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Initial Steps of Colicin E1 Import across the Outer Membrane of *Escherichia coli*▽

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Data suggest a two-receptor model for colicin E1 (ColE1) translocation across the outer membrane of *Escherichia coli*. ColE1 initially binds to the vitamin B_{12} receptor BtuB and then translocates through the TolC channel-tunnel, presumably in a mostly unfolded state. Here, we studied the early events in the import of ColE1. Using in vivo approaches, we show that ColE1 is cleaved when added to whole cells. This cleavage requires the presence of the receptor BtuB and the protease OmpT, but not that of TolC. Strains expressing OmpT cleaved ColE1 at K84 and K95 in the N-terminal translocation domain, leading to the removal of the TolQA box, which is essential for ColE1's cytotoxicity. Supported by additional in vivo data, this suggests that a function of OmpT is to degrade colicin at the cell surface and thus protect sensitive *E. coli* cells from infection by E colicins. A genetic strategy for isolating *tolC* mutations that confer resistance to ColE1, without affecting other TolC functions, is also described. We provide further in vivo evidence of the multistep interaction between TolC and ColE1 by using cross-linking followed by copurification via histidine-tagged TolC. First, secondary binding of ColE1 to TolC is dependent on primary binding to BtuB. Second, alterations to a residue in the TolC channel interfere with the translocation of ColE1 across the TolC pore rather than with the binding of ColE1 to TolC. In contrast, a substitution at a residue exposed on the cell surface abolishes both binding and translocation of ColE1.

The outer membrane of *Escherichia coli* acts as a barrier to the transport of macromolecules (34). To penetrate the cells, colicins and bacteriophages have evolved to parasitize various receptors in the outer membrane that are normally involved in the uptake of nutrient molecules, such as metals, sugars, vitamins, and nucleosides. Colicins are plasmid-encoded antibacterial toxins that are produced by *E. coli* to target other *E. coli* cells. They are generally divided into three structural and functional domains, with a central receptor (R) domain sandwiched between the N-terminal translocation (T) domain and the C-terminal cytotoxic (C) domain carrying pore-forming or nuclease activity (6). Colicins can be classified into two groups based on how they enter into the periplasmic space and access the inner membrane: the group A colicins (A, N, and E1 to E9) use the TolA system, whereas the group B colicins (Ia, Ib, B, and D) require the TonB system (4, 31). Both the Tol and Ton systems are coupled to the proton motive force across the inner membrane and provide the energy for the transmembrane import.

The mechanism by which colicins get across the outer membrane remains poorly understood. ColE1 belongs to the group A colicins and kills susceptible *E. coli* cells by forming ion channels in the inner membrane (10). Previous genetic and biophysical studies showed the requirement of both BtuB and TolC for ColE1 entry. As with all E colicins, cellular import of ColE1 is initiated by binding to the vitamin B_{12} (cyanocobalamin) receptor BtuB (17). BtuB is a minor outer membrane protein. It is made of 22 β-strands, with its interior occluded by an N-terminal globular "plug" domain (9). Based on the X-ray structure of the complex of BtuB and the ColE3 receptor binding domain (30) and the absence of any ion conductance of BtuB in the presence or absence of colicin (51), the function of initial binding is thought to concentrate the colicin on the membrane surface and deliver it to a neighbor protein for translocation. It has been known for some time that mutants of *E. coli* lacking the TolC protein exhibit pleiotropic phenotypes, which include tolerance to ColE1 and bacteriophage TLS (13, 20, 32), increased sensitivity to antibiotics, and impairment of toxin secretion (18, 27, 45). TolC is a trimeric protein embedded in the outer membrane by a β-barrel and spans the periplasm as an α-helical tunnel. The three assembled protomers form a single pore that is constitutively open to the cell exterior but constricted at the periplasmic entrance (1, 28). The resolution of the three-dimensional structure of TolC and previous genetic data suggest that the ColE1 and TLS binding sites are within the extracellular exposed surface (20). More recently, it has been shown that ColE1 occludes TolC channels reconstituted in planar bilayers (51).

Since colicins are large proteins (50 to 60 kDa), it is generally accepted that they must unfold to allow the translocation of the active C-domain through the outer membrane channels. The only published structures of full-length colicins are those of ColIa, ColB, and ColE3 (23, 41, 49). They show an elongated and highly flexible coiled-coil associated with the R domain. The formation of the BtuB-ColE3 complex results in the unfolding of the N-terminal receptor binding coiled-coil domain (30). In further support of the unfolding model, Penfold et al. showed that the introduction of a disulfide bond in the R domain of ColE9 leads to a loss of cytotoxicity that can be restored upon dithiothreitol reduction (36). However, recent reports are still somewhat controversial as to when such struc-

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tural changes occur. Some favor the idea that colicin unfolding is induced by initial receptor binding, while others claim that this reaction occurs after the assembly of the whole translocon (21, 24, 30).

E colicin-producing bacteria are protected from self-killing by cosynthesizing an immunity protein which binds to its cognate colicin's C-terminal cytotoxic domain and neutralizes its activity. In the case of ColE1, this binding occurs in the inner membrane and prevents the lethal pore formation, whereas in the case of enzymatic E colicins, it occurs in the cytoplasm with very high affinity and the resulting protein complex is then released to the extracellular medium. The mechanisms developed by the target bacteria to defend themselves against colicins have been historically attributed to spontaneous mutations that affect the receptor (resistance) or the components of the translocation pathway (tolerance). In 1990, the extracytoplasmic protease OmpT was shown to mediate the cleavage of colicins A, E1, E2, and E3 in culture supernatants. However, the biological relevance of this process has not been studied further (8).

