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The TolA Protein Interacts with Colicin E1 Differently than with Other Group A Colicins

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The 421-residue protein TolA is required for the translocation of group A colicins (colicins E1, E2, E3, A, K, and N) across the cell envelope of *Escherichia coli*. Mutations in TolA can render cells tolerant to these colicins and cause hypersensitivity to detergents and certain antibiotics, as well as a tendency to leak periplasmic proteins. TolA contains a long α-helical domain which connects a membrane anchor to the C-terminal domain, which is required for colicin sensitivity. The functional role of the α-helical domain was tested by deletion of residues 56 to 169 (TolAΔ1), 166 to 287 (TolAΔ2), or 54 to 287 (TolAΔ3) of the α-helical domain of TolA, which removed the N-terminal half, the C-terminal half, or nearly the entire α-helical domain of TolA, respectively. TolA and TolA deletion mutants were expressed from a plasmid in an *E. coli* strain producing no chromosomally encoded TolA. Cellular sensitivity to the detergent deoxycholate was increased for each deletion mutant, implying that more than half of the TolA α-helical domain is necessary for cell envelope stability. Removal of either the N- or C-terminal half of the α-helical domain resulted in a slight (ca. 5-fold) decrease in cytotoxicity of the TolA-dependent colicins A, E1, E3, and N compared to cells producing wild-type TolA when these mutants were expressed alone or with TolQ, -R, and -B. In cells containing TolAΔ3, the cytotoxicity of colicins A and E3 was decreased by a factor of >3,000, and K^+ efflux induced by colicins A and N was not detectable. In contrast, for colicin E1 action on TolAΔ3 cells, there was little decrease in the cytotoxic activity (<5-fold) or the rate of K^+ efflux, which was similar to that from wild-type cells. It was concluded that the mechanism(s) by which cellular uptake of colicin E1 is mediated by the TolA protein differs from that for colicins A, E3, and N. Possible explanations for the distinct interaction and unique translocation mechanism of colicin E1 are discussed.

Protein translocation across cell membranes is known to utilize a network of helper proteins which have been documented for secretion from bacteria and the endoplasmic reticulum and import into mitochondria and chloroplasts (33). The problem of the mechanism of protein import into bacterial cells is of interest for an understanding of the general mechanisms of colicin cytotoxicity.

The cell envelope of the gram-negative bacterium *Escherichia coli* presents a significant obstacle to the uptake of macromolecules exceeding the 600-Da size cutoff of the outer membrane porins (32). To cross this barrier and access its site of action, the cytoplasmic membrane for pore-forming colicins E1, A, B, N, Ia, Ib, and Col10, or cytoplasmic targets for colicins E2 and E3, a colicin molecule must accomplish three tasks: (i) bind to an outer membrane receptor, whose physiological purpose is metabolite (e.g., metal or vitamin) transport; (ii) translocate across the periplasmic space via interactions with either the Tol proteins (used by group A colicins A, E1, E2, E3, and N) or the TonB protein (used by group B colicins Ia, Ib, B, M, and Col10) family; and (iii) insert into the cytoplasmic membrane to form a highly conductive ion channel (colicins A, E1, N, Ia, Ib, and Col10) or enter the cytoplasm to degrade chromosomal DNA or rRNA (colicins E2 and E3) (7, 12).

Colicin molecules can be divided into three domains, each devoted to achieving one of the foregoing steps. The amino-terminal end of the colicin molecule facilitates translocation of the colicin across the periplasmic space aided by interactions with Tol proteins (2). The central domain of the protein is involved in the recognition and binding of the colicin to its outer membrane receptor (9), while the pore-forming or enzymatic activity resides in the carboxy-terminal third of the molecule (13).

TolA, TolB, TolQ, and TolR comprise the Tol protein system. The genes are contiguous and arranged on the chromosome in the order *tolQ tolR tolA tolB*. Mutations in the *tol* genes result in a tolerance to group A colicins and an increased cellular sensitivity to detergents and certain antibiotics and cause the outer membrane to leak periplasmic proteins to the extracellular medium (22, 42). Mutations in the chromosomal *tol* gene cluster can be complemented by a wild-type copy carried on a plasmid lacking an external promoter, suggesting that *tolQRA* may be part of a single operon (6, 40). TolQ and TolR are believed to be embedded in the cytoplasmic membrane by three and one transmembrane helices, with their COOH ends predicted to be in the cytoplasm and periplasm, respectively (18, 30, 39).

The translocation of group A colicins involves interaction with different combinations of the Tol family of proteins. All group A colicins require TolA, and to different extents TolQ and TolR (23, 39, 42), to traverse the cell envelope. Colicin E1 alone requires the outer membrane protein TolC but not TolB (31). The translocation of colicins E2, E3, A, and N is TolC independent but requires TolB. A portion of these Tol proteins can be localized at contact sites between the inner and outer membranes of *E. coli*. The fraction of TolQ and TolB at these contact sites increases approximately twofold after the

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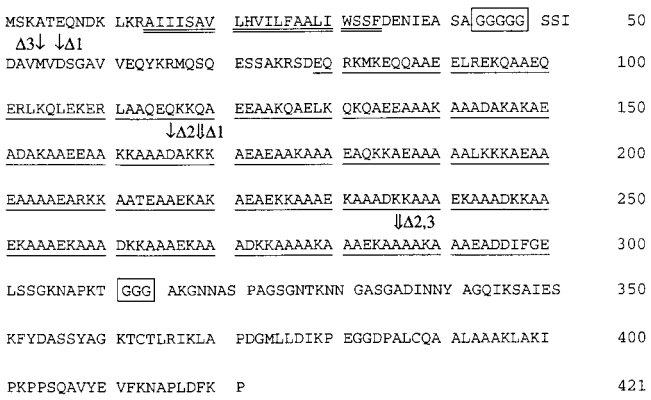

FIG. 1. TolA amino acid sequence. The starts (D56, D166, and M54) and ends (K169, A287, and A287) of the three deletions, TolAΔ1, -Δ2, and -Δ3 are denoted by ↓ and ⇓, respectively. The putative membrane-spanning segment is doubly underlined, and the glycine hinge regions are boxed. The residues predicted to be in the central α-helical domain are underlined. See Materials and Methods for changes in N termini of TolAΔ constructs.

addition of colicin A, suggesting that these adhesion zones may have an important role in colicin uptake (15).

