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Carboxy-Terminal Region Involved in Activity of *Escherichia coli* TolC

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The *Escherichia coli* TolC acts as a channel tunnel in the transport of various molecules across the outer membrane. Partial-deletion studies of *tolC* revealed that the region extending from the 50th to the 60th amino acid residue from the carboxy terminus plays an important role in this transport activity of TolC.

TolC, an outer membrane protein of *Escherichia coli*, functions as a transporter in multiple transport systems (3). For example, TolC interacts with the HlyD/HlyB complex to secrete hemolysin (20) and with the CvaA/CvaB complex to secrete colicin V (6). In addition to being involved in the secretion of these macromolecules, TolC functions in the export of small molecules such as antibiotics, sodium dodecyl sulfate (SDS), bile salts, and organic solvents by interacting with complexes such as EmrA/EmrB and AcrA/AcrB (2, 10, 12, 18).

These large and small molecules are secreted in a single step without stagnation in the periplasm. Recently, Yamanaka et al. (24) and Foreman et al. (4) found that TolC is also involved in the transportation of heat-stable enterotoxin I (STI) and STII of *E. coli*. These STs are synthesized as precursor proteins with an amino-terminal signal sequence and are translocated across the inner membrane via the Sec machinery (8, 14). In contrast to the secretory molecules which cross two membranes in a single step without stagnating in the periplasm, these STs, after being released into the periplasm, remain for a short period in this location (4, 13, 22). After being processed in the periplasm, these STs translocate across the outer membrane through the action of TolC. This suggests that TolC can also pump periplasmic components out into the medium.

In addition to having a secretory function, TolC participates in the uptake of colicin E1 (ColE1) from outside the cell into the periplasm (16, 21). However, the regions of TolC involved in these activities have not been established. In this study, we mutated the gene encoding the carboxy terminus of *tolC* and examined the efflux of STs and antibiotics and the influx of ColE1.

Function of mutant TolC. The *tolC* gene carried by pET11-STI-TolC (23) was mutated to delete amino acid residues at the carboxy terminus from the protein product. We generated a stop codon at target positions of TolC by PCR-based overlap extension mutagenesis using appropriate primers (5). The targets were the amino acid residues at positions 452, 442, 432, 422, and 412 of TolC. The mutations were verified by DNA sequencing. The mutant TolC proteins produced from these genes had deletions of 20, 30, 40, 50, and 60 amino acid residues from the carboxy terminus, respectively. The mutant plasmids were designated pET11-STI-TolC(ΔC20), pET11-STI-TolC(ΔC30), pET11-STI-TolC(ΔC40), pET11-STI-TolC (ΔC50), and pET11-STI-TolC(ΔC60), respectively.

The function of the mutant TolCs was examined by determining the sensitivity to acriflavine and novobiocin of the cells harboring these plasmids. Both antibiotics are excreted from cells by pumps which are composed of several proteins, including TolC (11). BL21-2, a derivative of BL21 whose *tolC* gene was mutated (24), was used as the host strain. Sensitivity was determined by an agar plate diffusion assay. Approximately 10^7 cells were spread on an L agar plate containing ampicillin (50 μg/ml). Sterile blank disks (6.4 mm in diameter) were placed on a lawn. A 20-μl solution of novobiocin (1 mg/ml; Sigma, St. Louis, Mo.) or acriflavine (1 mg/ml; Sigma) was pipetted onto each disk. The plates were incubated overnight at 37°C. The sensitivity of the cells to the substances was classified according to the size of the growth inhibition zone.

BL21-2 transformed with pET11-STI, which does not contain *tolC* (23), was sensitive to these inhibitors. In contrast, BL21-2 transformed with pET11-STI-TolC was tolerant (Table 1).

The cells transformed with pET11-STI-TolC(ΔC20), pET11-STI-TolC(ΔC30), pET11-STI-TolC(ΔC40), and pET11-STI-TolC(ΔC50) were tolerant to the inhibitors, indicating that a deletion of less that 50 amino acid residues does not affect the activity of TolC. In contrast, the cells transformed with pET11-STI-TolC(ΔC60) were sensitive to both inhibitors (Table 1), indicating that the region extending from the 50th to the 60th amino acid from the carboxy terminus is necessary for TolC to excrete the inhibitors.