The aim of this study was to obtain a better understanding of the sequential events in ColE1 cellular import. First, we found that the OmpT-mediated cleavage of ColE1 requires primary binding to BtuB, and it occurs before binding to TolC. We further identified the proteolytic cleavage site in ColE1 to be in the predicted N-terminal T domain. Interestingly, the N-terminal T domain of ColE1, which contains the TolQA and TolC binding regions, is essential for its translocation and cytotoxicity. We showed that OmpT is responsible for ColE1 inactivation by degradation and performs this function on the BtuB-engaged toxin at the external surface of the outer membrane. We propose that OmpT-mediated inactivation, also apparent for ColE2, could be a general defense mechanism against E colicin infections. We also describe the isolation and biochemical characterization of ColE1-resistant TolC mutants. These data point to specific TolC regions involved in ColE1 binding and translocation.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
Strains		
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*U139 rpsL150 flbB5301 ptsF25 deoC1 thi-1 rbsR relA*	7
RAM958	MC4100 *tolC*::Tn*10* Tet^r	43
RAM1129	MC4100 Δ*tolC*::Km^r	2
RAM1130	MC4100 Δ*tolC*::Cm^r	2
RAM1292	MC4100 Δ*ara174*	48
RAM1330	RAM1292 Δ*tolC*::Km^r	This work
RAM1342	RAM1292 Δ*tolA*::Cm^r	This work
CAG5052	*btuB3191*::Tn*10* Tet^r	40
RAM1343	RAM1292 *btuB*::Tn*10* Tet^r	This work
AD202	*ompT*::Km^r	33
RAM1344	RAM1292 *ompT*::Km^r	This work
RAM1345	RAM1130 *btuB*::Tn*10* Tet^r	
PLB3260	MC4100 Δ*lamB106* Φ*ompF′*::*lacZ*$^+$	S. Benson
RAM1346	PLB3260 *btuB*::Tn*10* Tet^r	This work
PLB3261	MC4100 Δ*lamB106* Φ*ompC′*::*lacZ*$^+$	S. Benson
RAM1347	PLB3261 *btuB*::Tn*10* Tet^r	This work
RAM789	MC4100 Δ*rfa*2057::Cm^r	35
RAM1348	RAM1343 Δ*rfa*::Cm^r	This work
K53	ColE1 colicinogenic strain	13
CA42	ColE2 colicinogenic strain	13
XL1 Red	*endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac mutD5 mutS mutT*::Tn*10*	Stratagene
JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*) (F′ *traD36 proABlacI*^{q}ZΔ*M15)*	Stratagene
Plasmids		
pTrc99A	Expression vector, Ap^r	Pharmacia
pTrc-TolC	Ap^r	47
pTrc-TolC(6His)	pTrc99A, *tolC-6his* Ap^r	This work
pTrc-TolC^{G43R}(6His)	Ap^r	This work
pTrc-TolC^{G43D}(6His)	Ap^r	This work
pTrc-TolC^{G43Y}(6His)	Ap^r	This work
pTrc-TolC^{Q281P}(6His)	Ap^r	This work
pTrc-OmpT	pTrc99A, *ompT* Ap^r	This work
pTrc-TRColE1(6His)	pTrc99A, *colE1(1-349)-6his* Ap^r	This work
pSF4000	pACYC184 containing *hlyABCD*, Cm^r Tet^r	43

MATERIALS AND METHODS

Bacterial strains, media, and biochemicals. The strains and plasmids used in this study are listed in Table 1. RAM1343 and RAM1344 were constructed by P1 transduction of RAM1292 with a phage lysate made from CAG5052 (*btuB*::Tn*10*) and AD202 (*ompT*::Km^r), respectively. RAM1346 and RAM1347 were constructed by P1 transduction of RAM1343 (*btuB*::Tn*10*) with a phage lysate made from PLB3260 (*ompF′*::*lacZ*) and PLB3261 (*ompC′*::*lacZ*), respectively; RAM1348 was constructed by P1 transduction of RAM789 (Δ*rfa*::Cm^r) with a RAM1343 (*btuB*::Tn*10*) lysate. The *tolA* gene was deleted from the chromosome of RAM1292 via the Datsenko and Wanner method by using the primers 5′-GAGAGCGGGTAACAGGCGAACAGTTTTTGGAAACCGAGACTGGAGCTGGAGCTGCTTCG-3′ and 5′-CTAAAATACACAAAACTACCAGAACCCCGTGGCAACCTGTAGGCATATGAATATCCTCCTTAG-3′ (12). Strains were routinely grown at 37°C in Luria broth (LB) or on agar (LBA). Antibiotics were added to the growth medium at the following final concentrations: ampicillin (Ap), 50 μg/ml; chloramphenicol (Cm), 12.5 μg/ml; kanamycin (Km), 25 μg/ml. Isopropyl-β-D-thiogalactoside (IPTG) was used at a final concentration of 0.4 mM for protein overexpression.

***tolC* manipulations.** The *tolC* gene was cloned under the control of an IPTG-inducible promoter into pTrc99A (Pharmacia) as described previously (2). The resulting plasmid, pTrc-TolC, was used for in vivo mutagenesis in the XL1-Red mutator strain according to the manufacturer's instructions (Stratagene). Briefly, transformants were selected on LBA plates supplemented with ampicillin. Plasmid DNA from five independent pools of Ap^r colonies was transformed into *E. coli* MC4100 *tolC*::Tn*10*, and transformants were screened for the desired *tolC* phenotype. The plasmid pTrc-TolC(6His) was constructed to allow purification by immobilized nickel affinity chromatography. The *tolC* gene was amplified from chromosomal DNA with primers 5′-CAGGAAACAGATCATGAGGAAATTGCTCCC-3′ and 5′-GGTCATAACCCTTTCCGTAACCATCACCATCACCATCACTAAGCTTCTAGAGC-3′. The forward primer contains a BspHI restriction site (underlined), which encompasses the ATG start codon of *tolC* (boldface), and the reverse primer contains a HindIII restriction site (underlined) and six consecutive histidine codons (italicized). Substitutions Q281P, G43D, G43R, and G43Y within TolC(6His)—the amino acid numbers are relative to the beginning of the mature TolC sequence—were created by site-directed mutagenesis using a QuikChange mutagenesis kit (Stratagene) according to the manufacturer's procedure.

Cloning of *ompT*. The *ompT* open reading frame was PCR amplified from genomic DNA of *E. coli* MC4100 with primers 5′-TGGAGAACTTTCATGAGGGCGAAAC-3′ and 5′-TGAAATGTCTAGATATTCCCCGGGGCGA-3′. The purified PCR fragment was digested with BspHI and XbaI (underlined) and inserted into the pTrc99A vector cut with NcoI and XbaI.

Cloning and purification of ColE1 fragments. ColE1$^{1-339}$ was cloned in the pTrc99A vector. The colicin construct contained a six-His tag at the C terminus to allow protein purification by immobilized nickel affinity chromatography. The ColE1$^{1-339}$-encoding fragment was amplified by using pColE1 as a template and the primers 5′-GAGGATTTTACCATGGAATTCGCGGTAGCG-3′ and 5′-CACAGATTAAGGATGCTGTTCACCATCACCATCACCATTAAGCTTCTAGAG-3′. The purified PCR fragments were digested with NcoI and HindIII and inserted into the pTrc99A vector cut with the same enzymes. The protocol for protein purification was adapted from the one provided by the manufacturer (Amersham Biosciences). Cell cultures were grown in LB broth to an optical density at 600 nm (OD_{600}) of 0.4 and induced with 0.4 mM IPTG for 3 h at 37°C. The cells were collected by centrifugation and stored at −80°C. The cell pellet

was resuspended in binding buffer (20 mM sodium phosphate buffer, 300 mM NaCl, 10 mM imidazole, pH 7.4). The mixture was passed through a French press and centrifuged at 8,000 × *g* for 30 min at 4°C to pellet unbroken cells and cellular debris. The supernatant containing soluble proteins was clarified by ultracentrifugation (40,000 × *g*, 20 min, 4°C) and passed through a 0.22-μm filter. The resulting lysate was loaded onto a 5-ml HiTrap chelating column charged with Ni^{2+} and equilibrated with 10 column volumes of binding buffer at a flow rate of 5 ml/min. The column was washed with 50 mM imidazole, and proteins were eluted with 250 mM imidazole. Protein-containing fractions were dialyzed overnight against 20 mM phosphate buffer (pH 7.4). Protein concentrations were determined using the Micro-BCA protein (Pierce). Purified $ColE1^{52\text{-}522}$ was a generous gift from William A. Cramer.

