

## Interaction between the $\alpha$ -Barrel Tip of *Vibrio vulnificus* TolC Homologs and AcrA Implies the Adapter Bridging Model

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(Received Nov 5, 2013 / Revised Dec 5, 2013 / Accepted Dec 6, 2013)

**The AcrAB-TolC multidrug efflux pump confers resistance to *Escherichia coli* against many antibiotics and toxic compounds. The TolC protein is an outer membrane factor that participates in the formation of type I secretion systems. The genome of *Vibrio vulnificus* encodes two proteins homologous to the *E. coli* TolC, designated TolCV1 and TolCV2. Here, we show that both TolCV1 and TolCV2 partially complement the *E. coli* TolC function and physically interact with the membrane fusion protein AcrA, a component of the *E. coli* AcrAB-TolC efflux pump. Using site-directed mutational analyses and an *in vivo* cross-linking assay, we demonstrated that the  $\alpha$ -barrel tip region of TolC homologs plays a critical role in the formation of functional AcrAB-TolC efflux pumps. Our findings suggest the adapter bridging model as a general assembly mechanism for tripartite drug efflux pumps in Gram-negative bacteria.**

**Keywords:** AcrA, TolC, TolCV1, TolCV2, multidrug efflux pump

### Introduction

Gram-negative bacteria use specialized efflux pump systems to expel drugs and toxic compounds across the inner and outer membranes separated by the periplasmic space (Zgurskaya and Nikaido, 1999; Federici *et al.*, 2005; Nikaido and Pages, 2012; Wang *et al.*, 2012). Well-studied examples of Gram-negative efflux pumps are the *Escherichia coli* AcrAB-TolC and the *Pseudomonas aeruginosa* MexAB-OprM pumps. The multidrug efflux pump AcrAB-TolC consists of the outer membrane factor (OMF) TolC, the inner membrane transporter (IMT) AcrB, and the membrane fusion protein (MFP) AcrA (Thanabalu *et al.*, 1998; Zgurskaya and

Nikaido, 1999; Lewis, 2000; Koronakis *et al.*, 2004; Kim *et al.*, 2010; Xu *et al.*, 2011b; Hobbs *et al.*, 2012). The IMT AcrB protein forms a trimer with structural asymmetry and uses proton motive force to pump substrates (Murakami *et al.*, 2002, 2006; Yu *et al.*, 2003; Seeger *et al.*, 2006; Sennhauser *et al.*, 2007; Murakami, 2008). The OMF TolC also functions as a trimer and provides a continuous conduit to the extracellular environment (Koronakis *et al.*, 2000; Fernandez-Recio *et al.*, 2004; Koronakis *et al.*, 2004). The MFP AcrA is known to interact directly with both AcrB and TolC and is necessary for the multidrug efflux function of the tripartite AcrAB-TolC system (Elkins and Nikaido, 2003; Gerken and Misra, 2004; Husain *et al.*, 2004; Lobedanz *et al.*, 2007; Ge *et al.*, 2009; Kim *et al.*, 2010; Xu *et al.*, 2011b). Conformational changes in AcrA have been proposed to be important in the assembly process of the tripartite AcrAB-TolC complex (Tikhonova *et al.*, 2011). Recently, it has been suggested that AcrA functions as a dimer that assembles into a hexamer in the AcrAB-TolC complex (Kim *et al.*, 2010; Xu *et al.*, 2011b). The electron microscopic study visualized the intermeshing cogwheel-like interaction between the TolC  $\alpha$ -barrel tip region and the AcrA  $\alpha$ -barrel (Xu *et al.*, 2011b), which is essentially same as the interaction between TolC and MacA (Xu *et al.*, 2011a). These studies proposed “adapter bridging model” for the AcrAB-TolC pump.

