Structure of the Tripartite Multidrug Efflux Pump AcrAB-ToIC Suggests an Alternative Assembly Mode

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Escherichia coli AcrAB-ToIC is a multidrug efflux pump that expels a wide range of toxic substrates. The dynamic nature of the binding or low affinity between the components has impeded elucidation of how the three components assemble in the functional state. Here, we created fusion proteins composed of AcrB, a transmembrane linker, and two copies of AcrA. The fusion protein exhibited acridine pumping activity, suggesting that the protein reflects the functional structure in vivo. To discern the assembling mode with ToIC, the AcrBA fusion protein was incubated with ToIC or a chimeric protein containing the ToIC aperture tip region. Three-dimensional structures of the complex proteins were determined through transmission electron microscopy. The overall structure exemplifies the adaptor bridging model, wherein the funnel-like AcrA hexamer forms an intermeshing cogwheel interaction with the α-barrel tip region of ToIC, and a direct interaction between AcrB and ToIC is not allowed. These observations provide a structural blueprint for understanding multidrug resistance in pathogenic Gram-negative bacteria.

INTRODUCTION

Multidrug resistance in pathogenic bacteria is one of the greatest threats to human health (Nikaido and Zgurskaya, 1999). AcrAB-ToIC is the major multidrug efflux pump in Escherichia coli, and its overexpression is common in multidrug-resistant clinical isolates (Morshed et al., 1995). This drug efflux pump is composed of three essential components: AcrA, AcrB, and ToIC (Lewis, 2000). The homotrimeric AcrB belongs to the resistance-nodulation-division (RND) family of proteins, is embedded in the inner membrane, and pumps out diverse substances using a proton gradient as an energy source (Lewis, 2000; Murakami et al., 2002). Crystal structures of AcrB show the molecular mechanisms underlying substrate transport through proton-motif energy (Murakami et al., 2002; Seeger et al., 2006). ToIC is also homotrimeric membrane protein that displays a long cylinder-like structure, which provides a conduit across the outer membrane (Koronakis et al., 2000). AcrA is an adaptor protein that connects AcrB and ToIC in the periplasm (Fralick, 1996; Ma et al., 1993). The ToIC channel remains closed without the other components, and channel opening is initiated by binding to AcrA and/or AcrB (Koronakis et al., 2000). Several structures have been determined for AcrA and its homologues MexA in the Pseudomonas aeruginosa MexAB-OprM pump as well as MacA in the macrolide-specific drug efflux pump MacAB-ToIC pump, which show that AcrA consists of an α-helical domain, lipoyl domain, β-barrel domain, and membrane-proximal domain (Mikolosko et al., 2006; Symmons et al., 2009). However, the oligomeric state of AcrA in the functional complex is controversial.

Binding between AcrA and ToIC as well as between AcrA and AcrB is likely dynamic. The binary complexes AcrA - ToIC and AcrB - AcrA were observable albeit with low affinity. However, the direct interaction between AcrB and ToIC is elusive (Touze et al., 2004). The interaction between AcrB and ToIC was detected when binding was fixed through cross-linking (Tamura et al., 2005). Based on this direct interaction between AcrB and ToIC, an ‘adapter wrapping model’ was proposed for AcrAB-ToIC pump assembly and supported by extensive binding mapping using Cys-mediated cross-linking experiments (Lobedanz et al., 2007; Symmons et al., 2009; Tamura et al., 2005). The key feature of the adapter wrapping model is that a tip-to-tip interaction between AcrB and ToIC and three AcrA protomers wraps the outer portion of the AcrB and ToIC binary complex with a stoichiometry of 3:3:3 (Hinchliffe et al., 2013). The stoichiometry and binding assembly of the AcrAB-ToIC pump has been under debate. Funnel-shaped hexmeric assembly of the adaptor protein was observed in the MacA crystal...
structure (Rouquette-Loughlin et al., 2005; Yum et al., 2009). Based on this structure, we proposed an ‘adaptor bridging model’, where the apical tip of the α-barrel consisting of six α-hairpins from the AcrA hexamer forms an intermeshing cogwheel interaction with the analogous six-bladed cogwheel of an α-barrel in the TolC trimer (Xu et al., 2011a; Yum et al., 2009). This model was further supported by extensive structural, biochemical, and genetic studies (Janganan et al., 2011; Kim et al., 2010; Xu et al., 2010; 2011a; 2011b; 2012; Yum et al., 2009). Structures of a chimeric protein containing the α-hairpin domain of MexA show a funnel-like hexameric arrangement (Xu et al., 2011a). In an electron microscopy study on a complex protein containing the adaptor protein and TolC, an intermeshing cogwheel interaction between the α-barrel tip regions of the adaptor protein and TolC was exhibited (Xu et al., 2010; 2011a; 2012). Independent research groups presented evidence for the pump assembly with stoichiometry among the components (Janganan et al., 2011; Mirna et al., 2007; Narita et al., 2003; Su et al., 2011).

