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REVIEW

Recent paradigm shift in the assembly of bacterial tripartite efflux pumps and the type I secretion system

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Tripartite efflux pumps and the type I secretion system of Gram-negative bacteria are large protein complexes that span the entire cell envelope. These complexes expel antibiotics and other toxic substances or transport protein toxins from bacterial cells. Elucidating the binary and ternary complex structures at an atomic resolution are crucial to understanding the assembly and working mechanism. Recent advances in cryo-electron microscopy along with the construction of chimeric proteins drastically shifted the assembly models. In this review, we describe the current assembly models from a historical perspective and emphasize the common assembly mechanism for the assembly of diverse tripartite pumps and type I secretion systems.

Keywords: multidrug resistance, multidrug efflux pump, structure, cryo-electron microscopy

Introduction

The tripartite efflux pumps of Gram-negative bacteria are large membrane protein complexes that expel structurally and chemically diverse toxic chemicals and antibiotics (Blair *et al.*, 2014). Understanding the assembly of the complex, which consists of three essential components spanning the inner membrane, the outer membrane, and the periplasmic space, proved to be difficult. AcrAB-TolC from *Escherichia coli* is a representative model efflux pump, and its homologues are commonly found in Gram-negative bacteria. Because they confer multidrug resistance to bacteria, the overexpression of efflux pumps in pathogenic bacteria is a worldwide emerging threat (Blair *et al.*, 2014). The expression of tripartite efflux pumps is regulated by two component systems, which were well reviewed (Neuberger *et al.*, 2018), and the clinical implications of the efflux pumps have been well summarized (Li *et al.*, 2016).

In our previous review, the so-called adaptor bridging model was presented as an alternative to the prevailing model (Song *et al.*, 2015). Determining the complete ternary complex structures posed a challenge due to the presence of two integral membrane proteins and the low and dynamic affinity between the components. Currently, several complete ternary complex structures at high resolutions are available due to recent advances in cryo-electron microscopy (cryo-EM). With the recent emergence of high-resolution structures of tripartite pumps, the adaptor bridging model is now regarded as the fundamental model for elucidating complex structures. We believe that it is a good example of scientific revolution characterized by a paradigm shift.

Recently, several well-written review papers describing the structure and mechanism of tripartite efflux pumps were published (Du *et al.*, 2018; Greene *et al.*, 2018; Neuberger *et al.*, 2018). Unfortunately, they do not focus on the paradigm shift in the assembly of tripartite efflux pumps. This review discusses the paradigm shift in the assembly of tripartite efflux pumps focusing on AcrAB-TolC, MacAB-TolC, CusBAC, and HlyDB-TolC from *E. coli*, which are representative models of different types of tripartite efflux pumps or the type I secretion system (T1SS). We also discuss the assembly model of YknXYZW, a counterpart in Gram-positive bacteria. Table 1 lists the names of each component in the representative models addressed in this review.

Individual components of tripartite efflux pumps

TolC homologues

TolC is a representative outer membrane factor (OMF) that forms the channel through the outer membrane. Different types of tripartite efflux pumps in *E. coli* employ TolC. The

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Table 1. Efflux pumps and their components

	Inner membrane protein	Periplasmic adaptor protein	Outer membrane channel protein	Accessory protein
E. coli AcrAB-TolC	RND-type AcrB (trimer)	AcrA (hexamer)	TolC (trimer)	AcrZ
E. coli MacAB-TolC	Noncanonical ABC-type MacB (dimer)	MacA (hexamer)	TolC (trimer)	
E. coli CusBAC	RND-type CusA (trimer)	CusB (hexamer)	CusC (trimer)	CusF
E. coli HlyDB-TolC	ABC-type HlyB (dimer)	HlyD (hexamer*)	TolC (trimer)	
S. aureus YknXYZ**	YknZ-YknY (dimer)	YknX (hexamer)	-	YknW

* The oligomeric state has not yet been experimentally determined.
** *St. pneumoniae* Spr0694-0694-0695 is the structural and functional homologue (Yang *et al.*, 2018).

AcrAB-TolC and MacAB-TolC pumps, as well as the T1SS HlyDB-TolC, commonly utilize TolC as the OMF component. The structural homologues of TolC participate in other tripartite efflux pumps. For instance, CusC serves as the outer membrane channel protein in the heavy metal efflux pump CusBAC (Kulathila *et al.*, 2011). In 2000, Koronakis *et al.* (2004) published a landmark paper elucidating the crystal structure of TolC from *E. coli* at 2.1 Å resolution. Later, several structures of TolC homologues were solved, including OprM from *Pseudomonas aeruginisa*, VceC from *Vibrio cholerae* (Akama *et al.*, 2004a; Federici *et al.*, 2005), and CusC from *E. coli* (Kulathila *et al.*, 2011).