Cell binding assays. Cells were grown at 37°C in LB broth to an OD_{600} of 1.0, harvested, washed with 10 mM HEPES buffer (pH 7.4), and resuspended in the same buffer. In the standard procedure, 10^9 bacteria in 1 ml buffer were added to 0.1 ml of colicin stock solution, and the mixture was incubated at 37°C for various time periods. Cells were harvested by centrifugation. Whole-cell extracts and supernatants were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. In the substrate competition assays, cells were incubated with dilutions of vitamin B_{12} for 10 min before the addition of colicin.

In vivo processing of ColE1 and N-terminal microsequencing. The purified $ColE1^{1\text{–}339}$ fragment was mixed with *E. coli* RAM1292 in 10 mM HEPES buffer (pH 7.4) and incubated for 2 h at 37°C. The cells were removed by centrifugation. The proteins in the supernatant were precipitated with 15% trichloroacetic acid, fractionated by SDS-PAGE, and then transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore). The bands corresponding to the full-size and the processed ColE1 were excised, and their N-terminal sequences were determined by Edman degradation on a Porton 2090 E gas-phase protein sequencer (Beckman).

Antibiotic, phage TLS, and colicin sensitivity assays. Susceptibilities to antimicrobials were determined by the agar diffusion method with antibiotic-soaked filter paper disks (Difco). The diameter of the zone of inhibition (in mm) was recorded after 8 h at 37°C. ColE1 and ColE2 were extracted from mitomycin C-induced colicinogenic *E. coli* K53 and CA42, respectively (52). LBA plates were overlaid with 4 ml of soft agar containing 100 μl of *E. coli* overnight culture. Serial 10- or 2-fold dilutions of ColE1, ColE2, and phage TLS were spotted in 10-μl drops onto the lawns, and the plates were incubated overnight at 37°C. Efficiency of killing (ColE1 and ColE2) and efficiency of plaguing (phage TLS) were taken as the reciprocal of the highest dilution that gave complete clearing of the lawn. Susceptibilities to colicin were also assayed using the double-layer test method adapted from that of Davies and Reeves (13). Briefly, the colicinogenic strain was streaked across an LB agar plate and grown overnight at 37°C. The colicinogenic strain was then killed with chloroform, and the plate was overlaid with LB agar. The *E. coli* mutant strains to be tested were then streaked across the original colicinogenic streak and grown overnight at 37°C. As the colicin diffuses out and up from the original colicinogenic streak, it will kill sensitive bacteria, forming a clear zone of growth. Any receptor (*btuB*) or tolerant (*tolA* or *tolC*) mutant will be unaffected by the colicin, and the growth will be continuous.

Hemolysin secretion assays. *E. coli tolC* mutants carrying the hemolysin plasmid were grown to an OD_{600} of 0.8 and harvested by centrifugation. The supernatant containing hemolysin was filtered through 0.22-μm (pore size) syringe filters and was used to examine hemolytic activities on blood agar plates.

In vivo cross-linking and copurification. In vivo cross-linking experiments were performed using *E. coli* RAM1130. Wild-type TolC and mutants were expressed from the recombinant plasmid pTrc-TolC(6His) or one of its derivatives. All TolC proteins were C-terminally six-histidine tagged and used as a bait to isolate protein complexes. Cell cultures (100 ml) were grown in LB broth to an OD_{600} of 0.3 and induced with 0.4 mM IPTG for 3 h at 37°C. The pellets were washed and concentrated 10-fold in 20 mM sodium phosphate buffer (pH 7.4). Cells were incubated for 10 min at 37°C in the presence of 1 ml ColE1 stock solution. Freshly prepared dithiobis(succinimidylpropionate) (DSP) cross-linker was then added to a final concentration of 0.5 mM, and cells were incubated for 30 min at 37°C with shaking. After quenching with 40 mM Tris, cells were harvested, resuspended in lysis buffer, and lysed by French press. The total membranes were collected by centrifugation and solubilized in PUTTS buffer (100 mM NaH_2PO_4, 8 M urea, 10 mM Tris-HCl, 1% Triton X-100, 0.2% Sarkosyl, pH 7.5) containing 5 mM imidazole. Protein complexes were purified as described previously (25) by affinity chromatography with an increasing concentration of imidazole. The eluted proteins were treated with sample buffer containing β-mercaptoethanol, boiled to cleave the DSP molecule, and then resolved by SDS-PAGE and immunoblotted with antibodies raised against TolC and ColE1.

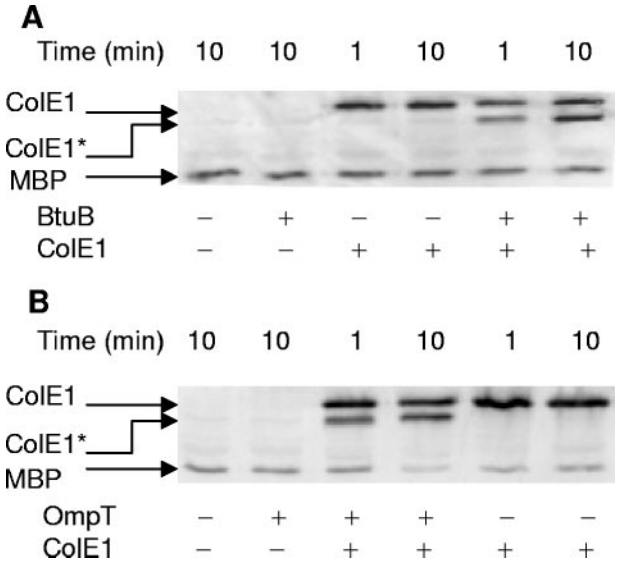

FIG. 1. Cleavage of ColE1 by OmpT during entry into *E. coli* cells. *E. coli* RAM1292 (MC4100 Δ*ara*), RAM1343 (*btuB*::Tn*10*) (A), and RAM1344 (*ompT*::Km^r) (B) derivatives were incubated in the presence (+) or absence (-) of ColE1. Cells were harvested by centrifugation at the indicated times (in min). Full-length ColE1 (57 kDa) and the large processed fragment (49 kDa) in whole-cell extracts were separated by SDS-PAGE and then detected by immunoblotting with antibodies raised against ColE1 fused to the maltose binding protein (MBP).

Western blot analysis. Whole-cell extracts were analyzed on SDS–polyacrylamide 11% mini gels and transferred onto polyvinylidene difluoride membranes. Membranes were probed with primary antibodies raised against TolC (1:5,000) or ColE1 (1:2,500). Goat anti-rabbit alkaline phosphatase-conjugated immunoglobulin G secondary antibodies and a chemiluminescence kit (Amersham) were used for detection. For histidine tag detection, membranes were probed with a 1:5,000 dilution of HisProbe-HRP (Pierce) according to the manufacturer's instructions. Protein levels were quantified with Quantity One software (Bio-Rad).