The TolA protein can be divided into three distinct regions (Fig. 1). The amino-terminal 40 residues contain a segment of 21 hydrophobic residues capable of forming a transmembrane α helix that anchors TolA in the cytoplasmic membrane (24). This segment also serves as a stop-transfer sequence for export to the periplasm and may mediate the interactions between TolA and the integral membrane helices of TolQ and TolR to form a Tol protein complex (14). The C-terminal (ca. 120 residues) periplasmic domain of TolA is believed to interact with the incoming colicin molecule because (i) a 4-bp deletion in *tolA* that causes a frameshift after residue 400 renders cells tolerant to group A colicins (25); (ii) a 40-residue deletion in this region (amino acids 337 to 378) abolished in vitro binding of colicins E1 and A to TolA (as detected by Western blotting), and cells expressing this form of TolA were insensitive to colicins A and E1 (4); and (iii) overproduction and export to the periplasm of a TolA fragment composed of residues 390 to 421 reduced sensitivity to colicins A, E1, and E3, implying that this TolA fragment competes with intact TolA for incoming colicin molecules (26).

Separating the N- and C-terminal domains are approximately 250 residues (Asp51 to Glu300), framed on either side by polyglycine sequences (boxed in Fig. 1) which may serve as flexible hinges (24). This 250-residue region has a highly repetitive sequence and is predicted to have a high α-helical content, based on its far-UV circular dichroism spectrum and the α-helix-forming propensity of alanine, which comprises 40% of the residues in this segment (24, 27) (Fig. 1). Thirteen repeats of the sequence KA_3(D/E) compose one-fourth of the α-helical domain. This i· · ·i + 4 arrangement of positively and negatively charged residues may promote the formation of salt bridges that lend stability to the protein in this region. The extended α-helical conformation could serve as a scaffold which places the C-terminal domain against the periplasmic face of the outer membrane, where it could act as a translocator for an incoming colicin molecule (42). The scaffold would be buttressed at the N-terminal hydrophobic anchor via interactions with the transmembrane segments of TolQ and TolR. If the α-helical domain extends across the periplasmic space, the length of the α-helical region would be important for proper positioning of the C-terminal domain relative to the outer membrane.

It is of interest to investigate whether the entire TolA α-helical region is necessary for the translocation of colicins, particularly colicin E1, from the outer to the inner membrane. Cytotoxicity studies indicated that deletion of an extensive region of the central helical domain of TolA did not eliminate the cytotoxicity of colicins A and E3 (19).

To gain more information on the role of the α-helical domain of TolA, the functional consequences of deletions in this region in the context of colicin sensitivity and cell envelope stability were determined. It was found that the TolA requirement of colicin E1 for translocation is different from that of the other group A colicins. Colicin E1 retained almost unimpaired cytotoxic and K^+ efflux activities toward cells containing an altered TolA protein, TolAΔ3, in which most of the TolA α-helical domain was removed.

MATERIALS AND METHODS

Bacterial strains. *E. coli* K17A1DE3, containing a mini-Tn*10* insertion near nucleotide 200 of *tolA* and a λDE3 lysogen encoding the isopropyl-β-D-thiogalactopyranoside-inducible gene for T7 RNA polymerase (24), was used as the host for plasmids carrying wild-type and TolA deletion mutants. pSKL10 (24) carries a partial copy of *tolR* and a complete copy of *tolA* under control of the T7 promoter. pTolAΔ1, pTolAΔ2, and pTolAΔ3 produce TolA with residues 56 to 169, 166 to 287, and 54 to 287 deleted, respectively. pQRAB carries an open reading frame of unknown function (*orf1*), complete *tolQ*, *tolR*, and *tolA*, and *tolB* truncated in the region encoding the C-terminal 66 residues. pQRAΔ1B, pQRAΔ2B, and pQRAΔ3B encode the corresponding TolA deletions as described above. K17DE3, which lacks the mini-Tn*10* insertion in *tolA*, was used to assay chromosomal *tolA* expression levels. Bacterial cultures were grown as described elsewhere (26, 44).

Colicin purification. Colicins E1 (44), E3 (16), A (11), Ia (28), and Col10 (6a) were purified as previously described. Purified colicin N was a generous gift from J. H. Lakey.

Construction of vectors expressing *tolA*. pTolAΔ2, expressing TolAΔ166-287, was created from pSKL10 by *Pst*I (New England Biolabs) digestion. Of the six *Pst*I sites contained in pSKL10, five are contained within *tolA*. Two of these sites frame the region of the gene encoding residues 166 to 287, which itself contains three other *Pst*I sites. An additional site is located in the β-lactamase gene. To avoid complications from the latter site, a 2.3-kb *Kpn*I-*Cla*I fragment of pSKL10 containing *tolA* and the flanking upstream region containing partial *tolR* was ligated into pSKE13.5, which contains no *Pst*I sites, previously digested with *Kpn*I and *Cla*I. This construct, pSK(3.5 + 2.3), then acted as a shuttle vector for the creation of the deletion mutants. The resulting 5.8-kb plasmid was then subjected to *Pst*I digestion and religated with T4 DNA ligase (Promega). The *Pst*I-deleted *Kpn*I-*Cla*I fragment was then isolated and ligated back into pSKL10 to yield pTolAΔ2. To create pTolAΔ3, a *Pst*I site was introduced between the codons for residues 53 and 54 of TolA, using the mutagenesis protocol of Kunkel (20), after which the same procedure used to make pTolAΔ2 was followed. Introduction of the *Pst*I sites in *tolA* resulted in a valine-to-alanine change for residue 53 of TolAΔ3. Residue 54, which was ultimately deleted, was changed from methionine to valine. Introduction by site-directed mutagenesis of two *Eco*RI sites into pSK(3.5 + 2.3) between the codons for TolA residues 55 and 56 and residues 169 and 170 facilitated the production of pTolAΔ1. The mutagenesis introduced the following amino acid changes: D56N, K170N, and A171S. This construct was then digested with *Eco*RI and religated. The *Kpn*I-*Cla*I fragment was then cloned back into pSKL10. The absence of the deleted segments was confirmed by restriction digestion and DNA sequencing.

