Membrane Insertion Characteristics of the Various Transmembrane Domains of the Escherichia coli TolQ Protein*

Tal M. Lewin‡ and Robert E. Webster§

From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

The Escherichia coli TolQ protein is a 230-amino acid integral cytoplasmic membrane protein required for the import of group A colicins, for infection by the filamentous phage, and for maintenance of the integrity of the bacterial envelope. TolQ is a polytopic protein with three membrane-spanning regions. The first membrane-spanning region has a 19-residue periplasmic NH₂-terminal tail, while the second and third membrane-spanning segments are separated by a short 17-amino acid periplasmic loop. To study the membrane assembly of TolQ, fusions of different membrane-spanning regions were examined for their ability to insert in the absence of functional SecA or the membrane potential. Fusions containing the first membrane-spanning region plus the adjacent cytoplasmic domain and a construct containing the “hairpin loop,” formed by the second and third membrane-spanning regions, insert in the absence of functional SecA. The fusion containing the second and third membrane-spanning regions required the membrane potential for insertion while the first membrane-spanning region was able to insert even in the absence of a membrane potential. Taken together, these results suggest that insertion of intact TolQ is not dependent on the Sec system, but does require the membrane potential.

The TolQRA proteins are components of a bacterial system that is required for infection by filamentous phage and entry of the group A colicins (1). In addition, mutations in the tolQRA genes render the cells sensitive to detergents and permeable to periplasmic enzymes suggesting a loss of integrity in the outer membrane (2, 3). TolR and TolA are type II bitopic inner membrane proteins that render the cell sensitive to detergents and permeable to the group A colicins (1). In addition, mutations in the tolQRA gene cluster make cells sensitive to detergents and permeable to periplasmic enzymes. The TolQRA proteins are components of a bacterial system that is required for infection by filamentous phage and entry of the group A colicins (1). In addition, mutations in the tolQRA genes render the cells sensitive to detergents and permeable to periplasmic enzymes suggesting a loss of integrity in the outer membrane (2, 3).

MATERIALS AND METHODS

Bacterial Strains and Plasmids—E. coli strain K17 (thr leu thi lac F supR) was obtained from N. Zinder, Rockefeller University. K17sca carries an azide-resistant mutation in SecA (az4) and was constructed by P1 transduction from D309 obtained from D. Oliver, Wesleyan University (19). K17(DE3) is K17 lysogenized with λ DE3 (obtained from F. W. Studier, Brookhaven National Laboratory) which carries the inducible gene for T7 RNA polymerase (20). Plasmids encoding TolQ-PhoA fusions at TolQ amino acids 36 (pJC7506), 128 (pQ128PhoA), and 155 (pQ155PhoA) were generated by inserts into the vector pTrc99A containing the left promoter of the lac operon under the control of the left promoter of the lac operon (21). Plasmid pTrc99A was purchased from Pharmacia Biotech Inc. and contains the strong IPTG-inducible trc promoter.

Plasmid Construction—Previously generated TolQ-PhoA fusions at

The abbreviations used are IPTG, isopropyl-β-D-thiogalactopyranoside; CCCC, carbonyl cyanide m-chlorophenylhydrazone; MBP, maltose-binding protein; PAGE, polyacrylamide gel electrophoresis.

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§ Supported by United States Public Health Service, NIGMS Grant GM18305. To whom correspondence should be addressed. Tel.: 919-684-3005; Fax: 919-684-8752.

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amino acids 36, 128, and 155 were cloned into the HpaI site of expression vector pJH1. Plasmid pTLQ36 contains the MluI-BstEII fragment from pJ7506 (from J. C. Lazzaroni). Plasmid pTLQ128 contains the 4.9-kilobase pair SmaI fragment of pQ128PhoA. Plasmid pTLQ155 contains the Klenow filled SmaI-BstEII fragment of pQ155PhoA. Plasmid pTLQ20 was constructed as follows. A 1.6-kilobase pair fragment encoding TolQ amino acids 113-155 fused to alkaline phosphatase was amplified by the polymerase chain reaction from plasmid pTLQ155 and inserted into the BamHI site of pTrc99A which provides a Shine-Dalgarno sequence. To construct plasmid pTLQ023, a 264-base pair fragment encoding TolQ amino acids 113-197 was amplified by polymerase chain reaction from the EcoRI-NcoI fragment of PTSP04 (23) and inserted into the BglII site of expression vector pJH12.

