; Janganan *et al*., 2011; Su *et al*., 2011; Lee *et al*., 2012, 2013, 2014; Du *et al*., 2014). Thus, the plausibility of the adapter wrapping model is now weakened (Du *et al*., 2015), although it is still supported by some researchers (Yamaguchi *et al*., 2015).

What has stymied research in the field of tripartite efflux pumps? The answer might be found in the inevitable drawbacks of chemical cross-linking experiments that have been largely ignored. Reaction of chemical cross-linkers with the lysine residue of the target protein would also be influenced by the reactivity of the lysine residue as much as the proximity. The reactivity and p*K*a value of the amino acid residues in proteins varies depending on the local concentration around the residue (Ha *et al*., 2001; Tomasio *et al*., 2013). Thus, if the remote lysine residue has a high reactivity it would be strongly cross-linked to the chemical cross-linker, leading to misinterpretation of the data. In this case, it would be challenging to distinguish this false positive signal from the real signals because the reactivity of the individual residues is not easily determined.

Moreover, the decreased or abolished functionality of AcrA and AcrB cysteine-variant proteins further affects the fidelity of results derived from *in vivo* cross-linking experiments (Lobedanz *et al*., 2007; Symmons *et al*., 2009). According to studies performed by an independent research group, the variants used for the *in vivo* chemical crosslinking experiments exhibited a severe loss-of-function phenotype (Table 1) (Kim *et al*., 2010; Xu *et al*., 2011a). These contradicting observations were somewhat surprising because Lobedanz *et al*. reported that all the variants conferred wild-type antibiotic resistance, but without showing the supporting data (Lobedanz *et al*., 2007). The chemical cross-linking experiments would be valid only if the variants used showed the same functionality as wild-type proteins. Otherwise, it would be practically impossible to know whether the results from the *in vivo* chemical cross-linking experiments were derived from a functional efflux-ready state or a non-functional assembly state.

Adaptor partial wrapping model of AcrAB-TolC pump

It has been challenging to obtain the stable ternary complex for structural analysis because of intrinsic dynamic binding between the components and the presence of detergents that can interfere with protein–protein interactions but are indispensable for stabilizing the integral membrane protein IMT and the OMPs. Very recently, two independent research groups overcame this technical issue by chimeric approaches, presenting 3-dimensional structural models using electron microscopy. The first structure was presented by Du *et al*. (2014) who devised two kinds of fusion proteins involving AcrA. In the first chimeric protein AcrA was inserted in a loop of the periplasmic domain of AcrB, whereas in the second protein AcrA was fused with AcrZ, a small accessory component that interacts with the transmembrane region of AcrB (Du *et al*., 2014). Kim *et al*. (2015) created a long chimeric protein containing one copy of AcrB, a transmembrane linker, and two copies of AcrA (AcrA-dimer). The AcrA-dimer, which was as functional as wild-type AcrA (Xu *et al*., 2011a), was designed to meet the 3:6 stoichiometry of AcrB and AcrA. The transmembrane linker was inserted between the C-terminus of AcrB and the N-terminus of AcrA to achieve the correct topology; the C-terminus of AcrB is located in the cytosolic region and the N-terminus of the AcrA dimer is at the periplasmic region (Kim *et al*., 2015).

The two groups presented essentially the same electron microscopy (EM) maps, leading to identification of drumstick-shaped particles that contradict the earlier 'adaptor wrapping model' (Fig. 2). The EM map strikingly matched the dimensions of the AcrAB-TolC pump model proposed based on the AcrA hexameric model and the intermeshing cogwheel interaction between AcrA and TolC (Xu *et al*., 2011a), in which a hexameric AcrA bridges the trimeric AcrB to the trimeric TolC without allowing direct interaction between AcrB and TolC. The EM maps were also in agreement with the homologous MexA-OprM complex from *P. aeruginosa* (Trepout *et al*., 2010; Xu *et al*., 2012) and with the 3:6 stoichiometry of the homologous CusA-CusB heavy-metal efflux complex (Su *et al*., 2011). Thus, the ternary complex models were built based on the EM maps, in which the key feature of the model is the AcrA hexamer. The AcrA hexamer resembles that of the homologous pro-