Construction of vectors coexpressing *tolA* with *tolQ*, *tolR*, and *tolB*. To create a vector which would coexpress wild-type TolA and the deletion mutants with TolQ, -R, and -B, a 4.2-kb *Eco*RI-*Bgl*II fragment from pTPS202 (25) carrying *tolQ*, *tolR*, *tolA*, and *tolB* was cloned into pT7-7 (37) digested with *Eco*RI and *Bam*HI to create pQRAB, which encodes the wild-type Tol protein cluster. The 4.2-kb *Eco*RI-*Bgl*II fragment of pTPS202, which complements mutations in *tolQ*, *-R*, *-A*, and *-B* (36), includes an upstream open reading frame (*orf1*) but lacks the region of *tolB* encoding the C-terminal residues 365 to 431. To facilitate creation of the TolA deletion mutants in this construct, the *Hin*dIII and *Pst*I sites existing in the multicloning region of pT7-7 were deleted by *Acc*I and *Cla*I digestion of pT7-7 and ligation of the compatible ends. pQRAΔ1B and pQRAΔ2B were created by exchanging a 0.35-kb *Hin*dIII-*Eag*I fragment of pQRAB (carrying wild-type *tolA*) with the corresponding regions of pTolAΔ1 and pTolAΔ2, respectively. pQRAΔ3B was constructed by exchanging the 0.75-kb *Hin*dIII-*Eag*I fragment with that of the pTolAΔ3 precursor, pSKE1(3.5 + 2.3)*Pst*I, and subsequently digesting with *Pst*I and religating. The absence of the deleted segments of *tolA* was confirmed by restriction digestion and DNA sequencing.

Quantitation of TolA by Western blotting. Equal amounts of cellular protein (as determined by bicinchoninic acid assay [Pierce]) from cells growing exponentially at 37°C in YT medium (8 g of Bacto Tryptone, 4 g of yeast extract, 2.5 g of NaCl) were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (10% acrylamide), transferred to nitrocellulose, and incubated with antibodies to the TolA C-terminal domain and ^{125}I-labeled protein A. The blot was exposed to a Fuji phosphorimaging screen and quantitated by using the Molecular Dynamics ImageQuant program.

Colicin cytotoxicity assays. Overnight cultures (50 μl) were transferred to 2×YT (5 ml; 16 g of Bacto Tryptone, 8 g of yeast extract, 5 g of NaCl) and incubated at 37°C with shaking until mid-log phase (optical density at 650 nm of 0.3 to 0.7) was reached. Spot test assays were performed as described elsewhere (35).

Colicin E1-induced K^+ efflux. Overnight cultures (5 ml) of K17A1DE3 with the appropriate plasmid were used to inoculate 2×YT (500 ml) supplemented with the appropriate antibiotic. The cells were grown at 37°C with shaking to mid-log phase (optical density at 650 nm of 0.3 to 0.7). They were then harvested, washed once with 100 mM sodium phosphate (pH 6.8), resuspended to a concentration of 3×10^9 cell/ml, supplemented with 0.4% glycerol (vol/vol), and stirred at room temperature prior to use. Colicin E1, N, or A was added to a final concentration of 20 to 400 ng/ml to a stirred cell suspension (15 ml). Colicin-induced K^+ efflux was measured as described elsewhere (44). Samples of the cell suspension were taken before and after colicin addition, diluted in 100 mM sodium phosphate buffer (pH 6.8), and plated. The survival level (S/S_0) and multiplicity (m) were determined by using the relationship $m = -\ln(S/S_0)$. Specific K^+ efflux rates were calculated relative to m, which describes only the population of colicin molecules that were successful in the formation of conductive channels.

DOC sensitivity. Overnight cultures (50 μl) were transferred to 6 ml of LB containing various concentrations of deoxycholate (DOC; Sigma). The optical density of the cultures was determined after incubation at 37°C for 3 h, using a Kontron 810 spectrophotometer in which the sample-detector distance was 5 cm.

RESULTS

Effect of TolA α-helical domain deletions on group A colicin cytotoxicity. The ability of plasmid-encoded TolA with shortened α-helical domains to restore colicin sensitivity to cells possessing chromosomal mutations in *tolA* that render it defective was first examined by assays of cytotoxicity. Strain K17A1DE3 (24) carries a mini-Tn*10* insertion just before the beginning of the α-helical domain. No TolA is detected in this strain by polyclonal antibodies to the α-helical and C-terminal domains, and the sensitivity of these *tolA* cells to group A colicins is reduced by a factor of 10^3 to 10^6 (26).

Complementation with plasmid-encoded wild-type TolA (pSKL10) restores the sensitivity to colicin E1, which is absent in K17A1DE3 (Table 1, assay A), to that of the wild-type strain, which is sensitive in spot tests to colicins E1, E3, and A added at 3 ng/ml or colicin N added at 200 ng/ml (Table 1, assay B). Residues 56 to 169 and 166 to 287 were deleted to form TolAΔ1 and TolAΔ2. Ninety percent of residues 166 to 287 deleted in TolAΔ2 are either alanine (50%), lysine (20%), or aspartate and glutamate (20%), and this deleted region contains all but 2 of the 13 KA_3(D/E) repeats. Although the segment from residues 56 to 169, deleted in TolAΔ1, contains 70% alanine, lysine, glutamate, and aspartate, these residues are not arranged in the i· · ·i + 4 pattern seen in the second half of the α-helical domain. Despite the different sequences of the deleted segments, the sensitivity of cells expressing plasmid-encoded TolAΔ1 and TolAΔ2 (Table 1, assays C and D) toward the group A colicins E1, E3, N, and A is approximately the same as that of the wild type, within a factor of 5.

