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ON THE MECHANISM AND PATHWAY OF COLICIN IMPORT ACROSS THE *E. COLI* OUTER MEMBRANE

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1. ABSTRACT

Colicins and phages parasitize outer membrane receptors whose physiological purpose is the transport of metabolites, metals, vitamins, and sugars. From mutagenesis studies, it is known that several colicins require the function of two outer membrane protein (Omp) receptors for cytotoxicity. A formidable list of problems associated with an understanding of a two receptor mechanism for colicin translocation includes the definition of the sites of initial binding and interactions of the colicin with the OM translocator protein, the working lumenal aperture of the translocator, the question of whether the colicin must be unfolded for translocation, the source of energy for unfolding and translocation, the order of colicin translocation, and the sites and mechanism of interaction of the colicins with the Tol-Pal proteins on the periplasmic side of the outer membrane. 3D crystal structures recently obtained of the cobalamin (vitamin B_{12}) receptor (BtuB), and of the complex of BtuB with the 135 residue receptor binding domain (R135) of colicin E3, have provided some new insights on the interactions between two Omp receptors that are necessary for translocation of colicins. Together with spectroscopic data on the R135-BtuB interaction and electrophysiological data on the colicin E3-OmpF interaction, this has led to a proposal for the utilization of two receptors, BtuB-OmpF, in an outer membrane translocon for colicin E3.

2. INTRODUCTION

A schematic pathway for the cellular import of A-type colicins, i. e., colicins A and E1-E9, is summarized [**Figure 1**; see Biochimie (2002) 84 (5/6) devoted to bacteriocins. The scheme shows that the colicins are first bound to integral membrane protein receptors in the outer membrane, and then are translocated using the Tol (A-group) components of the translocation network in the periplasm and cytoplasmic membrane to the cytoplasmic membrane or the cytoplasm, where they can perturb the cellular metabolism through one of several cytotoxic actions: membrane depolarization, or degradation of DNA, ribosomal RNA, or tRNA. The ultimate consequence in all cases is cytotoxicity. The present discussion focuses on the mechanism and pathway of translocation of colicin E3, and probably of the family of nuclease colicins, through the outer membrane. The proteins of the Tol network that facilitate translocation of type A colicins through the outer membrane by interaction with OM receptors/translocators are TolA, embedded in the inner membrane with an extensive periplasmic domain (1, 2), TolB located in the periplasmic space (3, 4), and Pal, a lipoprotein attached to the outer membrane from the periplasmic space (5, 6).

Some major questions about the pathway of import across the outer membrane are:

1. How many receptors are involved in binding/translocation, one or two? If only one outer membrane protein is involved, how does it accomplish both binding and translocation?
2. If two receptors are responsible for binding/translocation, how are they coupled?
3. Whether one or two receptors are involved, is the colicin translocated in a folded or unfolded state [an issue discussed previously (7)]?
4. If the colicin is unfolded prior to, or concomitant with, translocation, what is the trigger or mechanism that drives the unfolding?
5. Similarly, what is the source of energy for colicin unfolding and translocation?

Table 1. Groups A of colicins use Tol-Pal route to enter cells (Lazzaroni *et al.* (6))

Colicin	Receptor	Translocation	Cytotoxicity
A	BtuB	**OmpF**[a], TolQ,R,A,B	Pore
E1	BtuB	**TolC**, TolQ,R,A	Pore
E2,7-9	BtuB	**OmpF**, TolQ,R,A,B	DNase
E3,4,6	BtuB	**OmpF**, TolQ,R,A,B	16s rRNase
E5	BtuB	**OmpF**, TolQ,R,A,B	anticodon rRNase
N	**OmpF**	TolQ,R,A	Pore
K	Tsx	**OmpF**, OmpA, TolQRAB	Pore
U, 28b	OmpA	**OmpF**, LPS, TolQ,R,A,B	Pore
DF13	Iut	TolQ,R,A	16s rRNase

a, Omp possibly involved in translocon mechanism are marked in bold.

