Structure of the Complex of the Colicin E2 R-domain and Its BtuB Receptor
THE OUTER MEMBRANE COLICIN TRANSLCON

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The crystal structure of the complex of the BtuB receptor and the 135-residue coiled-coil receptor-binding R-domain of colicin E3 (E3R135) suggested a novel mechanism for import of colicin proteins across the outer membrane. It was proposed that one function of the R-domain, which extends along the outer membrane surface, is to recruit an additional outer membrane protein(s) to form a translocon for passage colicin activity domain. A 3.5-Å crystal structure of the complex of E2R135 and BtuB (E2R135-BtuB) was obtained, which revealed E2R135 bound to BtuB in an oblique orientation identical to that previously found for E3R135. The only significant difference between the two structures was that the bound coiled-coil R-domain of colicin E2, compared with that of colicin E3, was extended by two and five residues at the N and C termini, respectively. There was no detectable displacement of the BtuB plug domain in either structure, implying that colicin is not imported through the outer membrane by BtuB alone. It was concluded that the oblique orientation of the R-domain of the nuclease E colicins has a function in the recruitment of another member(s) of an outer membrane translocon. Screening of porin knock-out mutants showed that either OmpF or OmpC can function in the recruitment of another member(s) of an outer membrane translocon. Arg452 at the R/C-domain interface in colicin E2 was found to have an essential role at a putative site of protease cleavage, which would liberate the C-terminal activity domain for passage through the outer membrane translocon.

Protein transport across membranes in organelles and bacteria is known to involve multiprotein complexes (1, 2). Colicin import across the outer membrane of Escherichia coli has also been inferred to involve such a translocon (3–8). An experimentally useful attribute of colicin uptake for studies on protein transport across membranes is that the end result is cytotoxicity. Colicins are plasmid-encoded bactericidal proteins that are released in response to stress, enter the bacterial cell by appropriating its outer membrane nutrient-uptake machinery, and provide an advantage to colicin-resistant cells in the competition for nutrition (9). Colicins are produced in complex with a small immunity (~10 kDa) protein that binds to, and prevents, the colicin from killing the producing cell (10, 11). Nuclease colicins consist of three domains: an N-terminal T (translocation)-domain that functions in the import of the colicin across the outer membrane, a C-terminal C (catalysis or channel)-domain that contains the cytotoxic activity, and a central R (receptor-binding)-domain that functions in irreversible attachment to an outer membrane receptor.

Colicins have been divided into two groups, A and B, based on the intracellular protein translocation network that is utilized. “Group A” colicins utilize the Ton proteins TolA, TolB, TolQ, TolR, and Pal (4, 12, 13), whereas “Group B” colicins utilize the Ton proteins, TonB, ExbB, and ExbD (5, 14), to enter the bacterial cytoplasmic compartment and/or insert into the cytoplasmic membrane. Colicins E2 (a DNase) and E3 (a 16S rRNAse), both nuclease E colicins and members of Group A, utilize the same outer membrane receptor, BtuB, to bind to and enter the cell. BtuB, a 22-stranded antiparallel β-barrel integral outer membrane protein, functions physiologically to transport cobalamin (vitamin B₁₂) across the outer membrane (15). The interaction of nuclease E colicins with BtuB is neither TonB- nor energy-dependent. The central pore of the BtuB-barrel is occluded by an N-terminal 132-residue “plug” domain, which, in the absence of an energy input, would prevent the passage of any colicin. The crystal structure of a complex of the R-domain (E3R135)⁴ of colicin E3 in complex with BtuB, solved to a resolution of 2.75 Å, provides several clues to the mechanism of colicin import through the outer membrane (7).