*E. coli* TolC homologs have been identified in other Gram-negative bacteria (Paulsen *et al.*, 1997), including the TolCV1 and TolCV2 homologs from the marine pathogenic bacterium *Vibrio vulnificus* (Lee *et al.*, 2013). Although the amino acid sequences of the *V. vulnificus* proteins show 51.3% and 29.6% identity to that of the *E. coli* TolC (Park *et al.*, 2011; Lee *et al.*, 2013), TolCV1 and TolCV2 are significantly shorter than the *E. coli* TolC but share similar functional domains (Lee *et al.*, 2013). In this study, we investigated the interactions between TolC and AcrA using site-directed mutational analyses and *in vivo* cross-linking assays with *E. coli* TolC and its *V. vulnificus* homologs TolCV1 and TolCV2. Our results suggest that the  $\alpha$ -barrel tip regions of the TolC homologs have conserved function in the formation of tripartite efflux pumps.

### Materials and Methods

#### Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. Construction of BW25113 $\Delta$ acrAB $\Delta$ tolC210::Tn10 has been previously described (Kim *et al.*, 2010). Construction of pTolC1, pTolCV1, pTolCV2, and their derivatives (pTolC1-L373R, pTolCV1-L369R, and pTolCV2-

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**Table 1. Bacterial strains and plasmids used in this study**

Strains or plasmids	Relevant characteristics	Reference
<i>E. coli</i>		
DH5α	f80dlacZ DM15 <i>recA1 endA1 gyrA96 relA1 thi-1 hsdR17</i> (r <sub>K</sub> <sup>-</sup> m <sub>K</sub> <sup>-</sup> ) <i>supE44 deoR</i> Δ( <i>lacZYA-argF</i> )U169	Laboratory collection
BW25113Δ <i>acrABΔtolC210::Tn10</i>	<i>acrAB</i> and <i>tolC</i> deleted BW25113	Kim <i>et al.</i> (2010)
Plasmids		
pKAN6B	p15A <i>ori</i> , Km <sup>r</sup>	Yeom and Lee (2006)
pPM30	pSC101 <i>ori</i> , Amp <sup>r</sup>	Meacock and Cohen (1980)
pTolC1	p15A <i>ori</i> , Km <sup>r</sup> , <i>tolC</i> under P <sub>BAD</sub>	Kim <i>et al.</i> (2010)
pTolC1-L373R	pTolC1 with L373R mutation	Xu <i>et al.</i> (2011b)
pTolC1-L373G	pTolC1 with L373G mutation	This study
pTolC1-L373C	pTolC1 with L373C mutation	This study
pTolC1-L373D	pTolC1 with L373D mutation	This study
pTolC1-L373Q	pTolC1 with L373Q mutation	This study
pTolC1-Y362F/R367E	pTolC1 with Y362F/R367E mutations	Bavro <i>et al.</i> (2008)
pAcrAB2	pSC101 <i>ori</i> , Amp <sup>r</sup> , <i>acrAB</i> under PlacUV5	This study
pTolCV1	p15A <i>ori</i> , Km <sup>r</sup> , <i>tolCV1</i> under P <sub>BAD</sub>	Lee <i>et al.</i> (2013)
pTolCV1-L369R	pTolCV1 with L369R mutation	Lee <i>et al.</i> (2013)
pTolCV2	p15A <i>ori</i> , Km <sup>r</sup> , <i>tolCV2</i> under P <sub>BAD</sub>	Lee <i>et al.</i> (2013)
pTolCV2-I351R	pTolCV2 with I351R mutation	Lee <i>et al.</i> (2013)

