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THE COLICIN Ia RECEPTOR, Cir, IS ALSO THE TRANSLOCATOR FOR COLICIN Ia

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SUMMARY

Colicin Ia, a channel-forming bactericidal protein, uses the outer membrane protein, Cir, as its primary receptor. To kill *E. coli*, it must cross this membrane. The crystal structure of Ia receptor-binding domain bound to Cir, a 22-stranded plugged β -barrel protein, suggests that the plug does not move. Therefore, another pathway is needed for the colicin to cross the outer membrane, but no “second receptor” has ever been identified for TonB-dependent colicins, such as Ia.

We show that if the receptor-binding domain of colicin Ia is replaced by that of colicin E3, this chimera effectively kills cells, provided they have the E3 receptor (BtuB), Cir, and TonB. This is consistent with wild-type Ia using one Cir as its primary receptor (BtuB in the chimera) and a second Cir as the translocation pathway for its N-terminal translocation (T) domain and its channel-forming C-terminal domain. Deletion of colicin Ia’s receptor-binding domain results in a protein that kills *E. coli*, albeit less effectively, provided they have Cir and TonB. We show that purified T-domain competes with Ia and protects *E. coli* from being killed by it. Thus, in addition to binding to colicin Ia’s receptor-binding domain, Cir also binds weakly to its translocation domain.

Keywords

colicin; TonB; membrane translocation; TonB-dependent transporters

INTRODUCTION

Colicins are plasmid-encoded bactericidal proteins that are produced by many *E. coli* in times of stress. They kill competing *E. coli*, either by making a voltage-dependent channel in the inner membrane of the target cells or, in the case of some other colicins, by nuclease action in the cytoplasm or by degrading peptidoglycan (Braun *et al.*, 1994; Cascales *et al.*, 2007). They all must penetrate the outer membrane barrier of target cells in order to reach their site of action in susceptible *E. coli*. To do that, colicins have coopted a small number of receptors (FhuA, FepA, BtuB, and Cir) on the outer membrane that are normally involved in the uptake of essential nutrients, such as siderophore-bound iron or cobalamin. These receptor proteins belong to a family sometimes referred to as ligand-gated transporters and share common structural features; they are all 22-stranded β -barrel proteins with an N-terminal globular plug domain that faces the periplasm-exposed side of the protein in the outer membrane (Cao & Klebba, 2002; Buchanan, 2005). All of the transporters are

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dependent on the TonB protein for substrate uptake and have, near their N-termini, a seven-residue stretch called the TonB box. Mutations in this sequence abolish or reduce uptake of substrate by affecting interaction of the transporter with TonB in the periplasm. With its inner membrane partners, ExbB and ExbD, TonB is thought to energize the process and somehow remove or rearrange the plug domain, to allow substrate uptake. How a large protein toxin, such as colicin Ia, utilizes the receptor for a small nutrient, such as ferrichrome or vitamin B12, to gain access to the interior of the bacteria is the subject of the present work.

In addition to using these high affinity receptors, the colicins have also expropriated either of two groups of *E. coli* periplasmic and inner membrane proteins to facilitate their entry into susceptible cells. Group A colicins (E colicins, A, and N, *e.g.*) use the TolA, B, Q, R group of proteins for translocation across the outer membrane, whereas Group B colicins (Ia, Ib, B, D, and M) use TonB, ExbB, and ExbD, (Lazdunski *et al.*, 1998). All colicins have evolved to have a modular domain structure, with an N-terminal translocation (T) domain, a central receptor-binding (R) domain, and a C-terminal catalytic or channel-forming (C) domain (Braun *et al.*, 2002; Cascales *et al.*, 2007). Peptides consisting only of the R domain and adjacent coiled-coil sequences bind with high affinity to the appropriate receptor protein, and can thus protect cells from killing by the corresponding intact colicin (Penfold *et al.*, 2000), and co-crystallize with the receptor (Kurusu *et al.*, 2003; Buchanan *et al.*, 2007; Sharma *et al.*, 2007). Near their N-termini, in the T domain, colicins have short sequences, called the TolA box, the TolB box or the TonB box, that interact with the corresponding component of the *E. coli* translocation machinery; mutations in those “box” sequences reduce or abolish killing activity (Bouveret *et al.*, 1997), although the mutant colicins can still bind tightly to sensitive cells, and their killing activity (pore-formation or nuclease activity) is not impaired when tested *in vitro* (Mende & Braun, 1990; Buchanan *et al.*, 2007). Compensatory mutations in the corresponding target cell protein can restore killing activity by these T-domain colicin mutants, as shown for TonB (Heller *et al.*, 1988; Schoffler & Braun, 1989; Bell *et al.*, 1990; Braun *et al.*, 2002), implying that an interaction between the colicin and the Tol protein or TonB protein is necessary for killing to occur. In fact, in the case of the Group A colicins, physical binding between the translocation domains and components of the Tol apparatus have been measured. Isothermal titration microcalorimetry (ITC), surface plasmon resonance (SPR), and tryptophan fluorescence have been used to measure binding in the μM range of the C-terminal parts of TolA to the T domain of colicin N, which does not require TolB for cell killing (Raggett *et al.*, 1998; Gokce *et al.*, 2000). In the case of the enzymatic E colicins, as exemplified by colicin E9, direct binding of residues in the unstructured part of the N-terminal translocation domain to TolB was demonstrated by SPR, with a $K_D \sim 14 \mu\text{M}$ (Hands *et al.*, 2005). Binding of the colicin A T domain to both Tol A and Tol B has been detected, using *in vitro* cross-linking or detection of binding partners with monoclonal antibodies (Bouveret *et al.*, 1998; Journet *et al.*, 2001). Thus, the T domains of Group A colicins bind to one or more members of the Tol family in the periplasm in an essential step of colicin import. In fact, a portion of the natively disordered portion of the colicin E9 T domain was crystallized bound in the pocket of the TolB β -propeller where the Pal (peptidoglycan-associated lipoprotein) normally binds (Loftus *et al.*, 2006), confirming the physical interaction between a segment of the toxin and its periplasmic binding partner in the target cell.