Alkaline Phosphatase Activity—Alkaline phosphatase activity of the fusion proteins was measured essentially as described by Brickman and Beckwith (24). Five ml of bacteria grown to 2.5 x 10⁸ cells/ml were collected on a 0.45-μm filter and resuspended in an equal volume of 1 mM Tris-HCl, pH 8.0. Sigma 104 phosphatase substrate, p-nitrophenylphosphate, was added to a final concentration of 0.04%, and the reaction mixture was incubated at 37°C. One-ml samples were taken at 10, 20, 30, and 60 min, and the reaction was terminated by the addition of 0.1 ml of 1 M K₂HPO₄. Alkaline phosphatase activity was calculated based on the absorbance at 420 nm.

Analysis of Fusion Proteins—K17 or K17saE bacteria containing the plasmid encoding the appropriate fusion protein were grown in Luria Broth containing 10 mM K₂HPO₄ to 2.5 x 10⁹ cells/ml and then induced for 10 min with 1 mM IPTG or by temperature shift to 39 or 40°C in the presence or absence of 1 mM azide or 100 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP). Bacteria in a 1.5-ml sample were harvested and resuspended in 0.1 ml sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 4% SDS, 0.004% bromphenol blue). After boiling for 3 min, the samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose for Western blot analysis using antibodies to alkaline phosphatase or EcoRI endonuclease, and 125I-protein A.

Cellular Fractionation—A 200-ml culture of bacteria was grown to 2.5 x 10⁸ cells/ml, induced as described above, harvested by centrifugation, and resuspended in 20 ml of 10 mM HEPES-NaOH, pH 7.8. After the addition of 200 μl of lysozyme (10 mg/ml), an equal volume of 10 mM HEPES-NaOH, pH 7.8, 1 mM EDTA was added dropwise while the cell suspension was stirring on ice. After 2 min, spheroplasts were harvested by low speed centrifugation. Three ml of the supernatant were precipitated with 5% trichloroacetic acid and designated the cytoplasmic fraction. The membrane band at the interface was also collected and the inner and outer membrane fractions were pulled together and resuspended in 15 ml of 10 mM HEPES-NaOH, pH 7.8, 0.5 mM EDTA (HE buffer) or 15 ml of 2 mM NaBr in HE, and then lysed in a French pressure cell (20,000 p.s.i.). The lysed cells were layered over a 2-ml cushion of 55% (w/w) sucrose topped with 0.5 ml of 10 mM HEPES-NaOH, pH 7.8, 1 mM EDTA. A portion of the periplasm, cytoplasm, inner membrane, and outer membrane fractions corresponding to equal number of bacteria in the original culture were subjected to Western blot analysis as described above. The blot was exposed to a PhosphorImager screen and quantitated with the Molecular Dynamics Image Quant program.

Quantitation of Membrane-Localized TolQ Fusions—The protein concentration of each membrane sample (collected as described above) was determined by the bicinchoninic acid assay (Pierce). Equal amounts of total protein from each membrane sample were run on an 11% SDS-polyacrylamide gel, transferred to nitrocellulose for Western blot analysis, and quantitated as described above. Each experiment was repeated a minimum of three times.

Trypsin Digestion—100 ml of bacteria were grown to 2.5 x 10⁸ cells/ml, induced as described above, harvested by centrifugation, and resuspended in 10 ml of 20% (w/w) sucrose in 10 mM Tris-HCl, pH 8.0. Cells were lysed by addition of an equal volume of 10 mM Tris-HCl, pH 8.0, 1 ml EDTA dropwise while the cell suspension was stirring on ice. Aliquots of the plasmolyzed cells were incubated at room temperature for 30 min in the absence or presence of 10 μg/ml trypsin. Another fraction of plasmolyzed cells was broken in a French pressure cell and treated with trypsin as described above. All samples were then treated with 100 μl of 5 mg/ml trypsin inhibitor. The plasmolyzed cells were harvested by centrifugation and resuspended in sample buffer. An aliquot of each sample was subjected to Western blot analysis with antibody against TolQ.