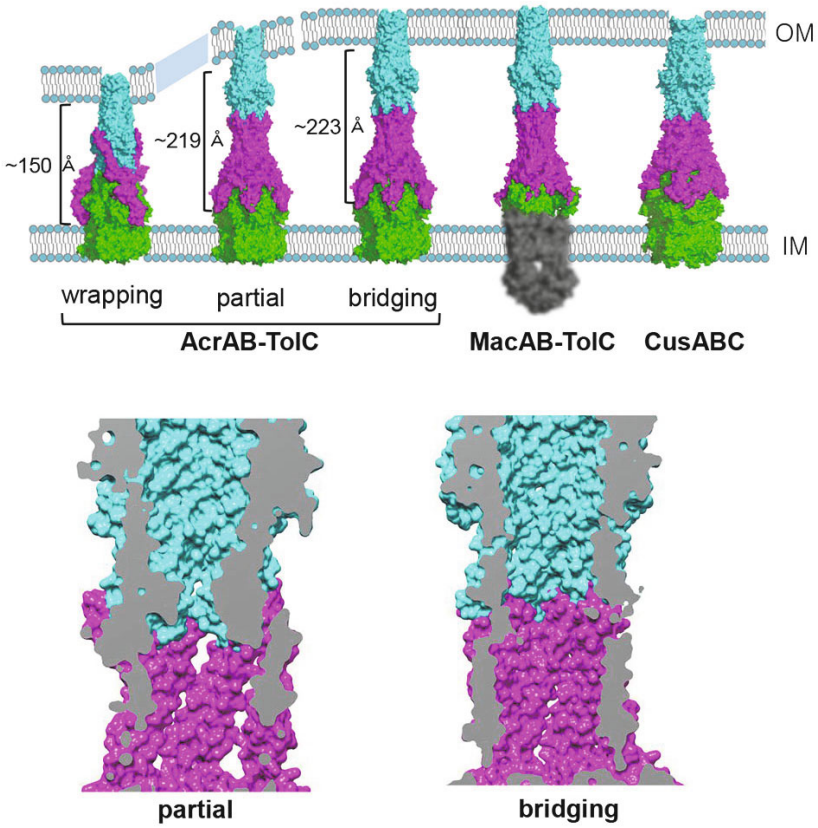

Fig. 2. Models for the tripartite drug efflux pumps. Three different complex models for the AcrAB-TolC pump (left three models), and the MacAB-TolC pump and CusABC pump, based on the adaptor bridging model. TolC or CusC are shown in cyan, the adaptor proteins (AcrA, MacA, and CusB) in magenta, and the inner membrane transporter (IMT; AcrB, MacB, and CusA) in green. Because of the lack of the full-length structure of MacB, the unknown transmembrane and cytosolic parts are shown in gray. There are also shown estimated distance (angstrom) between OM and IM spanned by each tripartite complex. Close-up views of the vertical sections of the AcrA-TolC assembly based on the adaptor partial wrapping model and the adaptor bridging model are shown at the bottom.

tein MacA, the structure of which was determined as the funnel-like hexameric form by X-ray crystallography (Yum *et al.*, 2009). In the models, the funnel mouth region formed by the lipoyl and β-barrel domain covers the docking domain of the AcrB trimer with a minimal motion. The flexible MP domains connected to the end of the β-barrel domain of AcrA are on the porter domain of the AcrB trimer. Additionally, this revealed that opening of the TolC channel is independent of the conformational change of AcrB because TolC maintains an open state regardless of the translocation stage of AcrB.

A vertical cross-section of both EM maps revealed a long conduit that runs from the AcrB substrate exit pore at the center of the docking domain to the transmembrane β-barrel of TolC through the α-barrels of the AcrA funnel stem and the TolC periplasmic region. The EM map at ~16 Å resolution produced by Du *et al.* (2014) showed a pore with an internal diameter of 34 Å at the docking interface of AcrA and TolC, allowing all substrates of the pump to pass through the central conduit of the complex. However, there is a marked difference between the two groups in the explanation of the binding interface of AcrA and TolC. In the former model, the AcrA α-barrel structure shows a flared conformation mimicking the α-hairpin end region of CusB structure complexed with CusA (Su *et al.*, 2011), which leads to widening of the AcrA α-barrel. The TolC α-barrel is partially inserted into the wider AcrA α-barrel and must therefore be narrowed at the binding interface between AcrA and TolC. Because AcrA partially wraps TolC, the model proposed by Du *et al.* was named the 'adaptor partial wrapping model' (Kim *et al.*, 2015) (Fig. 2). However, the narrowed channel at the binding interface was not consistent with the EM density maps from Du *et al.* (2014) and Kim *et al.* or with previous EM maps using the MacA chimeric proteins (see below) (Xu *et al.*, 2011a, 2011b, 2012; Kim *et al.*, 2015). Moreover, the model of Du *et al.* could not account for the importance of the conserved residues at the α-hairpin tip of the adaptor proteins since the tip region was exposed and did not make any interaction with TolC. Furthermore, the TolC channel appeared to be too narrow for substrates to pass through (Fig. 2).

