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The α-Barrel Tip Region of *Escherichia coli* TolC Homologs of *Vibrio vulnificus* Interacts with the MacA Protein to Form the Functional Macrolide-Specific Efflux Pump MacAB-TolC[§]

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TolC and its homologous family of proteins are outer membrane factors that are essential for exporting small molecules and toxins across the outer membrane in Gram-negative bacteria. Two open reading frames in the *Vibrio vulnificus* genome that encode proteins homologous to *Escherichia coli* TolC, designated TolCV1 and TolCV2, have 51.3% and 29.6% amino acid identity to TolC, respectively. In this study, we show that TolCV1 and TolCV2 functionally and physically interacted with the membrane fusion protein, MacA, a component of the macrolide-specific MacAB-TolC pump of *E. coli*. We further show that the conserved residues located at the aperture tip region of the α-hairpin of TolCV1 and TolCV2 played an essential role in the formation of the functional MacAB-TolC pump using site-directed mutational analyses. Our findings suggest that these outer membrane factors have conserved tip-to-tip interaction with the MacA membrane fusion protein for action of the drug efflux pump in Gram-negative bacteria.

Keywords: MacA, MacB, TolC, TolCV1, TolCV2, Type I secretion system

Introduction

Gram-negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella enterica* use tripartite pumps to export antibacterial drugs and other toxic compounds, which guide the diverse molecules across both inner and outer membranes (Lewis, 2000; Zgurskaya *et al*., 2011). These pumps are important for survival, particularly during infections where they contribute to multidrug resistance and virulence (Xu *et al*., 2011a; Lee *et al*., 2012).

Tripartite drug efflux pumps are typically composed of an inner membrane transporter (IMT), an outer membrane factor (OMF) and a periplasmic membrane fusion protein (MFP) (Thanabalu *et al*., 1998; Zgurskaya and Nikaido, 1999; Lewis, 2000; Koronakis *et al*., 2004; Kim *et al*., 2010). The IMT belongs to one of three structurally dissimilar protein superfamilies, including the major facilitator family, resistance-nodulation-cell division (RND) family, and the ATP-binding cassette (ABC) family (Misra and Bavro, 2009). The OMF connects to the IMT in the periplasm, providing a continuous conduit to the extracellular environment. This conduit uses the main channel, which is open only when the complex interacts with other components (Koronakis *et al*., 2004; Misra and Bavro, 2009). The MFP is an adaptor protein for the direct interaction between the IMT and OMF in the periplasm (Zgurskaya *et al*., 2009; Kim *et al*., 2010; Xu *et al*., 2011a, 2011b). The MFP contains a membrane proximal domain, a β-barrel domain, a lipoyl domain, and an α-hairpin domain, which are linearly arranged (Akama *et al*., 2004; Higgins *et al*., 2004; Mikolosko *et al*., 2006; Symmons *et al*., 2009; Yum *et al*., 2009). The MFP α-hairpin domain is responsible for binding to the OMF, whereas the other domains bind the IMT (Gerken and Misra, 2004; Lobedanz *et al*., 2007; Bavro *et al*., 2008; Kim *et al*., 2010; Xu *et al*., 2010, 2011a, 2011b). The crystal structure of TolC reveals that it is a 140 Å long axis, conduit-shaped protein consisting of a 12-stranded β-barrel, an α-helical domain, and a mixed α/β equatorial domain (Koronakis *et al*., 2000; Zgurskaya *et al*., 2011). The equatorial domain has been suggested to be required for interaction with MFP AcrA (Symmons *et al*., 2009).