The homotrimeric TolC forms a long channel over 140 Å in length that spans the outer membrane in the periplasmic space (Koronakis *et al.*, 2000). The TolC trimer is a β-barrel consisting of 12 β-strands in the transmembrane region, and it is largely a long α-barrel consisting of 12 α-helices in the periplasmic region. It has been proposed that the α-barrel of the TolC trimer is fitted to the perpendicular pore of peptidoglycan, based on the NMR structure of a synthetic peptidoglycan fragment (Meroueh *et al.*, 2006).

The periplasmic portion of each protomer largely consists of two internal repeats of α-hairpins (or antiparallel coiled-coil) (Koronakis *et al.*, 2004). One of the α-helices in the α-hairpin is interrupted by the equatorial domain and appears to provide structural flexibility to the α-hairpin. The overall structures of all the TolC homologues are very similar, and they exhibit high sequence similarity in the tip regions of the α-hairpins. OprM from *P. aeruginosa* has an N-terminal lipid moiety, and VceC from *V. cholerae* exhibits high electronegativity compared to TolC and OprM (Akama *et al.*, 2004a; Federici *et al.*, 2005). Although the sequence of CusC from *E. coli* is the least conserved in the α-helical tip region (Kulathila *et al.*, 2011), the overall structure is also very similar.

The α-barrel of the TolC trimer is partly closed at the periplasmic end by the inward twisting motion of the α-hairpins. The partly closed channel at the periplasmic end prevents unwanted influx from the external medium, and the channel should be dilated in the functional assembly (Koronakis *et al.*, 2004). The opening of the TolC channel in the functional assembly has been the most important structural feature for understanding the assembly and mechanisms of action of the tripartite pumps. The TolC trimer is connected to structurally diverse inner membrane transporters in the presence of periplasmic adaptor proteins, which are the third essential components.

AcrB and CusA, the RND superfamily proteins

The inner membrane proteins of the tripartite efflux pumps are structurally diverse and use different energy sources. They utilize the proton-motive force or ATP hydrolysis to drive the active transport of substrates in the functional efflux pumps (Murakami, 2008; Verhalen *et al.*, 2017; Du *et al.*, 2018; Greene *et al.*, 2018). The resistance-nodulation-cell division (RND) superfamily consists of homotrimeric integral membrane proteins powered by the proton-motive force across the inner membrane (Murakami, 2008; Seeger *et al.*, 2009; Eicher *et al.*, 2014). AcrB in a multidrug efflux pump, and CusA in a heavy metal efflux pump belonging to the RND superfamily. Some members (AcrD, AcrE, and MdtE) of the RND superfamily play roles in biofilm formation (Alav *et al.*, 2018).

The crystal structures of AcrB provided structural details and mechanism of action information (Murakami *et al.*, 2002; Seeger *et al.*, 2006). The protomer is divided into three domains: the transmembrane domain (TMD), the porter domain (or pore domain), and the top domain (or docking domain). The TMD consists of a bundle of 12 α-helices and functions as a passage for proton movement. The proton translocation in the TMD is coupled to the efflux of substrates in the porter domain. The porter domain consists of the four subdomains PN1, PN2, PC1, and PC2. The porter domain has two substrate binding cavities: the access and deep binding sites. The porter domain undergoes conformational changes upon proton translocation in the TMD and substrate binding. The porter domains in the trimer are asymmetric with different conformational states: the loose (L), tight (T), and open (O) states, whose cycling changes in an ordered manner and are driven by proton movement (Murakami *et al.*, 2002; Seeger *et al.*, 2006). This cycling conformational change in the porter domain was referred to as the "peristaltic mechanism". However, a recent study on CmeB, an AcrB homologue from *Campylobacter jejuni*, suggested that the cycling conformational changes are independent of the efflux process, which raised an alternative mechanism for the action of the RND superfamily (Su *et al.*, 2017).

The top domain, consisting of DN and DC subdomains at the periplasmic end of AcrB, was previously known as the TolC docking domain because it was believed to make direct contact with the TolC periplasmic end. The conformation of the top domain does not change upon proton translocation and substrate binding; thus, it may be more suitable for binding to the adaptor protein. However, the porter domain is also involved in binding to the adaptor protein (Du *et al.*, 2014; Kim *et al.*, 2015). The broad or poly substrate specificity of AcrB is the most important feature for conferring multidrug resistance to bacteria. AcrB transports structurally and chemically diverse substrates. The molecular nature of the poly substrate specificity may arise from the multiple bind-

ing sites with the same or different substrate binding pockets (Eicher *et al.*, 2012; Cha *et al.*, 2014). Several inhibitors of AcrB were developed to prevent the multidrug resistance of Gram-negative bacteria (Vargiu *et al.*, 2014; Opperman and Nguyen, 2015).

