MacAB Is Involved in the Secretion of *Escherichia coli* Heat-Stable Enterotoxin II[▽]

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The heat-stable enterotoxin (ST) produced by enterotoxigenic *Escherichia coli* is an extracellular peptide toxin that evokes watery diarrhea in the host. Two types of STs, STI and STII, have been found. Both STs are synthesized as precursor proteins and are then converted to the active forms with intramolecular disulfide bonds after being released into the periplasm. The active STs are finally translocated across the outer membrane through a tunnel made by TolC. However, it is unclear how the active STs formed in the periplasm are led to the TolC channel. Several transporters in the inner membrane and their periplasmic accessory proteins are known to combine with TolC and form a tripartite transport system. We therefore expect such transporters to also act as a partner with TolC to export STs from the periplasm to the exterior. In this study, we carried out pulse-chase experiments using *E. coli* BL21(DE3) mutants in which various transporter genes (*acrAB*, *acrEF*, *emrAB*, *emrKY*, *mdtEF*, *macAB*, and *yojHI*) had been knocked out and analyzed the secretion of STs in those strains. The results revealed that the extracellular secretion of STII was largely decreased in the *macAB* mutant and the toxin molecules were accumulated in the periplasm, although the secretion of STI was not affected in any mutant used in this study. The periplasmic stagnation of STII in the *macAB* mutant was restored by the introduction of pACYC184, containing the *macAB* gene, into the cell. These results indicate that MacAB, an ATP-binding cassette transporter of MacB and its accessory protein, MacA, participates in the translocation of STII from the periplasm to the exterior. Since it has been reported that MacAB cooperates with TolC, we propose that the MacAB-TolC system captures the periplasmic STII molecules and exports the toxin molecules to the exterior.

Gram-negative bacteria such as *Escherichia coli* have a cell envelope containing two lipid bilayer membranes, the inner membrane (IM) and outer membrane (OM). These membranes function as barriers to the transport of macromolecules. However, the bacteria in fact transport various kinds of macromolecules across these membranes to survive (8, 18). To achieve the efficient transport of macromolecules, the bacteria have developed special systems. The tripartite transport system is typical of such systems. This system generally consists of three proteins: the IM transporter, the OM channel, and the accessory protein, which functions as an adaptor connecting with both the IM and OM proteins (23, 35). The IM transporter acts as a pump protein. To achieve active transport, the three change their conformation, utilizing as a driving force the proton motive force or ATP hydrolysis (24). Although there is not enough energy in the OM, it is thought that the OM channel can express transport activity by working together with both the IM transporter and the accessory protein.

TolC is a major OM channel in *E. coli* that plays an important role in the excretion of a wide range of molecules, including antibiotics (18, 25, 27), bile salts (3, 40, 45), organic solvents (1), enterobactin (4), several antibacterial peptides such as

colicin V (11, 12) and microcin J25 (7), and also a large protein toxin, alpha-hemolysin (47, 48). Although TolC acts as a unique OM channel in these cases, various kinds of IM transporters and accessory proteins interact with TolC and enable *E. coli* to expel structurally diverse molecules. To date, it has been found that TolC can associate with at least 11 kinds of IM transporters and accessory proteins. They are AcrAB, AcrAD, AcrEF, MdtABC, MdtEF, EmrAB, EmrKY, MacAB, YojHI, CvaAB, and HlyBD. For instance, the AcrAB-TolC system functions as a major excretion apparatus for antibiotics, bile salts, and noxious organic solvents and provides intrinsic resistance to these compounds (1, 3, 18, 25, 27, 40, 45). CvaAB, YojHI, and HlyBD enable the bacterium to export proteinaceous factors such as colicin V, microcin J25, and alpha-hemolysin, respectively (7, 11, 12, 47, 48). Thus, TolC plays a central role in the extracellular transport of diverse molecules in cooperation with various kinds of translocators.