RESULTS

BtuB-bound ColE1 is cleaved by the outer membrane protease OmpT. During the course of investigating the cell entry mechanism of ColE1, we observed that ColE1 was cleaved into a smaller product when incubated with the *E. coli* MC4100 Δ*ara* strain. ColE1 fragments were identified in whole-cell extracts by SDS-PAGE followed by immunoblotting with ColE1 antibodies. Specifically, the 57-kDa full-length ColE1 was converted into a 49-kDa fragment (ColE1*) (Fig. 1A). ColE1 reaches the inner membrane of target cells by utilizing a number of envelope proteins. These include outer membrane receptors BtuB and TolC and the inner membrane energy transducer TolA. We examined the effect of *btuB* and *tolC* null mutations on the cleavage of ColE1. Results showed that ColE1 was cleaved in wild-type and *tolC* mutant cells but not in *btuB* mutant cells (Fig. 1A and 2A). Together with a time course analysis, the data indicated that the cleavage of ColE1 occurred early upon binding to BtuB (within the first minute), but before binding to TolC.

It is worth noting that a significant amount of unprocessed

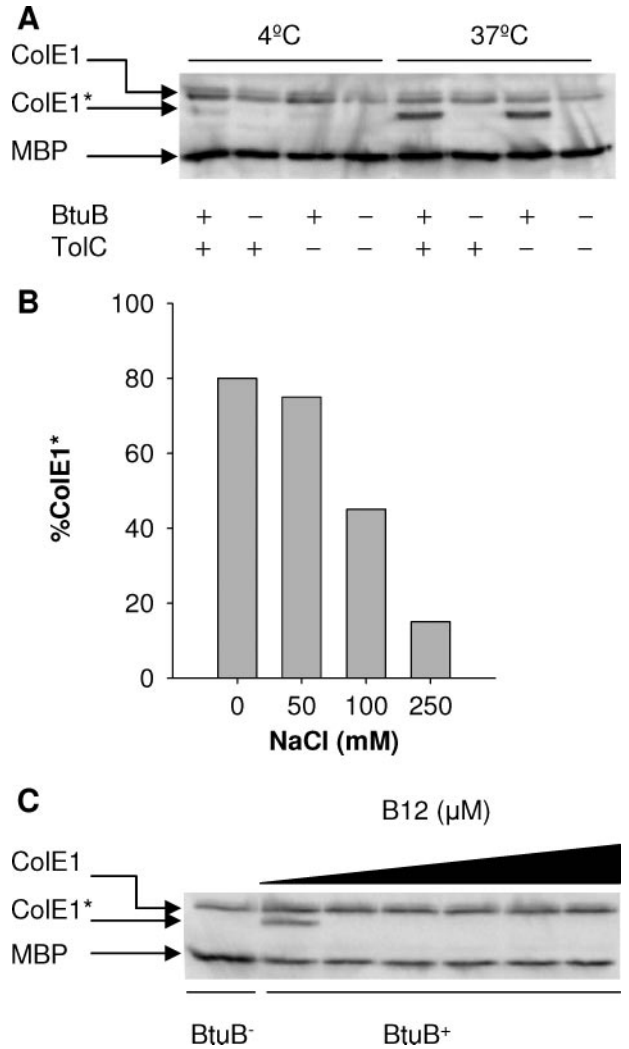

FIG. 2. Effects of temperature, ionic strength, and vitamin B_{12} on ColE1 cleavage. Cultures of *E. coli* RAM1292 (MC4100 Δ*ara*), RAM1130 (Δ*tolC*::Cm^r), RAM1343 (*btuB*::Tn*10*), and RAM1345 (Δ*tolC*::Cm^r *btuB*::Tn*10*) were incubated in the presence (+) or absence (−) of ColE1. (A) Incubation was carried out at either 4°C or 37°C. (B) Incubation was performed in binding buffer supplemented with NaCl at the indicated millimolar final concentrations. Protein bands corresponding to ColE1 and ColE1* were quantified using Quantity One (Bio-Rad). (C) For competition experiments, cells were preincubated in the presence of increasing concentrations of vitamin B_{12} (0 to 200 μM) prior to the addition of ColE1. After incubation, cells were pelleted, washed, and lysed by boiling in SDS sample buffer. Whole-cell extracts were analyzed by SDS-PAGE and immunoblotting with antibodies raised against colicin E1 fused to the maltose binding protein (MBP).

ColE1 was pulled down with the *btuB* mutant cells (Fig. 1A, lanes 3 and 4). We believe this represents BtuB-independent nonspecific binding that does not culminate in ColE1 proteolytic cleavage. To determine whether ColE1 was nonspecifically binding to lipopolysaccharide (LPS) or to major outer membrane porins, *rfa*, *ompF*, and *ompC* mutations were introduced into RAM1343 (*btuB*::Tn*10*). The chromosomal deletion Δ*rfa*-2057 results in the synthesis of truncated LPS molecules lacking the outer core (35). When cultures of these three resulting strains were incubated in the presence of ColE1, no decrease in the levels of cell-bound ColE1 was observed (data not shown), indicating that the outer core of LPS and majors outer membrane porins do not contribute to the observed nonspecific ColE1 binding. At present, we do not know the surface component responsible for the nonspecific ColE1 binding or the significance of this event.

A previous study demonstrated that colicins A, E1, E2, and E3 are likely processed in culture supernatants (8). The outer membrane protease OmpT was identified to be responsible for this processing (8). The proteolytic activity of OmpT on ColE1 was tested in vivo. As expected, cleavage of ColE1 was abolished in the *ompT* mutant but could be restored by a plasmid-borne copy of the *ompT* gene (Fig. 1B and data not shown). In agreement with Cavard and Lazdunski (8), BtuB/OmpT-dependent cleavage of ColE1 was also observed in the extracellular medium (data not shown).

To further characterize this event and binding specificity, we tested the cleavage of cell-bound ColE1 under various external conditions. It has been proposed that electrostatic interactions would initially guide the colicin over large distances to its cognate receptor in the outer membrane (50). All experiments described above were performed at 37°C in HEPES buffer without any additional NaCl. The majority of the colicin was present in the form of the 49-kDa cleaved fragment. However, as the salt concentration increased from 0 mM to 250 mM NaCl, levels of ColE1* dropped from 80% to 15% (Fig. 2B). Since the proteolytic activity of OmpT is not affected under high-saline conditions (8), the observed decrease in ColE1 cleavage could be attributed to a decrease in specific binding to BtuB. Incubation at low temperature (4°C) also inhibited BtuB-dependent ColE1 binding and resulted in decreased ColE1 cleavage (Fig. 2A). The binding of E colicins to BtuB is competitively inhibited by vitamin B_{12} (17). Indeed, preincubation of cells with 5 μM vitamin B_{12} inhibited cell killing by ColE1 and prevented ColE1 cleavage (Table 2 and Fig. 2C). This further supported the idea that ColE1 is not accessible to OmpT until it is bound to BtuB. Thus, initial binding of ColE1 to BtuB is a prerequisite to cleavage by OmpT.