The crystal structure of CusA was determined in the homotrimeric organization, sharing most of its structural features with AcrB. Interestingly, CusA contains a high number of methionine residues, and many methionine residues are paired up and arrayed in the crystal structure (Long *et al.*, 2010). Because methionine residues are involved in the coordination of Cu^+ or Ag^+ in copper tolerant proteins (Loftin *et al.*, 2007; Xue *et al.*, 2008), the methionine residues in CusA were proposed to form the Cu^+/Ag^+ binding pockets that are used for metal ion transport (Long *et al.*, 2010; Su *et al.*, 2011a). Consistently, multiple Cu^+/Ag^+ binding sites were observed in the CusA periplasmic domain (Yun *et al.*, 2010).

MacB and HlyB, the ABC superfamily

MacB in MacAB-TolC and HlyB in the T1SS belong to the ATP binding cassette (ABC) transporter superfamily. MacB is distinct from the classical ABC transporter and thus was recently called the MacB-type or tripartite-type ABC transporter (Okada *et al.*, 2017). MacB has a larger periplasmic domain compared to the classical ABC transporter. The transmembrane MacB was initially characterized as macrolide-pecific transporter (Kobayashi *et al.*, 2003). Further investigation revealed that it can also mediate the extrusion of heat-stable enterotoxin II, protoporphyrin IX, virulence factors, siderophores, and lipopolysaccharides (Nishino *et al.*, 2006; Yamanaka *et al.*, 2008; Imperi *et al.*, 2009; Lu and Zgurskaya, 2013; Turlin *et al.*, 2014).

The periplasmic region of the MacB structure was first solved (Xu *et al.*, 2009a) showing a dimeric feature in the functional assembly (Jo *et al.*, 2017; Okada *et al.*, 2017). The periplasmic region of MacB consists of two domains and shares structural similarity to the top domain of AcrB. Recently, two crystal structures of the full-length MacB from *Acinetobacter baumanii* (Okada *et al.*, 2017) and *Actinobacillus actinomycetemcomitans* (Crow *et al.*, 2017) were determined, and a mechanotransmission mechanism for MacB was proposed (Crow *et al.*, 2017; Greene *et al.*, 2018). In this mechanism, the MacB dimer accepts substrates from the periplasmic space using the large periplasmic domains and ejects them into the central channel of the MacA hexamer via a reversible dimerization motion driven by ATP hydrolysis in the cytoplasmic nucleotide binding domains (NBDs) (Crow *et al.*, 2017; Fitzpatrick *et al.*, 2017). Because the classical ABC transporters accept substrates in the cytoplasm and translocate them across the inner membrane, the mechanism for MacB is distinct from that of the classical ABC transporters.

The T1SS is widespread among Gram-negative bacteria and is related to the bacterial pathogenesis by secreting several virulence factors (Binet and Wandersman, 1995; Fuche *et al.*, 2015). The T1SS consists of three essential components, and it uses TolC as the OMF in *E. coli* (Buchanan, 2001). The substrate proteins of the T1SS are relatively large proteins containing a C-terminal secretion signal and the calcium binding "repeat-in-toxin" domain, which also contribute to transport via calcium-driven folding (Bumba *et al.*, 2016). In *E. coli*, hemolysin HlyA is transported from the cytosol to the external medium by the HlyDB-TolC system. HlyB belongs to the classical ABC transporter family and possesses an additional domain that possesses peptidase activity (Morgan *et al.*, 2017). HlyB is connected to TolC via the adaptor protein HlyD.

PrtD is the ABC transporter in the T1SS from *Aquifex aeolicus*, and its crystal structure was determined (Morgan *et al.*, 2017). Although the overall dimeric structures are similar to those of the classical ABC transporter, several distinct structural features were identified. The substrate entry window is just above the NBDs, and the highly kinked transmembrane helices frame the narrow channel in the dimeric interface. The structure suggests that the polypeptide translocation mechanism may be different from that where the alternative access is in either the inward or the outward conformations, which is typical for the classical ABC transporters (Lin *et al.*, 2015). Furthermore, the T1SS ABC transporter transports substrate through the inner membrane in the absence of the other components (Morgan *et al.*, 2017).

AcrA, MacA, CusB, and HlyD

The periplasmic components of the tripartite efflux pumps are called periplasmic adaptor proteins (Balakrishnan *et al.*, 2001; Yum *et al.*, 2009; Symmons *et al.*, 2015) and previously known as membrane fusion proteins (Zgurskaya *et al.*, 2009). The periplasmic adaptor proteins possess the signal sequence for secretion to the periplasm. Most adaptor proteins are anchored to the inner membrane at the periplasmic side via the N-terminal lipid moiety in AcrA, the uncleaved signal sequence in MacA or the transmembrane domain in HlyD (Koronakis *et al.*, 1992; Zgurskaya and Nikaido, 1999; Tikhonova *et al.*, 2007). The crystal structures of the adaptor proteins revealed that they consist of the linearly arranged α-helical domain, lipoyl domain, β-barrel domain, and the membrane proximal domain (Zgurskaya and Nikaido, 1999; Akama *et al.*, 2004b; Su *et al.*, 2009; Xu *et al.*, 2009b; Yum *et al.*, 2009; Kim *et al.*, 2016).

