J. Microbiol. Biotechnol. (2008), **18**(5), 845–851

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Expression and Biochemical Characterization of the Periplasmic Domain of Bacterial Outer Membrane Porin TdeA

Kim, Seulki[1], Soohwan Yum[1], Wol-Soon Jo[2], Bok Luel Lee[1], Min-Ho Jeong[2], and Nam-Chul Ha[1]*

[1]*College of Pharmacy and Research Institute for Drug Development, Pusan National University, Busan 609-735, Korea*
[2]*Donga-A University Medical Science Research Center, Busan 602-714, Korea*

Received: September 27, 2007 / Accepted: December 24, 2007

TolC is an outer membrane porin protein and an essential component of drug efflux and type-I secretion systems in Gram-negative bacteria. TolC comprises a periplasmic α-helical barrel domain and a membrane-embedded β-barrel domain. TdeA, a functional and structural homolog of TolC, is required for toxin and drug export in the pathogenic oral bacterium *Actinobacillus actinomycetemcomitans*. Here, we report the expression of the periplasmic domain of TdeA as a soluble protein by substitution of the membrane-embedded domain with short linkers, which enabled us to purify the protein in the absence of detergent. We confirmed the structural integrity of the TdeA periplasmic domain by size-exclusion chromatography, circular dichroism spectroscopy, and electron microscopy, which together showed that the periplasmic domain of the TolC protein family fold correctly on its own. We further demonstrated that the periplasmic domain of TdeA interacts with peptidoglycans of the bacterial cell wall, which supports the idea that completely folded TolC family proteins traverse the peptidoglycan layer to interact with inner membrane transporters.

Keywords: Membrane protein, TolC, bacterial multidrug resistance, peptidoglycan

Outer membrane porin protein TolC is ubiquitously expressed in Gram-negative bacteria, where it is important for bacterial resistance to antibiotics and for bacterial secretion of toxins [8, 138]. The TolC family of proteins is one of the three essential components of the efflux pathway that pumps out small noxious compounds such as detergents and antibacterial drugs, and that drives type I toxin secretion systems [5, 14]. TolC proteins provide a large exit channel that cuts across the peptidoglycan layer of the cell wall and opens out into the extracellular space. To create a channel that spans the periplasm, membrane-fusion proteins in the periplasm interact with transporters in the inner membrane and with TolC proteins in the outer membrane [14]. Crystal structures of a TolC protein reveal it to be a homotrimer with a long cylindrical shape and a central channel (Fig. 1A) [6]. The central channel comprises an α-helical barrel domain, 100 Å long, that extends across the periplasmic space, and a β-barrel domain, 40 Å long, that sits in the outer membrane [6].

TdeA belongs to the TolC family and is required for toxin and drug export in *Actinobacillus actinomycetemcomitans*, which is a pathogenic oral bacterium that causes localized aggressive periodontitis and extraoral infection [2]. The genes *macA*, *macB*, and *tdeA* are known to be organized as an operon for multidrug resistance and are expressed as a single transcript in *A. actinomycetemcomitans*, indicating that TdeA interacts with the membrane fusion protein MacA and the ABC-type exporter MacB [2]. TdeA, like TolC, is an integral membrane protein, which makes it difficult to express and purify.