I351R) has been previously described (Lee *et al.*, 2013). Plasmid pTolC1-Y362F/R367E and other pTolC1 derivatives (pTolC1-L373G, pTolC1-L373C, pTolC1-L373D, and pTolC1-L373Q) were constructed by overlap-extension PCR method using pTolC1 as a template. To construct these pTolC1 variants, *NotI* and *EcoRI* sites were created using the overlap-extension PCR method with two outside primers (5'-TTGCGGCCGCAAGGAGAGAATCATATGAAGAAATTGCTCCCATTCT and 5'-AAGAATTCTCAATGATGATGATGATGGTTACGGAAAGGGTTAT) and following primers for each mutation (mutated nucleotides are underlined) : 5'-TACGCGTACCATTGTTGATGTGGGCATGCGACCACCACGTTGTACA and 5'-TGTACAACGTGGTGGTTCGCATCGCCACATCAACAATGGTACGCGTA for pTolC1-L373G, 5'-TACGCGTACCATTGTTGATGTGTGCGATGCGACCACCACGTTGTACA and 5'-TGTACAACGTGGTGGTTCGCATCGCACACATCAACAATGGTACGCGTA for pTolC1-L373C, 5'-TACGCGTACCATTGTTGATGTGGATGATGCGACCACCACGTTGTACA and 5'-TGTACAACGTGGTGGTTCGCATCATCCACATCAACAATGGTACGCGTA for pTolC1-L373D, 5'-TACGCGTACCATTGTTGATGTGCAGGATGCGACCACCACGTTGTACA and 5'-TGTACAACGTGGTGGTTCGCATCCTGACATCAACAATGGTACGCGTA for pTolC1-L373Q, 5'-GCTTCTCGGTCGGTACGGAACACATTGTTGATGTGTGGAT and 5'-GTTTCCGTACCGACCGAGAAGCCCGTTCCATCGCGTCTAAT for pTolC1-Y362F/R367E. These resulting PCR products were digested with the *NotI* and *EcoRI* sites and cloned into the same sites in pTolC1. To clone *E. coli* *acrA* and *acrB*, we amplified the DNA segment encoding AcrA and AcrB from pAcrAB1 (Xu *et al.*, 2011b) by PCR using two primers (5'-CGCGGCCGACGATAATATAAACGCGAGCA and 5'-TCTAGAATCAATGATGATCGACAGTAT). A PCR-amplified DNA product

was digested with *NotI* and *XbaI* and ligated into the same sites in vector pPM30 to produce pAcrAB2.

#### Measurement of minimum inhibitory concentration (MICs) and the *in vivo* cross-linking assay

The procedures for MIC measurement and the *in vivo* DSP-mediated cross-linking assay have been previously described (Xu *et al.*, 2011b; Lee *et al.*, 2012, 2013). *E. coli* BW25113 Δ*acrABΔtolC210::Tn10* cells carrying pAcrAB2 and pTolC1, pTolCV1, pTolCV2, or their derivatives (pTolC1-L373R, pTolCV1-L369R, and pTolCV2-I351R) were grown in LB medium to an OD<sub>600</sub> = 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 AcrA and AcrB were used to detect the AcrA and AcrB proteins.

#### Trypsin digestion

*E. coli* BW25113Δ*acrABΔtolC210::Tn10* cells carrying pTolC-WT or pTolC-L373R that expressed wild-type TolC or its variants with C-terminal hexahistidine tag were grown in LB medium to an OD<sub>600</sub>=1.0. The bacterial cells were harvested and then washed with phosphate-buffered saline (PBS). For tryptic digestion, these cells were resuspended in buffer containing 20 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 20% sucrose. After treatment of the cells with trypsin (0.1, 1, or 10 mg/L) for 3 h at 37°C, proteolysis was terminated by boiling in SDS-sample buffer (Krishnamoorthy *et al.*, 2013). Total proteins were separated by 10% SDS-PAGE and probed with anti-His-tag monoclonal antibody.