The adaptor proteins were expected to be key in revealing the pump assemblies because they connect the other two components. However, the adaptor proteins' oligomeric state is under debate (Bavro *et al.*, 2008; Symmons *et al.*, 2009; Zgurskaya *et al.*, 2015; Jeong *et al.*, 2016; Travers *et al.*, 2018). A homotrimeric assembly previously prevailed , despite an accumulation of evidence supporting the homohexameric model (Piao *et al.*, 2008; Kim *et al.*, 2010; Janganan *et al.*, 2011; Xu *et al.*, 2011a, 2011b, 2017; Lee *et al.*, 2012, 2014; Song *et al.*, 2014; Symmons *et al.*, 2015).

The crystal structures of AcrA and its homologue MexA did not provide direct evidence for protein oligomerization (Akama *et al.*, 2004b; Mikolosko *et al.*, 2006). In 2009, MacA in the MacAB-TolC pump from *A. actinomycetemcomitans* and *E. coli* were determined (Yum *et al.*, 2009). MacA from *A. actinomycetemcomitans* has a greater tendency to form a hexamer in solution. The *A. actinomycetemcomitans* MacA was used as a scaffold to create the chimeric MacA protein that has TolC α-helical tip regions to investigate the role of TolC α-helical tips in the absence of detergents (Xu *et al.*, 2010, 2011a, 2011b, 2012; Lee *et al.*, 2013, 2014; Kim *et al.*, 2015; Song *et al.*, 2015; Jeong *et al.*, 2016; Jo *et al.*, 2017). The

most striking structural feature of the MacA hexamer is the funnel-like structure with a six-bladed cogwheel at the end of the funnel stem. The funnel stem, resembling a nanotube, is formed by the α-helical domain, and the funnel mouth is formed by the other domains containing the N- and C-termini. The flared funnel mouth portion is likely associated with the inner membrane proteins due to the inner membrane anchored N-terminus. Therefore, the funnel stem should directly interact with the outer membrane protein TolC. In the modeled open structure of TolC, the periplasmic end region appears very similar to the cogwheel structure of the MacA hexamer. The structural resemblance between the two cogwheels is indicative of the intermeshing cogwheel interaction for the binding of TolC and MacA (Yum *et al.*, 2009; Xu *et al.*, 2011b). The functional importance of both cogwheel regions was examined (Xu *et al.*, 2010).

The crystal structure of CusB revealed the unique α-helical domain consisting of three antiparallel α-helices, which is distinct from those in AcrA and MacA (Su *et al.*, 2009). Three Cu^{+}/Ag^{+} binding sites were observed in the crystal structure of the CusB protomer. The CusB hexameric assembly was also observed in the complex structure with CusA, and the shorter and thicker funnel stem portion was observed due to the four helical bundle structures in the α-helical domain. Despite the different α-helical domains, the CusB hexamer shares the cogwheel-like structure.

HlyD is the adaptor protein in the T1SS HlyDB-TolC from *E. coli*. HlyD was predicted to have an N-terminal cytoplasmic tail, a transmembrane domain, and a large C-terminal periplasmic domain (Schulein *et al.*, 1994; Balakrishnan *et al.*, 2001). The C-terminal periplasmic domains are homologous to the drug efflux pump adaptor proteins. Only one fragment of the C-terminal periplasmic domain, consisting of the lipoyl and α-helical domains, was structurally characterized (Kim *et al.*, 2016; Murata *et al.*, 2017). The crystal structures of the fragment showed a highly elongated α-helical domain and a lipoyl domain with significant structural flexibility between the two domains (Kim *et al.*, 2016). The α-helical domain is composed of one longer α-helix (α3) and two shorter helices (α1 and α2). The shorter helices partially overlap in a zigzag pattern. The α-helical tip region between α2 and α3 was noted due to its structural resemblance to the α-helical tip regions of AcrA and MacA, which are responsible for TolC binding. Based on the structural similarity to the MacA hexamer, the HlyD hexameric model was constructed (Kim *et al.*, 2016). The α-helical domain in the HlyD hexameric model exhibited a vase-like α-barrel with similar cogwheel structures at the α-helical tip region to those of the MacA hexamer (Fig. 1). The functional importance of the HlyD α-helical tip region was examined by conducting a mutagenesis study. Mutations in the α-helical tip region abolished binding to TolC as well as hemolysin secretion (Kim *et al.*, 2016). The oligomeric state of HlyD in the functional assembly still remains to be elucidated.