Localization of the ColE1 processing site. OmpT from *E. coli* belongs to a family of highly homologous outer membrane proteases called omptins, which are involved in the virulence of several pathogenic gram-negative bacteria (29). It exerts its proteolytic activity in the extracellular medium and preferentially cleaves substrates between two consecutive basic residues or after a basic residue followed by a nonpolar amino acid (14, 29). A recombinant fragment containing residues 1 to 339 of ColE1 (T+R domains) was cloned in frame with a C-terminal six-histidine tag and purified by affinity chromatography. When tested for cleavage, the 38-kDa ColE1$^{1–339}$ was converted into a smaller product (with a loss of approximately 10 kDa) after incubation with *E. coli* cells. The cleaved fragment was detected with antibodies directed against ColE1 and the C-terminal six-His tag, indicating that OmpT-mediated cleavage occurred in the N-terminal region of the T domain (Fig. 3A). To identify the site of cleavage in ColE1, the processed ColE1 product was extracted from the electrotransferred membrane and analyzed by N-terminal sequencing. This gave two different sequences of eight amino acids in approximately equal amounts: A_{85}NRDALTQ$_{92}$ (about 20 pmol) and D_{96}IVNEL

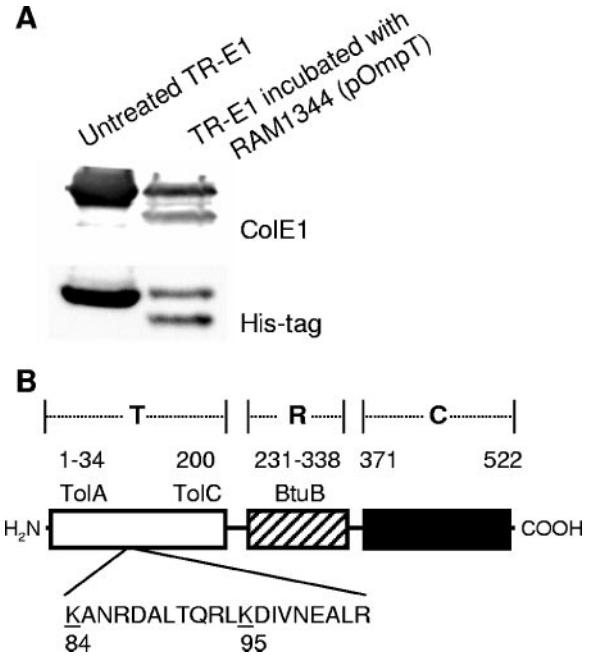

FIG. 3. Identification of the processing site of ColE1. The binding domains where the receptor protein (BtuB) and the translocation system (TolC/TolQA) bind on the ColE1 sequence are indicated as previously reported (5, 11, 21, 37). A recombinant fragment containing the translocation and receptor domains (residues 1 to 339) of ColE1 was used to locate the OmpT processing site. Purified ColE1$^{1-339}$ was incubated in the presence of *E. coli* RAM1292. After centrifugation, the proteins from the supernatant were precipitated with trichloroacetic acid, resolved by SDS-PAGE, and electrotransferred. (A) Proteins were detected by immunoblotting with antibodies raised against ColE1 (upper panel) or HisProbe-HRP (lower panel). (B) Proteins were stained with Coomassie blue, and bands of interest were excised from the membrane and submitted for N-terminal sequencing. The cleavage sites in a domain organization model of ColE1 are shown.

R_{102} (about 13 pmol). Therefore, OmpT cleaves at two sites—between K84 and A85 and between K95 and D96—located in the N-terminal T domain of ColE1 (Fig. 3B).

The T domain of ColE1 contains sequences that are required for interactions with the TolQA and TolC proteins (21, 37). A previous study showed that the region responsible for the TolQA-dependent uptake of ColE1 consists of residues 1 to 34 (37). Thus, OmpT processing of ColE1 at K84 and K95 removes an N-terminal fragment containing the TolQA binding region.

Cells expressing OmpT degrade and inactivate ColE1. Omptins have been implicated in the hydrolysis of various peptides and proteins in vitro. Many of these include recombinant proteins overexpressed in *E. coli* and are not likely targets in vivo (29). However, a number of recent studies have identified host proteins or peptides that are involved in bacterial invasion and virulence and also are candidate targets for the omptin protease family (22, 29, 42). We further investigated the role of OmpT on ColE1 activity in vivo. Cytotoxicity of ColE1 preincubated with *E. coli* cells was analyzed in a cell killing assay by spotting various dilutions of cell-free supernatants on agar plates seeded with the sensitive strain *E. coli* MC4100. ColE1 incubated with a suspension of the OmpT$^+$ strain MC4100 Δ*ara* (RAM1292) was 32- to 64-fold less active than the untreated ColE1 (Fig. 4A). However, we could not differentiate whether this drop in biological activity represented ColE1 adsorption to the cells, proteolysis of ColE1, or both. Incubation of ColE1 with an *ompT* null mutant (RAM1344) did not significantly lower its ability to kill target cells, ruling out that ColE1 had adsorbed to the outer membrane (Fig. 4A). Similar results were obtained when ColE1 was pretreated with a *btuB* null mutant (Fig. 4A). To confirm that the phenotype conferred by the disruption of *ompT* could be complemented, a plasmid expressing *ompT* (pTrc-OmpT) was introduced into RAM1344. Expression of *ompT* was induced with IPTG prior to the incubation with ColE1. High-level production of OmpT further inactivated ColE1, as evidenced by the appearance of only diffused killing zones in the lawn of sensitive cells (Fig. 4A).

The cleavage status of ColE1 in the extracellular medium after incubation with cells was analyzed by Western blotting. Almost all the ColE1 was cleaved when incubated with the strain RAM1344 (pTrc-OmpT), which expresses high levels of OmpT, while it remained intact when incubated with the *ompT* null mutant RAM1344 (Fig. 4B, lanes 3 and 4). An intermediate amount (approximately 65%) of ColE1 was cleaved when incubated with the parental strain RAM1292 (Fig. 4B, lane 2). Together, these results indicate that deletion of the N-terminal fragment of ColE1 by OmpT correlates with the inactivation of ColE1.

The previous observation that OmpT cleaves colicins A, E1, E2, and E3 (8) raised the possibility that this may be a general defensive mechanism in colicin-sensitive bacteria. We therefore performed the same experiments on ColE2, an enzymatic colicin with a different translocation pathway than ColE1. ColE2 was also cleaved and inactivated in vivo by OmpT-expressing cells (data not shown).

The direct effect of *ompT* deletion on sensitivity to several colicins was examined by spot assays, but strains with *ompT* deleted did not exhibit increased sensitivity to ColE1 and ColE2 (data not shown). One might speculate that in the environment, where colicins are more diluted, expression of OmpT is likely advantageous for a small number of bacteria that survive the initial exposure to the toxin. To test this hypothesis, we performed a double-layer plate test by cross-streaking *E. coli* mutant strains against ColE1 or ColE2 colicinogenic strains. Results confirmed that *ompT* deletion amplified ColE1 and ColE2 sensitivity (Fig. 4C and data not shown). The introduction of pTrc-OmpT restored growth to the level of the parental strain.

ColE1 with the N-terminal tail deleted cannot kill *E. coli*. It has been shown that the region responsible for the TolQA-dependent uptake of ColE1 extends from residues 1 to 34 (37). We used a truncated form of ColE1 with the first 51 N-terminal amino acids deleted in a cell killing assay. The affinity-purified ColE1$^{52-522}$ showed no killing activity (data not shown). These results unequivocally show that the 51 N-terminal amino acids are critical for the cell killing activity of ColE1.