In this study, we provide structures of the AcrAB-TolC pump using the AcrBA fusion protein. Our structure exemplifies the adaptor bridging model based on an intermeshing cogwheel interaction between the AcrA hexamer and TolC. Very recently, a complex structure of the AcrAB-TolC pump was reported, which essentially describes the same density envelop for the complex but exhibits a different binding interface between AcrA and TolC in their docked model (Du et al., 2014). Here, we compare the two models and discuss the confidence of the previous results compared with the adaptor-bridging model.

MATERIALS AND METHODS

Constructing the fusion proteins

To construct plasmids for the AcrBA fusion protein (AcrB-TM#-AcrA-AcrA) and measure the activity, three DNA fragments were sequentially inserted into the plasmid. First, DNA fragments encoding full-length E. coli AcrB were inserted into the pET22b vector (Novagen) Ndel and HindIII sites, which produced the vector pET22b-AcrB. DNA fragments encoding AcrA (residues 26-397), the Ser linker, and AcrA (residues 26-397 without the stop codon) were generated using the overlapping PCR technique. The DNA fragments were inserted into the pET22b-AcrB vector HindIII and XhoI sites, which produced the vector pET22b-AcrB-AcrB-AcrA-AcrA. Lastly, each TM region (Supplementary Table S1) was inserted between the AcrB and AcrA dimer using the vector pET22b-AcrB-AcrB-AcrA-AcrA HindIII site through the Cold Fusion ligation method in accordance with the manufacturer’s instruction, which yielded the vectors pET22b-AcrB-TM#(1-6)-AcrA-AcrA. We added the Precision protease recognition sequence (LEVLFQGP) and Streptococcus dysgalactiae Protein G (residues 302-427) to the AcrBA fusion protein C-terminus to facilitate purification through affinity chromatography. To add this sequence, a DNA fragment encoding the PreScission protease cleavage site as well as Protein G was synthesized (Bioneer, Korea) and inserted into the pET22b-AcrB-TM#(1-6)-AcrA-AcrA XhoI site using the Cold Fusion ligation method, which generated pET22b-AcrB-TM#5-AcrA-AcrA-ProG. To produce MacA-TolC-hybrid-dimer, the previously described plasmid pPRO-MacA-TolC (Koronakis et al., 2005; Yum et al., 2007; Narita et al., 2003; Su et al., 2011) was used. To produce the E. coli TolC protein, DNA fragments encoding E. coli TolC 1-452 (Koronakis et al., 2000) were inserted into the pET22b vector NcoI and XhoI sites, which produced the vector pET22b TolC. Sequences of the primers used in this study are listed in Supplementary Table S2.

Acridine pumping assay

The pET22b-AcrB-TM#(1-6)-AcrB-AcrA plasmids were transformed into the E. coli strain BW25113 (DE3) ΔacrAB, which was generated from the BW25113 ΔacrAB strain (Yum et al., 2009) using the ΔDE3 lysogenization kit (Merck, Germany). The acridine pumping assay was performed using the E. coli strains and based on the procedure previously described (Martins and Amaral, 2012). For the positive control, the AcrB AcrA gene cluster was inserted into pET22b.

Minimum inhibitory concentration

The procedure was previously described (Kim et al., 2009).

Transmission electron microscopy and Image processing

Five microliters of the AcrB-TM#5-AcrA-AcrA or MacA-TolC-hybrid-dimer complex protein at approximately 6 μg/ml was applied to a 300-mesh EM-grid covered with continuous carbon film. After 60 s of sample adsorption, the grid was washed 5 times using droplets of deionized water followed by negative staining with 5 μl of 0.75% uranyl formate and blotting excess solution with filter paper. The sample was visualized using a Tecnai G2 Spirit TWIN (FEI) transmission electron microscope operated at 120 kV. The images were recorded using an Ultrascan4000 charged-coupled device (CCD) camera (Gatan) under low-dose conditions at the nominal magnification x 52,000 and 0.8-1.0 μm under focus.