Residues 54 to 287 of TolA were deleted to create TolAΔ3. Residue 54 is 7 residues downstream from the pentaglycine segment that marks the beginning of the α-helical domain of TolA, and residue 287 is 30 residues upstream of three glycines that mark the end of the α-helical domain (Fig. 1). Cell fractionation of K17A1DE3 containing TolAΔ3 showed that essentially all of the TolAΔ3 protein is localized in the cytoplasmic membrane fraction (data not shown). Although this TolAΔ3 has fewer than 40 residues of the predicted helical region, cytotoxicity of colicin E1 was readily detectable. It was reduced approximately fivefold against TolAΔ3 compared to wild-type TolA (pSKL10), whereas the cytotoxicity of colicin E3, N, or A was decreased by a factor of at least 3,000 (Table 1, assays B and E). The TolAΔ3 cells were insensitive to colicins E3, N, and A (Table 1, assay E) at colicin concentrations as high as 10 μg/ml. No significant differences in the cytotoxicity of the TonB-dependent colicins Col10 and Ia toward K17A1DE3, producing wild-type TolA, relative to the TolAΔ1, -Δ2, and -Δ3 mutants were observed, reflecting the TolA independence of these colicins (Table 1, assays B to E). Similar results were obtained when these plasmids were expressed in a strain with *tolA592*, which has a chromosomal frameshift mutation in *tolA*, and K91A1, which has the same mini-Tn*10* insertion in *tolA* as K17A1DE3 (data not shown).

Coexpression of TolA deletion mutants with TolQ, -R, and -B. Plasmids pSKL10, pTolAΔ1, pTolAΔ2, and pTolAΔ3 ex-

TABLE 1. Cytotoxicity of group A colicins against cells producing plasmid-encoded TolA α-helical domain deletion mutants

Assay	Plasmid	Colicin	Clearing at colicin concn (μg/ml) of[a]:					
			10	2	0.4	0.08	0.016	0.003
A	None	E1	−	−	−	−	−	−
B	pSKL10	E1	+	+	+	+	+	±
		E3	+	+	+	+	+	+
		N	+	+	+	+	+	+
		A	+	+	+	+	+	+
		Ia	+	+	+	+	+	+
		Col10	+	+	+	+	+	−
C	pTolAΔ1	E1	+	+	+	+	+	+
		E3	+	+	+	+	+	+
		N	+	+	+	+	+	+
		A	+	+	+	+	+	−
		Ia	+	+	+	+	+	+
		Col10	+	+	+	+	+	−
D	pTolAΔ2	E1	+	+	+	+	+	−
		E3	+	+	+	+	+	−
		N	+	+	+	+	+	+
		A	+	+	+	+	+	−
		Ia	+	+	+	+	+	+
		Col10	+	+	+	+	±	−
E	pTolAΔ3	E1	+	+	+	+	+	−
		E3	−	−	−	−	−	−
		N	−	−	−	−	−	−
		A	−	−	−	−	−	−
		Ia	+	+	+	+	+	+
		Col10	+	+	+	+	±	−
F	pQRAB[b]	E1	+	+	+	+	+	+
		E3	+	+	+	+	+	±
		N	+	+	+	+	+	+
		A	+	+	+	+	+	+
G	pQRAΔ1B	E1	+	+	+	+	+	+
		E3	+	+	+	+	±	−
		N	+	+	+	+	+	±
		A	+	+	+	+	−	−
H	pQRAΔ2B	E1	+	+	+	+	+	+
		E3	+	+	+	+	+	±
		N	+	+	+	+	+	±
		A	+	+	+	+	−	−
I	pQRAΔ3B	E1	+	+	+	+	±	−
		E3	−	−	−	−	−	−
		N	−	−	−	−	−	−
		A	−	−	−	−	−	−

[a] Indicator strain, K17A1DE3; colicin N concentrations tested: 10, 2, 1, 0.6, 0.4, and 0.2 μg/ml. +, clear zone visible when 20 μl of colicin solution was spotted onto cell lawn; −, no clearing; ±, partial clearing.

[b] Group B colicins Ia and Col10 were not tested with the QRAB plasmids.

press TolA alone, while TolA is expressed from the chromosome as part of a gene cluster composed of open reading frame *orf1*, *tolQ*, *tolR*, *tolA*, and *tolB* (42). To determine whether the colicin sensitivity of cells is changed when the TolA deletion mutants are expressed as part of the Tol protein cluster, *tolA* and the deletion mutants were cloned into the vectors pQRAB (wild-type TolA), pQRAΔ1B (TolAΔ1), pQRAΔ2B (TolAΔ2), and pQRAΔ3B (TolAΔ3), which coexpress TolA with the other Tol proteins in a manner similar to that from the chromosome. The sensitivities to colicins E1, E3, and N of cells producing wild-type TolA, TolAΔ1, and TolAΔ2 were relatively unchanged when the Tol proteins were coexpressed (Table 1, assays B to D and F to H), with small (ca. 5-fold) changes in sensitivity shown at the lowest colicin concentrations tested. Coexpression of TolAΔ3 with TolQ, -R, and -B did not restore any sensitivity to colicins E3, N, and A (Table 1, assays E and I), which remained much lower than that of colicin E1. Colicin E1 activity toward cells with coexpressed TolAΔ3 was comparable to that seen for cells expressing TolAΔ3 alone (Table 1, assays E and I). It was inferred that at least one half of the TolA α-helical domain is necessary for the full activity of colicins E3, N, and A. However, substantial colicin E1 activity was retained even when most of the TolA α-helical domain was deleted.