AcrAB-TolC in *E. coli* and MexAB-OprM in *P. aeruginosa* are examples of tripartite efflux systems, where AcrB and MexB are RND-type IMTs, AcrA and MexA are MFPs, and TolC and OprM are OMFs (Poole *et al*., 1993; Ma *et al*., 1995; Nishino and Yamaguchi, 2001). MacAB-TolC in *E. coli*, which has been identified as a macrolide-specific efflux pump (Kobayashi *et al*., 2001), is an another example of a tripartite efflux system. The IMT MacB is a homodimeric ABC transporter that uses ATP hydrolysis as the driving force (Kobayashi *et al*., 2001, 2003; Tikhonova *et al*., 2007; Lin *et al*., 2009), and MFP MacA shares high sequence similarity with AcrA and MexA (about 44% sequence similarity) (Zgurskaya *et al*., 2009). MacA forms hexamers, and this funnel-like hexameric assembly of MacA is physiologically relevant during formation of the functional MacAB-TolC

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pump (Yum *et al.*, 2009). Our research group has recently investigated the assembly of tripartite efflux pumps and proposed a tip-to-tip interaction model between MFPs (AcrA, MacA, and MexA) and TolC (Yum *et al.*, 2009; Kim *et al.*, 2010; Xu *et al.*, 2010, 2011a, 2011b, 2012). According to this model, the α-helical tip region of the adapter protein makes cogwheel-to-cogwheel binding with the TolC α-barrel tip region.

Protein homologues of *E. coli* TolC have been identified in Gram-negative bacteria (Paulsen *et al.*, 1997). *E. coli* has developed to share a single *tolC* allele as their OMF, whereas tripartite efflux systems in many other bacteria encode their own cognate TolC homologue (Li *et al.*, 1995; Poole *et al.*, 1996; Kohler *et al.*, 1997; Bina *et al.*, 2000). The marine pathogenic bacteria *Vibrio vulnificus* also contains two open reading frames (ORFs), designated TolCV1 and TolCV2 (GenBank accession nos. NC_014965 and NC_014966, respectively), whose amino acid sequences show 51.3% and 29.6% identity to that of *E. coli* TolC (Park *et al.*, 2011). In this study, we provide experimental evidence showing that the conserved amino acids residues in the aperture tip region of TolC and its *V. vulnificus* homologs play a pivotal role in the formation and action of the MacAB-TolC efflux pump, indicating that OMFs in Gram-negative bacteria share a common mechanism for interacting with MFPs.

Materials and Methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Supplementary data Table S1, and the primers used in this study are listed in Supplementary data Table S2. Construction of BW25113Δ*acrAB*Δ*tolC210::Tn10* has been described previously (Kim *et al.*, 2010).

Construction of pTolC1 and its derivatives (pTolC1-L373R, pTolC1-D153E/V370G) expressing *E. coli* TolC wild or mutant types has been described previously (Kim *et al.*, 2010; Xu *et al.*, 2011a). The pTolCV1 plasmid was constructed by ligating polymerase chain reaction (PCR) DNA digested with *Not*I and *Eco*RI restriction enzymes into the same pKAN6B sites. The PCR-amplified fragment was digested with *Nde*I and *Xba*I to construct pTolCV2 and ligated into the same sites in pKAN6B. We synthesized PCR DNA fragments containing the TolCV1 and TolCV2 coding regions, with genomic DNA from *V. vulnificus* MO6-24/O as the template. The pTolCV1-D158E, pTolCV1-L369R, and pTolCV1-D158E/L369R plasmids were constructed by ligating *Not*I and *Eco*RI-digested PCR fragments containing the corresponding mutations using overlap-extension PCR into the same restriction enzymes sites in the pKAN6B vector. The pTolCV2-D146E, pTolCV2-I351R, and pTolCV2-D146E/I351R plasmids were constructed by ligating *Nde*I and *Xba*I digested PCR fragments containing the corresponding mutations using overlap-extension PCR into the same restriction enzymes sites in the pKAN6B vector. The PCR-amplified fragment encoding MacA and MacB was digested with *Not*I and *Xba*I to construct pMacAB2 and ligated into the same sites in pPM30 to produce pMacAB2.