Binary complexes

Obsolete AcrB-TolC complex

The crystal structures of the TolC and AcrB trimers revealed structural complementarity at both periplasmic end regions (Murakami *et al.*, 2002). This structural complementarity provided a strong bias for the direct interaction between TolC and AcrB. The periplasmic end (the top domain) of the AcrB periplasmic domain was initially designated as the TolC docking domain. Unlike the binding of AcrA and TolC and that

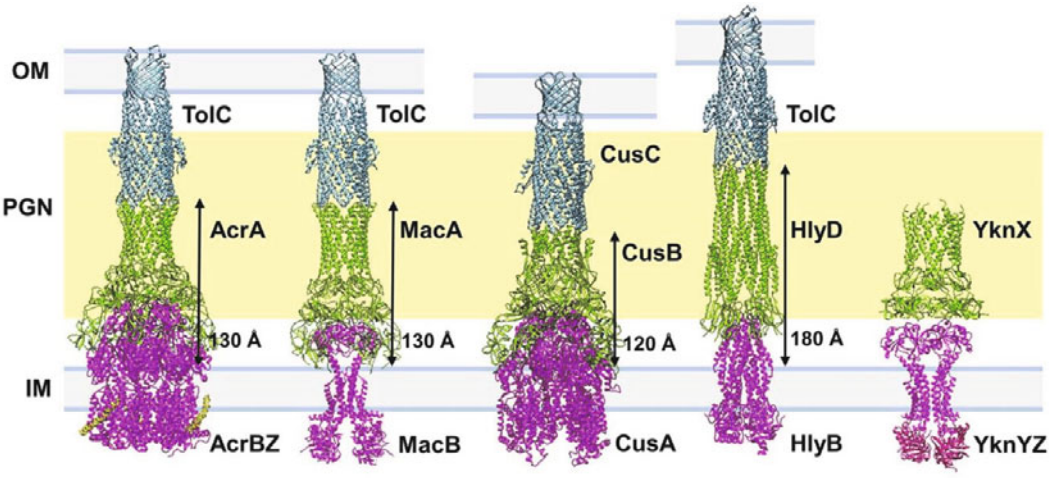

Fig. 1. Assembly structures of the AcrAB-TolC and MacAB-TolC pumps at (near) atomic resolution and the molecular models of the CusBAC pump, the HlyDB-TolC and the YknXYZ system. The inner membrane components are colored in magenta, the periplasmic components are in green, and the outer membrane components are in cyan. The inner membrane (IM) and outer membrane (OM) are indicated, and the peptidoglycan layer (PGN) is in the yellow shadow. The *E. coli* AcrAB-TolC pump was drawn based on the cryo-EM structure of AcrABZ-TolC (PDB code: 5O66). The accessory protein AcrZ is in yellow. The *E. coli* MacAB-TolC pump structure was drawn based on the cryo-EM structure at a 3.3 Å resolution (PDB code: 5NIK). The molecular model of the CusBAC pump was built based on the intermeshing cogwheel interaction between the CusB hexamer and the CusC trimer. The molecular model of the HlyDB-TolC type I secretion system was built using the HlyD hexameric model (PDB code: 5C21) (Kim *et al.*, 2016), and the crystal structure of the HlyB homologue PrtD (PDB code: 5L22). The assembly model of the YknXYZ pump was generated using the crystal structures of the Spr0694-0695 complex and Spr0693 (Yang *et al.*, 2018). The height of the periplasmic components is indicated.

of AcrA and AcrB, direct binding between TolC and AcrB was uncertain (Stegmeier *et al.*, 2006). Chemical cross-linkers were required to detect the binding of TolC and AcrB (Touze *et al.*, 2004; Tamura *et al.*, 2005). However, evidence of the direct interaction between TolC and AcrB was lacking in the absence of chemical cross-likers (Higgins *et al.*, 2004).

Based on the direct interaction between TolC and AcrB, the ternary complex model was proposed and further supported by *in vivo* site-specific cross-linking experiments in which numerous cysteine-substituted AcrA, AcrB, or TolC variants were constructed and compared to the mutants lacking the cysteines (Lobedanz *et al.*, 2007; Symmons *et al.*, 2009). Chemical cross-linkers that contain cysteine and amine-reactive moieties were treated with live bacteria and then the cross-linked protein complexes were isolated for analysis. In the complex, the introduced cysteine residues in one protein were covalently linked to adjacent lysine residues of the other protein. The interaction map was built by combining the information on the residues that are in close proximity to each other. Taken together, the assembly models, called adaptor wrapping (Song *et al.*, 2015) or the deep interpenetration model (Symmons *et al.*, 2015), were proposed, where AcrB and TolC form a tip-to-tip interaction and AcrA wraps around the outside of the AcrB-TolC binary complex with the α-helices of AcrA deeply penetrating into the groove formed by the TolC α-barrels. The models show a shorter and wider structure in the α-barrel portions because two α-barrels from AcrA and TolC overlap and form a double layered α-barrel. Longitudinal binding between the AcrA α-helices and the surface grooves of the TolC α-barrel was proposed to be the driving force for the TolC channel opening.