Yamanaka et al. and Foreman et al. found that TolC is also involved in the secretion of the heat-stable enterotoxins (STs; STI and STII) produced by enterotoxigenic *E. coli* (ETEC) (16, 50). Although in their primary structure these STs are quite different (32, 34, 44, 52), their maturation processes are similar. That is, these STs are synthesized as precursor proteins with an amino-terminal signal sequence and are translocated across the IM via the Sec machinery (16, 31, 51). After translocation, the toxins are released into the periplasm and folded to the mature forms via the actions of DsbA, a periplasmic disulfide oxidoreductase (9, 30, 49). The mature STs thus produced are ultimately transported from the periplasm into the

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TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Description	Source or reference
<i>E. coli</i> strains		
BL21(DE3)	F ⁻ <i>ompT hsdS_B(r_B⁻ m_B⁻) gal dcm</i> (DE3)	Novagen
BL21(DE3)Δ1	As for BL21(DE3) except <i>ΔacrEF</i>	This study
BL21(DE3)Δ2-1	As for BL21(DE3) except <i>ΔacrAB ΔacrEF</i>	This study
BL21(DE3)Δ2-2	As for BL21(DE3) except <i>ΔmacAB ΔyojHI</i>	This study
BL21(DE3)Δ3	As for BL21(DE3) except <i>ΔmacAB ΔyojHI ΔacrAB</i>	This study
BL21(DE3)Δ4	As for BL21(DE3) except <i>ΔmacAB ΔyojHI ΔacrAB ΔacrEF</i>	This study
BL21(DE3)Δ5	As for BL21(DE3) except <i>ΔmacAB ΔyojHI ΔacrAB ΔacrEF ΔemrAB</i>	This study
BL21(DE3)Δ6	As for BL21(DE3) except <i>ΔmacAB ΔyojHI ΔacrAB ΔacrEF ΔemrAB ΔemrKY</i>	This study
BL21(DE3)Δ7	As for BL21(DE3) except <i>ΔmacAB ΔyojHI ΔacrAB ΔacrEF ΔemrAB ΔemrKY ΔmdtEF</i>	This study
BL21(DE3)Δmac	As for BL21(DE3) except <i>macAB::kan</i>	This study
BL21(DE3)Δyoj	As for BL21(DE3) except <i>yojHI::kan</i>	This study
Plasmids		
pET11	<i>bla</i> (Ap ^r), pET expression vector	Novagen
pET11-STI	pET11 derivative with STI structural gene inserted in NdeI site	50
pET11-STII	pET11 derivative with STII structural gene inserted in NdeI site	33
pKD4	<i>bla</i> (Ap ^r), a plasmid carrying FRT-flanked adjacent <i>kan</i> (Km ^r) gene	6
pKD46	<i>bla</i> (Ap ^r), a helper plasmid for expression of Red λ recombinase	6
pCP20	<i>bla</i> (Ap ^r) <i>cat</i> (Cm ^r), a plasmid for expression of FLP recombinase	6
pACYC184	<i>tet</i> (Tet ^r) <i>cat</i> (Cm ^r), a general cloning vector compatible with ColE1 plasmid	Nippon Gene
pACYC-MacAB	pACYC184 derivative with <i>macAB</i> gene, including its own promoter inserted in HindIII site	This study

milieu through the TolC channel (9, 50). This evidence indicates the TolC channel is also involved in the excretion of periplasmic substrates such as STs. However, it remains unclear how STs produced in the periplasm are moved to the TolC channel. In several known efflux systems containing TolC, the components construct a tripartite transport system as described above. We therefore presume that a certain transporter in cooperation with TolC recognizes the periplasmic ST molecules and leads them to the interior of the TolC channel.

In this study, we examined whether several distinct types of IM translocators and their accessory proteins, which are already known to function with TolC, participate in the translocation of STs from the periplasm to the exterior. The transporters examined in this study are AcrAB, AcrEF, MdtEF, EmrAB, EmrKY, MacAB, and YojHI. It is known that AcrAB, AcrEF, and MdtEF are RND (resistance nodulation cell division) family transporters, EmrAB and EmrKY are MF (major facilitator) superfamily transporters, and MacAB and YojHI are ABC (ATP-binding cassette) family transporters (7, 27, 29, 39). The results revealed that an ABC transporter, MacAB, was involved in the translocation of STII from the periplasm into the milieu but not in the translocation of STI. This is the first report about a factor engaged in the export of an ST from the periplasm to the exterior in cooperation with TolC.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *E. coli* strains and plasmids used in this study are listed in Table 1. Strains were cultivated at 37°C in Luria-Bertani (LB) broth (10 g of Bacto-tryptone, 5 g of yeast extract, and 5 g of NaCl per liter) or LB agar. For gene disruption, strains were grown at 30°C in SOB medium (Difco). The Red helper plasmid pKD46 and other plasmids, pKD4 and pCP20, required for gene disruption were kindly provided by the *E. coli* Genetic Stock Center (Yale University). The antibiotics ampicillin (50 µg/ml), kanamycin (25 µg/ml), and chloramphenicol (12.5 µg/ml) were used for selection where indicated.