## Results

### TolCV1 and TolCV2 complement TolC in a functioning AcrAB-TolC multidrug efflux pump

To investigate the nature of the interactions between various MFPs and OMFs, we first tested whether TolCV1 and TolCV2 can functionally complement TolC in the action of the AcrAB-TolC multidrug efflux pump. To this end, we transformed an *E. coli* strain with the *acrAB* and *tolC* genes deleted (BW25113 $\Delta$ *acrAB* $\Delta$ *tolC210::Tn10*) with a plasmid expressing genes that encode a C-terminally hexahistidine-tagged TolCV1 or TolCV2 (pTolCV1 or pTolCV2) under control of the P<sub>BAD</sub> promoter and a pAcrAB2 plasmid that directs the expression of *acrA* and *acrB* under control of the *lacUV5* promoter. The resulting transformants were used to test the ability of the TolCV1 and TolCV2 proteins to form a functional AcrAB-containing multidrug efflux pump by measuring the degree of novobiocin resistance conferred to these transformants, since novobiocin is a well-known substrate for the AcrAB-TolC pump (Kim et al., 2010; Xu et al., 2011b). The results showed that the expression of TolCV1 and TolCV2 rendered *E. coli* cells resistant to novobiocin (MIC values of 100 and 25  $\mu$ g/ml, respectively), whereas *E. coli* TolC expression resulted in a higher novobiocin MIC (200  $\mu$ g/ml) (Table 2). BW25113 $\Delta$ *acrAB* $\Delta$ *tolC210::Tn10* harboring empty vectors (pKAN6B and pPM30) showed an MIC of 1.56  $\mu$ g/ml. These results indicate that TolCV1 and TolCV2 can partially complement TolC in the AcrAB-TolC multidrug efflux pump.

### Functional and physical interactions between the $\alpha$ -barrel tip region of TolC homologs and AcrA

We previously proposed a model for tripartite efflux pumps

**Table 2.** *In vivo* effects of an amino acid substitution in the  $\alpha$ -barrel tip region of TolC homologs on their function in antibiotic resistance

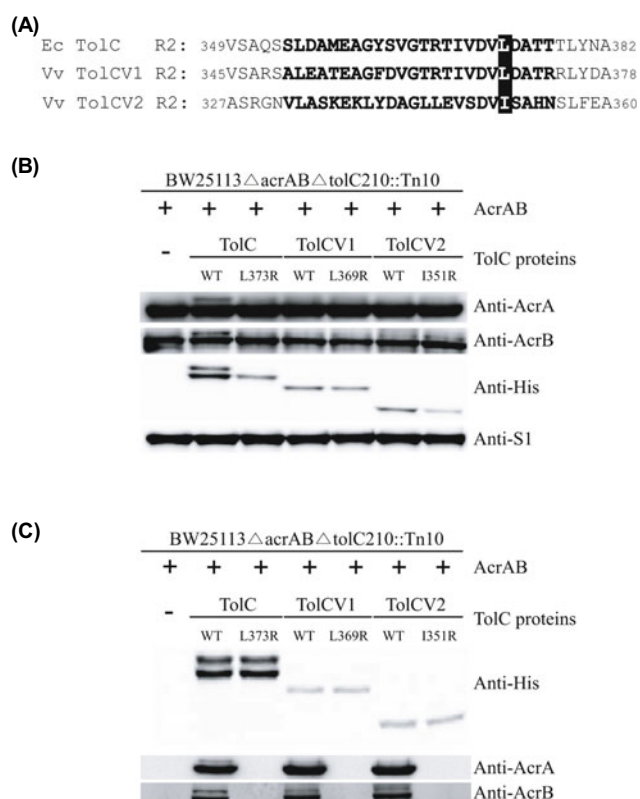
TolC proteins <sup>a</sup>	MIC ( $\mu$ g/ml)	
	Novobiocin <sup>b</sup>	Vancomycin <sup>c</sup>
None	1.56	1100.00
TolC-WT	200.00	500.00
TolC-L373R	25.00	450.00
TolC-L373G	100.00	600.00
TolC-L373C	100.00	600.00
TolC-L373D	100.00	650.00
TolC-L373Q	100.00	600.00
TolC-Y362F/R367E	100.00	250.00
TolCV1-WT	100.00	500.00
TolCV1-L369R	25.00	450.00
TolCV2-WT	25.00	550.00
TolCV2-I351R	1.56	500.00

<sup>a</sup> TolC, TolCV1, TolCV2 and their variants were expressed from pTolC1, pTolCV1, and pTolCV2. 'None' denotes that empty vectors pKAN6B and pPM30 were used instead of pTolC1 variants and pAcrAB2 respectively.