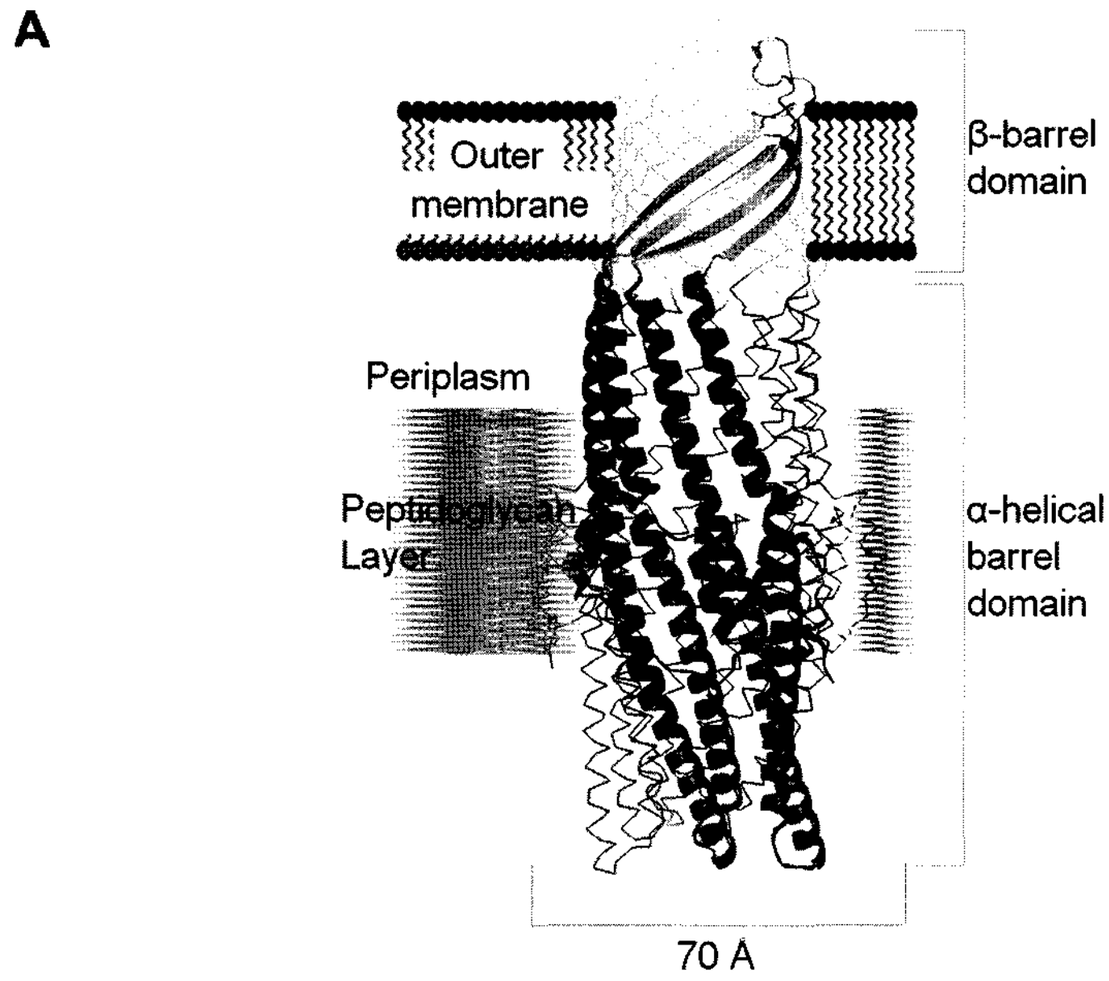
Based on the crystal structure of TolC [6], we hypothesized that the α-helical barrel and β-barrel domains of TdeA would fold independently, even though the membrane-embedded β-barrel domain is connected to the α-helical barrel domain through four loops that are separated in the primary structure (Fig. 1B). To test whether the α-helical barrel domain could properly fold without the β-barrel domain, we expressed and purified the periplasmic domain of TdeA by substituting the membrane-embedded domain with two soluble linkers.

Materials and Methods

Construction of Expression Plasmid for the Periplasmic Domain of TdeA from *A. actinomycetemcomitans*

The DNA sequence containing the α-helical barrel domain regions (residues 24-95, 142-301, 341-457) and the soluble linkers was