Thus, at least two distinct binding steps—high affinity binding by the R domain to an outer membrane receptor and a much weaker interaction of the T domain with one or more periplasmic proteins—occur before the final lethal step in target cell killing by a Group A colicin molecule. For TonB-dependent colicins, in addition to the high affinity binding of the colicin receptor-binding domain to its outer membrane receptor, two distinct interactions of TonB have been inferred—an interaction with the colicin’s outer membrane receptor and

an interaction with the colicin itself (Braun *et al.*, 2002; Buchanan *et al.*, 2007). Any model of colicin transport into sensitive bacteria must account for these two separate sets of interactions.

Until the high resolution structures of two different colicin receptor domains bound to their cognate receptors were solved, models for colicin penetration envisioned passage of some or the entire toxin through the barrel of the receptor prior to a step involving interaction of the T domain with a Tol protein or TonB. However, both the structure of BtuB with bound R domain of colicin E3 or E2 (Kurusu *et al.*, 2003; Sharma *et al.*, 2007) and the structure of Cir with bound R domain of colicin Ia (Buchanan *et al.*, 2007) revealed little or no change in the position of the plug domain upon colicin binding, so it no longer seemed possible that even an unfolded colicin molecule could pass through the receptor barrel into the periplasm. For the Tol-dependent (Group A) colicins, however, there was a plausible alternative pathway through the outer membrane. Co-receptors or translocons had been identified genetically many years ago, although their role in killing was obscure. *OmpF* mutants have been isolated which are insensitive to colicins E2, E3, E6–E9, and colicin A (Benedetti *et al.*, 1989), and *tolC* mutants are not killed by colicin E1 (Whitney, 1971; Davies & Reeves, 1975; Masi *et al.*, 2007), although binding of these colicins to BtuB is not affected. Recently, a complex of *OmpF*-BtuB-colicin E9 was reported, supporting the idea that the colicin uses its binding to the receptor to recruit *OmpF* (Housden *et al.*, 2005). In addition, channels formed by *OmpF* or *TolC* in planar lipid bilayer membranes are occluded by colicin E3 or colicin E1, respectively, but not by the other colicin (Zakharov *et al.*, 2004). It was therefore proposed that colicin E3 binds tightly to BtuB via its R domain and then uses its attachment at the membrane surface to search for a nearby copy of the *OmpF* porin, through which the T domain begins passage through the membrane (Zakharov *et al.*, 2006). In fact, *OmpF* has been crystallized with a short stretch of colicin E3 T domain in the interior of the pore, adding plausibility to a mechanism whereby E colicins pass through the porin into the periplasm (Yamashita *et al.*, 2008).

For the TonB-dependent colicins, however, no co-transporter or translocon has ever been identified (Cascales *et al.*, 2007; Buchanan *et al.*, 2007). We therefore investigated whether Cir acts as the high-affinity receptor from which colicin Ia searches for a second copy of Cir, which could then serve as the translocon for Ia to pass into the periplasm. In order to test this possible mechanism, we have separated the binding and translocation steps for colicin Ia, by replacing the colicin's receptor-binding domain with that from colicin E3, or by deleting the domain entirely. Our results are consistent with a model in which the N-terminal translocation domain of colicin Ia interacts with Cir independently of the former's receptor-binding domain, to initiate TonB-dependent translocation of the colicin.