The peripheral and oblique binding of E3 R-domain to BtuB, the complete absence of any colicin-induced current through BtuB incorporated into planar bilayers (16), and the fact that the plug domain of BtuB was stable with little or no conformational change upon interaction with the colicin R-domain (7), implied that the transfer of any domain of colicin E3 through the BtuB structure was unlikely. At least one additional embedded outer membrane protein participates in an outer mem-

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The atomic coordinates and structure factors (code 2YSU) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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4 The abbreviations used are: E2R135 and E3R135, 135-residue receptor-binding domain of colicins E2 and E3, respectively; MES, 2-(N-morpholino)ethanesulfonic acid; LDAO, N,N-dimethyl-dodecylamine-N-oxide; OMP, outer membrane protein.
bran translocon for colicin import through the outer membrane. It was hypothesized that the unusual elongate receptor-binding domain, with a length of ~100 Å for RNase colicin E3 (17), which is also inferred for structures of colicins E2, E6, E7, and E9 (18) on the basis of sequence similarity, and ~160 Å for colicin Ia (19), is associated with a “fishing rod” mechanism (16) for recruitment of an outer membrane protein in addition to BtuB to form a translocon. The additional protein utilized to form a translocon for the import of nuclease E colicins is inferred to be the OmpF porin in most studies on colicins E2, E3, E7, E9, and A (7, 16, 20–23). A dependence of the activities of nuclease E colicins on OmpF has also been noted (24, 25). Colicin E1 does not require OmpF, but it needs TolC (13, 26) to accomplish the first phase of protein import.

Nuclease E colicins have highly conserved T- and R-domains, with the major difference in their amino acid sequences residing in their C-domains and at the junction of the R- and C-domain, suggesting that they utilize a common pathway for entry into the bacterial cell. A question was posed as to whether the structure of the E3R135-BtuB complex could be applied to other nuclease E colicins. Alignment of the R-domain of colicin E3 and E2 revealed a difference of 12 of 135 residues, with all of the mismatches concentrated in the C terminus of the R-domain (Fig. 1). The structure of the E2R135-BtuB complex, obtained in the present study at a resolution of 3.5 Å, was found to be virtually identical to the corresponding structure of the E3R135-BtuB complex solved previously (7). Studies that bear on the import mechanism show that, under normal growth conditions, among the tested porins only OmpF or OmpC is necessary for colicin cytotoxicity and that the site of protease cleavage of the imported colicin E2 is similar or identical to that studied previously in colicin E7 (30).

**MATERIALS AND METHODS**

**Bacterial Strains—**The *E. coli* XL1 Blue strain was used as the host strain for cloning of E2R135. Cloning was done in the pET41b vector such that there was a His8 tag at the C terminus of the protein. *E. coli* BL21(DE3) was the host strain for expressing the protein. In the pET41b vector, protein expression is under the control of a strong isopropyl-1-thio-

**Purification of** E2R135—The E2R135 construct was created by cloning residues Thr311–Asp447, a 135-residue-long peptide, in the expression vector pET41b. The cloning was done using standard protocols. E2R135 was first amplified using upstream (Nde primer) and downstream (Xho primer) primers and then cloned between the NdeI-XhoI sites of pET41b in such a way that there was a His8 tag at the C terminus joined by a linker consisting of residues Lys and Glu, bringing the total length of the peptide to 145 residues. Purification of the over-expressed E2R135 construct was accomplished using an nickel-charged IDA-agarose column.

**Purification of BtuB—**BtuB was expressed in strain TNE012 (pJ3) and purified using a previously published protocol (31). BtuB was extracted from the outer membrane with 1.5% (w/v) β-d-octyl-glucoside, 50 mM Tris-HCl, 5 mM EDTA, phenylmethylsulfonyl fluoride/tosylphenylalanyl chloromethyl ketone (PMSF/TPCK), pH 8.0, and purified by ion-exchange chromatography in 0.1% (w/v) LDAO using a 0–0.8 M LiCl gradient in a DEAE fast protein liquid chromatography column. BtuB-enriched fractions were concentrated and further purified on a Superdex 200HR size exclusion column in 10 mM Tris-HCl, 0.1 M NaCl, 0.1% (w/v) LDAO, pH 8.0. BtuB concentrations were determined from its UV extinction coefficient: [ε (280 nm)] = 137 cm⁻¹ mol⁻¹⁻¹.