Overexpression of TolQ—K17(DE3) containing pTLQHis was grown in Luria broth to 2 x 10⁸ cells/ml and induced with 2 mM IPTG. Forty minutes following IPTG induction, the culture was harvested with maltose (0.4%). At 60 min after induction, 5 ml of culture (– and + maltose) was treated with 1 mM azide. Bacteria in 1.5 ml of culture were collected by centrifugation at 0, 20, 40, 50, 60, and 70 min after induction and resuspended in 0.1 ml of sample buffer. Samples were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue R-250 or transferred to nitrocellulose for Western blot analysis with antibody to maltose-binding protein and 125I-protein A.

RESULTS

TolQ is synthesized without a signal sequence and, once inserted into the cytoplasmic membrane, has very little of its sequence exposed to the periplasm (Fig. 1). One of these periplasmic regions is the amino-terminal 19 residues that still retains the initiating formylmethionine group (7). This observation suggests that the amino terminus and perhaps the other periplasmic region (residues 157–174) might be translocated across the membrane in a manner independent of the Sec system. To test this hypothesis, a number of TolQ fusion proteins were constructed (Fig. 1) and tested for their ability to be inserted into the membrane in the absence of functional SecA protein.

Insertion of the First Membrane-Spanning Region Is Sec-
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The TolQ128 fusion protein has the amino-terminal 128 amino acids of TolQ fused to alkaline phosphatase (Fig. 1). This places alkaline phosphatase 81 residues to the carboxyl side of the first transmembrane region. Therefore the membrane-spanning region of the fusion protein might be expected to be presented to the membrane for insertion in the same manner as for the intact TolQ protein. A cellular fractionation experiment showed that approximately 65% of the membrane protein is localized to the inner membrane (Table I). The absence of alkaline phosphatase activity in cells synthesizing TolQ128 (Table I) confirms the cytoplasmic localization of the alkaline phosphatase moiety.

To test the necessity of the Sec system for insertion of TolQ128, inner membranes were isolated from K17 and K17secA bacteria following expression of TolQ128 for 10 min in the presence and absence of 1 mM azide. This low concentration of azide selectively inhibits the ATPase activity of SecA (19). Equal amounts of protein from each inner membrane preparation were subjected to quantitative Western blot analysis (Fig. 2A). Quantitation of the radioactivity in the bands shows approximately 20% more TolQ128 is detected in the membranes isolated from the azide treated K17 cells (Fig. 2A, lane 2) as compared to that observed in untreated bacteria (Fig. 2A, lane 1). The TolQ128 present in azide-treated or untreated membranes was not removed by treatment with 2 M NaBr (data not shown), suggesting that TolQ128 is an integral membrane protein and spans the membrane with the proper orientation. The ability of TolQ128 to insert into the K17 cytoplasmic membrane when expressed in the presence of 1 mM azide, indicates that the first membrane-spanning region of TolQ does not require SecA for insertion. The presence of 1 mM azide inhibited normal SecA function as shown by the appearance of precursor to ribose binding protein in azide treated K17 bacteria (Fig. 2B, compare lanes 1 and 2). Ribose binding protein is constitutively expressed and any precursor produced was the result of synthesis only during the 10-min period of azide treatment.

The control strain, K17secA, carries a point mutation in secA (a24) which allows for normal SecA ATPase activity in the presence of 1 mM azide (19). This mutant secA bacteria also exhibits normal growth in the presence of 1 mM azide. Membranes isolated from azide treated K17secA bacteria contained 92% of the TolQ128 detected in membranes from the untreated cells (Fig. 2A, compare lanes 3 and 4). This shows that azide alone does not affect membrane insertion. Azide treatment of K17secA bacteria also does not affect normal protein secretion as demonstrated by the absence of precursor to ribose binding protein in K17secA bacteria treated with 1 mM azide (Fig. 2B, lane 4). Ribose binding protein requires both an intact membrane potential (see below, Fig. 2D) and the ATPase activity of SecA for proper translocation.