**Corresponding author*
Phone: 82-51-510-2528; Fax: 82-51-513-6754;
E-mail: hnc@pusan.ac.kr



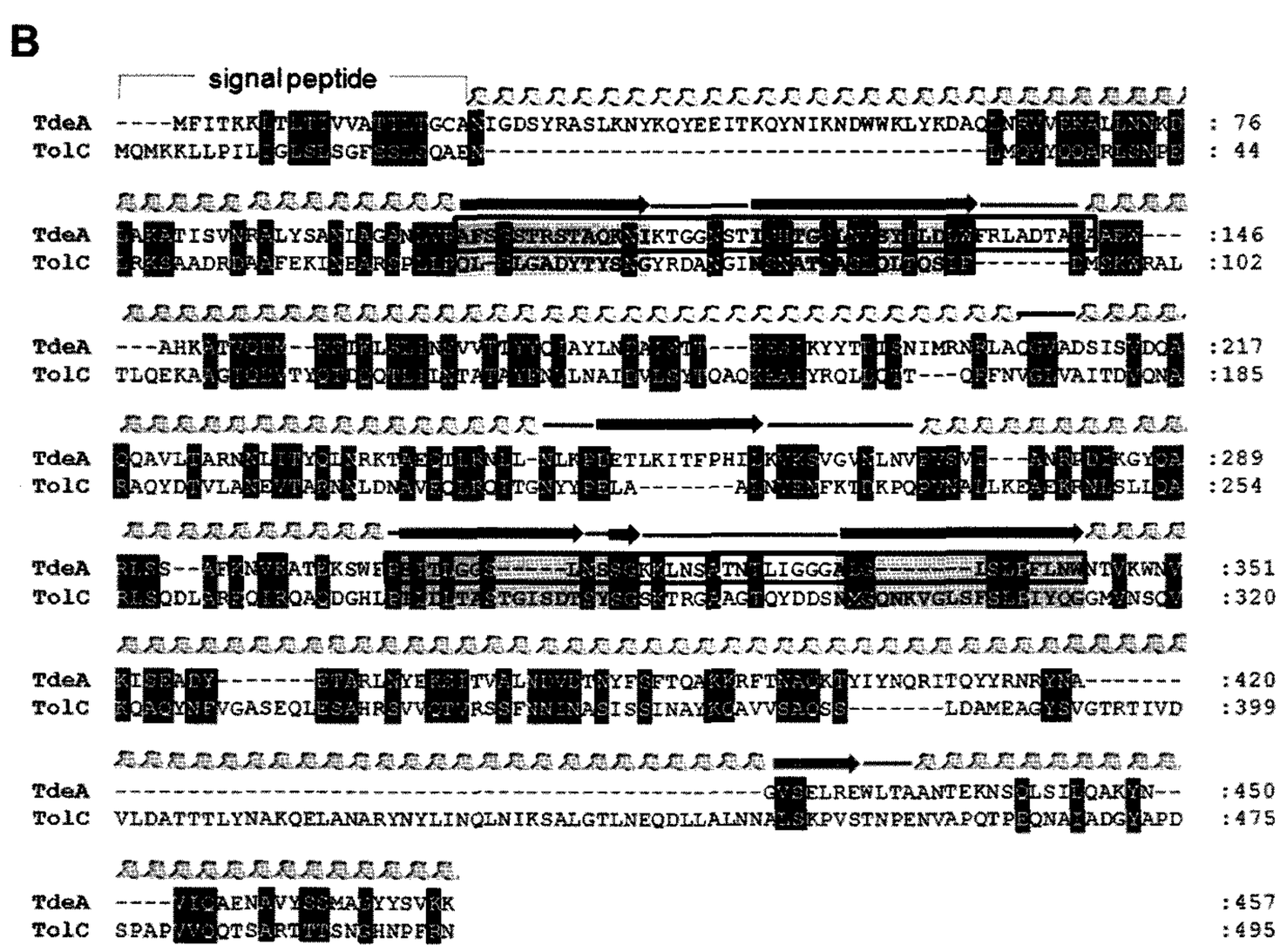


Fig. 1. Primary structure and domain architecture of TdeA.
A. Cα tracing of the TolC trimer [6] (PDB code: 1EK9). A protomer is highlighted in ribbon representation. Note that the β-barrel domain and α-helical barrel domain are independently folded. This figure was made with PYMOL [3]. **B**. Sequence alignment of *A. actinomycetemcomitans* TdeA (TdeA) with *E. coli* TolC (TolC). The alignment was performed using CLUSTALW [15]]. The secondary structure of TolC is shown above the sequences. Membrane-embedded regions are shaded, and highly conserved residues are highlighted. The residues lying in the β-barrel domain of TdeA are indicated by boxes, and these residues were substituted with soluble linkers in the construct synthesized for the present work. The helices, arrows, and lines above the amino acid sequence indicate α-helix, β-barrel, and loop regions, respectively.

chemically synthesized (GENSCRIPT, U.S.A.; Fig. 1B). The synthesized DNA fragment was digested and ligated into the NcoI and XhoI sites of pProEX HTa (Novagen), which contains an N-terminal hexahistidine tag. The sequence of the resulting plasmid (pProEX HTa-TdeA) was confirmed using the Thermo Sequenase II dye terminator cycle sequencing premix kit from Amersham Pharmacia Biotech.

Expression of the Recombinant Protein

The recombinant plasmid pProEX HTa-TdeA was transformed into BL21(DE3) to produce the α-helical barrel domain of TdeA. A starter cell culture was grown overnight at 37°C in a 50-ml conical tube containing 25 ml of LB broth supplemented with 50 μg/ml ampicillin. This starter culture was added to 3 l of LB broth with the same concentration of antibiotic and grown at 37°C until an OD_{600}

of 0.6–0.8. Protein expression was induced by adding 0.4 mM isopropyl-D-thiogalactoside to the culture and reducing the temperature to 30°C. Cells were harvested by centrifugation for 4 h after induction, and the cell pellets were stored frozen at −80°C until use.