<sup>b</sup> *E. coli* strain BW25113 $\Delta$ *acrAB* $\Delta$ *tolC210::Tn10* over-expressing AcrA and AcrB was used to test whether TolC, TolCV1 and TolCV2 variants are capable of forming functional AcrAB-TolC pump. Novobiocin concentrations used to measure MICs were 0, 0.78, 1.56, 3.13, 6.25, 12.50, 25.00, 50.00, 100.00, and 200.00  $\mu$ g/ml.

<sup>c</sup> *E. coli* strain BW25113 $\Delta$ *acrAB* $\Delta$ *tolC210::Tn10* that did not express AcrA and AcrB was used to test whether TolC, TolCV1 and TolCV2 variants form a leaky channel to vancomycin. Vancomycin concentrations used to measure MICs ranged from 0, to 1100.00  $\mu$ g/ml by an increment of 50.

in the type I secretion system of Gram-negative bacteria in which the  $\alpha$ -barrel tip region of OMFs plays a key role in interacting with the MFP units (Piao et al., 2008; Yum et al., 2009; Kim et al., 2010; Xu et al., 2010, 2011a, 2011b, 2012; Lee et al., 2012, 2013). Based on our observation that TolCV1 and TolCV2 contain structural motifs similar to those of the  $\alpha$ -barrel tip region of TolC (24 amino acids, residues 354-377), which functionally and physically interacts with MacA (Lee et al., 2013), we hypothesized that the  $\alpha$ -barrel tip regions of TolCV1 and TolCV2 would also be involved in the formation of a functional AcrAB-TolC pump (Fig. 1).



**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  $\alpha$ -barrel tip region of TolC proteins. The repeat sequences of the  $\alpha$ -barrel tip region are in bold. Black boxes indicate the conserved residues in TolC, TolCV1, and TolCV2 that were subjected to mutational analyses. Ec and Vv indicate *E. coli* and *V. vulnificus*, respectively. (B) Expression levels of AcrAB-TolC pump components in *E. coli* strain BW25113 $\Delta$ *acrAB* $\Delta$ *tolC210::Tn10*. The strains used for measuring minimum inhibitory concentrations (MICs) in Table 2 were grown, and Western blot analysis was performed to measure AcrA, AcrB, and TolC (TolC, TolCV1, TolCV2, and their variants) protein levels. The S1 protein is shown as a loading control. (C) *In vivo* interactions between AcrA and TolC proteins. *In vivo* interactions between AcrA and TolC proteins (TolC, TolCV1, TolCV2, and their variants) were detected using the chemical cross-linking agent dithiobis(succinimidyl propionate) (DSP). The *E. coli* BW25113 $\Delta$ *acrAB* $\Delta$ *tolC210::Tn10* strains listed in Table 2 were cultured and treated with DSP. Affinity-purified TolC proteins (TolC, TolCV1, TolCV2, or their variants) and cross-linked AcrA protein were separated by SDS-PAGE and detected by Western blotting. AcrA and AcrB were detected using polyclonal antibodies to AcrA and AcrB. TolC, TolCV1, TolCV2, and their variants were visualized using anti-his-antibody.