To examine the requirement for the membrane potential in the insertion of the first membrane-spanning region of TolQ128, half the culture was treated with 50 μM CCCP at the time of induction. Inner membranes were isolated and equal amounts of protein from each inner membrane sample were analyzed by Western blot with antibody to ribose binding protein (RBP). TolQ128 containing plasmid pTLQ128 were induced in the absence (lanes 1 and 3) and presence (lanes 2 and 4) of 1 mM azide. A, cytoplasmic membranes were isolated from an isopycnic sucrose gradient, and equal amounts of total protein from each membrane sample were subjected to 11% SDS-PAGE and Western blot analysis with antibody to alkaline phosphatase. B, a sample of the harvested bacteria was subjected to 11% SDS-PAGE and Western blot analysis using antibody to ribose binding protein (RBP). Bottom, K17 containing plasmid pTLQ128 were induced in the absence (lane 1) and presence (lane 2) of 50 μM CCCP. C, cytoplasmic membranes were isolated from an isopycnic sucrose gradient, and equal amounts of total protein from each membrane sample were subjected to 11% SDS-PAGE and Western blot analysis using antibody to alkaline phosphatase. D, a sample of the harvested bacteria was subjected to 11% SDS-PAGE and Western blot analysis using antibody to ribose binding protein (RBP).

### Table I

| Fusion protein  | Plasmid       | AP activity | Localization |
|----------------|---------------|-------------|--------------|
| TolQ36         | pTLQ36        | ++          | C = 50, P = 50, IM = OM |
| TolQ128        | pTLQ128       | +           | C = 35, P = 65, IM = OM |
| TolQ155        | pTLQ155       | + +         | C = 35, P = 65, IM = OM |
| TolQII         | pTLQ020       | + +         | C = 40, P = 60, IM = OM |
| TolQ11/II      | pTLQ023       | N/A         | C = 30, P = 50, IM = OM |

*Alkaline phosphatase activity of intact cells (average from five experiments) corresponds to units of p-nitrophenylphosphate cleaved/min/cell/ml. – is <5; ++ is 20 to 40; +++ is 40 to 60; N/A = not applicable.

*Bacteria were fractionated into cytoplasm (C), periplasm (P), inner membrane (IM), and outer membrane plus inclusion bodies (OM) in the presence or absence of 2 M NaBr. Each fraction, containing material from the same number of cells, was analyzed by Western blot and quantitated by phosphorimaging as described under "Materials and Methods."
Plasmid pTLQ36 were induced in the absence (bars) and presence (3 secA K17) alone does not inhibit alkaline phosphatase activity, since in 14146 M NaBr does not affect the amount of TolQ36 detected in inner membranes following induction in the presence or absence of 1 mM azide (Fig. 3, lane 1 and 2) and K17 secA (bars and lanes 3 and 4) containing plasmid pTLQ36 were induced in the absence (bars and lanes 1 and 3) and presence (bars and lanes 2 and 4) of 1 mM azide. A, alkaline phosphatase activity corresponds to units of p-nitrophenyl phosphate cleaved/min/cells/ml. B, cytoplasmic membranes were isolated from an isopycnic sucrose gradient and equal amounts of total protein from each membrane sample were subjected to 11% SDS-PAGE and Western blot analysis with antibody to alkaline phosphatase. The NH₂ terminus and first membrane-spanning region of TolQ look like a typical signal sequence. Expression of TolQ36 results in the bacteria expressing alkaline phosphatase activity (Table I). This activity is not due to translocation of the entire TolQ36 fusion to the periplasm, since no anti-alkaline phosphatase reactive species is detected in the periplasmic fraction (Table I). Since 60% of TolQ36 is localized to the inner membrane (Table I), it would seem that some TolQ36 can be inserted into the membrane in the opposite orientation to that determined for the NH₂ terminus of wild type TolQ or TolQ128, resulting in the alkaline phosphatase moieties being exposed to the periplasm.