Model docking

The fully open TolC structure was constructed as described previously (Xu et al., 2011b). Briefly, residues 106-187 and 324-404 were moved through rigid body movement onto the corresponding region of the E. coli MacA structure (PDB code 2FPP; residues 103-177), and the backbone conformations was adjusted to produce an α-hairpin structure. To construct the AcrA hexamer model, we first used the partial AcrA hexamer model that was previously reported and contains the α-barrel domain, lipoyl domain, and α-hairpin domain (Xu et al., 2011a). The AcrA membrane proximal domain (residues 26-52 and 298-397) derived from the MexA structure (PDB code 2V4D; residues 13-27 and 264-339) (Symmons et al., 2009) was attached to the partial AcrA hexamer model as a template of the MacA hexamer crystal structure (PDB code 4DK0) (Xu et al., 2012). The MacA-TolC-hybrid dimer was constructed as described previously (Xu et al., 2011b). To dock the structures into the EM reconstructions, the AcrA hexamer and fully open TolC (or the MacA-TolC-hybrid-dimer) structures were regarded as individual rigid bodies. These three rigid bodies were automatically fitted into the EM map through sequential fitting using the program UCSF Chimera (Goddard et al., 2007) and then manually adjusted into the map using the program COOT (Emsley and Cowtan, 2004).

Expression and purification of the efflux pump assembly

The construct pET22b-AcrB-TM#5-AcrA-AcrA-ProG was transformed into E. coli strain BL21 (DE3) ΔacrAB. The cells were induced with 0.1 mM IPTG at 18°C overnight, harvested and resuspended in lysis buffer 1 (20 mM HEPES (pH 7.5), 10% glycerol, 1 mM PMSF, and 1 mM EDTA). The cells were disrupted using a continuous cell disruptor (Constant Systems, UK), and the cell debris was removed through centrifugation at 10,000 g for 30 min. The cell lysate was ultra centrifuged at 100,000 g for 3 h, and the membrane fraction (pellet) was obtained. The membrane fraction was solubilized in 40 ml of lysis buffer 2 (20 mM HEPES (pH 7.5), 10% glycerol, 1 mM PMSF,
and 2% dodecylmaltoside (DDM) and then dialyzed against 800 ml of a buffer containing 20 mM HEPES (pH 7.5) and 10% glycerol to reduce the DDM concentration. The AcrBA fusion protein was purified using bovine IgG-coupled resin. The protein sample was incubated with bovine IgG-coupled resin and subsequently washed with washing buffer 1 [20 mM HEPES (pH 7.5), 10% glycerol, 0.02% DDM, and 300 mM NaCl] and washing buffer 2 [20 mM HEPES (pH 7.5), 10% glycerol, 0.02% DDM, and 150 mM NaCl]. Next, the purified TolC or MacA-ToLCu-hybrid-dimer protein was incubated at 30°C for 1 h to allow the proteins to form a complex in the resin. After sequential washing with washing buffer 1 and 2, PreSission protease was added overnight at room temperature in the presence of 2 mM 2-mercaptoethanol. The complex proteins were eluted with washing buffer 2 and then concentrated to 1 mg/ml using a Centricone concentrator.

**Results**

**Construction of the AcrBA fusion proteins**

A major barrier to structural work on the AcrAB-ToLC pump was low affinity between the proteins. Moreover, the detergents required to stabilize the membrane proteins may obstruct the protein-protein interactions. To overcome this obstacle, we created fusion proteins that contain AcrA and AcrB. Evidence supporting a 3:6:3 ratio for AcrB:AcrA:TolC has accumulated; however, the functional assembly stoichiometry remains controversial (Janganan et al., 2011; Mima et al., 2007; Narita et al., 2003; Stegmeier et al., 2006; Su et al., 2011; Xu et al., 2009; 2010; 2011a; 2011b; 2012; Yum et al., 2009). As supporting evidence for the AcrA hexamer, an AcrA dimer (two AcrA proteins fused in a single polypeptide chain) exhibited the activity as high as the wild type AcrA (Xu et al., 2011a). To drive the binding and satisfy the stoichiometry between AcrB and AcrA, we designed a long fusion protein, wherein the functional AcrA dimer was fused to the C-terminus of AcrB. Because the AcrB C-terminus is located in the cytoplasmic space (Murakami et al., 2002), and the AcrA is anchored to the periplasmic face of the inner membrane, a transmembrane linker was required between AcrB and the AcrA dimer to bring the AcrB C-terminus to the periplasmic space. Six candidates for the transmembrane linker were selected from transmembrane helices (TM) of other known transmembrane proteins, which yielded the arrangement.