Colicin-induced K^+ efflux. Colicin-induced efflux of intracellular K^+ provided information on the effect of the TolA deletion mutants on in vivo channel activity of colicins E1, N, and A. The average initial specific rates of colicin E1-induced K^+ efflux from K17A1DE3 expressing either plasmid-encoded wild-type TolA, TolAΔ1, or TolAΔ2 alone (Fig. 2A) or with TolQ, -R, and -B (data not shown) are similar. This rate, 1×10^6 to 2×10^6 K^+ channel^{-1} s^{-1}, is comparable to in vitro values of colicin E1 single-channel conductivity in 0.1 M salt (10). The lag time, the period after colicin addition during which no K^+ efflux is detectable, is approximately 40 to 60 s. This lag time presumably represents the time needed for the colicin molecule to locate its receptor and translocate across the periplasmic space, aided by interactions with the Tol proteins (5). The rate of K^+ efflux induced by colicin E1 from cells expressing TolAΔ3 (Fig. 2A, trace d) is similar to that seen from cells producing either wild-type TolA, TolAΔ1, or TolAΔ2 (Fig. 2A, traces a to c). The only difference is that the lag time with TolAΔ3 is increased two- to threefold to 100 to 150 s, and the multiplicity of killing caused by addition of 30 ng of colicin E1 per ml was reduced from 6 to 2.2. The rate of K^+ efflux from TolAΔ3 cells is approximately unaffected because, even with the latter multiplicity, active colicin E1 molecules are bound to 90% of the cells. Some effect on the lag time was sometimes observed with TolAΔ2 (Fig. 2A, trace c). The increased lag time always observed with TolAΔ3 implies that there is a population of TolAΔ3 which is in a proper position to receive an incoming colicin molecule, but this population is smaller than for cells producing wild-type TolA, TolAΔ1, or TolAΔ2. Thus, a longer time is required for colicin E1 to locate the TolAΔ3 molecules that are properly situated, but once they are found, translocation and subsequent channel formation can proceed. Once the channel is formed, it conducts K^+ ions at the same rate as observed for TolA, TolAΔ1, and TolAΔ2.

Both colicin N and colicin A were able to form conductive channels in the cytoplasmic membrane of K17A1DE3 expressing wild-type TolA, TolAΔ1, or TolAΔ2 either alone (Fig. 2B and C, traces a to c) or with TolQ, -R, or -B (data not shown), although the lag time for TolAΔ1 and TolAΔ2 was increased (Fig. 2B and C). In contrast to colicin E1, colicin N (400 ng/ml) and colicin A (30 ng/ml) were unable to induce any K^+ loss from cells producing TolAΔ3 (Fig. 2B and C, trace d). An absence of K^+ efflux was also observed when colicin N was added at concentrations up to 2 μg/ml (data not shown). The

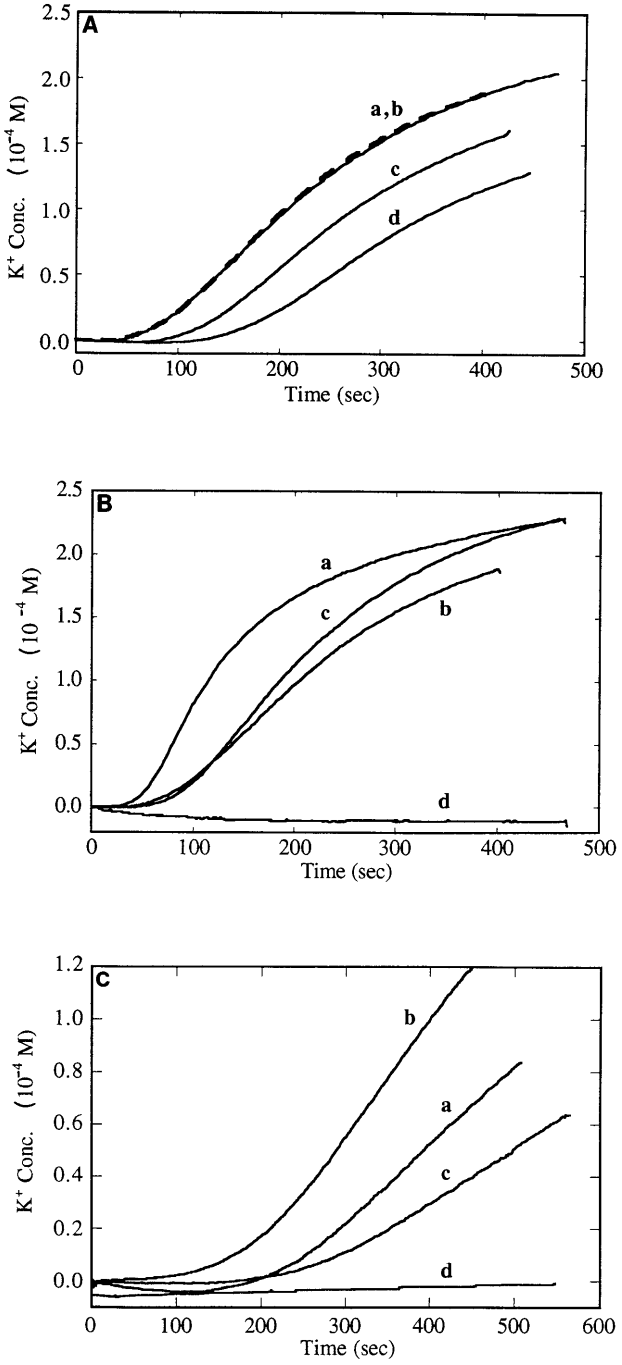

FIG. 2. Colicin E1 (A)-, N (B)-, and A (C)-induced K^+ efflux from K17A1DE3 carrying pSKL10 (wild-type TolA) (trace a), pTolAΔ1 (trace b), pTolAΔ2 (trace c), or pTolAΔ3 (trace d). The ordinate shows the concentration of free K^+ released into the extracellular medium. Colicins E1 and A were added to a final concentration of 30 ng/ml, and colicin N was added to 400 ng/ml. The colicin-induced K^+ efflux was detected for a period of 5 to 7 min after colicin addition at time zero. Samples of the cell suspension were taken before and after colicin addition, diluted in 100 mM sodium phosphate buffer (pH 6.8), and plated. The colicin multiplicities were 6.4, 6.6, 4, and 2.2 for traces a to d, respectively, in panel A, 6.4, 6.6, 6.2, and 0 for traces a to d in panel B, and 5.3, 5.8, 3.9, and 0.3 for traces a to d in panel C.

K^+ efflux traces of colicin A shown in Fig. 2C, curves a to c, are all indicative of high activity. The differences in the traces are not qualitatively significant and are the result of the longer and more variable lag times associated with colicin A.