Adaptor bridging model of MacAB-TolC

In the previous studies, the macrolide-specific efflux pump MacAB-TolC provided us with the insights necessary to discuss the adaptor bridging model (Fig. 2). In 2009, the hexameric forms of MacA from *E. coli* and *A. actinomycetemcomitans* in MacAB-TolC pumps were characterized structurally (Yum *et al.*, 2009). Subsequent studies revealed that the funnel-like hexameric forms of MacA represented the functional state of MacA (Kim *et al.*, 2008; Yum *et al.*, 2009). It was found that the propensity of *A. actinomycetemcomitans* MacA was greater than that of other periplasmic adaptor proteins and that it behaved as a hexamer in solution, unlike other adaptor proteins (Yum *et al.*, 2009). Using this unique feature of *A. actinomycetemcomitans* MacA, a chimeric MacA protein containing the 24 amino acids of the TolC α-hairpin tip region was constructed. The struc-

ture of the *A. actinomycetemcomitans* MacA-TolC hybrid protein revealed a funnel-like hexamer similar to MacA in solution, as expected, and the hybrid displayed an extremely high affinity toward the MacA protein *in vitro* with dissociation constant (KD) of ~0.2 nM (Xu *et al*., 2011b). Subsequent electron microscopy studies of the complex of MacA and MacA-TolC hybrid protein revealed that MacA assembles with TolC in a cogwheel-like tip-to-tip manner, which is a key feature of the adaptor bridging model (Xu *et al*., 2011b). The accumulated genetic and biochemical evidence strongly support the adaptor bridging model for the MacAB-TolC efflux pump (Yum *et al*., 2009; Xu *et al*., 2009b, 2010, 2011a, 2011b, 2012; Lee *et al*., 2013).

Partial structures of MacB are available, although the oligomeric state of MacB in the complex of MacAB-TolC remains to be solved (Xu *et al*., 2009a). Biophysical experiments and sequence analysis suggest that MacB comprises a dimeric or multimeric protein of the dimer, since the NBD of MacB should make a dimer (Lin *et al*., 2009; Du *et al*., 2015). In a simple *in vitro* GST pull-down assay MacB did not affect the binding of MacA and TolC, confirming the adaptor bridging model for the MacAB-TolC pump (Xu *et al*., 2009a).

Adaptor bridging model of RND tripartite pumps

Chimeric approaches are useful for understanding the binding modes of the RND-type efflux pump proteins to their cognate OMPs, considered as the shared characteristics, even though the lengths of the α-hairpin domains are different. When the α-hairpin domain of ArcA was substituted with the corresponding regions of *P. aeruginosa* MexA and *E. coli* MacA, the chimeric ArcA proteins were functional similarly as wild-type AcrA (Stegmeier *et al*., 2006; Kim *et al*., 2010). Similar studies were performed with the CusABC pump. A chimeric MacA protein containing the inner helical region of the adaptor protein CusB had a high specific affinity to the OMP CusC α-hairpin tip region (Jun S and Ha NC, unpublished observation), as observed in AcrA and TolC (Xu *et al*., 2011a). These results indicate that the assembly mode of the RND tripartite pumps AcrAB-TolC, MexAB-OprM, and CusABC are similar with that of the ABC tripartite pump MacAB-TolC.

Recent data indicated that the hexamerized form of MexA α-hairpin domains exhibited a strong affinity for both OprM and MexB. Additionally, the high-resolution crystal structure and a low-resolution EM structure of two *A. actinomycetemcomitans* MacA chimeric proteins, one with a MexA α-hairpin domain and the other with a OprM periplasmic tip region, revealed that MexA and OprM binding is same as that for AcrA and TolC (Xu *et al*., 2012). In this complex model of OprM and MexA α-hairpin tips, the conserved residues Arg119, Leu123, and Ser130 of the α-hairpin tip region of MexA are responsible for the binding to the tip region of OprM, as observed in MacA/TolC (Xu *et al*., 2010) and AcrA/TolC (Xu *et al*., 2011a). The complex structures also suggested the detailed binding interface between MexA and OprM (Xu *et al*., 2012). The most prominent feature of the binding model is the hydrophobic interaction mediated by the leucine residue of MexA and the conserved motif VGV of the OprM α-hairpin end region, which is reminiscent of a leucine zipper. These residues are mostly conserved among adaptor proteins, even in MdsA of the *Salmonella*-specific RND-type multidrug efflux pump that does not exist in *E.coli* (Song *et al*., 2014) and in HlyD of the HlyDB-TolC pump, a type I secretion hemolysin translocator (Lee *et al*., 2012). The conserved regions in the α-barrel tip region of TolC homologs in *Vibrio vulnificus* also play an essential role in the formation of a functional efflux pump with *E.coli* AcrAB (Lee *et al*., 2014, 2015b).