**Knock-out Strains—**Strains having single, double, and triple porin knock-outs were used to study the role of these porins in the colicin import. Knock-out strains were produced by using a previously published method (32). PCR primers were used to provide homology to the targeted gene(s). The chromosomal sequence was replaced with a kanamycin resistance gene generated by PCR by using primers with 36-nucleotide homology extensions. This is accomplished by Red-mediated recombination in these flanking homologies. The Red helper plasmid, a temperature sensitive replicon, was cured by growth at 37 °C (32).

**Cytotoxicity Assays—**190 μL of LB broth with or without 1 mM colicin E3 was inoculated with 10 μL of overnight cultures of porin knock-out strains in a 96-well plate. The cells were allowed to grow over a period of 5 h, and growth was monitored by measuring optical density at 410 nm at 30-min intervals.

The cytotoxicity of wild type E2 and E2 R452A was assayed using a “spot test.” Spot tests involve plating colicin-sensitive pT7-7 cells (Amp’ cells) onto a LB Amp plate to which different concentrations of colicin are applied in 20-μL drops. Lysis of the bacterial cells is seen as clear spots in a lawn of bacteria. The lowest inhibitory concentration was defined as the smallest concentration that would generate a clear zone of inhibition.

**Crystallization—**Crystallization screening was done in a 96-well plate utilizing a Genomic Solutions robot. E2R135 and BtuB were mixed at a ratio of 1.6:1 (mol:mol) and allowed to react for ~30 min. The protein was dissolved in 20 mM Tris-HCl, pH 8.0, 0.1 M NaCl, and 0.1% LDAO buffer. The reservoir solution contained 10% dioxane, 0.1 M MES, and 1.6 M ammo-
Aucanum sulfate. Crystals of the E2R135-BtuB complex were obtained by hanging drop vapor diffusion, mixing 0.7 μl of the protein solution with 0.7 μl of reservoir buffer at 20 °C. Crystals grew within 1 week.

Structure Determination and Refinement—X-ray diffraction data at 3.5-Å resolution were collected at 100 K at the SBC X-ray source (APS, SBC-19ID) at Argonne National Laboratory. Data reduction and scaling were performed using the program HKL2000 (33). The crystals belonged to the orthorhombic space group P2_12_12_1, with a = 76.2 Å, b = 80.7 Å, c = 235.9 Å. The unit cell of the E2R135-BtuB crystal was similar to that of E3R135-BtuB (a = 76.9 Å, b = 80.1 Å, c = 233.6 Å; Protein Data Bank accession code 1UJW). The structure was determined by rigid body refinement with the program REFMAC5 in CCP4 (34) using the published structure of the E3R135-BtuB complex (1UJW) as an initial model. Structure refinement and model construction used the programs REFMAC5 and O (35). A summary of the crystallographic data and refinement statistics is presented in Table 1. The programs Molscript (36), Povscript (37), and Raster3D (38) were used for molecular graphics presentations. The program SURFACE in CCP4 was used to calculate surface areas.

Oclusion of OmpF—The occlusion by colicin E2 of OmpF channels incorporated into planar bilayers was studied as described previously (16). The synthetic phospholipids DOPC and DOPE were mixed in a 1:1 molar (total concentration, 10 mg/ml) ratio, dissolved in n-decane, and then used to form planar bilayers (39, 40). Bilayers were formed by painting lipids using a “brush technique” (41) on a 0.2-mm aperture in a partition separating two 4-ml chambers containing 10 mM HEPES, pH 7.0, 0.1 M KCl, 23 °C. Occlusion was studied by measuring the current across this aperture with Ag/AgCl electrodes immersed into the two chambers, using a Warner BC-525C amplifier (Hamden, CT). To form channels, OmpF (1 pg/ml-1 ng/ml) was added to the cis-side of the membrane, and the solution was stirred until channels appeared. A negative trans-membrane potential was applied to the cis-side, with the trans-side at electrical ground. Colicin (0.5–1 μg) was added to the trans-side of the aperture.