Isolation and characterization of ColE1-resistant TolC mutants. After binding to BtuB, specific sequences in the N-terminal domain of ColE1 must contact the TolA protein in the periplasm, and ultimately the C-terminal pore-forming domain will insert into the inner membrane. A central question

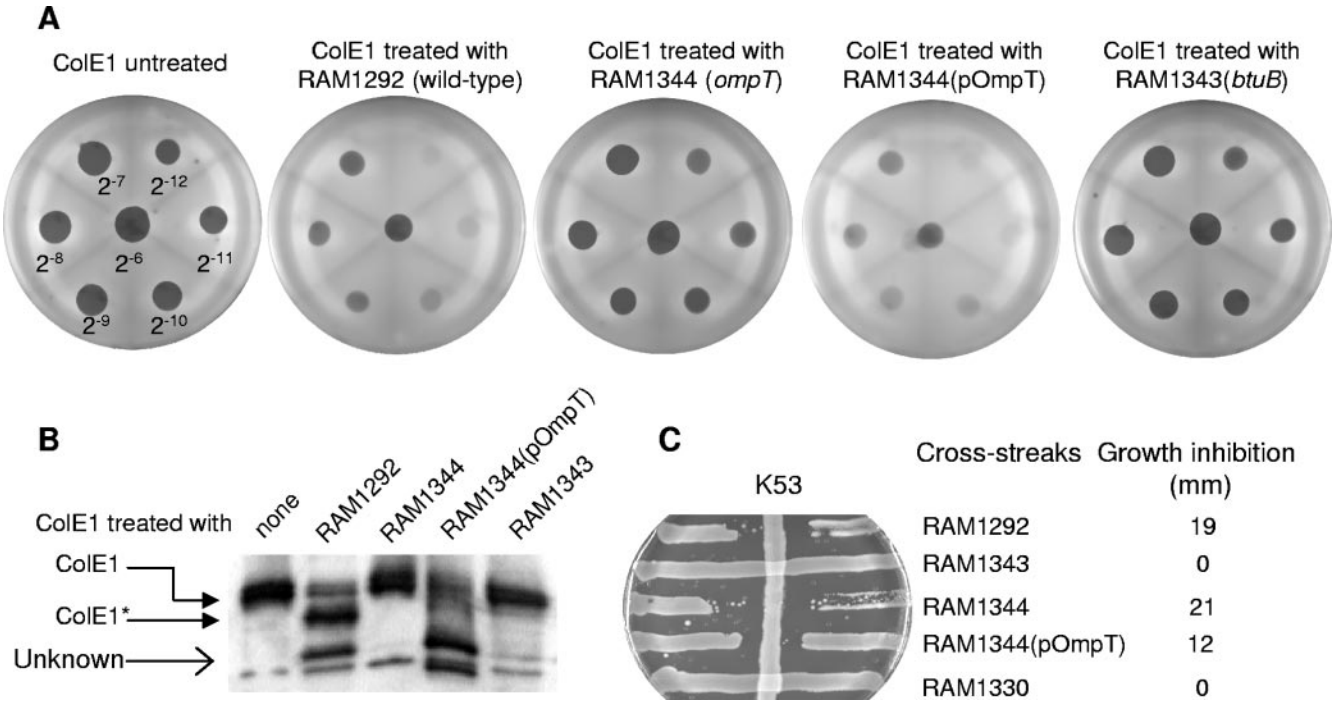

FIG. 4. Effect of OmpT on ColE1 cell-killing activity. (A) Cultures of *E. coli* RAM1292 (wild type), RAM1343 (*btuB*::Tn*10*), RAM1344 (*ompT*::Km^r), and RAM1344 (pTrc-OmpT) were incubated for 2 h at 37°C with ColE1 at a final dilution of 2^{-6} in 10 mM HEPES buffer (pH 7.4). Cells were removed by centrifugation, and the supernatants were passed through a 0.22-μm filter. Twofold dilutions of the supernatants were spotted onto agar plates spread with the indicator *E. coli* MC4100 strain. The growth inhibitory activities of the untreated and treated ColE1 are shown. A clear zone in the lawn of cells indicates the colicin titer at that dilution. (B) The contents of the supernatants from the assay in panel A were analyzed by immunoblotting with antibodies raised against ColE1. (C) Double-layer ColE1 sensitivity test plate. An indicator strain (MC4100), *btuB* (RAM1343), *tolA* (RAM1342), *tolC* (RAM1130), and *ompT* (RAM1344) derivative mutants, and a pTrc-OmpT (RAM1344)-expressing strain were cross-streaked against the ColE1-producing strain K53. Results were scored after 6 to 8 h at 37°C and are expressed as the length of the zone of inhibition (in mm).

then is how these domains get across the outer membrane. *tolC* and *btuB* null mutants are resistant to ColE1, indicating that both TolC and BtuB are required for ColE1 cytotoxicity (17, 32). While the regions of BtuB for ColE1 binding have been identified (19), little is known about which TolC regions are involved in ColE1 binding and translocation (20). Yet, direct involvement of TolC in ColE1 uptake is supported by specific occlusion of TolC channels in planar bilayers by ColE1 (51). To address this issue genetically, we sought ColE1-resistant (ColE1^r) mutants that contained alterations within the TolC protein. To avoid ColE1^r mutations mapping in the *btuB* and *tolA* genes, we mutagenized the plasmid pTrc-TolC carrying the *tolC* gene, which produces TolC at a level similar to the chromosomal level. Random *tolC* mutations were generated by propagating pTrc-TolC in the XL1 Red mutator strain. ColE1^r mutations were isolated by exploiting *tolC* null mutant's sensitivity to bile salts such as deoxycholate (Doc). This demand for double positive phenotypes avoided the isolation of antimicrobial-efflux sensitive TolC mutants (Doc^s). Mutagenized plasmids from five independent pools were introduced into a *tolC*::Tn*10* strain (Doc^s E1^r), and transformants were replica plated on LB agar plates supplemented with Doc (0.05%) and ColE1. Of the more than 10,000 transformants screened by replica plating, only 10 displayed the desired phenotype of ColE1^r Doc^r. DNA sequence analysis of the plasmid *tolC* gene from six of these mutants indicated the presence of a single missense mutation resulting in a G43D or S257P substitution. These two substitutions were located in the transmembrane channel domain of TolC (Fig. 5).