The alkaline phosphatase moiety in TolQ36 is translocated in a SecA-dependent manner since the alkaline phosphatase activity observed in K. bakeri is dramatically reduced in the presence of 1 mM azide (Fig. 3A, compare bars 1 and 2). Azide alone does not inhibit alkaline phosphatase activity, since in K17 secA bacteria treated with 1 mM azide, the alkaline phosphatase activity only decreases 10% (Fig. 3A, bars 3 and 4). The loss of alkaline phosphatase activity observed in the presence of azide in K17 indicates that the translocation of alkaline phosphatase fused to residue 36 of TolQ proceeds in a SecA-dependent manner. However, TolQ36 is still inserted into the membrane in the presence of azide. Western blot analysis of K17 samples shows the same amount of TolQ36 is detected in the cytoplasmic membrane following treatment with azide (Fig. 3B, lane 2) or without azide (Fig. 3B, lane 1). Treatment with 2 M NaBr does not affect the amount of TolQ36 detected in inner membranes following induction in the presence or absence of azide (data not shown), suggesting that TolQ36 spans the membrane.

The second Membrane-spanning Region Can Function as an Internal Signal Sequence—TolQ155 has alkaline phosphatase fused to residue 155 which lies at the COOH terminus of the second membrane-spanning region (region B in Fig. 1). Cellular fractionation shows that TolQ155 is found primarily (65%) in the inner membrane (Table I). Bacteria expressing TolQ155 have alkaline phosphatase activity (Table I) indicating a periplasmic localization for the alkaline phosphatase moiety.

The SecA dependence for the insertion of the second membrane-spanning region in TolQ155 was examined by monitoring alkaline phosphatase activity following induction of TolQ155 in the presence and absence of 1 mM azide. In K17 bacteria, no alkaline phosphatase activity is observed when TolQ155 is expressed in the presence of 1 mM azide (Fig. 4A, bar 2), suggesting that SecA is required for the translocation of the alkaline phosphatase moiety into the periplasm.

We also examined the role of the membrane potential in translocation of the alkaline phosphatase moiety of TolQ155. No alkaline phosphatase activity is observed when TolQ155 is expressed in the presence of 50 μM CCCP (data not shown). The lack of alkaline phosphatase activity is not due to a block in protein synthesis, as analysis of whole bacteria shows that CCCP treated bacteria synthesize 80% of the amount of TolQ155 detected in untreated cells (data not shown). These results suggest that the membrane potential is required for the translocation of the alkaline phosphatase moiety of TolQ155 into the periplasm.

The data presented above suggest that the Sec system is used for the insertion of the second membrane-spanning region of TolQ155 into the cytoplasmic membrane. These experiments do not address the role of the first membrane-spanning region in this process. To test whether the first membrane-spanning region affects the insertion of the second membrane-spanning region, an amino-terminal truncation of TolQ155 was made, generating TolQII (Fig. 1). Cellular fractionation shows that 60% of the TolQII synthesized is detected in the inner membrane (Table I). TolQII also has alkaline phosphatase activity (Table I) indicating a periplasmic localization for the alkaline phosphatase moiety.

In K17 bacteria, induction of TolQII results in a two-fold increase in alkaline phosphatase activity above the uninduced level. When TolQII is induced in the presence of 1 mM azide no increase in alkaline phosphatase activity above the uninduced level is observed (data not shown). The absence of additional alkaline phosphatase activity following expression of TolQII in the presence of 1 mM azide, indicates that SecA is required for the translocation of the alkaline phosphatase moiety into the periplasm. This result is consistent with the result obtained for TolQ155 and suggests that the absence of the first membrane-spanning region of TolQ has little effect on the insertion of the second membrane-spanning region fused to alkaline phosphatase.

Although the above data are consistent with SecA being required for insertion of the second membrane-spanning region of TolQ155 into the membrane, it does not address the question of whether insertion of this transmembrane domain affects the mode of insertion of the first membrane-spanning region. Therefore, we analyzed the cellular location of TolQII pro-
The absence of azide (Fig. 4, lane 2) or plasmid pC1S (lanes 1 and 4) or presence (lanes 2, 3, and 5) of 10 μg/ml trypsin. Equal amounts of cellular material were subjected to 11% SDS-PAGE and Western blot analysis with antibody against EcoRI endonuclease. The open arrow indicates the position of the 38-kDa C1S protected fragment.