Measurement of minimum inhibitory concentration (Kohler *et al.*) and *in vivo* cross-linking assay

The procedure for measurement of MIC and *in vivo* cross-linking assay has been described previously (Xu *et al.*, 2011a; Lee *et al.*, 2012). *E. coli* BW25113Δ*acrAB*Δ*tolC210::Tn10* cells carrying pMacAB2 and pTolC1, pTolCV1, pTolCV2, or their derivatives (pTolC1-L373R, pTolC1-D153E/V370G, pTolCV1-D158E, pTolCV1-L369R, pTolCV1-D158E/L369R, pTolCV2-D146E, pTolCV2-I351R, and pTolCV2-D146E/I351R) were grown in LB medium to OD_{600}=0.7 and used for cross-linking experiments. Anti-His monoclonal antibody was used to detect TolC, TolCV1, and TolCV2 with a C-terminal hexahistidine tag. Polyclonal antibodies to MacA and MacB were used to detect the MacA and MacB proteins.

Results

Sequence alignment analysis of the TolC homologues in *V. vulnificus*

We sought to identity and characterize TolC homologues in *V. vulnificus*. Potential *tolC* candidate genes were identified by TBLASTX search of the *V. vulnificus* genome (Park *et al.*, 2011) with *E. coli* TolC. The results of this search identified two ORFs (VVM0602608 and VVM0604400) in which the translated products possessed amino acid sequence similarity with 51.3% and 29.6% identity to *E. coli* TolC, respectively. We renamed these genes TolCV1 and TolCV2 (TolC of *V. vulnificus* 1 and 2) (Supplementary data Fig. S1). TolCV1 and TolCV2 consisted of 419 and 399 amino acid residues, respectively. These proteins were significantly shorter than that of *E. coli* TolC (471 residues) but shared similar functional domains according to the structure-based sequence alignment. The TolCV1 protomer had a structural repeat (H1-H2-S1-S2-H3-H4 and H5-H6-S4-S5-H7-H8), which is noted in the family of bacterial OMFs. The TolCV2 protomer also has a structural repeat (Supplementary data Fig. S1) (Federici *et al.*, 2005). The repeat chain of each protomer seemed to construct the long axis and passed from β-strands (S1, S2, S4, and S5) into α-helices (H2, H3 and H4, H6, H7, and H8). The mixed α/β equatorial domain was comprised of β-strands S3 and S6 and α-helices H1, H5, and H9. However, TolCV1 and TolCV2 had shorter C-terminal domains by 48 and 50 amino acid residues, respectively, which are part of the mixed α/β equatorial domain elements. These results suggest that, similar to TolC, TolCV1, and TolCV2 may play a role as outer membrane proteins during formation of tripartite efflux pumps.

TolCV1 and TolCV2 can functionally replace TolC during formation of the macrolide-specific efflux pump MacAB-TolC

To test whether TolCV1 and TolCV2 can functionally complement TolC during action of the MacAB-TolC efflux pump, we transformed an *E. coli* strain with deleted *acrAB* and *tolC* genes (BW25113Δ*acrAB*Δ*tolC::Tn10*) with a plasmid expressing a C-terminally hexahistidine-tagged TolCV1 or TolCV2 (pTolCV1 or pTolCV2) under the control of the P_{BAD}

promoter and a pMACAB2 plasmid that directs synthesis of MacA and MacB under control of the *lacUV5* promoter. The resulting transformants were used to test the ability of TolCV1 and TolCV2 to form a functional MacAB-containing drug efflux pump by measuring erythromycin MICs of these transformants. The BW25113Δ*acrAB*Δ*tolC::Tn10* strain was used for this experiment, because the macrolide drug efflux function of MacAB-TolC can be easily measured in the absence of the major multidrug resistance gene *acrAB* (Lee *et al.*, 2012). The results showed that expression of TolCV1 and TolCV2 rendered *E. coli* cells resistant to erythromycin (MIC = 40 and 10 μg/ml, respectively), whereas TolC expression resulted in a higher MIC (80 μg/ml) (Table 1). BW25113Δ*acrAB*Δ*tolC::Tn10*, harboring empty vectors (pPM30 and pKAN6B), showed an MIC of 1.25 μg/ml.