Construction of mutants whose target gene(s) was disrupted. A direct mutagenesis using PCR gene fragments was performed as originally described by

Datsenko and Wanner (6). The kanamycin resistance gene (*kan*) of the plasmid pKD4 was amplified using primers P1 and P2, which are shown in Table 2. These primers were designed with 39 nucleotides (nt) homologous to the region immediately upstream (P1) or downstream (P2) of the target gene followed by the 20-nt sequence derived from sequence upstream or downstream of *kan* encoded on pKD4 (Table 2). A 100-ng aliquot of the PCR product was mixed with 50 µl of electrocompetent *E. coli* BL21(DE3) cells carrying pKD46, and the mixed solution was transferred into an ice-cold 0.2-cm cuvette (Bio-Rad Laboratories). Electroporation was conducted using a Gene Pulser Xcell electroporation system (Bio-Rad Laboratories) at 2.5 kV, 25 mF, and 200 Ω and was immediately followed by the addition of 1 ml of SOC medium (SOB medium containing 20 mM glucose). We then incubated the culture for 2 h at 37°C. A 100-µl aliquot of the cell suspension was spread onto LB agar containing kanamycin and incubated for 16 to 18 h at 37°C to select for kanamycin-resistant recombinants.

Verification of the gene disruption. To verify whether appropriate homologous recombination occurred, we carried out a PCR analysis using the primers k1 (CAGTCATAGCCGAATAGCCT), k2 (CGGTGCCCTGAATGAAGTGC), and kt (CGGCCACAGTCGATGAATCC) (6) and also primers for the regions upstream and downstream of each target gene (Table 2).

Elimination of the antibiotic resistance gene. To make multiple gene knockout (KO) mutants, we eliminated the *kan* gene on the chromosome introduced by the homologous recombination. We utilized pCP20, a temperature-sensitive plasmid that shows thermal induction of FLP (flip-out) recombinase (6). The *kan* replacement mutants were transformed with pCP20, and the transformants were selected at 30°C on LB agar containing both ampicillin and chloramphenicol, after which a few were colony purified once, nonselectively, at 43°C and then tested for loss of all antibiotic resistance. We confirmed the loss of *kan* on the chromosome by means of a PCR analysis using primers for the regions upstream and downstream of each target gene (Table 2). The deletion mutants thus obtained were used in the second direct mutation for the next target gene. Repeating these procedures, we made multiple KO mutants: BL21(DE3) Δ2-1, Δ2-2, Δ3, Δ4, Δ5, Δ6, and Δ7 (Table 1).

Pulse-chase experiment. The pulse-chase experiment using L-[³⁵S]cysteine (ICN Biomedicals) was performed according to our previously described method (33, 50). After the pulse-chase period (pulse for 3 min, chase for 3 min), the culture was fractionated into culture supernatant and periplasmic fractions as described previously (33, 50). Both fractions were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using a 20% acrylamide gel. Radiolabeled STs were visualized by autoradiography using BioMax MS X-ray film (Kodak).

Insertion of *macAB* into the plasmid pACYC184. The *macAB* gene was amplified from the chromosomal DNA of *E. coli* BL21(DE3) using two primers, MacAB-up (5'-AAAAGTGACAAGCTTCTGAGAGATTCA-3') and MacAB-