In the crystal structure of the CusAB complex, the hexameric structure of the adaptor protein is on the docking domain of CusA (Su *et al*., 2011). However, CusB has a short α-hairpin region and flares outward slightly, just like the MacA hexamer. In a simple docking experiment, the CusB hexamer makes an intermeshing cogwheel-like interaction with CusC in a putative open conformation without any structural adjustment of CusB (Fig. 2).

Recently, Kim *et al*. (2015) have asserted that the EM maps support an 'adaptor bridging model' as previously proposed for AcrAB-TolC, MexAB-OprM, and MacAB-TolC pumps (Yum *et al*., 2009; Xu *et al*., 2011a, 2011b, 2012). In the adaptor bridging model, the α-hairpin tip region of the hexameric AcrA makes intermeshing cogwheel interactions with the tip region of the TolC trimer (Fig. 2). Since both the tip regions of the AcrA hexamer and the TolC trimer display six-bladed cogwheel structures, the two proteins could achieve tight packing without a gap, inducing opening of the TolC channel. This model appears to better fit the EM map, including the dilated feature at the junction of AcrA and TolC, and results from previous biochemical and genetic studies (Xu *et al*., 2009a, 2009b, 2010, 2011a, 2011b, 2012; Yum *et al*., 2009; Kim *et al*., 2010, 2015; Lee *et al*., 2012, 2013, 2014). Taken together, these data suggest the 'adapter bridging model' can be considered as a general assembly mechanism for RND-type tripartite drug efflux pumps in Gram-negative bacteria.

Adaptor bridging model of the MFS tripartite pump EmrAB-TolC

The crystal structure of EmrA, the adaptor protein of EmrAB-TolC belonging to the MFS efflux pump family in *Aquifex aeolicus* has been determined (Hinchliffe *et al*., 2014). Since EmrA has the similar structure at the α-hairpin tip region as those of MacA, AcrA, and MexA, the binding mode of EmrA and TolC is expected to be similar as those of that the MacAB-TolC, AcrAB-TolC, and MexAB-OprM pumps. However, further studies are necessary to validate the adaptor bridging assembly model of the MSF tripartite pumps.

Concluding remarks

Multidrug resistance of Gram-negative bacteria is becoming an increasingly serious issue. Because of its clinical relevance, extensive studies have been performed to decipher the assembly and action mechanism of the tripartite drug efflux pumps in Gram-negative bacteria to control multidrug resistance of these bacteria. Most studies have concentrated on the inhibition of AcrB (Dastidar *et al*., 2007; Sennhauser *et al*., 2007; Nakashima *et al*., 2011; Vargiu *et al*., 2014). In this review, we describe the three classes of

tripartite drug efflux pump, which employ different kinds of IMTs that are structurally distinct and use different energy sources whereas the OMPs have a common structural architecture. The periplasmic adaptor proteins that connect the IMTs to OMPs have diverse structural features at the region for binding to IMPs, but the same structural motif (a six-bladed cogwheel structure) for binding to OMPs. From these observations, it is possible to draw the simple conclusion that the binding mode between the adaptor proteins and OMPs is shared among the tripartite drug efflux pumps. In this review, we discuss the plausibility of the so-called 'adaptor bridging model' as a common assembly model for these kinds of efflux pump. Furthermore, since the OMPs are also employed in the type I secretion system responsible for secretion of protein toxins, it is likely that this secretion system exhibits a similar assembly mode as the tripartite drug efflux pumps. In particular, type I secretion systems are critical for virulence and biofilm formation in the human pathogen *Vibrio vulnificus* (Choi *et al.*, 2002; Lee *et al.*, 2015a). Structural and functional studies on tripartite assembly proteins propose that it is necessary to explore and elucidate the mechanism and target for the inhibition to binding between the adaptor protein and the OMPs, which will counteract multidrug resistance and virulence of some pathogenic bacteria.

Tripartite efflux pumps still have many unknown properties. For example, it remains unknown how the ABC and MFS transporters are assembled in the ternary complex and how the central channel opens subsequently with the complexed substrates. This information will definitely help developing a new drug and chemotherapy in order to prevent a multidrug resistance caused by the functional assembly of the complex.

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