Results showed that strains expressing mutant TolC (G43D or S257P) had a ColE1 titer approximately 100- to 1,000-fold lower compared to that expressing wild-type TolC. TolC levels were quantified from total membranes by Western blot analysis. Both mutants produced TolC at levels similar to that of the strain expressing wild-type protein (Table 2). These results indicated that the reduced ColE1 sensitivity was not due to a decrease in TolC protein level. In fact, cells will not become resistant to ColE1 until TolC expression from a plasmid drops below 25% of the chromosomal level (43). None of the mutants showed hypersensitivity toward novobiocin, which was expected since the isolation strategy demanded efflux competence. The ability to form hemolytic zones on blood agar plates was affected in the mutant harboring the S257P substitution. It is worth noting that this mutant has been previously isolated among both α-hemolysin secretion-defective mutants (43) and phage TLS-resistant mutants (20). As S257 is located at the top of the β-strand domain, it is conceivable that its replacement by a proline would affect the surface structure of TolC, resulting in abortive transport of α-hemolysin, TLS, and ColE1. Interestingly, the two TolC mutants produced varying degrees of phage TLS resistance. Whereas both the G43D and S257P substitutions abolished ColE1 sensitivity, phage sensitivity was

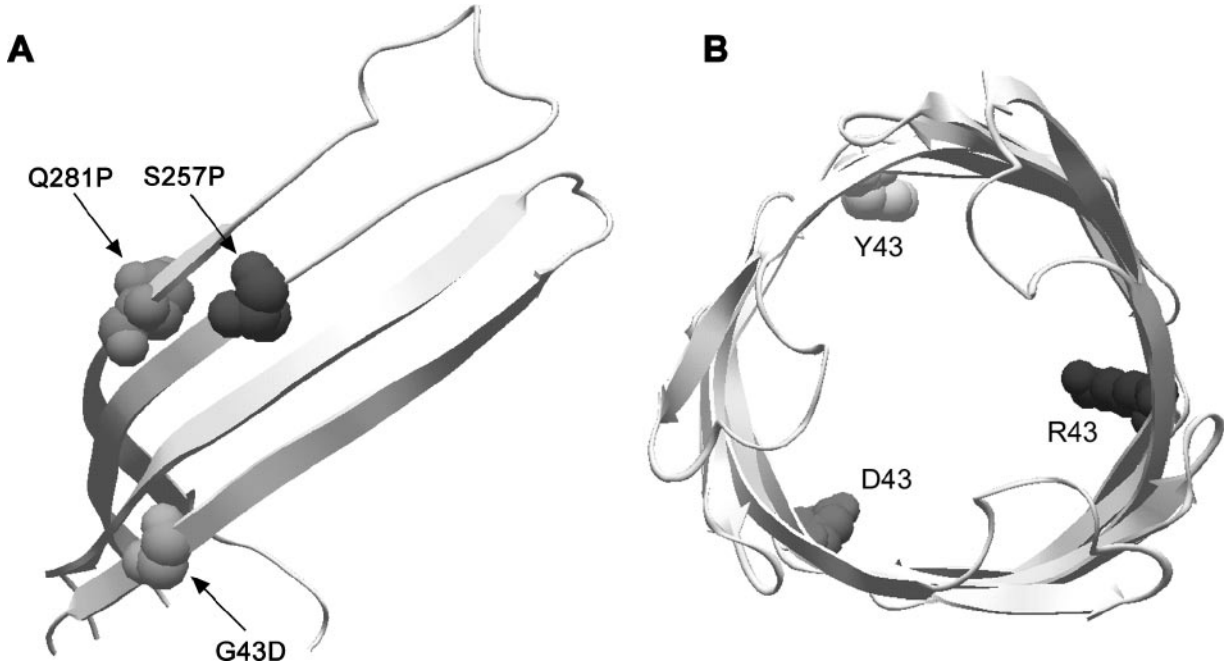

FIG. 5. TolC alterations conferring ColE1 resistance. (A) Only the β-sheet region of one TolC protomer is shown. Sites of amino acid substitutions are identified by space-fill representations. (B) Top view of the extracellular entrance of TolC. Three protomers, each carrying one of the three alterations engineered at residue 43, were assembled in a composite illustration. Replacement of the small and nonpolar glycine residue at position 43 with a tyrosine, aspartate, or arginine residue alters the interior of the TolC channel. For clarity, only the 12 β-stands contributing to the channel domain are shown. The figures were generated using the WHAT IF online software (http://swift.cmbi.kun.nl/WIWWWI/mutate1.html) and SwissProt PBD Viewer, using 1EK9 as the template (27).

affected only by the S257P substitution. This suggested that the functions of TolC with respect to ColE1 uptake and phage TLS infection are genetically separable.

In vivo interaction of ColE1 with TolC. Currently, there is no evidence that cell-bound ColE1 is able to recruit TolC. To establish in vivo interactions between TolC and ColE1, *E. coli* strain RAM1130 was transformed with the recombinant plasmid pTrc-TolC(6His), in which *tolC* expression is inducible by IPTG. The recombinant His-tagged TolC conferred ColE1 sensitivity similar to that of the TolC protein expressed from the wild-type MC4100 chromosome (Table 2). Induced cultures were incubated in the presence of ColE1. Transient protein complexes were stabilized with the chemical cross-linker DSP, which has a fixed 12-Å spacer arm that connects primary amine groups of two adjacent proteins and contains a disulfide bond cleavable under reducing conditions. Cells were lysed, and protein complexes were isolated by affinity chromatography via the His-tagged component. The eluted complexes were treated with sample buffer containing β-mercaptoethanol and boiled to cleave the DSP molecule and release the individual components, which were resolved by SDS-PAGE and identified by immunoblotting.

TABLE 2. Phenotypic characterization of *btuB*, *ompT*, *tolA*, and *tolC* cells and TolC ColE1^r mutants

Strain and alteration[a]	TolC level[b]	TLS (EOP)[c]	E1 (titer)[c]	Nov[d]
RAM1292	ND[e]	1	1	ND
RAM1292 + 5 μM B_{12}	ND	ND	10^{-3}	ND
RAM1343, *btuB*::Tn*10*	ND	1	$>10^{-4}$	ND
RAM1129, Δ*tolC*::Km^r	ND	10^{-9}	$>10^{-4}$	ND
RAM1342, *tolA*:: Cm^r	ND	1	$>10^{-4}$	ND
RAM1344, *ompT*:: Km^r	ND	1	1	ND
RAM1344 (pTrc-OmpT)	ND	1	1	ND
RAM1130, Δ*tolC*::Cm^r	0	10^{-9}	$>10^{-4}$	15
pTrc-TolC(6His)	1	1	1	7
pTrc-$TolC_{G43R}$(6His)	0.6	1	10^{-3}	7
pTrc-$TolC_{G43D}$(6His)	0.9	1	10^{-2}	7
pTrc-$TolC_{G43Y}$(6His)	0.8	1	10^{-1}	8
pTrc-$TolC_{Q281P}$(6His)	0.8	10^{-9}	$>10^{-4}$	11

[a] Chromosomal mutants and parental strains are indicated. Substitutions in TolC refer to the mature protein sequence.
[b] Levels of TolC variants were analyzed from whole-cell extracts and Western blotting. Quantification is relative to the parental wild-type protein.
[c] Sensitivities to phage TLS (efficiency of plaquing [EOP]) and ColE1 (titer) were determined by spotting 10-fold serial dilutions.
[d] Nov, novobiocin (30 μg). Growth inhibition diameters are in millimeters. The diameter of the disk was 6 mm.
[e] ND, not determined.

Without in vivo cross-linking, only a small amount of ColE1 was copurified with TolC (Fig. 6, lane 1). With DSP, a significantly elevated amount of cross-linked TolC and ColE1 was clearly isolated (Fig. 6, lane 2). However, ColE1 failed to recruit TolC when BtuB was absent (Fig. 6, lane 3). Our data provide strong evidence that TolC interacts with ColE1 and that secondary binding of ColE1 to TolC is dependent on its primary binding to BtuB.