3. FUNDAMENTAL INFORMATION

3.1. Genetics & mutations

At least 12 colicins are known, from genetic and mutation studies (6, 8), to require two outer membrane receptors for cytotoxicity (Table 1). All are group A (Tol-dependent) colicins, including colicins A, E1- E9 and K. It is curious that a dual receptor requirement has not been reported thus far for any group B, TonB-requiring colicins. There are only a few known combinations for the dual receptor requirement. Colicin A, the nuclease colicins E2-E9, and E6-E9 are known to utilize the outer membrane proteins, BtuB and the OmpF porin. Colicin E1 utilizes BtuB/TolC. One can note that for these cases, at least one of the Omp required for colicin action, OmpF or TolC, possesses an ion channel activity that is associated with a relatively large channel aperture. As an exception from the dual receptor requirement, it seems that only one OM protein, OmpF, is utilized by colicin N to cross the outer membrane. The ubiquitous use of OmpF in colicin translocation is presumably a consequence of (a) its great abundance in the outer membrane (~ 10^5 copies per cell (9)) and (b) the relatively large aperture of its channel lumen (7 Å x 11 Å (10)).

3.2. Structures

3.2.1. Outer membrane proteins, candidates for receptors, and Tol proteins

The 16 strand □-barrel crystal structure of OmpF (10) shows each channel of the tripartite porin to be limited by a loop, the "L3 loop" that sets an 7 Å x 11 Å limitation on the cross-section of the channel lumen (Figure 2A). Crystal structure data is also available for TolC (11) (Figure 2B), TolB (3, 4) (Figure 2C), and the C-terminal domain of TolA (2). TolB is particularly relevant in the present discussion because colicin E9, a homologue of colicin E3, is known to have an N-terminal recognition sequence for TolB (12).

The 22 strand β-barrel BtuB (13) (Figure 3A) is a recent addition to the family of outer membrane receptors that have a 22 strand β-barrel structure plugged by an N-terminal globular domain. This family includes FhuA (14) (Figure 3B), FepA (15) (Figure 3C), and FecA (16, 17) (Figure 3D).

3.2.2. Colicins

Crystal structures are also available for the major part of three colicins, Ia (18), E3 (19), and N (20) (Figures 4A-C), as well as of the C-terminal activity domain of colicins A (21), E1 (22), E7 (23, 24) and E9 (25, 26) (Figure 5A-C). The crystal structures of the intact colicins Ia and E3 show the catalytic (C), receptor binding (R), and translocation (T) domains, and also the tightly bound immunity protein in the case of colicin E3 (19). A prominent feature of the structures is the elongated coiled-coil, 100 Å and 160 Å in length, respectively, in the structures of colicins E3 and Ia. This coiled-coil overlaps the polypeptide segment that is known from mutation analysis to be associated with the function of receptor-binding (27), as well as the 76 residue receptor binding peptide of colicin E9 (28). This overlap leads to additional questions:

6. How much of the coiled-coil is involved in direct binding to the receptor?

7. Does this extended coiled-coil structure have any additional function besides the initial binding?

3.2.3. Structure of the complex of the BtuB with colicin receptor-binding domain

The 2.75 Å structure, derived from crystals formed in detergent, of the complex between the cloned 100 Å long 135 residue coiled-coil ("R135") of colicin E3 (19) and the BtuB receptor (29), might provide some answers to questions 1, 5, and 6 above. The structure shows that only one-fifth of the 135 residue coiled-coil of the putative receptor-binding domain is actually involved in direct binding interactions with the BtuB receptor in its extracellular loop region (29). The peripheral nature of the binding interaction of the coiled-coil with BtuB does not, however, imply that the binding is weak. The dissociation constant of the R135 coiled-coil is 0.5 -1.0 nM, as determined by surface plasmon resonance (29) and microbiological spot tests. The latter assay has shown that the K_d for the intact colicin E3 with BtuB is at least 100 times smaller and the binding correspondingly tighter. Another striking feature of the structure of the BtuB-R135 complex is the oblique orientation of the bound R135 that would result in the significant extension (~ 70 Å) of the 100 Å coiled-coil laterally along the membrane plane from the center of BtuB (Figure 6).

4. TRANSLOCATION ACROSS THE OUTER MEMBRANE

The interaction of R135 primarily with the extracellular loop domain of BtuB contrasts with our initial "nail" hypothesis (cited in (7)), based (i) on the appearance