Purification of the Periplasmic Domain of TdeA

Frozen cells were thawed on ice and disrupted by sonication in 60 ml of 20 mM Tris (pH 8.0) buffer containing 150 mM NaCl and 2 mM β-mercaptoethanol. The soluble lysate was centrifuged at 13,000 rpm for 30 min and the supernatant was mixed with Ni-NTA affinity resin (Qiagen, The Netherlands) that had been pre-incubated with the Tris buffer, and the mixture was stirred for 1 h at 4°C. After the slurry was loaded into the column, unbound proteins were washed with 400 ml of the Tris buffer supplemented with 20 mM imidazole. Recombinant TdeA with the hexahistidine tag was eluted with the Tris buffer supplemented with 200 mM imidazole. Eluted fractions (total 14 ml) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Five fractions (10 ml) containing TdeA were pooled and concentrated to 2 ml using Centriprep (Millipore, U.S.A.). Subsequently, the TdeA was purified using HiLoad Superdex 200 (Amersham Pharmacia, U.S.A.) pre-equilibrated with 20 mM Tris (pH 8.0) containing 150 mM NaCl and 2 mM β-mercaptoethanol. Fractions (7 ml) containing the TdeA periplasmic domain were diluted 3-fold with 20 mM Tris (pH 8.0) buffer, and then loaded onto a HiTrapQ column (Amersham Pharmacia, U.S.A.). The protein was eluted from the column using a 0–1 M NaCl linear gradient in 20 mM Tris buffer (pH 8.0). The fractions (1 ml for each fraction) were collected from size-exclusion chromatography and anion-exchange chromatography at the flow rate of 1 ml/ml. Total proteins were analyzed by SDS-PAGE on a 15% gel and stained with Coomassie blue. The purity of the protein sample was estimated through the protein band intensities on the Coomassie-blue-stained gel. The purified protein was stored at 4°C for use within a week, or stored frozen at −80°C until use.

Circular Dichroism (CD) Spectroscopy

The purified protein was diluted to 0.6 mg/ml in 20 mM sodium phosphate (pH 7.5) buffer containing 100 mM NaCl. The CD spectrum was collected on a Jasco J810 spectropolarimeter at 25°C. The protein concentration was measured using a Bradford protein assay kit (Biorad, U.S.A.).

Electron Microscopy

The purified protein was diluted to 0.1 mg/ml in 20 mM Tris buffer (pH 8.0) containing 150 mM NaCl and 2 mM β-mercaptoethanol. Then, the sample was adsorbed to a thin carbon foil and negatively stained with 2% uranyl acetate for 1 min. Transmission electron microscopy was carried out using a Tecnai G2 Spirit Twin transmission electron microscope (FEI, The Netherlands) at a magnification of 100,000×.

N-Terminal Sequencing

N-terminal amino acid sequence determination was carried out after blotting onto a polyvinylidene difluoride membrane by automated Edman degradation.

Purification of Peptidoglycan from *Escherichia coli*

Peptidoglycan from *E. coli* was purified according to the methods of Leuc *et al.* [7] and Marcyjaniak *et al.* [9] with modifications. Briefly, *E. coli* strain XL1-Blue was grown at 37°C in LB medium and harvested at an OD_{600} of ~0.7. The cell pellet was suspended in 10 ml of distilled water, mixed with an equal volume of 8% SDS, and incubated for 30 min at 100°C. After standing at room temperature overnight, the sample was centrifuged at 30°C for 30 min at 20,000 ×*g* (13,000 rpm) in a high-speed centrifuge (Hanil, Korea). The gel-type pellet was suspended in 1 ml of water, then washed by four cycles of resuspension and recentrifugation in water to remove traces of SDS, and finally resuspended in 0.5 ml of 10 mM Tris buffer (pH 7.5). The insoluble material was treated with DNase at 37°C for 30 min, subsequently with α-amylase at 37°C for 16 h, and than with Pronase at 50°C for 16 h. The used enzymes were removed from the peptidoglycan solution by boiling and washing using centrifugation. The purified peptidoglycans were finally resuspended in 0.5 ml of 10 mM Tris buffer (pH 7.5).

***In Vitro* Binding Assay for Peptidoglycan**

Five μg of the purified TdeA periplasmic domain (30 μg of BSA for a control experiment) and 30 μl of the resuspended peptidoglycan solution were incubated in 300 μl of 10 mM Tris buffer (pH 7.5) for 1 h at 4°C. The mixture was then centrifuged for 30 min at 20,000 ×*g* (13,000 rpm) in a high-speed centrifuge (Hanil, Korea). The pellet was collected, washed in 500 μl of 10 mM Tris buffer (pH 7.5) four times, and resuspended in 150 μl of the Tris buffer. To analyze the protein bound to peptidoglycan, 20 μl was taken for SDS-PAGE from the resuspended solution.

RESULTS

Plasmid Construction, Cloning, Expression, and Purification of the Recombinant α-Helical Barrel Domain of TdeA

The high degree of homology (21% in sequence identity) between *E. coli* TolC and *A. actinomycetemcomitans* TdeA allowed us to identify the membrane-spanning β-barrel domain of TdeA by alignment with the TolC amino acid sequence (Fig. 1B). The distance between both ends of the α-helices to be linked was measured in the crystal structure of TolC [6], and the appropriate lengths of the flexible linkers to replace the β-strands were determined based on this distance (Fig. 1B). Two linkers replacing four β-strands spanning the membrane were designed with amino acid sequences SGSGS and SGSGSG, respectively (Fig. 1B). In order to reduce the time-consuming molecular cloning work, we ordered the resulting *tdeA* gene with the substitutions from a custom *de novo* gene synthesis service, as shown in Fig. 1B. To overexpress the periplasmic domain of TdeA cytosolically in *E. coli*, we inserted the synthesized DNA fragment without the signal sequence into an expression vector containing the N-terminal hexahistidine tag for purification.