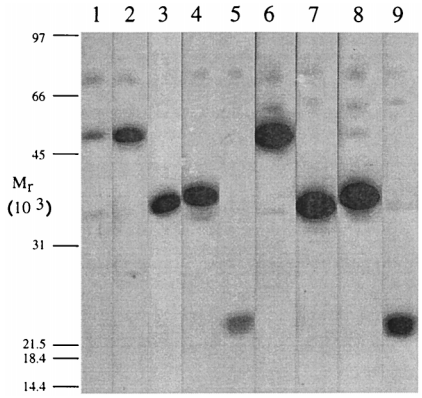

FIG. 3. Western blots of K17A1DE3 expressing TolA alone or with TolQ, -R, and -B in comparison to chromosomal *tolA* expression. Equal amounts of protein obtained from cells growing exponentially in YT at 37°C were subjected to SDS-PAGE (10% acrylamide), transferred to nitrocellulose, and blotted with antibodies to the TolA C-terminal domain. Reactive bands were then detected with ^{125}I-labeled protein A and quantitated with a Fuji phosphorimager. (A) Lane 1, K17DE3 (wild-type chromosomal expression); lane 2, K17A1DE3 (pSKL10 [wild-type TolA]); lane 3, K17A1DE3(pTolAΔ1); lane 4, K17A1DE3 (pTolAΔ2); lane 5, K17A1DE3(pTolAΔ3); lane 6, K17A1DE3(pQRAB); lane 7, K17A1DE3(pQRAΔ1B); lane 8, K17A1DE3(pQRAΔ2B); lane 9, K17A1DE3 (pQRAΔ3B). Relative molecular weights of Coomassie blue-stained protein standards are shown at the left. The order of the lanes in the original blot was changed for clarity of presentation.

Concentrations of plasmid-encoded TolA mutant proteins relative to levels of chromosomal expression. To determine if the difference in colicin sensitivity of cells expressing the TolA deletion mutants could be attributed to a smaller content of TolAΔ3, the cellular content of the different TolA protein species was quantitated by Western blotting of K17A1DE3 lysates with antibodies against the TolA C-terminal domain. Antibody binding was assayed with ^{125}I-labeled protein A, and the relative amounts were quantitated with a phosphorimager. Using equal amounts of cellular protein, wild-type TolA and TolAΔ1 to -Δ3 are all expressed at levels at least as high as the level of chromosomal expression of TolA in K17DE3 (Fig. 3; compare lanes 2 to 5 with lane 1 [chromosomal expression]). No TolA expression was detectable in K17A1DE3 in the absence of plasmid-encoded TolA (not shown), as expected from the presence of the mini-Tn*10* transposon in *tolA*. The expression levels relative to K17DE3 (chromosomal expression of wild-type TolA [lane 1]) are approximately 8-fold (Fig. 3, lane 2, wild-type TolA), 20-fold (lane 3, TolAΔ1), 10-fold (lane 4, TolAΔ2), and 2-fold (lane 5, TolAΔ3) greater than the chromosomal expression levels (Table 2, assay A). It is important to note that the cellular level of TolAΔ3 (Fig. 3, lane 5) is higher than that of the chromosomally expressed wild-type TolA (Fig. 3, lane 1), as also summarized in Table 2.

Larger amounts of TolA, TolAΔ1, TolAΔ2, and TolAΔ3 are produced from pQRAB, pQRAΔ1B, pQRAΔ2B, and pQRAΔ3B, respectively (Fig. 3, lanes 6 to 9; Table 2, assay B). The constructs pQRAB, pQRAΔ1B, and pQRAΔ2B express their forms of TolA at levels that are 40-fold higher than that detected for chromosomal TolA (Fig. 3, lanes 6 to 8), while the expression level of TolAΔ3 from pQRAΔ3B was 6-fold higher than chromosomal expression levels. The coexpressed levels of TolA, TolAΔ1, TolAΔ2, and TolAΔ3 are five-, two-, four-, and threefold, respectively, higher than those of the corresponding vectors expressing only TolA (Fig. 3; compare lanes 6 to 9 with lanes 2 to 5). There are small (two- to threefold) changes in cytotoxicity associated with increased levels of coexpressed TolA protein, but the changes consist of both increases and decreases of cytotoxicity (Table 1) and do not imply any clear consequence for a dependence of cytotoxicity on the relative levels of TolA expression. In particular, the decreased cytotoxicity toward cells producing TolAΔ3 by colicins A, E3, and N cannot be attributed to smaller amounts of TolAΔ3 relative to wild-type TolA, TolAΔ1, or TolAΔ2, as it was also observed with coexpressed TolAΔ3, whose expression level is similar (Table 2, assay B) to that from pSKL10 expressing wild-type TolA alone (Table 2, assay A).

Influence of α-helical domain deletions on outer membrane integrity. In addition to being tolerant to the lethal action of the group A colicins, mutations in *tolA* also cause cells to leak periplasmic proteins (e.g., alkaline phosphatase and RNase) into the extracellular medium and to become hypersensitive to detergents such as SDS and DOC (42). This phenotype has led to the suggestion that the physiological role of TolA involves maintenance of cell envelope integrity (42). The growth of K17A1DE3 was compared with that of K17A1DE3 complemented with pSKL10 (wild-type TolA) or pTolAΔ1, -Δ2, or -Δ3 in LB broth containing various concentrations of DOC.

An increase in the sensitivity to detergents of the TolA deletion mutants was apparent. The sensitivity of the growth of K17A1DE3(pTolAΔ1 or pTolAΔ2) to DOC was intermediate between K17A1DE3(pSKL10) and K17A1DE3(pTolAΔ3) (Fig. 4). At DOC concentrations above 0.05%, growth of TolAΔ3 cells was severely restricted and was comparable to cells that of cells that do not produce TolA. K17A1DE3 complemented with vectors which expressed TolA or the TolA deletion mutants with TolQ, -R, and -B showed the same general trends (data not shown). Thus, removal of half the region between residues 54 and 287 is sufficient to destabilize interactions between the inner and outer membranes mediated by TolA that affect permeability to detergents.