TABLE 2. PCR primers designed for gene disruptions^a

Primer	Sequence
AcrEF-P1	5'-ATGACGAAACATGCCAGGTTTTCTCTCTGCCCTCTTTGTGTAGGCTGGAGCTGCTTC-3'
AcrEF-P2	5'-CGTCACTGTTTACGCGCAGGGTCACTTTGCCG AATTCTTCATATGAATATCCTCCTTAG-3'
AcrAB-P1	5'-GAATGGCACGCTGAAACAAGAGAACGGCAAA GCCAAAGTGTGTAGGCTGGAGCTGCTTC-3'
AcrAB-P2	5'-TTAGCTGGAATTTGTTACGCTCATTCGGGTTT ATCCAGACATATGAATATCCTCCTTAG-3'
EmrAB-P1	5'-ATGAGCGCAAATGCGGAGACTCAAACCCCGCA GCAACCGTGTAGGCTGGAGCTGCTTC-3'
EmrAB-P2	5'-TTAGCCAGCGTTAGCTTTTACGATATCGTCGA TCAGTTTCATATGAATATCCTCCTTAG-3'
EmrKY-P1	5'-ATGGAATTAGAAGACATGATTAGTACAGATGA CGCTATGTGTAGGCTGGAGCTGCTTC-3'
EmrKY-P2	5'-GGTGCCGGAGTTGATTTAGTGATTGCCATTAA AGTTGTCCATATGAATATCCTCCTTAG-3'
MacAB-P1	5'-TTGGCATATGGAGTATTCAGAATATTTATGAA AAAGCGGGTGTAGGCTGGAGCTGCTTC-3'
MacAB-P2	5'-TCATTGCGCAGCTCCTGGTTTGGCCTACCAA TCACCACCATATGAATATCCTCCTTAG-3'
YojHI-P1	5'-CTGCGGGCTGTAATTCATTGTCCGGGTTTTCT GCGTGTAGGCTGGAGCTGCTTC-3'
YoiHI-P2	5'-GACGGCAGCAGTCCGCCAGGTCAGCGTCGTA ATACATATGAATATCCTCCTTAG-3'
MdtEF-P1	5'-ATGAACAGAAGAAGAAAAGTGTAAATACCGTT GTTATTCGTGTAGGCTGGAGCTGCTTC-3'
MdtEF-P2	5'-AGCCATGAAAGTCCCTTAAGCCTGCAACGTT ATTGTTTCATATGAATATCCTCCTTAG-3'

^a The P1 and P2 primers contain the nucleotide sequences to be amplified in the kanamycin resistance gene (underlined) from the plasmid pKD4 (6). To verify the gene mutation, primers for the region upstream and downstream of each target gene (bold portions of sequences) were also designed. Underlined portions of sequences are the 20-nt sequence upstream or downstream of *kan* encoded on pKD4.

down (5'-GCAGTCGCATAAAGCTTCTGTCTCATTGTG-3'). These primers cover the regions upstream and downstream of *macAB*, respectively. Both primers contain a HindIII site (underlined). The *macAB* gene amplified with these primers encompassed both the promoter and structural regions of the gene. The amplified gene fragments were digested with HindIII. The resulting gene fragments were inserted into a HindIII site of the plasmid pACYC184. The plasmid obtained was designated pACYC-MacAB (Table 1).

Sensitivity test. The sensitivity of cells to erythromycin was examined by halo assay on an LB agar plate using a Tridisk (Eiken, Japan). A drop of bacterial suspension (approximately 10⁸ cells/ml) was spread on an LB agar plate. A Tridisk attached to three filters containing 0.5, 2.0, and 10.0 µg/ml of erythromycin was carefully placed onto the plate. The plate was allowed to incubate overnight at 37°C. We then observed the halo (growth inhibition zone) formed around each filter.

Immunoblotting against TolC. Preparation of the OM fraction and detection of the TolC band on the SDS-PAGE (12.5% acrylamide) gel by immunoblotting were performed as described previously (53). To detect TolC, an antiserum prepared by injecting a peptide (ELRKSAADRDAAFEK) conjugated with ovalbumin into rabbits was used. The generated immune complexes were detected with a chemiluminescence reagent (Amersham Biosciences, United Kingdom).

RESULTS AND DISCUSSION

The STs STI and STII are major pathogenic factors produced by ETEC that evoke watery diarrhea (22). To investigate how STs are released into the milieu, therefore, is very

important for understanding and controlling the pathogenicity of ETEC. In this study, we searched for a factor participating in the TolC-dependent translocation of STs from the periplasm to the exterior, because TolC usually forms a tripartite transport system with the IM translocator and its accessory protein.

Secretion of STs in multiple KO mutants. Since all standard *E. coli* strains can release STs to the exterior when transformed with the plasmid containing the ST gene alone, we postulated that an intrinsic mechanism contributes to the secretory process. To date, it has been found that nine IM translocators and their accessory proteins, both encoded on the *E. coli* chromosome, can construct a tripartite transport system in cooperation with TolC. They are AcrAB, AcrAD, AcrEF, MdtABC, MdtEF, EmrAB, EmrKY, MacAB, and YojHI (7, 21, 27, 28). We therefore focused upon these transporters in this study.

We made multiple KO mutants (Δ1 to Δ7) derived from BL21(DE3) as shown in Table 1 and analyzed the extracellular secretion of STs in these mutants by conducting pulse-chase experiments. As shown in Fig. 1A, the amount of TolC in the

FIG. 1. Secretion of STs from cells of *E. coli* BL21(DE3) and derivative deletion mutants. TolC in the OM of each mutant was detected by immunoblotting using anti-TolC antiserum (A). To examine the secretion of STs, a pulse-chase experiment was carried out as described in the text. The culture supernatant and periplasmic fractions were resolved by SDS-PAGE (20% acrylamide gel), and STs in the gels were detected by autoradiography. (B and C) Secretion of STI (B) and STII (C) from cells.