RESULTS

Structure of the E2R135-BtuB Complex—Crystals of the E2R135-BtuB complex diffracted to 3.5 Å. The structure was solved as described under “Materials and Methods” using the E3R135-BtuB structure as a reference (Protein Data Bank code 1UJW). The structure reveals 123 residues of E2R135 (residues 321–443), in a coiled-coil conformation, bound to BtuB (Fig. 2A). A total of 12 residues distal to the BtuB binding site, eight N-terminal and four C-terminal, are disordered and not resolved in the structure. This extent of disorder differs slightly from the total of 19 residues (10 N-terminal and 9 C-terminal) of E3R135 that are disordered in the E3R135-BtuB structure. Otherwise, the crystal structure of E2R135-BtuB is very similar to that of E3R135-BtuB (Fig. 2B (7)). In a recently published structure of Cîr and the receptor-binding domain of colicin Ia, a single C-terminal residue is disordered and not resolved (42). The BtuB-R135 interaction surface, defined by a 4.5-Å van der Waals distance of closest approach, is similar for the E2 and E3 structures: (i) in all, 28 residues of E2R135 (and E3R135) interact with 29 residues of BtuB; (ii) Met383 and Pro382, respectively, of E2R135 and E3R135 are in contact with six residues of the plug domain (Thr55, Gln56, Asn57, Leu63, Ser64, and Ser65); (iii) for both colicins, the buried area of E2R135 is 1262 Å², whereas the corresponding figure for E3R135 is 1287 Å²; (iv) the principal axis of the R-peptide resides at an identical angle of ~44° relative to the membrane surface for both colicins; (v) the number of hydrogen bonds between the plug and the barrel of BtuB is 61, 56, and 65 for E2R135-BtuB, E3R135-BtuB, and apo-BtuB crystallized in surfo (Protein Data Bank code 1NQE) structure, respectively; (vi) the accessible surface area of the plug domain is also essentially identical, as the area for the apo-BtuB (1NQE), for E2R135-BtuB (2YSU) and E3R135-BtuB (1UJW) is 2768, 2900, and 2904 Å², respectively (the above calculation includes the surface area of the BtuB plug domain/R-domain interface for the E2R135-BtuB and E3R135-BtuB structures); (vii) R-domain residues involved in crystal contacts (Leu330, Asn331, Asn334, Ala338, Gln414, Gln414, Ala432, Arg432, and Glu436 of E2R135) are identical in the case of both structures; (viii) all of the crystal contacts of the R-domain for both the E2- and E3R135 complexes are contained in extracellular loops 5–6 and 7–8 of BtuB.

It is inferred that the entry of the colicin does not occur through the pore of BtuB because: (i) there is no detectable distortion of the plug domain in the E2R135 and E3R135 structures relative to that of apo-BtuB (Fig. 2C); (ii) no ionic current (>1.0 × 10⁻¹² Å) was induced by the addition of colicin E3 to BtuB incorporated into planar bilayers (16).

As was the case for E3R135-BtuB, binding of E2R135 affects the loops of BtuB in the E2R135-BtuB structure when compared with both the in surfo (Fig. 2D) (15) and in meso (Fig. 3) structures of apo-BtuB (43). Loops 5–6 and 7–8, which are disordered in the in surfo apo-BtuB structure, are ordered in the

TABLE 1

| Data collection | X-ray source | APS |
|-----------------|--------------|-----|
| Wavelength (Å)  | 0.97934      |     |
| Resolution (Å)  | 3.50 (3.63-3.50) |     |
| Measured reflections | 127,245 (10,186) |     |
| Unique reflections | 18,963 (1,787) |     |
| Redundancy      | 6.7 (5.7)    |     |
| I/σ(I)          | 16.3 (3.7)   |     |
| Completeness (%)| 99.3 (95.5)  |     |
| Rmerge (%)      | 10.8 (37.7)  |     |

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E2R135-BtuB structure (Fig. 2D). This can be compared with the Cir-colicin Ia structure in which there is a large outward movement of loops 7 and 8 (42). In E2R135-BtuB there is a change in the position of loop 19–20, and the location of the partial order in loop 3–4 is altered. When compared with the \textit{in}

\textit{meso} BtuB structure, the disordered loops 5–6 and 21–22 become ordered upon binding of E2R135 (Fig. 3). There is also a difference in the position of the loops 9–10, 13–14, 15–16, and 19–20, and the location of the partial order in loop 3–4 is altered. In addition, unlike the Cir-Ia complex structure (42), where the binding of the R-domain of colicin Ia does not elicit a change in the N-terminal \textit{tonB} box, binding of the R-domain of both colicins E2 and E3 causes ordering of residues Ser\textsuperscript{4} and Pro\textsuperscript{5} in the \textit{tonB} box on the periplasmic side of BtuB.