DISCUSSION

Domain structure of TolA. The sequence of TolA suggests that the protein may be divided into three domains, each serving a specific purpose in the function of the protein (Fig. 1). The amino-terminal 47 residues contain a segment of hydrophobic amino acids that is capable of forming a transmembrane α helix which anchors the TolA protein in the cytoplas-

TABLE 2. Expression levels of TolA deletion mutant proteins[a]

Assay	Plasmid	Mol wt	Relative mobility (M_r [10^3])	Expression level (dpm [10^4])[b]	Relative expression level[c]
A	TolA (chromosomal)	43,130	54	3.2	1.0
	pSKL10 (wild-type TolA)	43,130	54	26	8.1
	pTolAΔ1	30,674	39	33	10.3
	pTolAΔ2	31,332	41	64	20.0
	pTolAΔ3	18,896	24	6.1	1.9
B	pQRAB (wild-type TolA)	43,130	54	134	41.8
	pQRAΔ1B	30,674	39	133	41.5
	pQRAΔ2B	31,332	41	144	45.0
	pQRAΔ3B	18,986	24	20	6.3

[a] Data derived from Fig. 3. Equal amounts of cellular protein from exponential-phase K17A1DE3 complemented with the given plasmid were subjected to SDS-PAGE (10% acrylamide), transferred to nitrocellulose, and incubated with antibodies specific for the TolA C-terminal domain; reactive bands were detected with ^{125}I-labeled protein A.

[b] Phosphorimager units proportional to disintegrations per minute (dpm).

[c] Expression relative to chromosomal expression (defined as 1.0) detected in K17DE3.

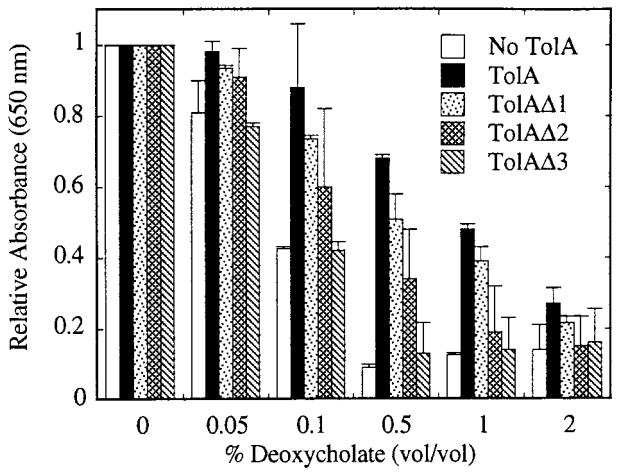

FIG. 4. DOC sensitivity of K17A1DE3 expressing TolA alone. The optical density of the cultures was determined after incubation and growth at 37°C for 3 h. Data were normalized to a value of 1.0 for 0% DOC. Error bars represent the standard deviations of three trials.

mic membrane. The C-terminal domain is believed to be involved in interaction with the periplasmic face of the outer membrane and in the recognition of incoming colicin molecules (26). The role of the central 260 residues is less well defined. From its striking structural features, >50% alanine content, and high α-helical content (24), a scaffold-like function has been proposed (42). This section of the protein could extend toward the outer membrane of the cell envelope to tether the C-terminal domain near the outer membrane. If this is indeed the case, then varying the length of this central section should result in changes in cell sensitivity to group A colicins and to detergents.

TolA was previously predicted to span the periplasmic space with the α-helical domain in an extended conformation (24). If the α-helical domain exists as a single, uninterrupted 230-residue α helix, it would be nearly 35 nm in length (1.5 Å rise/residue). An α-helix of this length could be accommodated if the periplasm were as wide (between 30 and 70 nm) as that predicted by several studies (19, 38). Alternatively, the α-helical domain of TolA may be arranged as a bundle of several α helices and localized at the adhesion zones of the cell envelope (15) where the width of the periplasmic space would be narrowed considerably.

Uniqueness of cytotoxicity mechanism of colicin E1. Cells producing plasmid-encoded TolA in which either the first (TolAΔ1) or second (TolAΔ2) ca. 120 residues in the putative intermembrane helical domain were deleted exhibited a small decrease in colicin sensitivity when TolA was expressed from a plasmid either alone (Table 1, assay B to E) or with TolQ, -R, or -B (Table 1, assays F to I), implying that neither the first or second half of the α-helical domain of TolA is uniquely required for colicin import.

Deletion of residues 54 to 287 (TolAΔ3), nearly 90% of the α-helical domain, essentially abolished cytotoxicity of the group A colicins E3, N, and A (Table 1, assays E and I). In contrast, the sensitivity of TolAΔ3 cells to colicin E1 was reduced far less, approximately 5-fold, compared with at least 50-fold for colicin N and 3,000-fold for colicins E3 and A (Table 1, assays E and I). In addition, the rate of colicin E1-induced K^+ efflux was not inhibited in TolAΔ3 cells compared to that of wild-type TolA (Fig. 2A; compare traces d and a). For cells producing TolAΔ3 alone or with TolQ, -R, or -B, colicin E1 induces a K^+ efflux rate similar to that of cells producing TolA, with a longer time delay required to produce this efflux rate (Fig. 2A, trace d). This finding suggests that there is a smaller population of these shortened TolA molecules available, or properly positioned, to accomplish the import of the colicin E1 molecule. In contrast, neither colicin N nor colicin A had detectable channel activity against cells expressing TolAΔ3 (Fig. 2B and C, curves d). It was concluded that the requirement of colicin E1 cytotoxicity for aspects of the TolA structure is qualitatively different from that of the other group A colicins, E3, N, and A. This conclusion is consistent with the finding that only a sixfold-greater amount of colicin E1 was necessary to kill cells producing fewer than 50 molecules of whole TolA compared to cells producing normal amounts of TolA (~400 to 800/cell). At the same lowered TolA concentration, the reduction in activity of colicins E3 and A was 1,000-fold (26). Other unique aspects of colicin E1 import are (i) the absence of a requirement for TolB (42) and (ii) the utilization of TolC (31).