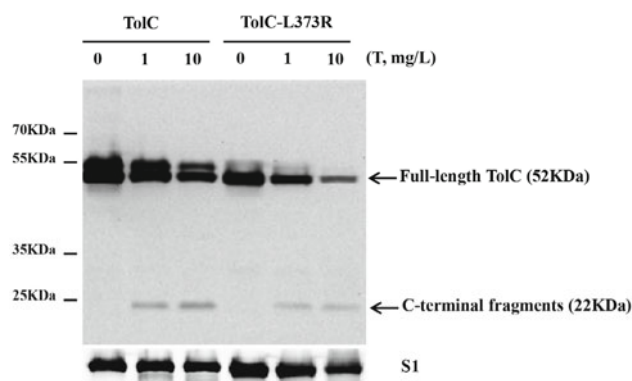
To test this hypothesis, an amino acid substitution was introduced to replace the conserved leucine residue at position 373, located in the  $\alpha$ -barrel tip region of TolC and in the corresponding residues of TolCV1 (Leu369) and TolCV2 (Ile351) (Fig. 1A). The resulting TolC variant proteins were produced in BW25113 $\Delta$ acrAB $\Delta$ tolC210::Tn10 harboring pAcrAB2 (Fig. 1B) and tested for their ability to render *E. coli* cells resistant to novobiocin. The results showed that these amino acid substitutions abolished novobiocin resistance in *E. coli* (Table 2). These results indicate the functional importance of Leu (or Ile) residues in the  $\alpha$ -barrel tip region of TolC proteins for the formation of functional AcrAB-TolC pumps.

Since the importance of the TolC  $\alpha$ -barrel tip region in the binding of MFPs was identified for the AcrAB-TolC multidrug efflux pump (Thanabalu *et al.*, 1998; Xu *et al.*, 2011b), we further investigated whether the novobiocin-sensitive phenotypes of the TolCV1 and TolCV2 variants were a result of their inability to physically interact with AcrA. The same strains used for measuring MICs were grown to the early-log phase in the presence of IPTG (1 mM) and arabinose (0.2%) to induce the production of the AcrAB and TolC proteins, respectively. To stabilize the transient protein complexes, the cultures were treated with the chemical cross-linker dithiobis (succinimidyl propionate) (DSP), which connects the primary amine groups of adjacent proteins. This cross-linker has a fixed 12-Å spacer arm that contains a cleavable disulfide bond under reducing conditions. AcrAB-TolC complexes were isolated using a Ni-NTA resin column via the his-tagged TolC proteins, treated with DTT, and boiled to cleave the DSP molecules. The components of the AcrAB-TolC complexes were identified by immunoblotting (Fig. 1C). The TolCV1 and TolCV2 proteins were cross-linked to AcrA, whereas the TolCV1 and TolCV2 variants were not. AcrA was copurified when *E. coli* TolC and AcrA were coexpressed, as reported previously (Xu *et al.*, 2011b; Lee *et al.*, 2013). These results show that the introduction of an amino acid substitution at the  $\alpha$ -barrel tip region of the TolC homologs resulted in a direct loss of interaction with

AcrA, indicating that the TolCV1 and TolCV2 aperture region makes physical contact with AcrA.

### Effects of the L373R and the corresponding mutations on the functional state of the $\alpha$ -barrel tip region of TolC homologs

The conserved hydrophobic residue (L373) in the *E. coli* TolC is directly adjacent to the aspartic acid residues 371 and 374, which are key residues of the secondary bottleneck of the TolC aperture and are involved in maintaining a closed aperture of TolC (Pei *et al.*, 2011). Therefore, it is also possible that a drastic conformational change occurred in the TolC homologs harboring the mutation, thus resulting in a loss of interaction with AcrA. For this reason, we tested vancomycin sensitivity of *E. coli* strains that expressed wild-type TolC homologs or their variants in the absence of AcrA and AcrB expression, as *E. coli* becomes sensitive to this large antibiotic when the TolC aperture has been permanently altered to an open state. To exclude the effects of interaction with AcrAB on the ability of TolC variants to allow the diffusion of vancomycin, we used *E. coli* strains that did not express AcrA and AcrB for these experiments. The results showed that the expression of TolC variants containing the L373R or the corresponding mutations did not significantly affect the resistance of *E. coli* cells to vancomycin, while cells expressing the 'leaky' TolC mutant containing the Y362F/R367E mutations became susceptible to this antibiotic, as previously reported (Bavro *et al.*, 2008) (Table 2). These results indicate that the L373R and the corresponding mutations did not disturb the maintenance of a closed aperture in the TolC homologs. Next, we used limited proteolysis with trypsin to compare the conformations of the TolC variants. Consistent with the results from the vancomycin susceptibility test, the proteolytic profile of the wild-type TolC was similar to that of TolC-L373R (Fig. 2). In the presence of amounts of trypsin (1.0 mg/L), the 22-kDa fragment that corresponds to the C-terminal half of TolC was cleaved following R267, located in the extracellular loop (Koronakis *et al.*, 1997). Furthermore, the expression of TolC variants harboring an amino acid substitution of L373 to glycine, cysteine, aspartate, or glutamine resulted in the increased susceptibility of *E. coli* cells to novobiocin, while these mutations did not significantly affect the degree of vancomycin resistance (Table 2). Once again, these results indicate that a loss of TolC function by the L373R mutation did not result from vast conformational changes within the TolC protein due to the addition of a charge residue (arginine) or the loss of the hydrophobic residue (leucine) at L373. Based on these results, we concluded that a loss of TolC interaction with AcrA is not attributable to a drastic conformational change in TolC proteins by the mutation.