There are some differences between the R-domain of the two structures, with most of these differences located at the termini of the coiled-coil. The major difference is that the E2R135 structure is longer by two residues at the N terminus and by five residues at the C terminus. Also, compared with the corresponding residues of E3R135-BtuB structure (7), the C-\alpha positions of the N-terminal seven residues 323–329 of E2R135 show a gradual increase in displacement as a function of increasing distance from BtuB along the coiled-coil, with the N-terminal residue, Tyr\textsuperscript{323}, having a maximum displacement of 5.2 Å.

\textbf{Occlusion of OmpF Channels by E2—}OmpF channels incorporated into planar bilayer membranes were occluded by colicin E2 (4 \mu g/ml) added to the \textit{trans}-side in the presence of a \textit{cis}-negative potential (data not shown) in a manner similar to the occlusion by colicin E3 (7, 16). This is consistent with the 100% identity of the N-terminal 83 residues of colicin E3 and E2.

\textbf{In Vivo Cytotoxicity Assays—}A library of indicator knock-out strains each missing one or more porins (44) was tested for colicin sensitivity. No single porin knock-out was resistant to colicin E3 (Fig. 4A). However, the A\textit{ompR} strain, in which the OmpF and OmpC positive regulator is knocked out and thus is missing both OmpF and OmpC, was resistant to E3 (Fig. 4B). This implies that either OmpF or OmpC, but no other OMP, can function in the translocon for the nuclease E colicins under normal growth conditions.
Protease Cleavage Site—The high affinity binding of the nuclease E colicins to the primary BtuB receptor implies that the catalytically active C-domain must be released from the bound colicin in order for it to be imported through the outer membrane. A protease cleavage site between the R- and C-domains has been demonstrated for the DNase colicin E7 (30). On the basis of sequence similarity in this region (90%; 27 of 30 residues) between colicin E2 and E7 (Fig. 5), the site Arg452 was postulated to be the corresponding site for cleavage in colicin E2. Mutagenesis of residue 452 of colicin E2 (R452A) resulted in a 330-fold loss of colicin activity (Fig. 6), implying that this residue has a critical role in the protease cleavage site of all E colicin DNases. Initial experiments on colicin E3, and a much smaller degree (40%; 12 of 30 residues) of sequence similarity at the putative protease cleavage site region (Fig. 5), suggest that the location of the cleavage site and/or the properties of the protease are different for the RNase compared with the DNase colicins.

DISCUSSION

BtuB Is a Receptor but Not a Translocator for the Nuclease E Colicins—BtuB serves as the high affinity receptor that transfers the colicin from three-dimensional to the two-dimensional space of the outer membrane surface. However, it does not provide the pore, nor does it function alone to translocate the colicin across the outer membrane. This is inferred from (i) structure and (ii) electrophysiological data. (i) Displacement of the plug domain that would be necessary for entry of the colicin through the central pore was not seen in either the E2R135-BtuB or the E3R135-BtuB structures. There are some small changes in the accessible area of the plug domain and the number of H-bonds between the plug and the barrel, but none of these changes suggest removal of the plug domain. (ii) Furthermore, the addition of colicin E3 to planar bilayers into which BtuB was incorporated did not generate any ion channel activity that would be associated with displacement of the plug domain (16). It was concluded that BtuB is a receptor but not a translocator for the nuclease E colicins.