OM was almost the same in all KO mutants, suggesting that these gene disruptions did not affect the production of TolC.

Results regarding the secretion of STI from the cells harboring pET11-STI are presented in Fig. 1B. Almost all the STI synthesized in these KO mutants ($\Delta 1$ to $\Delta 7$) was efficiently secreted into the culture supernatant (lanes 3 to 10), and the amount of STI remaining in the periplasm was very small (lanes 13 to 20), as observed in the wild-type cells (lanes 2 and 12). This result indicates that deletion of the transporter genes did not affect the extracellular secretion of STI. We therefore conclude that the transporters encoded by these genes (AcrAB, AcrEF, EmrAB, EmrKY, MacAB, MdtEF, and YojHI) are not engaged in the secretion of STI.

In contrast, we also examined the secretion of STII in these mutant strains harboring pET11-STII. The results are shown in Fig. 1C. In both BL21(DE3) mutants $\Delta 1$ and $\Delta 2$ -1, almost all the STII produced in the cells was released into the culture supernatant (lanes 3 and 4) and the amount remaining in the periplasm was very small (lanes 13 and 14), as observed with the wild-type strain (lanes 2 and 12). However, in the other mutants ($\Delta 2$ -2 to $\Delta 7$), the extracellular secretion of STII was markedly disturbed (lanes 5 to 10) and a large amount remained in the periplasm (lanes 15 to 20). This result indicates that the translocation of STII from the periplasm to the exterior was largely decreased in these mutants. Since both *macAB* and *yoyHI* genes were knocked out in all of these mutants, we speculate that either MacAB, YojHI, or both participate in the translocation of STII from the periplasm to the exterior.

Secretion of STs in the *macAB::kan* or *yoyHI::kan* mutant. To examine the involvement of MacAB and/or YojHI in the extracellular transportation of STII, a pulse-chase experiment was carried out using a single-gene-disrupted mutant, *macAB::kan* [BL21(DE3) Δ mac] or *yoyHI::kan* [BL21(DE3) Δ yoy]. In these mutants, the immunoblotting analysis clearly revealed that the production of TolC was not affected by the mutation of the target gene (Fig. 2A).

As expected, disruption of either *macAB*, *yoyHI*, or both did not affect the extracellular secretion of STI at all (Fig. 2B). On the other hand, disruption of *macAB* caused a marked decrease in the extracellular secretion of STII (Fig. 2C, lane 2) and the STII accumulated in the periplasm (Fig. 2C, lane 8), as observed with the mutant $\Delta 2$ -2 harboring pET-STII (Fig. 2C, lanes 5 and 11). Disruption of *yoyHI*, however, did not affect the secretion of STII (Fig. 2C, lanes 3 and 9). These results indicate that MacAB is closely involved in the translocation of STII from the periplasm into the culture supernatant.

Restoration of STII secretion in the *macAB::kan* mutant by the introduction of pACYC-MacAB. To confirm the participation of MacAB in the transportation of STII from the periplasm to the exterior, we introduced the compatible plasmid pACYC184 containing *macAB* (pACYC-MacAB) into BL21(DE3) Δ mac harboring pET-STII. First, we estimated the expression of MacAB from pACYC-MacAB. SDS-PAGE (12.5% acrylamide gel) of the sonicated cell preparation revealed that both MacA and MacB were expressed in the mutant transformed with pACYC-MacAB (Fig. 3A, lane 2) but not in the mutant without the plasmid (Fig. 3A, lane 1). Next, we assessed the activity of MacAB expressed from pACYC-MacAB. Since MacAB participates in the excretion of macrolides such as erythromycin, we performed a sensitivity test

FIG. 2. Effects of disruption of the *macAB* and *yoyHI* genes on translocation of STs from the periplasm to the exterior. TolC in the OM of each mutant was detected by immunoblotting using anti-TolC antiserum (A). To examine the secretion of STs, a pulse-chase experiment using cells harboring the indicated plasmid(s) was performed as described in the text. The culture supernatant and periplasmic fractions were resolved by SDS-PAGE (20% acrylamide gel), and STs in the gels were detected by autoradiography. (B and C) Secretion of STI (B) and STII (C) from cells.

using a Tridisk containing erythromycin. In this test, we used the mutant $\Delta 3$ because AcrAB also functions in the excretion of macrolides. As shown in Fig. 3B (lane 2), the mutant $\Delta 3$ was highly sensitive to erythromycin. In contrast, the mutant $\Delta 3$ transformed with pACYC-MacAB became resistant to erythromycin (Fig. 3B, lane 3) as observed with the wild type (Fig. 3B, lane 1), suggesting that MacAB expressed from pACYC-MacAB was actually functional.