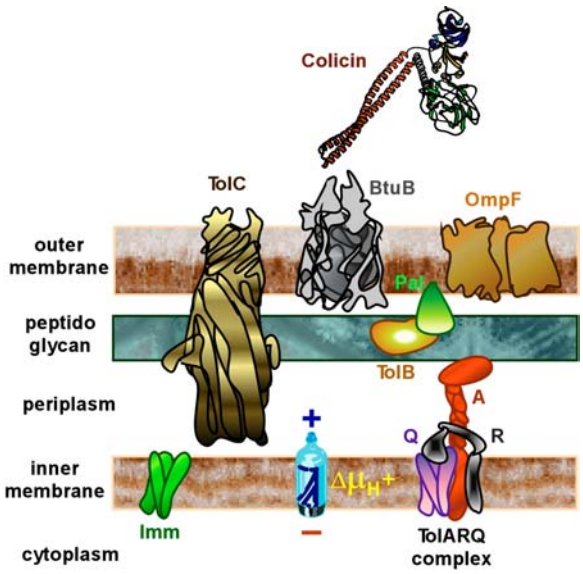

Figure 1. Components of Tol-Pal cellular import pathway for colicins of group A (**Table 1**).

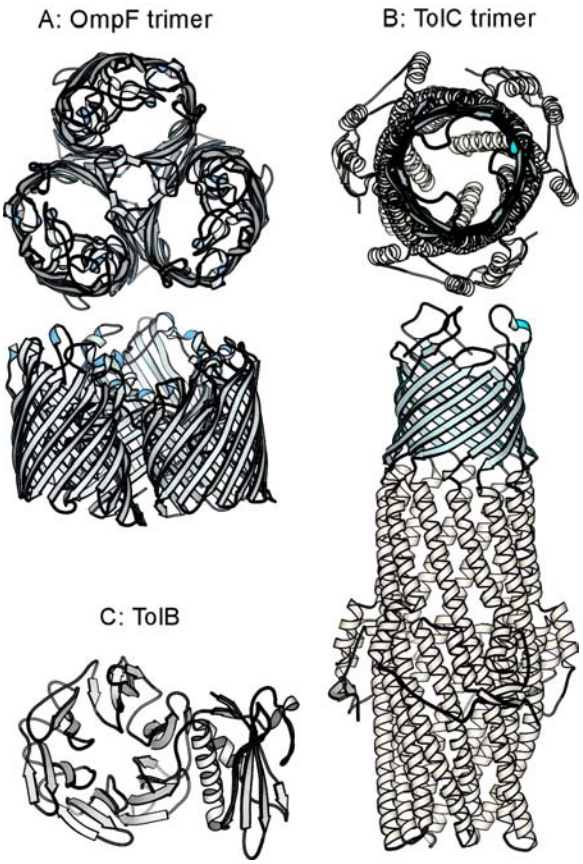

Figure 2. Ribbon diagrams of (A) OmpF trimer, (B) TolC, and (C) TolB. Top (upper) and side views are shown for OmpF and TolC.

of the elongate coiled-coil, and (ii) the assumption that the translocation mechanism involved only one OM receptor, implying that translocation of the colicin must therefore involve passage through BtuB. The tight binding that barely extends beyond the peripheral region of BtuB implies, however, that the primary role of BtuB is only to bind the colicin and thereby to concentrate it in the 2-dimensional space of the outer membrane surface. The other set of experimental data that argue against passage of the colicin through BtuB is the absence of large ion channels (> 20 pSiemens) associated with BtuB inserted into planar bilayers (Eroukova *et al*., manuscript in preparation). It is noted here that preliminary measurements of such a channel activity in BtuB were initially confused by a small contamination of OmpF. An absence of large channel activity has also been found with purified FepA outer membrane receptor (provided by P. Klebba) incorporated into planar bilayers (Eroukova *et al*., manuscript in preparation).

If colicin E3 is not translocated through BtuB, how does the colicin cross the outer membrane and, referring to question 3 above, is the colicin translocated in a folded or unfolded state (7)? The requirement for OmpF porin, shown by loss of colicin cytotoxicity in an $OmpF^-$ mutant (30), suggested the possibility that the porin may provide the avenue for transfer. OmpF is the "universal pore" or channel through the *E. coli* outer membrane for small hydrophilic solutes with molecular weight ≤ 600 Da (9). It contains a tripartite channel structure in which each of the three channels has a lumen cross-section of 7 Å x 11 Å (10). The role of the OmpF channels as a pore has been considered for the transfer of colicin N (31).