The Elongate Projection of the Elongate R-domain—The angle of oblique binding of the E2R135 peptide (Fig. 2A) was found to be identical, within experimental error, to that of E3R135 peptide (Fig. 2B). Because of the high degree of sequence similarity between the nuclease E colicins in the T- and R-domains, colicins E2, E3, E6, E7, and E9, and the similarity of the E2R135 and E3R135 structures, all of these colicins should bind with approximately the same oblique orientation to BtuB, as shown in Fig. 2, A and B. A similar oblique binding

5 O. Sharma, unpublished data.
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E2 R452A

wt E2

Studies of the complex of colicin R-domain with their primary receptor have recently been expanded to colicin Ia and Cir (42). It was inferred that Cir, with its plug domain displaced, is the probable channel through which colicin Ia crosses the outer membrane (42) and that the oblique binding represents the initial stage of attachment of colicin Ia to Cir. The hypothesis is supported by the fact that no secondary receptor is known for the Ton-dependent colicins. The mechanism of colicin insertion may be different for the Ton-dependent colicins. However, without additional protein interactions it is difficult to understand how the N-terminal end of colicin Ia, which is 160–200 Å removed from Cir, would enter this receptor to interact with the periplasmic TonB protein.

OmpF or OmpC Contributes to the Translocation Pores for Nuclease E Colicins—Involvement of OmpF (7, 16, 21, 22) and OmpC (24) in the import of nuclease E colicins has been suggested previously. We note that the current method of creating single gene “knock-outs” (32) is different and superior to the previously applied method of screening for mutants resistant to phages or colicins (45). Although we (7, 16) and others (21, 22) have focused on the role of OmpF in the uptake of nuclease E colicins, the role of OmpC in colicin import has mostly been neglected, the exception being the studies in Refs. 46 and 24. In the present study, we have found that OmpC has a significant role in the uptake of colicin E3. Single knock-out mutants of OmpT, OmpA, LamB, OmpF, and OmpC did not affect colicin E3 cytotoxicity (Fig. 4A). However, when OmpR, a positive regulator of OmpF and OmpC, was knocked out, cells were completely resistant to colicin (Fig. 4B). Thus, the two porins, OmpF and OmpC, can substitute for each other in the import of nuclease E colicins. At least under the normal growth conditions used in this study, no outer membrane protein other than OmpF and OmpC could confer sensitivity to these colicins.

Crystal Contacts—The hypothesis that the oblique orientation of the R-domain to its receptor has a significant function requires consideration of the structure perturbation that would be caused by crystal contacts, because such crystal contacts could conceivably provide the necessary force required to affect the angle between BtuB and E3R135 and thus to generate the oblique binding seen in Fig. 2, A and B. Although Arg<sup>135</sup> is involved in crystal contacts, all of the crystal contacts with the R-domain in the two structures are between loops 5–6 and 7–8 of BtuB. These loops are highly flexible, as suggested by loop 5–6 being disordered in both the in surfo BtuB structures and loop 7–8 being disordered in the in meso BtuB structure. The 7–8 loop is ordered in the in meso BtuB structure, as it participates in crystal contacts. Participation of these loops in crystal contacts might be one of the reasons that the loops are ordered in the E2R135-BtuB and E3R135-BtuB structures. However, because of the flexibility of the loops, it is believed that these interactions are not the cause of the oblique binding of the R-domain to BtuB. The near identity of the oblique orientation of the R-domain with respect to the membrane plane in the E2R135 and the E3R135 complexes, as well as an oblique orientation of a receptor-binding fragment of colicin Ia with its Cir receptor (42), also implies that the oblique orientation of the R-domain binding to BtuB or Cir is a consequence of primary structure interactions and not crystal contacts.