**Electron microscopy analyses of the AcrBA fusion protein and TolC**

To efficiently isolate the fusion protein AcrB-TM#5-AcrA-AcrA, we fused Protein G to the C-terminus of the fusion protein with the PreScission protease recognition site. The protein was purified using bovine IgG-coupled resin and incubated on the resin with the independently purified ToLC protein. Next, the protein complex was eluted from the resin by cleaving the pre-protease recognition site. The resulting protein complexes were then subjected to negative-staining electron microscopy (EM), which readily identified rocket-like particles (Fig. 2A). Subsequent class averaging enhanced visualization of the particles in...
and MacA is in green. The dilated binding interface between AcrA and the TolC \(\alpha\)-hairpin tip is indicated by an arrow. The scale bars are 100 nm and 10 nm in (A) and the inset, respectively.

various orientations, from which a three-fold symmetry was apparent along the longest axis. These data are consistent with the notion that AcrB and TolC have a three-fold symmetry, and AcrA has been predicted to have a three- or six-fold symmetry. Accordingly, the 3D density map was produced with a three-fold symmetry imposed (Fig. 2B).

At a moderate 26 Å, the EM map showed an elongated feature with the AcrB trimer, AcrA hexamer, and TolC trimer (Fig. 2C). To dock the crystal structures of TolC, AcrA, and AcrB, we created a AcrA hexamer model that includes the membrane proximal domain and used the structural model of TolC in the fully-opened conformation (Xu et al., 2011b) as well as the AcrB crystal structure (Murakami et al., 2002). The structures were fitted into the EM map with manual adjustments using rigid body movement; the results were consistent with the density envelop (Fig. 2C). The docked model of the AcrAB-TolC pump has a 770 kDa protein mass and spans the inner membrane, periplasmic space, and outer membrane; it is similar to the modelled structures that exemplify the adaptor bridging model (Xu et al., 2011a; 2011b; 2012; Yum et al., 2009). The cross-section through the 3D-reconstituted density map and docked model exhibit a long channel that runs from the substrate exit region on top of the AcrB to the external medium via the central channels of AcrA and TolC, which is consistent with the homologous MexAB-OprM pump dimensions (Trepout et al., 2010; Xu et al., 2012). However, a docked structure based on the adaptor wrapping model were contradicted to the EM map (Fig. 2D), which demonstrates that the adaptor wrapping model is not consistent with the experimental data for the AcrAB-TolC pump.

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Electron microscopy analysis of the AcrBA fusion protein and TolC α-hairpin tip region

Although the AcrBA fusion protein and TolC EM density map provide an important implication for the AcrAB-TolC pump assembly, how TolC in the fully-opened structure interacts with AcrA remains unclear due to the limited resolution of the EM map. Prior to this study, we investigated the fully-opened structure of TolC using a chimeric protein from *A. actinomycetemcomitans* (Aa), MacA, that contains TolC α-barrel tip region, which we referred to as MacA-TolCα-hybrid-dimer (Xu et al., 2011a; 2011b; 2012). Because the MacA-TolCα-hybrid-dimer protein exhibited a strong affinity to MacA and AcrA in vitro (Kd value of 0.7 nM (Xu et al., 2011b)), this hybrid protein was considered to exhibit a TolC structure that specifically binds AcrA or MacA. To test whether the hybrid protein represented the fully opened TolC structure for binding to the AcrBA complex, we produced a protein complex comprising the AcrBA fusion protein as well as this hybrid protein and performed a similar electron microscopic analysis.