The recombinant α-helical barrel domain of TdeA was successfully expressed at 30°C in the cytosol of *E. coli*. Initial purification of the hexahistidine-tagged TdeA domain was performed by Ni-NTA affinity chromatography in a

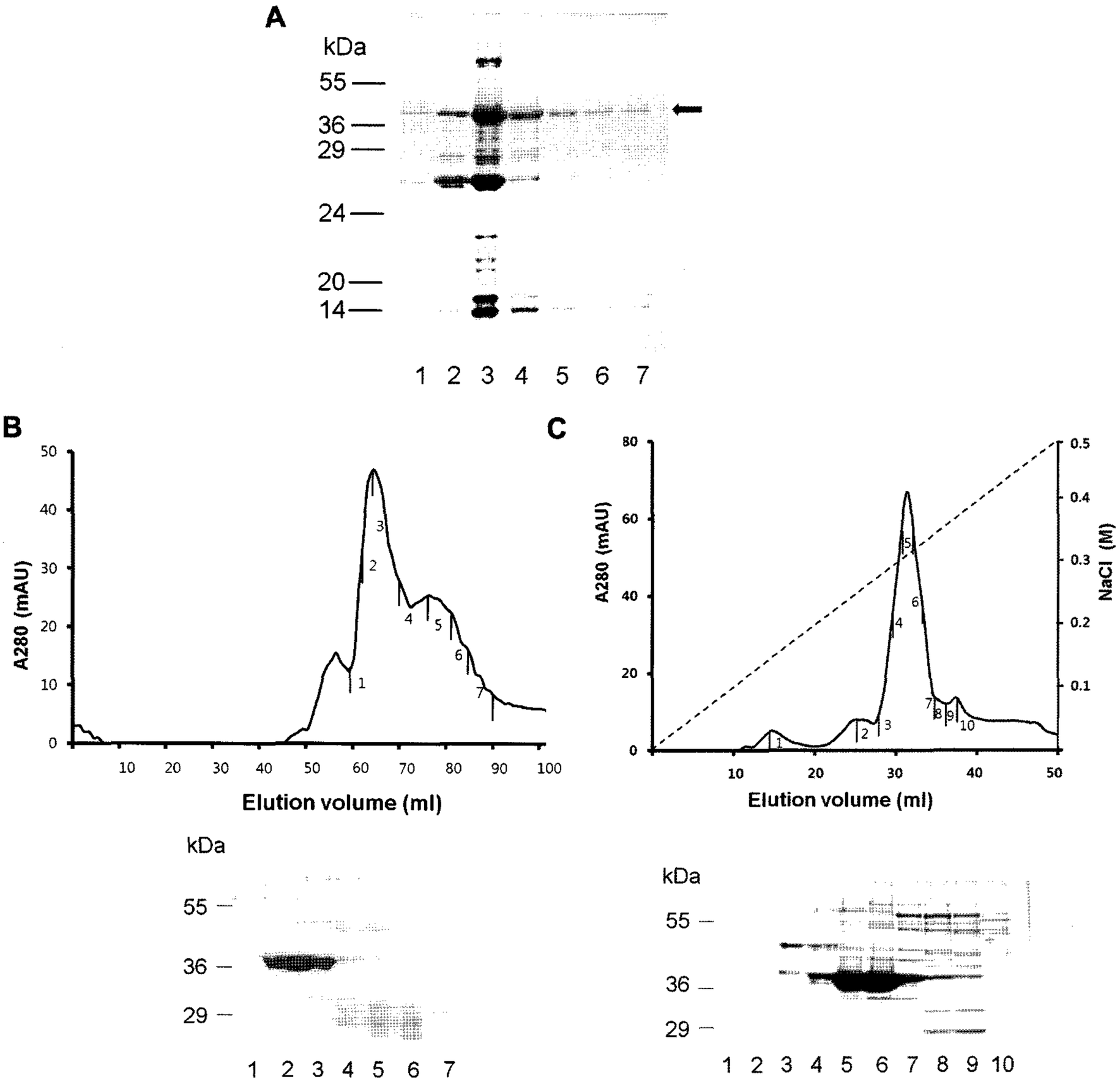


Fig. 2. Purification of the α-helical barrel domain of TdeA.
A. SDS-PAGE gel showing the eluted fractions from the Ni-NTA column. The arrow indicates the α-helical barrel domain of TdeA. Twenty μl was taken from each fraction (2 ml) and loaded onto the 15% SDS-PAGE gel for analysis (Lanes 1–7). The gel was stained with Coomassie blue. The positions of the molecular mass markers are indicated on the left. **B**. Size-exclusion chromatography of the recombinant TdeA protein. Representative profile of a size-exclusion chromatography run using a HiLoad Superdex 200 16/60 column (*Upper*) and SDS-PAGE analysis of the eluted fractions (*Lower*). Each lane number corresponds to the fractions indicated on the chromatogram, and the fractions 2 and 3 were pooled for the further purification. **C**. Further purification by anion-exchange chromatography. Fractions 2 and 3 from the size-exclusion column were pooled and applied to a HiTrapQ column (5 ml). The absorbance at 280 nm is shown as a solid line, and the NaCl gradient (right *y* axis) is shown as a dashed line (*Upper*). Eluted fractions were analyzed by SDS-PAGE (*Lower*), and the fractions 5 and 6 were pooled and stored.