The sensitivity of the transformed cells to ColE1 was also examined by the disk assay. The concentration of ColE1 (Sigma) used was 100 μg/ml. As shown in Table 1, truncations at the 20th, 30th, 40th, and 50th amino acid residues did not affect ColE1 sensitivity, but the truncation at the 60th residue induced a complete loss of ColE1 sensitivity.

Assembly of mutant TolCs and association with the outer membrane. The native TolCs associate with the outer membrane and assemble to form a trimer (7). To examine whether TolC(ΔC60)s form trimers and associate with the outer mem-

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TABLE 1. Sensitivities to antimicrobial agents of strains of an *E. coli* BL21 *tolC* mutant (BL21-2) harboring the indicated plasmids

Plasmid	Formation of a halo in the presence of[a]:		
	Acriflavine	Novobiocin	ColE1
pET11-STI	+++	+++	−
pET11-STI-TolC	−	−	+++
pET11-STI-TolC(ΔC20)	−	−	+++
pET11-STI-TolC(ΔC30)	−	−	+++
pET11-STI-TolC(ΔC40)	−	−	+++
pET11-STI-TolC(ΔC50)	−	−	+++
pET11-STI-TolC(ΔC60)	+++	+++	−
pET11-STI-TolC(L412P)	+++	+++	−

[a] A drop of cell suspension (approximately 10^8 cells/ml) was spread on an L agar plate containing ampicillin (50 μg/ml). Sterile blank disks (6.4 mm in diameter) were placed on a lawn. The solution containing antimicrobial reagent was pipetted onto each disk. The plates were allowed to incubate overnight at 37°C. The halos (growth inhibition zone) obtained were classified into four groups according to size. −, diameter of less than 8 mm; +++, diameter greater than than 18 mm.

brane, we did cross-linking and membrane fractionation experiments.

BL21-2 cells harboring the plasmids were gently sonicated, and 300 μl of the sonicated suspension containing 5 mg of protein was removed to a new tube. One hundred microliters of 25 mM dimethyl suberimidate (DMS), a cross-linking reagent (19), was added to the tube, which was then incubated at 37°C for 10 min. The reaction was quenched by the addition of Tris-HCl (pH 7.4) to a final concentration of 50 mM. The samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (9), and the TolC on the gel was detected by immunoblotting using the anti-TolC antiserum, which was prepared by the injection of a peptide (ELRKSAADRDAAFEK), corresponding to residues 16 to 30 from the amino-terminal end of TolC, into a rabbit.

The sample from BL21-2/pET11-STI-TolC was placed in lanes 1 and 2 of the gel shown in Fig. 1 and analyzed. A 51-kDa band was detected in the sample not treated with DMS (lane 1). The calculated molecular weight of TolC is 51,454. In the sample treated with DMS (lane 2), a band of 155 kDa, presumably representing TolC trimers, appeared.

The TolC(ΔC60) sample not treated with DMS (lane 5) migrated to the 45-kDa position. TolC(ΔC60) treated with DMS (lane 6) produced a band of 135 kDa. This result showed that the mutant TolC(ΔC60)s associated to form a trimer.

To examine the association of TolC(ΔC60) with the outer membrane, the crude membrane fractions of BL21-2 harboring pET11-STI-TolC(ΔC60) were centrifuged through sucrose density gradients spanning 24 to 70%. A previous study showed that the outer membrane and inner membrane were recovered from the fractions containing 50 and 30% sucrose, respectively (15).

After centrifugation, the solution in the centrifugation tube was divided into fractions. All fractions were examined for the presence of TolC by immunoblotting. Both TolC(ΔC60) and wild-type TolC were located in tubes containing 50 to 58% sucrose (data not shown).

Secretion of STs through the mutant TolCs. Secretion of STs into the medium through the actions of mutant TolCs was examined by pulse-labeling (17). BL21-2 cells transformed with the derivatives of pET plasmids (Fig. 2) were labeled for 3 min with [^{35}S]cysteine. After further incubation for 3 min (chase period), the cells were separated by centrifugation. The periplasmic fractions were prepared by treatment with polymyxin B (23). The culture supernatant and periplasmic fractions obtained were resolved by SDS-PAGE, and STs in the gels were detected by autoradiography.