We then carried out a pulse-chase experiment using the mutants transformed with pACYC-MacAB. As shown in Fig. 2C, the extracellular secretion of STII in the mutant Δ mac was completely restored by the introduction of pACYC-MacAB (lane 4) and the amount of STII remaining in the periplasm

FIG. 3. Functional analysis of MacAB expressed from pACYC-MacAB. (A) SDS-PAGE (12.5% acrylamide gel) analysis of the sonicated cell preparation obtained from cells harboring the indicated plasmid. The positions of molecular mass markers are shown on the left. The arrows on the right indicate the positions of MacA and MacB. (B) Bioassay for erythromycin efflux by the MacAB expressed from pACYC-MacAB. A sensitivity test using a Tridisk containing erythromycin (0.5, 2.0, and 10 μ g/ml) was performed as described in the text.

was markedly decreased (lane 10). Similarly, the secretion of STII from the mutant $\Delta 2-2$ was also restored by the introduction of pACYC-MacAB (Fig. 2C, lanes 6 and 12). These results strongly support that MacAB contributes to the translocation of STII from the periplasm to the exterior, although it does not participate in the secretion of STI. Thus, the secretory processes from the periplasm to the outside differ between STI and STII.

It has been reported that MacAB is an ABC transporter that collaborates with TolC (14, 15). Although MacAB was first recognized as a transporter that participates in the excretion of macrolides such as erythromycin (15), the results of our study suggest that it can transport other substrates, including STII.

FIG. 4. Schematic model of secretion of STs to the exterior.

For *Salmonella enterica* serovar Typhimurium, Nishino et al. recently reported that deletion of *macAB* attenuated virulence (26). It is, therefore, likely that MacAB also functions as a specific export system in *E. coli* that is closely related to bacterial pathogenicity.

How does MacAB engage in the export of STII in *E. coli*? Although further study is needed to fully answer this question, MacAB must be able to capture the substrates from the periplasm and translocate them into the center of the tunnel formed by TolC, because the STII precursor is released into the periplasm, where it is converted into the mature toxin (30). Our previous study indicated that STII was localized in the periplasmic fraction, not in the IM fraction containing MacAB, when the toxin was produced in a *tolC*-deficient strain (53). It is therefore likely that the interaction of STII with MacAB may be weak or transient, although the interaction must occur specifically. In the efflux of some β -lactams through an RND family transporter, AcrB, it has been reported that drugs in the periplasm might be collected in the central cavity through vestibules that are opened to the periplasm (19, 20, 43) and the adaptor protein AcrA might play an important role in the transfer of energy provided by AcrB to the TolC channel to be opened (2, 17). Furthermore, in the MacAB system, Tikhonova et al. recently reported that the adaptor protein MacA stimulates the ATPase activity of MacB and acts as a functional subunit of the MacB transporter (46). It is, therefore, likely that similar molecular mechanisms, as expected in the AcrAB-TolC system, are involved in the transportation of the periplasmic STII molecules through the MacAB-TolC apparatus.

In gram-negative bacteria, six different secretion systems (types I to VI) have been identified as specific to extracellular proteins (5, 13, 36, 38, 41, 42). The type II secretion system usually functions in the transport of products that have been released and have matured in the periplasm. In this system, the periplasmic products are translocated across the OM via a multimeric protein complex called the secretion (10, 37). In the secretion of STII, however, the periplasmic toxin molecules are transported to the exterior via a distinct intrinsic transport system, MacAB-TolC, although they are released into the periplasm through the Sec machinery as seen with the type II secretory process. At this point, we should emphasize that STII is secreted via a unique pathway different from that for other protein toxins.

In conclusion, we propose a schematic model for the secretion of STs (Fig. 4) in which MacAB recognizes and transports periplasmic STII but not STI into the entrance of the TolC channel and the STII molecules are ultimately released to the exterior through the channel. However, we have not found any

evidence for the interaction of STII with MacAB. Several molecular approaches will be required to resolve this process. Elucidation of the tertiary structure of MacAB may also help to consider the molecular mechanism behind this secretory process. Further research is in progress in our laboratory.

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