detergent-free buffer as described in Materials and Methods. The purity of protein was determined on a Coomassie-stained SDS-polyacrylamide gel (Fig. 2A). Two major protein bands were seen on the gel: one at approximately 40 kDa, which was identified as the TdeA periplasmic domain by N-terminal amino acid sequencing, and another at 27 kDa (Fig. 2A). Further purification was achieved by size-exclusion chromatography, which removed most of the 27 kDa protein (Fig. 2B). Final purification was achieved by anion-exchange chromatography, producing 3.3 mg with about 90% purity from a 3-l culture (Fig. 2C and Table 1).

The Recombinant α-Helical Barrel Domain is Correctly Folded

We checked whether the TdeA periplasmic domain can form a correct homotrimer on its own, given the possibility that the β-barrel domain may be important for this process. From the size-exclusion chromatography during the purification, the molecular size of the recombinant

Table 1. Purification yields of the recombinant protein from a 1-l culture.

Purification step	Total protein recovered (mg)	Yield (%)
Ni-NTA chromatography	1.6	–
HiLoad Superdex 200	1.25	78.12
HiTrapQ	1.1	88

protein was measured as approximately 230 kDa. This is larger than the calculated value for a TdeA trimer (108 kDa), and the difference is likely due to its bulky shape with the big hollow in the α-helical barrel domains. These results suggest that even in the absence of the α-barrel domain, the α-helical barrel domain of TdeA correctly assembles into a homotrimer with a hollow central channel.

To ascertain the structural integrity of TdeA lacking the β-barrel domain, we employed circular dichroism (CD) spectroscopy and electron microscopy. The secondary structural characteristics of the TdeA periplasmic domain were determined using CD spectroscopy. The CD spectrum was very similar to myoglobin, which is composed of only α-helices – in good agreement with the expected all α-helical structure of the TdeA periplasmic domain. Next, the overall shape and dimension of the TdeA α-helical domain were measured by negative-stain electron microscopy. As shown in Fig. 3B, the domain forms a ring-shaped structure with an outer diameter of 40 Å, which is consistent with the structure of *E. coli* TolC. Taken together, these results indicate that the periplasmic domain of TdeA, and presumably the periplasmic domain of all members of the TolC protein family, can properly fold in the absence of the membrane-embedded domain.

α-Helical Barrel Domain of TdeA Binds to Peptidoglycan *In Vitro*

The bacterial cell wall component peptidoglycan is thought to be a meshwork containing numerous pores that are perpendicular to the inner and the outer membranes *in vivo* [4]. *E. coli* TolC protein has been observed to be loosely associated with peptidoglycan *in vivo* [11], and the periplasmic domain of TolC proteins is known to contact the tip of the inner membrane transporter, thereby forming an efflux channel connecting the cytoplasm and the exterior of the bacterium [10]. Taking into account the outer diameter of TolC-family proteins, the diameter of the peptidoglycan pores should be greater than 70 Å [10]. It remains unclear, however, if the completely folded TolC-family proteins encompass the peptidoglycan pores *in vivo*. To address this question, we performed an *in vitro* binding assay with the

Fig. 3. Structure of the α-helical barrel domain of TdeA.
A. CD spectroscopic analysis of the recombinant TdeA protein. The data indicate that the secondary structure of the TdeA domain is composed of mostly α-helix, as predicted. **B**. Electron micrographs of the TdeA α-helical domain. A selected particle is displayed with a scale bar representing 10 nm (*Left*). They resemble the periplasmic domain of TolC (*Right*), which is shown protruding from the outer membrane and positioned perpendicularly to the membrane.