As described previously, the Q281P substitution confers resistance to both phage TLS and ColE1, without affecting TolC's efflux or secretion activity (20). In contrast, the G43D substitution was shown to confer resistance to only ColE1. The G43 residue in TolC was replaced with an aspartate (D), an arginine (R), or a tyrosine (Y) residue. Among these three

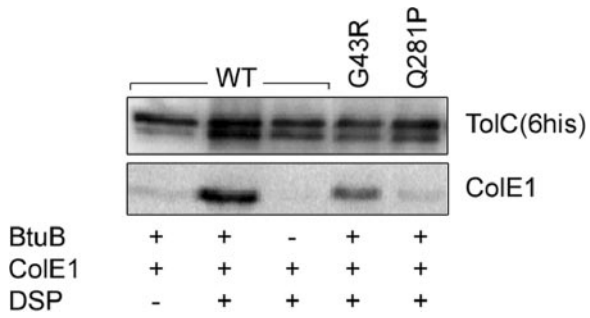

FIG. 6. In vivo engagement of TolC by ColE1. The expression of wild-type or mutant TolC(6His) in cultures of *E. coli* RAM1130 transformants was induced with IPTG. After in vivo cross-linking with DSP in the presence (+) or the absence (−) of ColE1, protein complexes were purified using a Ni^{2+} column. Cross-linked proteins were released by cleaving DSP with β-mercaptoethanol at 95°C, resolved by SDS-PAGE, and identified by immunoblotting with antibodies raised against TolC and ColE1.

substitutions, G43R most effectively prevented cell killing by ColE1, probably due to both the bulk and the net positive charge of the R residue (Table 2; Fig. 5B). In light of the three-dimensional structure of TolC, we postulate that the substitutions at residues close to the cell surface (Q281 and S257) would influence binding of ColE1 and TLS proteins, while substitutions at residues located deep inside the transmembrane channel (G43) may affect the subsequent translocation of ColE1 (Fig. 5). Consistent with this view, we found that ColE1 still recruited $TolC^{G43R}$, but not $TolC^{Q281P}$ (Fig. 6, lanes 4 and 5). The replacement of the Q281 residue with a proline likely affects the surface conformation that is important for recognition by ColE1, rather than a pairwise interaction with a ColE1 residue.

DISCUSSION

In vivo assays showed that ColE1 is cleaved during entry into *E. coli* cells. This cleavage was (i) dependent on specific binding to the BtuB receptor, but not to TolC, and (ii) mediated by the outer membrane protease OmpT. Interestingly, OmpT is not needed for ColE1 activity, but instead OmpT inactivates ColE1. This noticeably differs from the enzymatic ColD, E3, and E7, whose cleavage is an essential step for import and cell killing (16, 39). Proteolytic processing of nuclease colicins is likely to occur in the periplasmic space, leading to the delivery of the active C domain into the cytoplasm of target bacteria. Omptin family members are widespread among gram-negative bacteria (29). Recent data shed new light on the structure and the activity of the archetypal *E. coli* OmpT. OmpT forms a β-barrel that protrudes far from the outer membrane into the extracellular medium, with its active site located in a groove at the top of the barrel (44). This orientation raised the possibility that OmpT may contribute to cell protection against harmful compounds present in the environment by hydrolysis. Consistent with this idea, *E. coli* OmpT and the homologous PgtE from *Salmonella enterica* serovar Typhimurium detoxify cationic antimicrobial peptides and protect the bacteria against the innate immune system (22, 42). Here, we presented evidence demonstrating that OmpT promotes cell protection against E colicins. Once associated with its specific receptor BtuB, the exogenous ColE1 is cleaved by OmpT. It is striking that without binding to BtuB, ColE1 is inaccessible for cleavage from the extracellular medium. We hypothesized (i) that upon binding to BtuB, ColE1 undergoes conformational changes that incidentally expose OmpT cleavage sites, or (ii) that primary binding to BtuB is needed to bring the predicted disordered N-terminal T domain to close proximity with OmpT. The processing step results in a 49-kDa ColE1 fragment carrying the C-terminal pore-forming domain but with the N-terminal translocation domain (residues 1 to 95) deleted. The TolQA binding region that is essential for colicin import and killing activity extends from residues 1 to 34 in the T domain of ColE1 (37). To date, it is not obvious whether or not the cleaved ColE1 is able to cross the outer membrane. The detection of the cleaved fragment in supernatants supports the possibility that the cleaved colicin is released from BtuB without being translocated through TolC.

Recognition of the target cell surface receptor is the first stage in colicin-mediated killing of *E. coli*. Previous studies have established that E colicins bind to the BtuB receptor and that Tol proteins are required for their translocation into the periplasm (17, 31, 32, 46). However, the sequence of events that occurs after receptor binding and the overall dynamics of the system remain to be elucidated. As a first step, we aimed to study the translocation of ColE1 across the outer membrane TolC channel. Here, we showed that TolC is recruited directly to the BtuB-ColE1 complex. This supports the current hypothesis of a "colicin translocon." In this model, BtuB works with a secondary receptor, which is TolC in the case of ColE1 and OmpF in the case of colicins E3 and E9. The ColE3-BtuB three-dimensional structure suggests that BtuB binding places the translocation domain close to the outer membrane surface, in a conformation where it can "fish" for OmpF (30). Likewise, engagement of ColE9 to BtuB has been shown to induce bridging of OmpF (24). We set out to study how TolC binds and translocates ColE1. Two different classes of TolC mutants whose expression conferred resistance to ColE1 were selected for further study. Substitutions S257P and Q281P presumably abolished both binding and translocation of ColE1. Interestingly, these substitutions also conferred resistance to bacteriophage TLS, suggesting they influence cell surface-exposed regions that participate in the receptor function of TolC. In contrast, TolC mutants with alterations at the G43 residue retained their full ability to bind ColE1 and were sensitive to TLS. Yet, these alterations conferred ColE1 resistance, most likely by impeding or preventing the transport of ColE1 due to steric hindrance inside the channel domain of TolC. A similar mechanism has been proposed to describe an OmpF mutant resistant to ColN (26).

The unique structure of the trimeric TolC outer membrane protein forms a 20-Å-diameter channel that extends 100 Å into the periplasm. This wide channel tapers to a narrow opening (4 Å) on its periplasmic end, restricting the passage of substrates (27, 28). TolC can accommodate the export of large proteins such as the 110-kDa hemolysin HlyA. According to the model proposed by Cramer and colleagues, passage of ColE1 through the TolC channel would occur with the T domain first and then the active C domain in a mostly unfolded state (50, 51). Opening the periplasmic entrance is key to the function of TolC and

its associated assembled machineries. It has been proposed that TolC's aperture opens by an allosteric "iris-like" realignment of the entrance helices during substrate translocation (1, 28). After passing through TolC, further translocation of ColE1 is achieved by the inner membrane Tol system, whose function remains to be elucidated. All group A colicins require TolA and, to different extents, TolQ and TolR (31, 46). ColE1 does not require TolB and interacts with TolA differently than other group A colicins (38). The literature implies that the C-terminal periplasmic domain of TolA binds to the incoming ColE1 (3). The central domain of TolA has been shown to interact directly with outer membrane proteins such as OmpF, OmpC, PhoE, and LamB (15). Experiments are in progress to investigate the putative interactions between TolA and TolC. Such interactions may play a role in proximal opening of the TolC tunnel.

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