The second and third membrane-spanning regions insert in a Sec-independent manner—The experiments with TolQ155 suggest that the second membrane-spanning region behaves as a Sec-dependent internal signal sequence. However, the TolQ155 construct does not mimic the actual membrane topology for TolQ, which have the second and third membrane-spanning segments (Fig. 1, regions B and C) connected by a short (17-amino acid) periplasmic loop. It has recently been shown that Sec-independent insertion of such hairpin loops requires both membrane-spanning regions (26). To test the insertion characteristics of the combined second and third membrane-spanning regions of TolQ, amino acids 113–197 were inserted into the middle of EcoRI endonuclease to create TolQII/III (Fig. 1). Cellular fractionation indicated that at least 50% of TolQII/III is inserted into the cytoplasmic membrane (Table I). Treatment with 2 M NaBr does not remove TolQII/III from the membrane suggesting that TolQII/III spans the inner membrane. The 20% found in the outer membrane fraction was the result of inclusion body formation.

The membrane topology of TolQII/III was verified by a trypsin accessibility experiment. Previous studies have shown that TolQ is only partially susceptible to trypsin at very high concentrations (>100 μg/ml) (5, 7) and that EcoRI endonuclease is trypsin sensitive (22). Therefore, if TolQII/III adopts a hairpin loop conformation in the membrane it should be resistant to digestion at low concentrations of trypsin. To test this hypothesis, bacteria expressing TolQII/III were either plasmolyzed or lysed, and then treated with 10 μg/ml trypsin. A Western blot with antibody against EcoRI endonuclease shows that trypsin was unable to digest TolQII/III in plasmolyzed cells (Fig. 5, lane 2), while trypsin completely digested the fusion protein in lysed cells (Fig. 5, lane 3). As a control, C1S (22), an EcorI fusion protein with a single membrane-spanning region (Fig. 1), was subjected to the same trypsin digestion. Trypsin treatment of plasmolyzed cells resulted in digestion of the periplasmically exposed COOH terminus of the 51-kDa C1S protein to yield a 30-kDa protected fragment (Fig. 5, compare lanes 4 and 5). These results suggest that TolQII/III adopts a membrane conformation similar to that predicted for the second and third membrane-spanning regions of TolQ.

TolQII/III was used to examine the SecA-dependence of the insertion of the second and third membrane-spanning regions of TolQ. Quantitative Western blot analysis of inner membranes from azide treated K17 cells, showed that they contained 90% of the amount of TolQII/III detected in membranes from untreated cells (Fig. 6A, compare lanes 1 and 2). Membrane-associated TolQII/III in azide-treated bacteria was not removed by treatment with 2 M NaBr (data not shown) and is trypsin-resistant in plasmolyzed bacteria. This suggests that TolQII/III expressed in the presence of azide adopts the correct membrane topology. In azide-resistant cells, azide treatment results in only a 20% decrease in the amount of inner membrane TolQII/III detected (Fig. 6A, lane 4). The ability of TolQII/III to insert properly in the absence of functional SecA indicates that the second and third membrane-spanning regions of TolQII/III insert in a SecA-independent manner.

K17 bacteria expressing TolQII/III were also treated with 50 μM of the protonophore CCCP. Inner membranes were isolated and equal amounts of protein from each inner membrane preparation were subjected to Western blot analysis. No TolQII/III was detected in the CCCP treated sample (Fig. 6B, lane 2). CCCP treatment did not affect the expression of TolQII/III as illustrated by the presence of a band corresponding to TolQII/III in a whole cell sample (Fig. 6B, lane 4). The absence of inner membrane TolQII/III following CCCP treatment indicates that the membrane potential is required for the insertion of the hairpin loop formed by the second and third membrane-spanning regions.

Overexpression of TolQ Does Not Affect Correct Protein Localization—Our experiments with the TolQ fusions suggest...
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that TolQ is inserted into the cytoplasmic membrane independently of functional SecA protein. To assess the effects of TolQ overexpression on the translocation of Sec-dependent proteins, K17(DE3) bacteria containing plasmid pTLQHis were treated with 2 mM IPTG to induce synthesis of TolQ. Forty minutes following induction, TolQ can be detected on an SDS-polyacrylamide gel showing overexpression of TolQ. Lower panel, Western blot with antibody to maltose-binding protein (MBP). AZ indicates 10 min azide treatment (1 mM) at 60 min following induction.