Notably, TolCV1 and TolCV2 expression levels in *E. coli* cells were 63.4 and 85.7% lower than those of TolC, respectively. As the ORFs of these proteins were cloned into the same vector (pKAN6B) and their mRNAs share the same 5′- and 3′-untranslated region, the difference in MICs may stem from decreased stability of TolCV1 and TolCV2 pro-

Table 1. ***In vivo*** **effects of TolCV1 and TolCV2 variants**[a]

TolC proteins[b]	MIC (μg of erythromycin/ml)[c]
None[d]	1.25
TolC-WT	80
TolC-L373R	2.50
TolC-D153E/V370G	2.50
TolCV1-WT	40
TolCV1-D158E	2.50
TolCV1-L369R	2.50
TolCV1-D158E/L369R	1.25
TolCV2-WT	10
TolCV2-D146E	0.63
TolCV2-I351R	0.63
TolCV2-D146E/I351R	0.63

[a] *E. coli* strain BW25113Δ*acrAB*Δ*tolC210::Tn10* over expressing MacA and MacB was used to test whether TolC, TolCV1, and TolCV2 variants are capable of forming functional MacAB-TolC pump.
[b] TolC, TolCV1, TolCV2 and their variants were expressed from pTolC1, pTolCV1, and pTolCV2.
[c] Erythromycin concentrations used to measure MICs were 0, 0.63, 1.25, 2.50, 5.00, 10.00, 20.00, 40.00, and 80.00 μg/ml.
[d] Empty vectors, pKAN6B and pPM30, were used.