As noted above, OmpF is the only required outer membrane protein for colicin N (Table 1). This is of interest in connection with the fact that the extended coiled-coil domain of colicins E3 and Ia (Figures 4A,B) is replaced by a single helix of much shorter length in colicin N (Figure 4C). Penetration of colicin N into or through the lumen of OmpF was suggested by colicin N resistance of an OmpF mutant strain with a Gly119Asp substitution in the L3 loop. This residue was shown in the structure analysis to participate in the definition of the 7 x 11 Å aperture through the channel lumen (32) that would be used for translocation of any polypeptide. It was specified in this study that the colicin would have to be unfolded to be transferred through the porin channel. It was further noted that colicin A, which also utilizes OmpF (Table 1), must be unfolded to utilize this channel because, with 592 residues, it is much larger than the 385 residue colicin N. The proposal that colicin A must be unfolded for passage through the OM had been made previously by others (33, 34). In addition, Bainbridge *et al.* (35) raised the interesting questions as to whether the L3 loop had to be displaced for colicin to penetrate the channel. They approached this problem by testing the effect of disulfide cross-linking of L3 on the colicin cytotoxicity of OmpF site-directed Cys mutants. As in the studies of (32), the activity of colicin N or A could be inhibited by a single cysteine substitution in loop L3 at positions 107 and 168. These residues were different than were used for mutagenesis in (32). It was argued that the loop-wall double

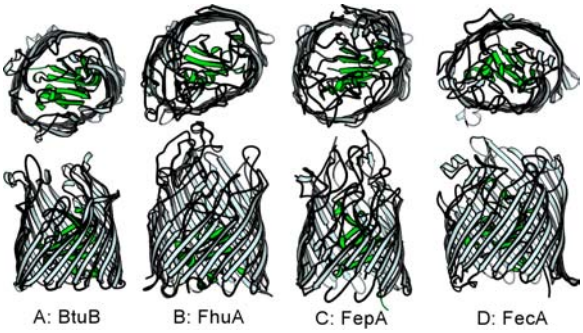

Figure 3. Ribbon diagrams of structures of outer membrane receptors of E.coli, (A) BtuB, (B) FhuA, (C) FepA and (D) FecA, shown in top and side views.

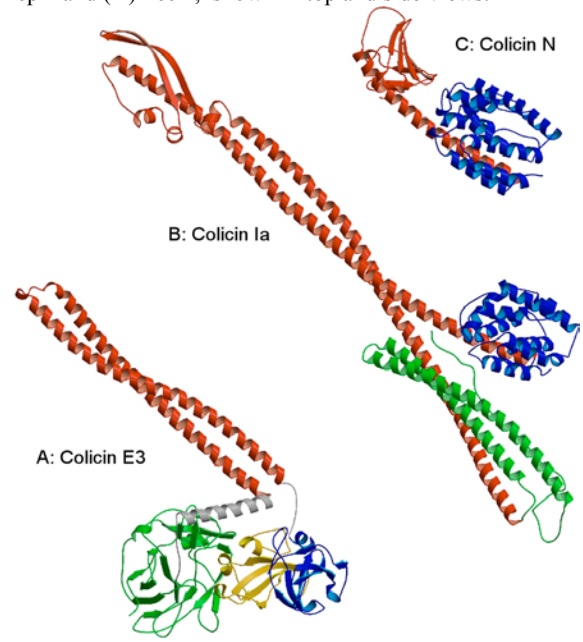

Figure 4. X-ray crystal structures of colicins (A) E3, (B) Ia and (C) N. Colors of translocation (green), receptor-binding (red) and activity (blue) domains are based on the domain organization of colicin E3, taking into account the role of the extended coiled-coil discussed in the text.

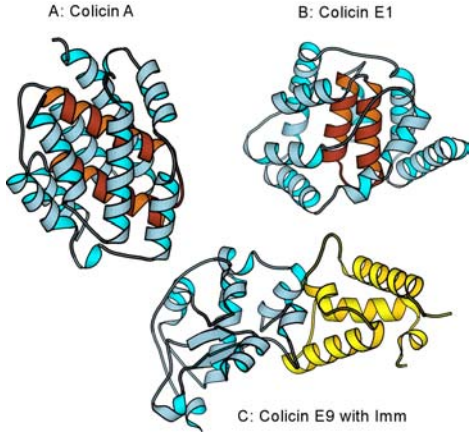

Figure 5. X-ray crystal structures of the C-terminal activity domains of colicins (A) A, (B) E1 and E9 with (C) the immunity protein.

substitution C117/C312, which caused a small inhibition of colicin N activity, was a result of the disulfide cross-link. However, the possibility that the inhibition arose from the cumulative effect of two mutations with individual small effects cannot be excluded, since there was no change in the electrophoretic mobility of the putative cross-linked mutant. Whether these experiments imply that small conformational changes of L3 are necessary for colicin translocation through the OmpF lumen was not established. However, colicin passage is certainly sensitive to perturbation by these mutations.