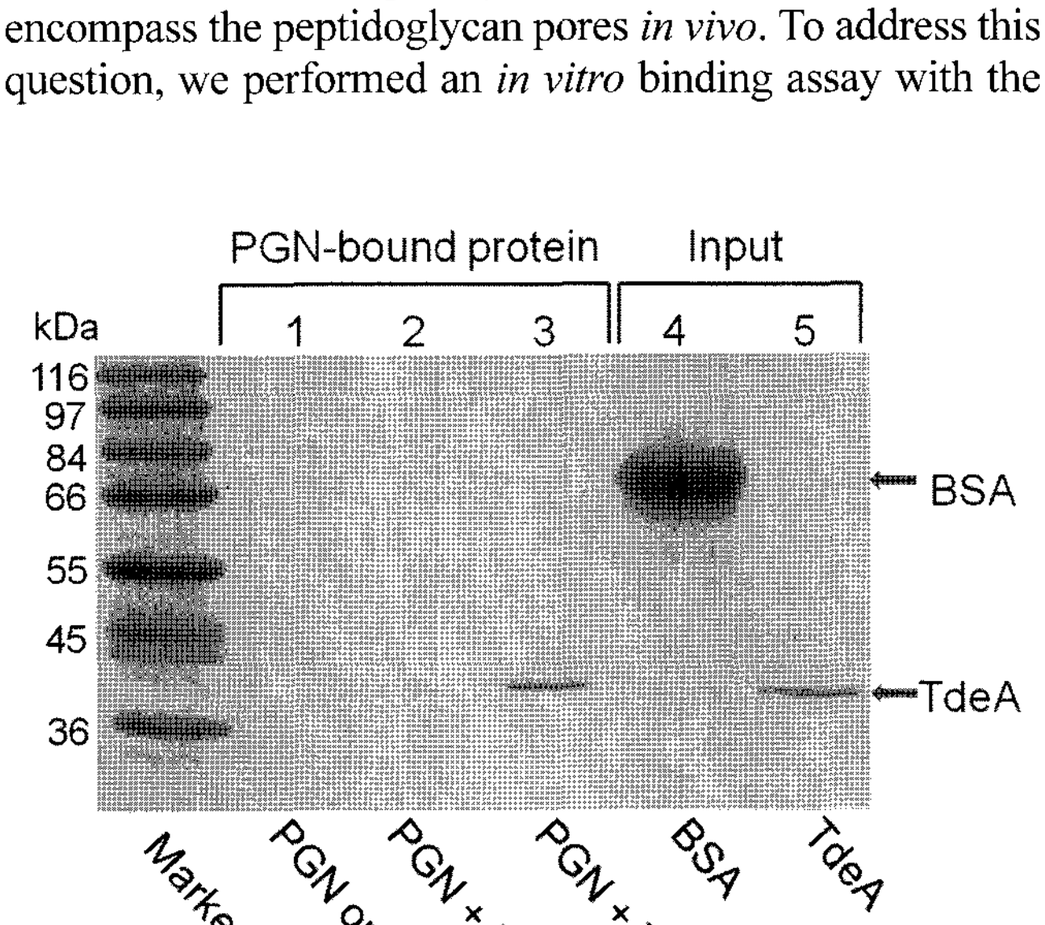


Fig. 4. The purified TdeA α-helical domain interacts *in vitro* with peptidoglycan.
The purified TdeA domain or BSA (Lanes 4 and 5, respectively) was incubated with peptidoglycan (*PGN*), and then the peptidoglycan-bound proteins (Lanes 2 and 3) were analyzed by SDS-PAGE. The SDS-polyacrylamide gel was stained with Coomassie blue. Any enriched band was not detectable in lane 3 except the TdeA protein, compared with the loading sample in lane 4, which excludes a possibility that a contaminant may mediate the interaction between the TdeA protein and peptidoglycan. To confirm that the peptidoglycan used did not contain any protein, the peptidoglycan alone was loaded in lane 1. The positions of the molecular mass markers are indicated on the left.

TdeA periplasmic domain and a protein-free preparation of peptidoglycan.

We first prepared highly purified peptidoglycan from *E. coli* by combining two methods described previously [7, 9]. The purity of the peptidoglycan material was significantly increased by adding a DNase-treatment step into the purification method; this degrades DNA in the gel-type material formed by SDS treatment. Precipitated white material was obtained at the end, and confirmed to be peptidoglycan by using *Tenebrio molitor* peptidoglycan recognition protein-SA that specifically binds peptidoglycan [12] (data not shown). SDS-PAGE analysis of the purified peptidoglycans failed to reveal any obvious protein contaminants (Lane 1 in Fig. 4).

To test whether the periplasmic domain of TdeA can interact with peptidoglycans, the recombinant TdeA protein was incubated with the purified peptidoglycan, the mixture was washed extensively to remove unbound and non-specifically bound proteins, and it was finally analyzed by SDS-PAGE. In a control experiment, a greater amount of bovine serum albumin (BSA) was used. The TdeA domain, but not BSA, was found to bind peptidoglycan (Fig. 4). This result shows that the periplasmic domain of TdeA binds directly to purified peptidoglycan, independently of the membrane-embedded domain. It also suggests that TolC-family proteins can interact with the preformed meshwork of peptidoglycan after the proteins are completely folded.