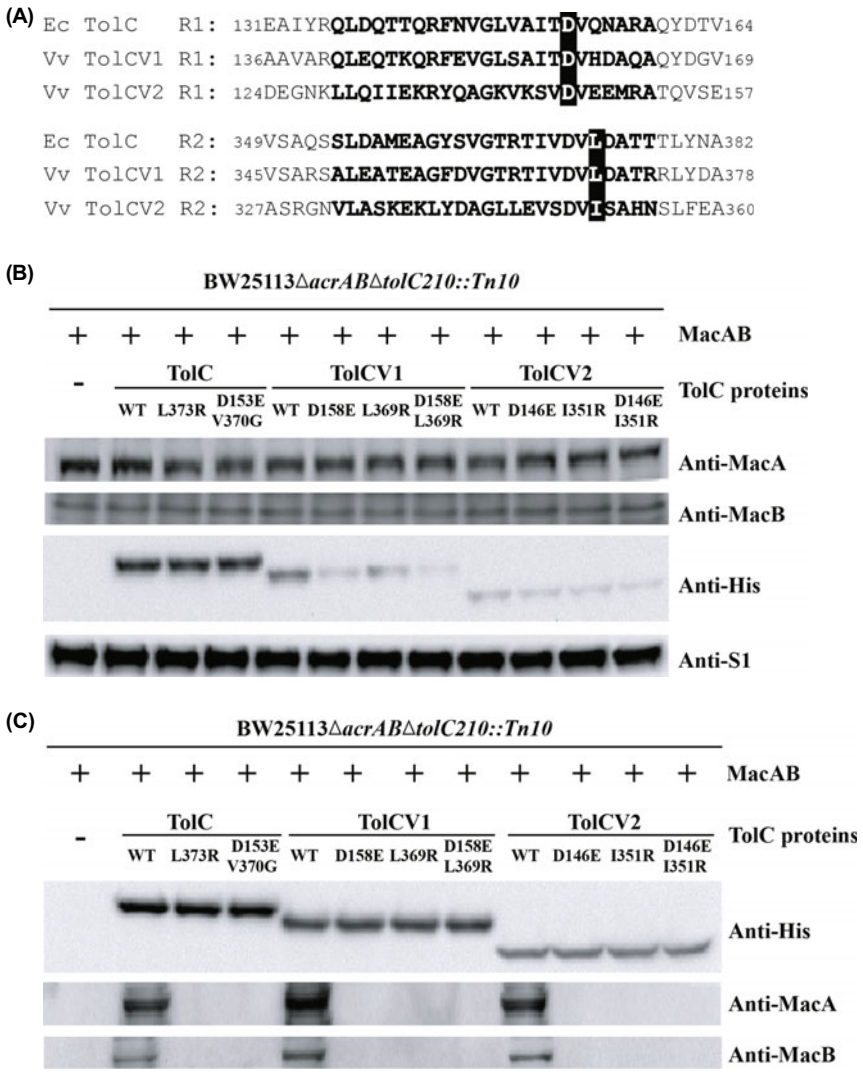

Fig. 1. Functional role of the conserved amino acid residues in the aperture tip region of TolC proteins. (A) Conserved amino acid residues in the aperture tip region of TolC proteins. The repeat sequences of the aperture tip region are in bold. Black boxes indicate the conserved residues in TolC, TolCV1, and TolCV2 that were subject to mutational analyses. Ec and Vv stand for *E. coli* and *V. vulnificus*, respectively. (B) Expression levels of MacAB-TolC pump components in *E. coli* strain BW25113Δ*acrAB*Δ*tolC210::Tn10*. The strains used for measuring minimum inhibitory concentrations (MICs) in Table 1 were grown and Western blot analysis was performed to measure of MacA, MacB, and TolC (TolC, TolCV1, TolCV2, and their variants) proteins expression levels. The S1 protein is shown as a loading control. (C) *In vivo* interactions between MacA and TolC proteins. *In vivo* interactions between MacA and TolC proteins (TolC, TolCV1, TolCV2, and their variants) were detected using the chemical cross-linking agent dithiobis [succinimidylpropionate] (DSP). *E. coli* BW25113-Δ*acrAB*Δ*tolC210::Tn10* strains in Table 1 were cultured and treated with DSP. Affinity-purified TolC proteins (TolC, TolCV1, TolCV2, or their variants) and cross-linked MacA protein were separated by SDS-PAGE and immunoblotted. MacA and MacB were detected using polyclonal antibodies to MacA and MacB. TolC, TolCV1, TolCV2, and their variants were visualized using anti-his-antibody for Western blotting.

teins compared to that of TolC in *E. coli*. It is possible that inefficient transport of these proteins into the outer membrane and/or poor assembly of TolCV1 or the TolCV2-containing MacAB efflux pumps might have caused this result. Lower steady-state levels of these proteins might have consequently resulted in lower MICs. Nevertheless, these results show that TolCV1 and TolCV2 can partially complement TolC during formation of a functional MacAB-TolC macrolide-specific efflux pump.

Functional and physical interactions between *E. coli* TolC homologs of *V. vulnificus* and MacA protein

Previous studies from our research group have suggested that the α-barrels from the MFP units make a cogwheel-to-cogwheel interaction in a tip-to-tip manner with the aperture tip region of OMFs in the type I secretion system (Piao *et al*., 2008; Yum *et al*., 2009; Kim *et al*., 2010; Xu *et al*., 2010, 2011a, 2011b, 2012; Lee *et al*., 2012). We observed that TolCV1 and TolCV2 contain structural motifs similar with α-barrels in the aperture tip region of TolC (24 amino acids, residues 136–159 or 354–377). This observation led us to hypothesize that aperture tip region TolCV1 and TolCV2 α-barrels may be involved in MFP binding (Fig. 1A).

We performed a site-directed mutagenesis experiment for TolCV1 and TolCV2 to examine whether the TolCV1 and TolCV2 aperture tip region is functionally related to assembly of the MacAB-TolC pump. Based on our previous results showing inability of TolC variants containing an amino acid substitution at positions 153 or 373 (D153E or L373R) in the aperture tip region to form a functional AcrAB-TolC pump (Xu *et al*., 2011a), amino acid substitutions at these corresponding positions were introduced into TolCV1 and TolCV2 (Fig. 1A). The resulting variants were expressed along with MacA and MacB in BW25113Δ*tolC::Tn10* and tested for their ability to render *E. coli* cells resistant to erythromycin. The results showed that these amino acid substitutions abolished the resistance of *E. coli* cells to erythromycin (Table 1), indicating that the aperture tip region of these proteins is functionally related to the action of the MacAB-containing drug efflux pump.