A model for translocation of colicin N proposed that insertion of colicin N into the OmpF receptor could be initiated and directed by insertion and threading of the disordered and unfolded 66 residue N-terminal translocation domain that would be recognized in the periplasm by TolB (Figure 6 in (20)). It was assumed that the C-terminal channel-forming domain could not be unfolded because of insufficient energy available and therefore could not be translocated through the OmpF pore. It was also proposed that the colicin N receptor-binding domain remained on the extracellular side of the outer membrane during colicin translocation (20), based on trypsin reversal effects on cytotoxicity of colicins E2, A, K, and E1, and reversal of colicin inhibition of cellular metabolism (34, 36, 37). The proposal for translocation of the channel domain through the lipid-lipopolysaccharide region of the outer membrane peripheral to OmpF (20), besides having the problem of demonstrated energetic feasibility (shared by all models), also seems somewhat vaguely defined in terms of its structural basis.

5. UNFOLDING; THE ENERGY SOURCE.

5.1. Colicins have an unfolded N-terminal segment

As discussed above, the N-terminal 66 residues of colicin N are believed to be disordered. The glycine-rich N-terminal 83 residue segment of colicin E3 is disordered and not seen in the electron density (19). Similarly, NMR analysis of colicin E9 showed an absence of secondary structure in the majority of the 83 N-terminal amino acids (38). Furthermore, 65 of the N-terminal 82 residues of colicin Ia were not resolved in its structure determination (18). Thus, it may be inferred that a disordered and readily unfolded conformation is a structural characteristic of the N-terminal segment of the translocation domain of the colicins.

Recent studies on the binding of immunity protein to colicin E9 (a homologue of E3) and its C-domain alone showed that the affinity of the T-domain for immunity protein is small ($K_d \approx 10^{-2}$ M) (38). Thus, the tight binding of the R-domain to BtuB ($K_d \approx 10^{-9}$ M) could provide sufficient energy to release and unfold the T-domain. As proposed by Vetter *et al.* (20), there is no obvious steric barrier to the entry of an unfolded segment of the translocation domain into the OmpF lumen. It is, however, unlikely that this N-terminal domain can thread through OmpF without energy input. Based on our finding that OmpF channels in planar bilayers are occluded by

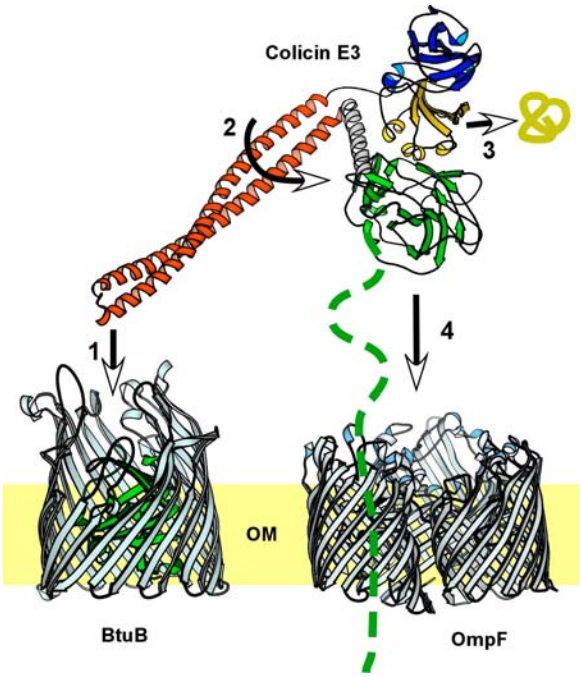

Figure 6. "The fishing pole" model of colicin translocation through outer membrane. Steps and structural transition in colicin E3 include: (1) binding of colicin E3 with BtuB through coiled-coil receptor-binding domain (red); the 100 Å coiled-coil domain is shown in its approach to the binding site in the extracellular loop region of BtuB (29). In its bound state , 1/5 of the coiled-coil is in direct contact with BtuB and the axis of the coiled-coil makes an angle of 40^{o} with the membrane plane (29), as shown. (2) Tight binding induces partial unfolding of the receptor-distal segment of coiled-coil. (3/4) The flexible T-domain is delivered to the membrane by the extended coiled-coil and, because of its flexibility, calculated from formulae presented in ref. (47), and the disorder in the N-terminal 80 residues of the translocation domain, is able to "fish" for OmpF. The unfolding of the coiled-coil, the interaction of OmpF with the T-domain, and tension exerted by TolB pulling on the T-domain, may exert forces that weaken the C-domain interaction with Imm, resulting in Imm release and unfolding of the C-domain.