The adaptor wrapping model prevailed, and it was believed to represent the functional assembly and highly influenced this field. Although the experimental results seemingly contradicted the adaptor wrapping model, the literature ultimately supported the adaptor wrapping model (Trepout *et al.*, 2007; Su *et al.*, 2011b). However, recent advances in cryo-electron microscopy resulted in the paradigm shift to the adaptor bridging model, which was first proposed based on the MacA hexameric structures in 2009 (Yum *et al.*, 2009).

MacA-TolC complex

Because MacA has a higher tendency to form a hexamer, an initial attempt was made to isolate the TolC and MacA complex (Xu *et al.*, 2011b). The binding of MacA and TolC is dynamic and unstable, and the detergents used for stabilizing the integral membrane protein TolC may interfere with the binding of the two proteins. To isolate the MacA and TolC complex, a chimeric TolC protein was constructed by considering the structural resemblance between the MacA α-hairpin and the TolC α-hairpin regions. The chimeric proteins were constructed first by combining two MacA proteins in a tandem manner and the MacA α-helical tip regions. This tandem linking strategy was used to obtain the ternary complex in later studies (Du *et al.*, 2014; Song *et al.*, 2014; Kim *et al.*, 2015; Jeong *et al.*, 2016; Jo *et al.*, 2017; Xu *et al.*, 2017).

The TolC chimeric protein formed a strong and tight complex with MacA via hexamerization induction, and it was subjected to an electron-microscopic study (Xu *et al.*, 2011a). The low-resolution EM structure revealed a dumbbell-like structure that supported the intermeshing cogwheel interaction in the adaptor bridging model.

CusB-CusA complex

The CusB adaptor protein from the heavy metal CusBAC efflux pump possesses a unique property. CusB does not have the membrane anchoring motif, unlike the other adaptor proteins. To compensate for the absence of the membrane anchoring motif, it appears that the affinity of CusB for the inner membrane protein CusA is higher compared to the other tripartite efflux pumps. Su *et al.* determined the crystal structure of the CusB-CusA complex, the first binary complex of the tripartite efflux pump to be solved (Su *et al.*, 2011b). The structure presented a stable complex of the CusB hexamer and the CusA trimer. CusB formed a hexameric ring on top of CusA, and the flower-like α-helical domain flared out. Su *et al.* proposed an assembly model with the OMF CusC based on the adaptor wrapping model.

Ternary complex structures

In *E. coli*, TolC is commonly used as the outer membrane porin protein among the tripartite efflux pumps and the T1SS. This finding strongly indicates that there is one common assembly mechanism among all of the tripartite efflux pumps and the T1SS, although local variations may exist. Simplicity is often the best solution.

TolC and all of its homologues have a six-bladed cogwheel structure at the periplasmic tip region, and most periplasmic adaptor proteins have six-bladed cogwheel structures when bound to their cognate inner membrane transporters. It is interesting that the oligomerization number (six) for periplasmic adaptor proteins is actually the least common multiple of three for the trimeric RND transporters and two of the dimeric ABC transporters. This finding suggested that the hexameric periplasmic adaptor proteins could potentially adapt to both trimeric and dimeric transporters (Yum *et al.*, 2009; Xu *et al.*, 2011a, 2011b, 2012). Indeed, the complex structures available demonstrated that all of the adaptor proteins form a hexameric ring on the trimeric RND or the dimeric ABC transporters (Du *et al.*, 2014, 2015, 2018; Kim *et al.*, 2015; Song *et al.*, 2015; Jeong *et al.*, 2016; Fitzpatrick *et al.*, 2017; Jo *et al.*, 2017; Su *et al.*, 2017; Wang *et al.*, 2017; Xu *et al.*, 2017; Greene *et al.*, 2018; Neuberger *et al.*, 2018).

AcrAB-TolC complex

Fusion proteins were constructed to isolate the complete complex. The first attempt focused on the AcrA from *E. coli*. The purified AcrA protein behaved as a monomer in solution, and the protein did not form a stable complex with TolC. However, the AcrA dimer, which was created by linking two AcrA proteins with a linker sequence, behaved as a hexamer in solution and was functionally comparable to the wild type AcrA (Xu *et al.*, 2011a). Du *et al.* (2014) constructed a fusion protein by combining AcrB and AcrA, and additional AcrA was added in the co-expression experiment. These efforts resulted in the formation of the complete AcrAB-TolC complex in the presence of the small additional protein AcrZ.

Kim *et al.* (2015) constructed the fusion protein AcrB-AcrA-AcrA to isolate the complete complex containing TolC or the chimeric TolC protein. Both research groups visualized the EM structures of the AcrAB-TolC complex at low resolutions. However, the atomic models were built differently at the interface between the AcrA hexamer and the TolC trimer. Du *et al.* claimed that AcrA partially penetrated into the TolC α-barrel, while Kim *et al.* presented the adaptor bridging model with the intermeshing cogwheel interaction (Song *et al.*, 2015). Later, cryo-EM structures at pseudoatomic (Jeong *et al.*, 2016) or near-atomic resolutions (Wang *et al.*, 2017) established the adaptor bridging model as the final assembly to date. The adaptor bridging model was further re-confirmed without using fusion proteins (Daury *et al.*, 2016). The cryo-EM structure at near-atomic resolution further revealed a quaternary structural change by coupling the ligand binding with the channel opening of TolC (Wang *et al.*, 2017).