**FIG. 7. Effect of TolQ overexpression on Sec-dependent translocation.** K17(DE3) containing plasmid pTLQHis were induced for the synthesis of TolQ with 2 mM IPTG. At 40 min following induction, maltose was added to the culture. One and a half ml samples were collected by centrifugation at 0, 20, 40, 50, 60, and 70 min after induction. Upper panel, Coomassie Brilliant Blue R-250 staining of gel showing overexpression of TolQ. Lower panel, Western blot with antibody to maltose-binding protein (MBP). AZ indicates 10 min azide treatment (1 mM) at 60 min following induction.

DISCUSSION

The characteristics of TolQ membrane insertion were examined using various TolQ fusions. Fusion TolQ128, which contains the first membrane-spanning region, and TolQ11/111, which contains the hairpin loop formed by the second and third membrane-spanning regions, insert into the cytoplasmic membrane independent of functional SecA. Since these fusion proteins mimic the membrane topology observed for TolQ, we propose that TolQ inserts Sec independently. This is consistent with the observation that wild type TolQ synthesized in the presence of azide is localized only to the inner membrane (data not shown). Attempts to prove the topology of TolQ in azide treated bacteria were inconclusive due to the normal incomplete digestion of the short periplasmic domains and the apparent fragility of azide treated bacteria.

Experiments with TolQ128 suggested that the first membrane-spanning region of TolQ is able to insert into the cytoplasmic membrane in the absence of functional SecA and a membrane potential. In azide treated bacteria, treatment with 2 mM NaBr does not remove TolQ128 from the membrane. This suggests that TolQ128 spans the inner membrane via the 18-residue hydrophobic domain (Fig. 1, region A). Since the alkaline phosphatase moiety is fused 81 residues to the carboxyl side of the first transmembrane domain, we propose that the membrane-spanning region of TolQ128 might be presented to the membrane for insertion in the same manner as intact TolQ. We have previously shown that overexpressed TolQ purified from the inner membrane contains the initiating formylmethionine (7) which suggests that the first membrane-spanning region is rapidly inserted into the cytoplasmic membrane.

The SecA-independent insertion of the first membrane-spanning region is consistent with results from studies on the translocation of the NH2-terminal tails of Pf3 coat protein, ProW, and Pf3-Lep fusions (16–18). Interestingly, insertion of the NH2-terminal tails of Pf3 coat, ProW, and 38Pf3-Lep required an intact membrane potential while the first membrane-spanning region of TolQ was able to insert even in the absence of the membrane potential. It has been proposed that the membrane potential facilitates translocation of negatively charged amino acids and hinders translocation of positively charged ones (27). Studies on the NH2-terminal tails of ProW and Pf3-Lep showed that mutations that placed basic amino acid residues in the periplasmic tails abolished translocation (17, 18). However, the periplasmic amino-terminal 19 residues of TolQ contain two lysines at amino acid positions 12 and 18, and yet this region is easily translocated into the periplasm. Thus, features other than basic amino acids may determine insertion in the absence of a membrane potential.

Experiments with a TolQ-PhoA fusion that only contained the first 36 residues of TolQ (TolQ36) gave us two interesting results. First, the ability of TolQ36 to insert in the presence of azide indicated that the first 36 amino acids of TolQ are sufficient to drive the SecA-independent insertion of the NH2-terminal tail. Second, in the absence of azide, the membrane orientation is inverted and the alkaline phosphatase moiety is translocated to the periplasm in a Sec-dependent manner. This result suggests that the highly charged cytoplasmic domain of wild type TolQ (residues 39–135, see Fig. 1) may act as a strong topological determinant. It has been proposed that charged amino acids, especially positive ones, are energetically unfavorable for translocation (15, 27, 28). In TolQ, the first cytoplasmic domain contains 27 charged side chains with three basic amino acids in the region immediately following the first membrane-spanning region (Fig. 1) (23). Therefore, when this cytoplasmic region is absent, as in TolQ36, the first membrane-spanning region together with alkaline phosphatase can be recognized as a typical Sec-dependent signal sequence. Although the highly charged cytoplasmic domain appears to influence the membrane orientation of the first membrane-spanning region, it is not required for SecA-independent insertion.