The asymmetric dumb-bell shaped particles were identified in negative-staining EM images, which were processed to create the 3D-reconstitution at a 24 Å resolution (Figs. 3A and 3B). The overall structure and size were similar to the AcrBA fusion protein and TolC. Compared with the dumb-bell structure of the AcrA (or MacA) and the MacA-TolCα-hybrid-dimer protein complexes previously reported (Xu et al., 2011a; 2011b; 2012), this complex structure is similar, except for the AcrB, which generated asymmetry in the dumb-bell structure. The AcrB trimer was docked to the binary complex model comprising the AcrA hexameric model and MacA-TolCα-hybrid-dimer using the complex comprising the AcrBA fusion protein and TolC as a reference (Fig. 3C). Because this AcrBA and MacA-TolC hybrid protein complex structure is consistent with the AcrBA fusion protein and TolC complex, we conclude that the binding interface between AcrA and the TolC α-barrel tip region in this study as well as in previous reports using the MacA-TolCα-hybrid-dimer protein represent the actual AcrAB-ToIc pump assembly (Xu et al., 2011a; 2011b; 2012). In particular, the TolC trimer α-barrel tip region is twisted by approximately 45° similar to the corresponding region of the AcrA (or MacA) hexamer to form the intermeshing cogwheel interaction with the AcrA cogwheel in this binding model and the previous EM studies (Fig. 3D) (Xu et al., 2011a; 2011b; 2012). Combined with the AcrBA-TolC structure, this structure largely confirms the adaptor bridging model based the intermeshing cogwheel interaction between AcrA and TolC as well as the fully-opened TolC structure.

**DISCUSSION**

In this study, we constructed an AcrBA fusion protein because the components bind with low affinity, and we present the EM structures for the AcrAB-ToIc pump assembly using the fusion proteins. The AcrAB-ToIc pump structure exemplifies 'adaptor bridging model', which is an alternative to the prevailing 'adapter wrapping model'.

Du et al. (2014) recently published a structure of the AcrAB-ToIc pump using a different combination of chimeric and fusion proteins. They co-expressed the AcrB-AcrA chimeric protein and AcrA-AcrZ fusion protein, which showed partial activity similar to our fusion protein (Du et al., 2014). They produced a cryo-EM density map and modeled the components in the electron density based on hexameric organization of MacA from A. *actinomycetemcomitans* (Xu et al., 2012; Yum et al., 2009) as well as the interaction between *E. coli* CusA and CusB in the metal-efflux pump (Ju et al., 2011). The overall EM map from Du et al. is essentially the same as our map, but higher in resolution. In particular, the EM map exhibited a dilated feature at the binding interface between AcrA and TolC, which was also apparent in our two EM maps. Because this dilated feature at the binding interface is common among the previously reported AcrA (or MacA) and TolC tip region (Xu et al., 2010; 2012; Yum et al., 2009), this feature is not likely due to experimental errors from the relatively low resolution of the EM density maps. The proposed binding mode between AcrA and TolC in Du et al.'s docked model differs from our model. In their model, the AcrA α-barrel structure cogwheel shows a flared conformation similar to 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 was partly inserted into the wider AcrA α-barrel. Due to these structural features, we call this model 'adaptor partial wrapping model'. According to 'adaptor partial wrapping model', the TolC channel should be narrowed at the binding interface between AcrA and TolC. However, neither
their EM density map nor ours shows the narrowed channel at the binding interface. Both density maps rather display the dilated channel at the binding interface (Fig. 4). Thus our docking model based on the intermeshing cogwheel interaction between AcrA and TolC appears to be more consistent with the EM density map (Fig. 4).

Our docking model based on the intermeshing cogwheel interaction better accounts for the previously reported biochemical and genetic results. The three conserved residues at the ω-hairpin tip region of the adaptor proteins were crucial for binding to the TolC ω-hairpin tip region (Xu et al., 2010; Yum et al., 2009); vice versa, 24 amino acids in the TolC ω-hairpin tip region were sufficient to bind the adaptor proteins (Lee et al., 2012; Xu et al., 2011a; 2012). However, further high resolution structures are necessary to discern the binding assembly between AcrA and TolC as well as to determine how the TolC channel is opened. In this study, we present structural features of the AcrAB-TolC pump, which shows the adaptor bridging model based on the intermeshing cogwheels interaction between AcrA hexamer and TolC trimer. Because binding between the components is crucial to the multidrug efflux pumps, compounds that inhibit protein-protein interactions between the components are good candidates for preventing multidrug resistance in pathogenic bacteria.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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