As shown for the physical interaction of the TolC aperture region with AcrA (Thanabalu *et al*., 1998; Xu *et al*., 2011a; Lee *et al*., 2012), we further investigated whether an erythromycin-sensitive phenotype of the TolCV1 and TolCV2 variants results from loss of physical interaction with MacA. The same strains used for measuring MICs were grown to a mid-log phase (Fig. 1B) for this experiment, and transient protein complexes were stabilized by treating with the chemical cross-linker DSP, which has a fixed 12 Å spacer arm that connects the primary amine groups of adjacent proteins and contains a cleavable disulfide bond under reducing conditions. *E. coli* cells were lysed, and protein complexes were isolated using a nickel-nitrilotriacetic acid resin column via the His-tagged protein (TolC, TolCV1, TolCV2 or their variants). The eluted complexes were treated with sample buffer containing DTT and boiled to cleave the DSP molecule and release the individual components. These complexes were resolved by 10% SDS-PAGE and identified by immunoblotting using anti-His for TolC, TolCV1, TolCV2 or their variants and polyclonal antibodies for MacA and MacB. As shown in Fig. 1C, the TolCV1 and TolCV2 proteins were cross-linked to MacA, whereas TolCV1 and TolCV2 variants were not, indicating that the TolCV1 and TolCV2 aperture region makes specific contact with MacA. Co-expressed TolC and MacA resulted in strong copurification as reported previously (Xu *et al*., 2011b). These results show that the TolCV1 and TolCV2 aperture tip regions are not only functionally related, but physically interact with MacA.

Discussion

In this study, we demonstrated that TolCV1 and TolCV2 are functional homologs of *E. coli* TolC during formation of the MacAB-containing drug efflux pump, and that the aperture region of these proteins plays an important role in the physical interaction with MacA. Considering the degree of amino acid sequence conservation between *E. coli* TolC and its *V. vulnificus* homologs (51.3% and 29.6% identity for TolCV1 and TolCV2, respectively), the effects of amino acid substitutions in the aperture region of these proteins were very strong on their physical interactions with MacA and consequent assembly of the functional MacAB-TolC tripartite pump. This finding supports the "adapter bridging model" for the MacAB-TolC pump, of which the key feature is tip-to-tip binding between MacA and TolC (Yum *et al*., 2009; Xu *et al*., 2010).

Many crystal structures of OMFs (TolC, OprM, and VceC) have similar long C-terminal residues that consist of mixed α/β equatorial domain elements (Koronakis *et al*., 2000; Akama *et al*., 2004; Federici *et al*., 2005), which are a possible recognition site for OMF-MFP interactions in another AcrAB-TolC pump assembly model (Lobedanz *et al*., 2007; Bavro *et al*., 2008; Symmons *et al*., 2009). In this model, the AcrA hairpins surround the outside of the TolC α-barrel, which is distinct from the adaptor bridging model. However, the sequence alignment of OMFs with TolCV1 and TolCV2 does clearly define the difference in length of the C-terminal residues that consist of the equatorial domain (Supplementary data Figs. S1 and S2). TolCV1 and TolCV2 have a 48 and 50 residue shorter C-terminal domain than that of TolC.

We think that it is likely that MFPs exhibit similar interactions with TolC, based on our biochemical and genetic evidence implicating a tip-to-tip interaction between the MacA and *V. vulnificus* OMFs TolCV1 and TolCV2 in the assembly of the functional tripartite efflux pump, and the observations showing that the conserved residues in the MacA aperture region and other MFPs such as AcrA and HlyD are important in TolC binding and the formation of functional tripartite pumps (Xu *et al*., 2011a, 2012; Lee *et al*., 2012).

Although further structural and functional studies are required to better understand the specific functions of TolCV1 and TolCV2 and the assembly mechanism of tripartite efflux pumps in *V. vulnificus*, our results provide information for understanding the type I secretion system, which involves TolC-like proteins in pathogenic bacteria.

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