colicin E3, and that the occlusion *in vitro* is dependent upon a membrane potential (*cis*-negative relative to the side of OmpF addition, and *trans*-negative relative to that of colicin addition), we propose that threading energy is provided by a potential on the order of 50 mV, negative on the periplasmic side across the outer membrane. It has been argued that the existence of such a potential is unlikely because of the high conductance of the outer membrane pores (39). On the other hand, the outer membrane, with its lipid/LPS asymmetry, is predicted to contain an asymmetric dipole potential and thereby an intrinsic trans-membrane potential of approximately 80 mV, periplasmic side negative (40).

5.2. Unfolding of the C-terminal domain

Certainly, the unfolding of colicin E3 has a formidable energy barrier because of the need to remove the immunity protein before the colicin enters the cytoplasm (41). This was inferred from studies on the proteolytic processing of nuclease colicins by LepB protease. Key findings were that the protease acts before the C-domain enters the cytoplasm and the immunity protein protects the colicin molecule against proteolytic cleavage. However, if the counterpart of this logic is that the immunity protein must be removed prior to translocation across the outer membrane (41), then the C-terminal domain must be unfolded. The energy that drives the unfolding may come from two sources: (i) threading of the N-terminal translocation domain that is driven by an electrophoretic process whose source is the potential across the outer membrane. The threading through the OM would inevitably unfold the remainder of the colicin. (ii) In addition, the 2.75 Å structure of the R135-BtuB complex, along with difference far-UV circular dichroism analysis, showed that the coiled-coil receptor binding domain is partially unwound and unfolded upon binding to BtuB (29). Unfolding of the R-domain would tend to trigger unfolding of the remainder of the colicin (42).

6. A MODEL FOR TRANSLOCATION OF COLICIN E3 ACROSS THE OUTER MEMBRANE

(i) Colicin E3 binds with sub-nM affinity to the BtuB receptor, using only its receptor-proximal tip for direct binding (Figure 6), and is thereby concentrated in the 2-dimensional space of the outer membrane surface. (ii) The complex diffuses in the outer membrane, as does OmpF. Although the surface density of BtuB is low (200-400/cell), OmpF is very abundant (~ 10^5 molecules/cell), which implies a surface density of approximately $1/10^4$ $Å^2$). Thus, BtuB readily collides with an OmpF. (iii) The extended coiled-coil acts like a "fishing rod" to contact the OmpF neighbor, and like a "crane" to deliver the colicin translocation domain to the extracellular domain of OmpF, thus forming a two receptor "translocon" (Figure 6). (iv) Contact would be made through sequences of the colicin and OmpF that are not yet identified. (v) The unfolded 83 residue N-terminal end of colicin E3 enters the OmpF lumen and is threaded through one of the three channels of OmpF into the periplasm using an electrophoretic mechanism based on the potential across the outer membrane. (vi) The energy available for threading is increased when the colicin is bound in the periplasm by TolA or TolB. It is known that TolB interacts with the T-domain of colicin E9, using the pentapeptide sequence (DGSGW, residues 35-39) for a recognition, and presumably, as an interaction site (3, 38). (vii) The total threading energy would pull on the T domain that remains on the extracellular side, and causes release of the immunity protein and unfolding of the C-terminal activity domain. (viii) The colicin remains bound to BtuB as it is translocated. However, the colicin R-domain must ultimately be released from BtuB, perhaps as a consequence of the interaction between BtuB and OmpF. It would subsequently be freed by cleavage in the periplasm, as was shown for colicin D that is processed by inner membrane peptidase LepB (43). Binding of the C-domain to OmpF was shown for colicin N (44).

A discussion of the mechanism by which the colicin E3 C-domain can cross the cytoplasmic membrane

is beyond the scope of the present review. We note, however, that the ability of colicin E9 to form channels in planar bilayer membranes (45) is relevant to this problem.

7. ACKNOWLEDGMENT

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Abbreviations: LPS, lipopolysaccharides; OM, outer membrane; Omp, outer membrane protein; R, receptor-binding domain; R135, 135 residue coiled-coil of colicin E3 that includes its receptor-binding domain; T, translocation domain; Tol, tolerant

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