**Fig. 2.** *In vivo* proteolytic profiles of TolC-WT and TolC-L373R. Cell extracts of *E. coli* strains that overexpress TolC-WT or TolC-L373R were treated with trypsin at various concentrations (0.1, 1, 10 mg/L). Partially digested proteins were separated by SDS-PAGE and detected using monoclonal anti-His-tag antibody to compare proteolytic patterns of TolC-WT and TolC-L373R protein.

## Discussion

Numerous studies have investigated the action of the AcrAB-TolC multidrug efflux pump complex (Koronakis *et al.*, 2000; Akama *et al.*, 2004; Seeger *et al.*, 2006; Lobedanz *et al.*, 2007; Kim *et al.*, 2010; Xu *et al.*, 2011b). However, the process of the assembly of this complex remains unclear. In

the present study, we provided experimental evidence showing that the  $\alpha$ -barrel tip regions of TolCV1 and TolCV2, despite having limited overall homology (~30 to 50%) to the *E. coli* TolC, play an important role in both the physical and functional interactions with AcrA. Our findings are consistent with the notion that the structures of various OMFs (TolC, OprM, and VceC) are remarkably similar, even though their amino acid sequence identity is quite low (Koronakis et al., 2000; Akama et al., 2004; Federici et al., 2005). Although the *V. vulnificus* TolCV1 and TolCV2 OMFs share structural domains with other OMFs, the lengths of the C-terminal domains that constitute the  $\alpha/\beta$  equatorial domain are 48 and 50 residues shorter in TolCV1 and TolCV2, respectively, than the C-terminal domain of TolC (Lee et al., 2013). These missing residues equate to a significantly shortened  $\alpha/\beta$  equatorial domain, which has been proposed as a possible recognition site for OMF-MFP interactions. The functional complementation of TolC by TolC homologs with shortened  $\alpha/\beta$  equatorial domains is not unusual, since it has been reported that a C-terminally truncated TolC containing a deletion of 43 residues was able to complement a *tolC* null strain fully (Bavro et al., 2008). Thus, it is not clear how much the  $\alpha/\beta$  equatorial domain contributes to OMF-MFP interactions.

Based on our results in this study and the observation that the conserved residues in other MFPs such as MacA and HlyD are important for physical interactions with TolC and the formation of functional tripartite efflux pumps (Xu et al., 2011a; Lee et al., 2012, 2013), we suggest that MFPs exhibit similar interactions with TolC and that adapter bridging model is generally applicable for assembly model for tripartite efflux pumps. The identification of the binding partners of TolCV1 and TolCV2 in *V. vulnificus* and the characterization of their molecular interactions will further enhance our understanding of the assembly mechanism of tripartite efflux pumps in Gram-negative bacteria.

## Acknowledgements

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2013R1A1A2006953) and the Chung-Ang University Research Scholarship Grants in 2011.

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