Discussion

Since structural biologists are keenly interested in membrane proteins because of their importance in biological systems, diverse approaches have been taken to solubilize them. Expression of the full-length version of membrane proteins can be the best choice because it reflects the native state of the proteins. However, the expression yield is usually too low for structural studies, and the detergent used for solubilization may interfere with protein-protein interactions in biochemical studies. Thus, many researchers have used an alternative method in which the membrane-bound region or domain of the membrane protein is truncated or substituted in order to make the proteins more soluble [1]. Although this method has proven effective for structural and biochemical studies, it has been applied mainly to membrane proteins spanning the membrane once or twice. In this study, we extended the truncation method to multipass membrane proteins whose membrane-embedded domain is independently folded. We successfully expressed and purified the periplasmic domain of TdeA by truncating the membrane-embedded region. The periplasmic domain behaved as a well-folded protein, its secondary structure and overall shape under electron microscopy were as expected, and it interacted with peptidoglycan *in vitro*. These results confirm that the periplasmic domain of TdeA can fold properly in the absence of the membrane-embedded domain. Our approach may be applicable to other multipass membrane proteins.

Acknowledgments

This project was supported by a Korea Research Foundation Grant (KRF-2006-312-C00355) to N.C.H. This study was also supported by a grant from the Research Institute for Drug Development, Pusan National University. We thank the staff members at the Korea Basic Science Institute (Daejeon, Korea) for assistance with the electron microscopy.

References

1. Choi, J. H., J. I. Choi, and S. Y. Lee. 2005. Display of proteins on the surface of *Escherichia coli* by C-terminal deletion fusion to the *Salmonella typhimurium* OmpC. *J. Microbiol. Biotechnol.* **15:** 141–146.
2. Crosby, J. A. and S. C. Kachlany. 2007. TdeA, a TolC-like protein required for toxin and drug export in *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans*. *Gene* **388:** 83–92.
3. DeLano, W. 2002. *The PyMOL User's Manual*. DeLano Scientific, Palo Alto.
4. Dmitriev, B., F. Toukach, and S. Ehlers. 2005. Towards a comprehensive view of the bacterial cell wall. *Trends Microbiol.* **13:** 569–574.
5. Koronakis, V., J. Li, E. Koronakis, and K. Stauffer. 1997. Structure of TolC, the outer membrane component of the bacterial type I efflux system, derived from two-dimensional crystals. *Mol. Microbiol.* **23:** 617–626.
6. Koronakis, V., A. Sharff, E. Koronakis, B. Luisi, and C. Hughes. 2000. Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export. *Nature* **405:** 914–919.
7. Leduc, M., D. Joseleau-Petit, and L. I. Rothfield. 1989. Interactions of membrane lipoproteins with the murein sacculus of *Escherichia coli* as shown by chemical crosslinking studies of intact cells. *FEMS Microbiol. Lett.* **51:** 11–14.
8. Lee, K. E., J. S. Bang, C. H. Baek, D. K. Park, W. Hwang, S. H. Choi, and K. S. Kim. 2007. IVET-based identification of virulence factors in *Vibrio vulnificus* MO6-24/O. *J. Microbiol. Biotechnol.* **17:** 234–243.
9. Marcyjaniak, M., S. G. Odintsov, I. Sabala, and M. Bochtler. 2004. Peptidoglycan amidase MepA is a LAS metallopeptidase. *J. Biol. Chem.* **279:** 43982–43989.
10. Meroueh, S. O., K. Z. Bencze, D. Hesek, M. Lee, J. F. Fisher, T. L. Stemmler, and S. Mobashery. 2006. Three-dimensional structure of the bacterial cell wall peptidoglycan. *Proc. Natl. Acad. Sci. USA* **103:** 4404–4409.
11. Morona, R., P. A. Manning, and P. Reeves. 1983. Identification and characterization of the TolC protein, an outer membrane protein from *Escherichia coli*. *J. Bacteriol.* **153:** 693–699.

12. Park, J. W., B. R. Je, S. Piao, S. Inamura, Y. Fujimoto, K. Fukase, S. Kusumoto, K. Soderhall, N. C. Ha, and B. L. Lee. 2006. A synthetic peptidoglycan fragment as a competitive inhibitor of the melanization cascade. *J. Biol. Chem.* **281:** 7747–7755.
13. Paulsen, I. T., J. H. Park, P. S. Choi, and M. H. Saier Jr. 1997. A family of Gram-negative bacterial outer membrane factors that function in the export of proteins, carbohydrates, drugs and heavy metals from Gram-negative bacteria. *FEMS Microbiol. Lett.* **156:** 1–8.
14. Thanabalu, T., E. Koronakis, C. Hughes, and V. Koronakis. 1998. Substrate-induced assembly of a contiguous channel for protein export from *E. coli*: Reversible bridging of an inner-membrane translocase to an outer membrane exit pore. *EMBO J.* **17:** 6487–6496.
15. Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins. 1997. CLUSTALX Windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25:** 4876–4882.