The adaptor bridging model presents a longer ternary complex than the adaptor wrapping model. The hexameric periplasmic adaptor proteins interfere with the OMF and the inner membrane transporter and prevents the direct interaction between the OMF and the inner membrane. The adaptor proteins' α-helical domain forms an α-barrel consisting of six α-hairpins, and the remaining adaptor protein domains form the flared cone, which is optimal for binding to the periplasmic region of the inner membrane transporter. The key interaction between the periplasmic adaptor protein and the OMF is the intermeshing between two six-bladed cogwheels. To form the intermeshing interaction, the cogwheels are slightly more dilated than α-barrels. The conserved leucine residue in the α-hairpin tip regions of AcrA (or the periplasmic adaptor protein) are bent up into the conserved motif in the α-hairpins of AcrA (or the OMF) (Xu *et al.*, 2012). The six intermeshing interactions between the six-bladed cogwheels can be augmented cooperatively, and thus the interaction would become much stronger than six-fold the single hook-up interaction. Since the open conformation of the OMF is capable of forming the intermeshing cogwheel interactions with the periplasmic adaptor hexamer, the OMF channel opening and the functional complex formation would be coupled.

The adaptor bridging model can explain previously unresolved questions. The OMF channel opening mechanisms are intuitively understood. The adaptor wrapping model attempted to explain the insertion of the adaptor protein α-helices into the groove on the outside of the OMF α-barrel as the driving force for opening the OMF channel. However, the model was unreasonable because the OMF surface grooves would disappear in the open state. The diameters of the α- barrels in the OMF trimer and the adaptor hexamer are identical. How could the α-barrel of the OMF be opened via insertion into the adaptor α-barrel? Another question arises from the mismatch between the heights of the bacterial periplasmic space and the ternary complexes proposed based on the adaptor wrapping model. However, the ternary structures of the adaptor bridging models agree with the height of the periplasmic space.

MacAB-TolC complex

To isolate the complete complex of MacAB-TolC, a MacB-MacA fusion protein was constructed (Fitzpatrick *et al.*, 2017). Cryo-EM structures of MacB-MacA and TolC at pseudo-atomic resolution were determined, revealing the complete complex structure of MacAB-TolC from *E. coli*. Due to a mismatch in stoichiometry between MacB and MacA, the redundant four subunits of MacB were disordered. The overall assembly is similar to the AcrAB-TolC pump in the adaptor bridging model. The dimeric MacB periplasmic domain resides in the dome structure of the β-barrel and the membrane proximal domain of the MacA hexamer, and the α-helical tip region of MacA is in the cogwheel of the TolC trimeric tip region. The stoichiometry for the dimeric MacB was subsequently confirmed independently (Jo *et al.*, 2017).

Model of CusBAC complex

To date, there are no structures of the complete CusBAC heavy metal efflux pump complex available. However, the inner (first) two α-helices in the three antiparallel helices of the CusB α-helical domain form a cogwheel, as observed in MacA and AcrA. According to unpublished results (Ha *et al.*), a chimeric MacA protein containing the inner two α-helices formed a stable complex with the CusC α-helical tip regions. A mutation in the helical tip region of the CusB inner helices abolished CusC binding. A structural model constructed by shrinking the inner helices of the CusB hexamer formed the six-bladed cogwheel. These findings suggest that the six-bladed cogwheel structure formed by the inner helices of CusB give rise to the intermeshing cogwheel interaction with the CusC trimer (Fig. 1).

Model of the HlyDB-TolC complex

Although the structures of the individual components are available, no complex structure has been determined experimentally. However, given the common structural feature of HlyD at the α-helical tip region, the complex model was built as shown in Fig. 1 (Kim *et al.*, 2016). The toroidal HlyD containing the six-bladed cogwheel at the periplasmic end connects the HlyB ABC transporter to TolC. The overall architecture is similar to the MacAB-TolC pump in the adaptor bridging model. However, additional evidence is needed to elucidate the assembly model of the T1SS.