STI synthesized by the TolC-deficient cells transformed with pET11-STI was not released into the external medium and remained in the periplasm (Fig. 2A, lanes 1 and 5). In contrast, almost all STIs synthesized by the cells transformed with pET11-STI-TolC were secreted into the medium and the amount of STIs remaining in the periplasm was very small (lanes 2 and 6). Deletion of 50 amino acid residues at the carboxy terminus of TolC did not affect the secretion of STI (lanes 3 and 7). However, deletion of 60 residues markedly reduced the amount of STI secreted into the culture supernatant and the amount of STI remaining in the periplasm was very large (lanes 4 and 8).

The secretion of STII was also examined. The mutant plasmid pET11-STII-TolC(ΔC60) was obtained by mutagenesis from pET11-STII-TolC, which was prepared by inserting *tolC* into the *Hin*dIII-*Eco*RI site of pET11-STII (13). STII was not secreted from BL21-2/pET11-STII-TolC(ΔC60), and the STIIs remained in the periplasm (Fig. 2A, lanes 11 and 14).

Effect of replacement of Leu-412. The 60th amino acid residue from the carboxy-terminal end of TolC is leucine at position 412. We replaced the bases encoding Leu-412 of appro-

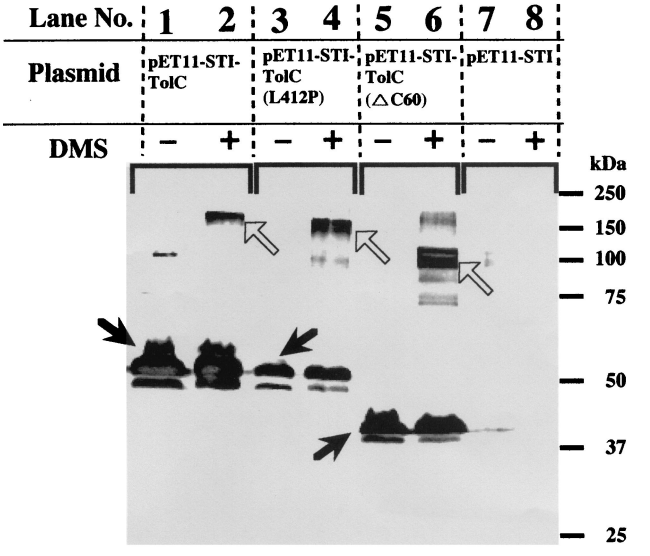

FIG. 1. Cross-linking of TolC exists in cells. Cells of *E. coli* BL21-2, the *tolC* mutant strain, transformed with the indicated plasmids were grown to the exponential phase in L broth at 37°C. The cultured cells were collected by centrifugation and suspended in phosphate-buffered saline. They were then disrupted by sonication. The solution containing the cell debris was treated with DMS to generate cross-linkages between associated proteins. The sample obtained was separated by SDS-PAGE, and the TolCs on the gel were detected by immunoblotting as described in the text. The bands indicated by black arrows are monomers of TolC, and those indicated by open arrows are trimers. Numbers along the right side indicate molecular masses in kilodaltons.