TolC dependence of colicin E1. How is colicin E1 able to exert a toxic effect on cells producing TolAΔ3 when the other group A colicins E3, N, and A cannot? The lowered activity of colicin E1 with TolAΔ3 compared to TolAΔ1 and -Δ2 might be attributed to a lower TolA content. However, the cellular content of TolAΔ3 is greater than that of chromosomally expressed TolA (Fig. 3; compare lane 5 with lane 1). In addition, although the level of TolAΔ3 is less than that of TolAΔ1 or -Δ2, the activity of colicin E1 is decreased very little compared to the large decrease of colicin E3 and N cytotoxicity. Colicin E1 seems to be particularly efficient in locating the C terminus of TolA, even if the C terminus is attached almost directly to the membrane-spanning region, as with TolAΔ3. This efficiency may be explained by the different outer membrane receptor complex utilized by E1. Colicins E1, E3, and A all require the vitamin B_{12} receptor, BtuB, for initial binding to the outer membrane. Colicin N requires only OmpF for its receptor, while colicins E3 and A also require OmpF to enter the periplasm (3). Colicin E1 is unique in its independence of TolB (42), additional dependence on TolC (31), and requirement for a much lower level of TolA than other group A colicins (26). A special role that might be assumed by TolC in the translocation of colicin E1 could explain the previously documented requirement for low levels of TolA compared to the other TolA-dependent colicins, as well as the ability to use a much shortened TolA documented in this work.

TolC, like BtuB, is a minor component of the protein content of the outer membrane of *E. coli*, present at <1,000 copies/cell (29). OmpF, in contrast, is a major component of the *E. coli* outer membrane and is expressed to high levels, approximately 10^5 copies/cell (32). TolC is believed to be involved in Sec-independent export of α-hemolysin and colicin V (41, 43). As a result of this function, it may be localized to adhesion zones (1) where the outer and inner membranes are in close contact. TolC may then provide a direct route to the TolQRAB protein complex which has been shown to preferentially reside at adhesion zones (15). It is reasonable that OmpF, present at much higher concentrations, is not in direct contact with adhesion zones. The OmpF-dependent colicins may therefore need a longer TolA molecule to bridge the periplasm for effective translocation.

The inference that the different interaction of TolA with colicin E1 is a consequence of the unique requirement for OmpF instead of TolC as a receptor has recently received support from a study by Derouiche et al. (14a). These authors found that TolA can form complexes, through its central domain, with several outer membrane complexes, including

OmpF. Therefore, deletion of the central domain of TolA, as done in this study, would impair the activity of the other A group colicins, consistent with the data of Fig. 2B and C and Table 1.

A similar TolA deletion mutant: TolAΔh. During the course of these studies, a TolA deletion mutant, TolAΔh, that removed residues 68 to 290 was described (14). This mutant contained an insertion of residues 8 to 25 of colicin A which forms an epitope for monoclonal antibody 1C11, which allows its specific detection by Western blotting (4). TolAΔh was unable to relieve the leaky phenotype of *tolA* cells, but in contrast to the TolAΔ3 mutant, TolAΔh could still participate in the translocation of colicins E3 and A, although with a 10-fold reduction in cytotoxicity. The insertion of colicin A residues 8 to 25, which are from the glycine-rich region common to all group A colicins and have the sequence GKGDG TGWSSERGSGPEP, may result in a tertiary structure of TolAΔh that differs from that of TolAΔ3. A second difference in the TolAΔh construct is simply that the fraction of the residual central domain remaining in the deletion mutants is larger in TolAΔh (22% including the colicin A insertion) than in TolAΔ3 (11%), and TolAΔh may be long enough to function for colicins A and E3. The explanation for the difference in absolute activity of colicins A and E3 toward TolAΔh and TolAΔ3 is uncertain at present. The main finding in this work is the difference in activities of colicin E1 and the other group A colicins.

TolA α-helical domain deletions affect *E. coli* cell envelope stability. Cells producing TolAΔ3 behaved as though they were producing no TolA, while those expressing TolAΔ1 and TolAΔ2 had an intermediate detergent sensitivity (Fig. 4). This result suggests that the α-helical domain is important for maintenance of cell envelope stability. The stabilizing effect of this region of TolA could be attributable to the $i \cdots i + 4$ arrangement of positively charged lysines and negatively charged aspartates and glutamates, allowing the formation of salt bridges which would provide added stability to the α-helical region of TolA (24, 27). Wild-type TolA contains 13 such KA_3(D/E) repeats, and TolAΔ2 lacks all but two. From these results, it appears that the TolA α-helical domain works in conjunction with the C-terminal domain of TolA to contribute stability to the cell envelope (26).

Comparison of TonB and TolA central domains. The counterpart to TolA in the Ton system is TonB. TonB functions in intermembrane energy transduction and may serve as a means for the cell to couple the cytoplasmic membrane potential to the active transport of vitamin B_{12} and Fe(III)-siderophore complexes across the cell envelope (34). The *E. coli* TonB has a length of 239 residues, approximately half that of TolA (421 residues), and contains an unusual, proline-rich region, residues 60 to 108, in which more than 50% of the residues are proline (41). This proline-rich region confers on TonB a rigid, extended structure that presumably allows TonB to span the periplasmic space, where its C terminus could interact with outer membrane receptor proteins such as FhuA and FepA (8, 17). TonB from which residues 66 to 100 were deleted was still able to interact with the outer membrane receptor, FepA, and also participate in the uptake of the TonB-dependent colicin B and phage ϕ80 (21). However, a shift from low to high osmolarity resulted in a lowered phage ϕ80 adsorption for cells producing TonBΔ66-100. These results suggest that the proline-rich central region of TonB is dispensable to colicin, phage, and iron uptake except under conditions of osmolar stress, when the width of the cell envelope would be effectively widened, and this extended segment may provide TonB with an extra reach to the outer membrane. It is of interest that while TonBΔ66-100 and TolAΔ3 are similar in size (197 and 187 residues, respectively), the activity of TonBΔ66-100 resembles that of the wild type, while that of TolAΔ3 is defective with respect to activity of colicins E3, N, and A and stability of the cell envelope. This difference suggests that the TolA α-helical domain is necessary for maintenance of cell envelope integrity and translocation function, while the analogous region of TonB may be required under stress conditions.

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