In TolQ155 and TolQ111, which have alkaline phosphatase fused to the COOH terminus of the second membrane-spanning region, our results show that SecA is required for translocation of the alkaline phosphatase moiety to the periplasm. We also demonstrated that the membrane potential is required for translocation of the alkaline phosphatase moiety of TolQ155. These data are consistent with the hypothesis that periplasmic domains > 60 residues require the Sec system for translocation (9, 15). In the context of the TolQ155 and TolQ111 constructs we propose that the second membrane-spanning region functions as a signal sequence, and promotes the Sec-dependent translocation of the alkaline phosphatase moiety.

The fact that absence of functional SecA prevented insertion of the second membrane-spanning region in TolQ155 allowed
us to examine whether insertion of the first membrane-spanning region was dependent on insertion of the second membrane-spanning region. In the presence of azide, which inhibits SecA, the amount of TolQ155 detected in the cytoplasmic membrane was reduced to 55% of that inserted in the absence of azide. Since experiments with TolQ36 and TolQ128 suggest that normal insertion of the first membrane-spanning region is not affected by azide treatment, it seems that insertion of the second membrane-spanning region of TolQ155 exerts some influence on the efficient insertion of the first membrane-spanning region.

Experiments with TolQ155 suggested that the second membrane-spanning region functioned as a Sec-dependent signal sequence. However, in TolQ, the second membrane-spanning region is part of a membrane traversing hairpin loop, which includes the third membrane-spanning domain of TolQ. Therefore, TolQII/III was constructed to encode this hairpin loop sandwiched between the amino and carboxyl regions of TolQ. This construct inserts independently of functional SecA, which suggests that the second and third membrane-spanning regions of TolQ may act together during Sec-independent insertion. This is consistent with the "helical hairpin" hypothesis (29) and the results of Cao et al. (26), which suggest that two hydrophobic domains act synergistically in the translocation of a central hydrophilic domain.

The role of the membrane potential in the insertion of the hairpin loops is somewhat ambiguous. We have shown that the membrane potential is required for insertion of the hairpin loop in TolQII/III, and Date et al. (30) have shown that insertion of M13 procoat is dependent on an intact membrane potential. The periplasmic region in M13 coat is acidic (net charge of minus 3) and has a glutamic acid residue positioned at the COOH terminus of the translocation signal. Replacement of this Glu with Gin, Ser, Tyr, or Leu allows insertion to occur in the absence of a membrane potential (31). This is consistent with results found in studies of inverted leader peptidase which suggested that the membrane potential facilitates the translocation of negative charges proximal to the first membrane-spanning region (27). Since, in TolQ, a lysine is positioned immediately after the second membrane-spanning region and an intact membrane potential is required for insertion of the hairpin loop formed by the second and third membrane-spanning regions, it would appear that more than one mechanism can be proposed for helical hairpin insertion.

Overexpression of Sec-dependent proteins often leads to mislocalization of the overexpressed protein, accumulation of precursors to periplasmic proteins, and an increase in SecA synthesis (32-34). Overexpressed TolQ correctly localizes to the inner membrane (7) and does not affect maltose-binding protein translocation (Fig. 7) or SecA levels (data not shown). This is another indication that TolQ assembles into the cytoplasmic membrane independent of the Sec system. This is consistent with the proposed Sec-independent mechanism for insertion of NH2-terminal tails and hairpin loops. The data also suggest that the membrane potential is required for insertion of the second and third membrane-spanning regions of TolQ, but not the first membrane-spanning region. These results did not entirely agree with previous studies on the requirement of the membrane potential during membrane protein assembly. Further study on the roles of charged amino acids, character of the transmembrane segments, and roles of other proteins may help elucidate the requirements for insertion of cytoplasmic membrane proteins.

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