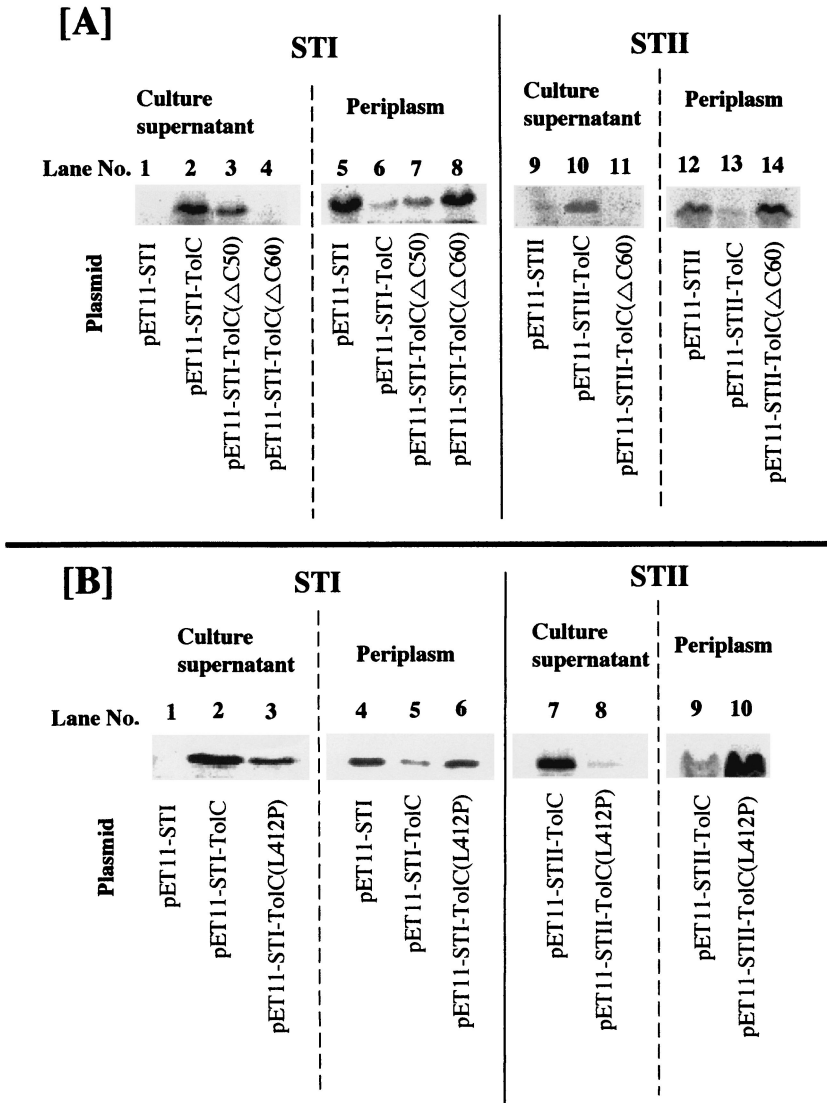

FIG. 2. Secretion of STI and STII from cells. The secretion of STs from cells was examined by the labeling method. Exponentially growing cells of BL21-2 harboring the indicated plasmid were treated with IPTG (isopropyl-β-D-thiogalactopyranoside) and then labeled for 3 min with L-[^{35}S]cysteine. The labeling was terminated by the addition of cold cysteine to the culture. Incubation was continued for 3 min (chase period), and then the culture supernatant was obtained by centrifugation. The periplasmic fractions of the cells were prepared by treatment with polymyxin B. These prepared fractions were treated with an SDS-dye solution and heated at 100°C for 10 min. The samples thus prepared were resolved by SDS-PAGE. Radioactive bands were detected by image analysis.

priate plasmids with those encoding proline and examined the properties of the mutant TolC [TolC(L412P)].

As shown in lane 4 of Fig. 1, TolC(L412P) formed trimers. Association of TolC(L412P) with the outer membrane was also confirmed by ultracentrifugation through a sucrose density gradient (data not shown).

E. coli BL21-2 transformed with the plasmid was sensitive to acriflavine and novobiocin and tolerant to ColE1 (Table 1). The activity of the mutant cells for the secretion of STI and STII into the culture supernatant was low compared with that of the wild type (Fig. 2B, lanes 3 and 8). A large amount of STs remained in the periplasm (Fig. 2B, lanes 6 and 10).

In this study, we showed that the region extending from the 50th to the 60th amino acid from the carboxy-terminal end is indispensable to TolC for expressing its export-import activity, although it is not required for the formation of the TolC trimer.

The TolC molecule can be divided into three domains: a β-domain, an α-domain, and a mixed α-β domain (1, 7). The β-domain is embedded in the outer membrane. In contrast, the α-domain penetrates the periplasm and forms a 12-stranded antiparallel α-barrel. Some side chains of residues constituting the mixed α-β domain interact with the α-domain through hydrogen bonds and van der Waals contacts. The region extending from the 50th to the 60th amino acid from the carboxy terminus is a part of the mixed α-β domain that interacts with two strands of the α-domain (H3 and H4) in the periplasm. Our results suggest that this interaction may be important for TolC to express its activity.

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