FIGURE 6. In vivo cytotoxicity analysis of E2 R452A (top) and wild type E2 (bottom). The lowest inhibitory concentration was 31 pm and 10 nm for wild type E2 and E2 R452A, respectively. The numbers on the plates are normalized to 1 = 1 nm. Thus, mutation of Arg<sup>452</sup> to Ala decreases colicin E2 cytotoxicity by a factor of~330.

orientation has been found for a somewhat smaller R-domain fragment of colicin Ia and its receptor, Cir (42). The consequence of the oblique angle of binding to the BtuB receptor (44° relative to plane of the membrane) is that the projection of the R-domain of colicins E2 and E3 in the plane of the membrane is ~70 Å. The projection of the entire colicin molecule, together with the C- and T-domains, is ~100 Å (Fig. 7). Based on these structure data, the requirement of colicin activity for the presence of OmpF or OmpC (Fig. 7), and the defined occlusion of OmpF channels by colicin E3 (7, 16), its C-domain (8), and colicin E2 (data not shown), it is proposed that the purpose of the elongate R-domain and the oblique orientation of receptor bound colicin is to allow the colicin T-domain to search for a secondary receptor/translocator, OmpF or OmpC for the nuclease E colicins, which is necessary to form the translocon structure needed for colicin import across the outer membrane (6–8, 16).
**Proteolytic Cleavage Frees C-domain**—Because the nuclease E colicins bind very tightly to BtuB (7), it is necessary for the C-domain to be proteolytically cleaved from the rest of the colicin for it to enter the cytoplasm. A periplasmic protease acting on residue Arg\textsuperscript{447} in the linker region connecting the R- and C-domain to be proteolytically cleaved from the rest of coli-

E colicins bind very tightly to BtuB (7), it is necessary for the C-domain to be proteolytically cleaved from the rest of coli-

E colicins (Fig. 5). On the basis of high sequence homology among

DNase colicins, E2, E7, E8, and E9 (Fig. 5). Based on the structure of the C-terminal activity domain of colicin E9 (47), residue Arg\textsuperscript{447} of colicin E9, homologous to Arg\textsuperscript{453} of colicin E2 and Arg\textsuperscript{452} of colicin E7, was predicted to be a part of the active site that functions by contacting the bound nucleotide phosphate (48). Thus, there might be concern that the decrease in cytotoxicity seen on mutating this residue results from its role in DNA hydrolysis. However, the in vitro endonuclease assay done with colicin E7 (30), which was con-

firmed by our experiments with full-length colicin E2 (data not shown), showed that the colicin retains its activity on mutation of the residue implicated in proteolysis. Interestingly, when

Arg\textsuperscript{447} was mutated in a colicin E7 C-domain construct, the activity decreased to 10–15% of that of the wild type C-domain (30). This de-

crease was mostly because of a lower binding affinity of the mutated C-domain and its substrate DNA and not because of a reduction in DNase activity of the nuclease domain (30). This may be a conse-

quence of Arg\textsuperscript{453} being located in a region with a different fold in full-

length colicin E9 compared with the C-domain construct where it is close to the N terminus (47).

**Mechanism of Uptake of Nuclease E Colicins**—Because the target for the nuclease E colicins is present in the cytoplasm, the C-domain (activity domain) of all of these colicins must enter the cytoplasm after crossing the outer and inner mem-

branes. The nuclease E colicins E2, E3, E6, E7, and E9 have very similar T- and R-domains, and the mecha-

nism of uptake should therefore be similar for these colicins (18).

A model for the import of nuclease E colicins is proposed in which the colicin binds to BtuB in the outer membrane as the first step (7); Fig. 7). This binding serves to sequester the colicin from the media, or aqueous extracellular phase, and to localize it on the surface of the cell. The oblique angle of binding to BtuB places the colicin appropri-

ately for the natively unfolded N terminus to interact with, and insert through OmpF (16, 21, 22) or OmpC (24) and to interact with TolB in the periplasm. The interaction of the N-terminal translocation (T-) domain of the nuclease E colicins with OmpF and that of the “TolB box” with the TolB protein (28, 49) leads to release of the immunity protein (46), resulting in an unfolding of the C terminus (8). The unfolded C terminus is now free to interact with and insert through OmpF (8). The C terminus is then proteolyzed between the R- and C-domain by a protease (30), which allows the C-terminal domain of nuclease colicins such as E2 and E3 to pass through the outer mem-

brane and subsequently to use additional components of the Tol network to pass through the cytoplasmic membrane and enter the cytoplasm where they exert their cytotoxic nucleolytic activity (50).

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