MacAB-like pump of Gram-positive bacteria

Gram-positive bacteria possess MacAB-like pumps that are involved in the resistance to antibiotics and protein toxins by the efflux of substrates. In *Bacillus subtilis*, the cannibalistic toxin SPD kills a subset of *Bacillus* (Lamsa *et al.*, 2012) and is expelled by the four-component efflux pump YknWXYZ (Yamada *et al.*, 2012). Of the four components, YknX and YknZ-YknY exhibit high sequence similarity to MacA and MacB, respectively. YknZ and YknY form a tight complex, where YknY corresponds to the nucleotide binding domain of MacB and YkZ to the transmembrane domain and the extracellular (periplasmic) domain of MacB. The crystal structure of the extracellular domain of YknZ from *Bacillus* revealed high structural similarity to that of MacB (Xu *et al.*, 2016), and the low resolution electron density map of YknX exhibited a funnel-like hexamer similar to that of MacA (Xu *et al.*, 2017).

Recently, homologues of *Streptococcus pneumoniae* were structurally characterized at atomic resolutions (Yang *et al.*, 2018). Consistently, crystal structures of the full-length Spr0694-0695 complex (YknZ-YknY homologue, MacB-like protein) exhibited MacB-like structural features. Moreover, the crystal structure of Spr0693 (YknX homologue, MacA-like protein) exhibited a funnel-like hexamer. Based on these high resolution structures, the authors proposed a MacAB-like assembly model based on the MacAB-TolC structure (Fitzpatrick *et al.*, 2017) (Fig. 1).

Although Gram-positive bacteria do not require the outer membrane factor due to the lack of the outer membrane, the cooperative role between the MacB-like protein and the MacA-like protein likely exists. The MacA-like protein Spr0693 augmented the ATPase activity of the MacB-like protein Spr0694-0695 (Yang *et al.*, 2018). Furthermore, the chimney-like feature was proposed for the MacA-like protein YknX to send it out farther by utilizing the nanotube of the α-barrel to penetrate the peptidoglycan layer of the bacteria (Xu *et al.*, 2017).

Interaction of peptidoglycan with the α-barrels of the outer membrane channel proteins and the periplasmic adaptor proteins

The cell envelope structure of Gram-negative bacteria is characterized by two phospholipid bilayers of the inner and outer membranes. The peptidoglycan layer is located between the membrane layers. The peptidoglycan interacts with the α-barrels of the TolC homologue (Kim *et al.*, 2008), the adaptor proteins AcrA (Xu *et al.*, 2012), and YknX (Xu *et al.*, 2017). Importantly, it should be noted that *E. coli* peptidoglycan, but not *Staphylococcus aureus* peptidoglycan, augmented binding between AcrA and TolC (Xu *et al.*, 2012). To understand the dynamic assembly of the tripartite efflux pumps, peptidoglycan should be considered as an important environmental factor that affects the binding between the components.

Conclusion

Additional evidence is required to understand the fundamental changes in basic concepts because scientific works are conducted on the basis of currently dominating ideas. The adaptor wrapping model appeared to dominate this field until several years ago due to extensive biophysical evidence, including *in vivo* site-directed cross-linking results (Tamura *et al.*, 2005; Lobedanz *et al.*, 2007; Symmons *et al.*, 2015; Yamaguchi *et al.*, 2015). However, few researchers suspected that the fidelity of the results using the chemical cross-linkers was inaccurate (Song *et al.*, 2015). They noted that the results may have contained experimental artifacts, which could not be distinguished from real interactions (Song *et al.*, 2015). Furthermore, a conflict was reported regarding the functionality of the mutant strains used for the site-directed cross-linking experiments (Kim *et al.*, 2010; Xu *et al.*, 2011b; Song *et al.*, 2015). Several researchers have used *E. coli* strains harboring the AcrB mutant lacking cysteine to investigate the assembly of the tripartite efflux pumps (Lobedanz *et al.*, 2007; Symmons *et al.*, 2009; Tikhonova *et al.*, 2011) because the AcrB mutant strain lacking cysteine was reported to exhibit similar activity to the wild type AcrB (Lobedanz *et al.*, 2007). However, the functionality of these mutants was severely decreased or abolished when the mutants were reproduced by an independent research group (Kim *et al.*, 2010; Xu *et al.*, 2011b; Song *et al.*, 2015). To clarify this issue, the functionality of the original mutant strains should be retested. Given that the adaptor wrapping, or the deep or partial interpenetration models, did not represent the functional assembly of the pumps, all of the biophysical results supporting the models should be re-evaluated and reinterpreted to prevent the further propagation of the obsolete models.

On the other hand, it is a pity that the initial contributions to the adaptor bridging model before the paradigm shift were ignored by the main contributors of the recent advances in the tripartite efflux pumps (Du *et al.*, 2014, 2018; Neuberger *et al.*, 2018). The recent high-resolution cryo-EM structures of the tripartite efflux pumps are remarkable and play a critical role in the recent paradigm shift. However, it is also true that the conclusions of the recent works largely confirm the idea proposed in 2009 (Yum *et al.*, 2009). This review summarizes the assembly of tripartite efflux pumps and the T1SS by focusing on the adaptor bridging model. Tripartite efflux pumps are almost completely understood, although the current assemblies may be changed or refined in final models. We are looking forward to evaluating the final models in the near future.

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