RESULTS

A hybrid colicin with E3 R domain and Ia T and C domains requires Cir and TonB for killing

We created a new colicin (called IaE3R) by replacing the receptor-binding domain of colicin Ia and adjacent coiled-coil residues (residues 250 to 407) (Mankovich *et al.*, 1986; Wiener *et al.*, 1997) with a comparable 135-residue R-domain segment from colicin E3 which binds to BtuB (residues 312 to 447) (Masaki & Ohta, 1985; Kurisu *et al.*, 2003) (Figure 1). The newly-created protein was expressed and purified following normal procedures for purifying colicin Ia. Remarkably, this hybrid colicin had very high killing activity per mg on wild-type indicator *E. coli*, comparable to the specific activity of colicin E3, and only one order of magnitude lower than that of wild-type colicin Ia (Fig. 2). (It is possible that the ~10-fold reduction in specific activity of the chimera vs. wild-type colicin Ia is simply a reflection of the ~20-fold lower number of BtuB receptors per cell, 200 (Sabet & Schnaitman, 1973), compared with about 5,000 Cir receptors per cell (Konisky & Cowell, 1972)). As was

expected, this new colicin required an intact BtuB receptor protein for full activity; killing was reduced by 3 orders of magnitude on a *btuB* mutant that does not bind E3R. Surprisingly, however, its killing activity was not zero on this *btuB* mutant; the hybrid colicin could be diluted to 1 µg/ml and still inhibit growth of a lawn of *btuB* mutant cells. Colicin E3, by contrast, did not kill these *btuB* mutant cells even at the highest concentration tested, 1 mg/ml (Fig. 2). Despite having acquired a requirement for the BtuB receptor protein for full activity, the IaE3R hybrid had no killing activity at all on a *cirA* deletion mutant, just as wild-type colicin Ia had no activity on this *cir* mutant. It was further demonstrated that the hybrid IaE3R colicin still required the TonB protein for killing, but did not require TolA, which is absolutely required for killing by colicin E3 (Fig. 2). On the *tonB* mutant with a complete deletion of the gene, OKN1 (Ma *et al.*, 2007), no killing was detected at any concentration of either colicin Ia or the chimeric IaE3R. On another *tonB* mutant, CGSC #11229, characterized as having a kanamycin transposon within the *tonB* gene, there was detectable killing at 100 µg/ml of either colicin (not shown).

Colicin Ia does not require a receptor-binding domain to kill *E. coli*

The fact that IaE3R had measurable, albeit greatly reduced, killing activity on the *btuB* mutant indicator strain, which is completely insensitive to colicin E3, suggested that another domain of the colicin besides its R domain can bind to the outer membrane and initiate translocation of the toxin into a susceptible cell. To test whether a receptor-binding domain is absolutely required for killing by colicin Ia, the R domain and adjacent residues from the coiled-coil, from residues 251 to 408, were deleted from colicin Ia, joining the T domain directly to the channel-forming domain (Fig. 1). The resulting protein (IaΔR) had the same specific activity on the *btuB* mutant cells as the hybrid IaE3R, and had measurable activity on wild-type *E. coli*, 10⁴-fold less than the activity of wild-type colicin Ia on the same bacteria (Figure 2). This colicin without a receptor-binding domain still required both the Cir and TonB proteins for killing, however (Fig. 2). Since the channel-forming domain alone cannot kill *E. coli* (Fig. 2), binding and translocation of IaΔR must be initiated by the T domain, which implies that T domain can bind independently to Cir, albeit with a greatly reduced efficiency that is reflected in the reduced killing efficiency of the IaΔR relative to that of intact colicin on wild-type cells.

Isolated T domain protects *E. coli* from killing by colicin Ia, IaE3R, and IaΔR

Four different N-terminal fragments of colicin Ia were cloned and purified. The first, T225, comprised the first 225 residues of the protein, the entire T domain from the crystal structure (α-helices T1, T2 and T3, which form a side-by-side antiparallel helical sheet) (Wiener *et al.*, 1997), which was synthesized and purified with a C-terminal histidine tag. A second construct consisted of just the first 64 amino acids of colicin Ia (T64) with a C-terminal histidine tag, representing the largely unstructured N-terminal portion of the colicin and containing the TonB box sequence. In addition, T domains with deletions of residues 2-64 (TΔ2-64) and of the TonB box (residues 23-27, TΔTonB box) were constructed, cloned, and expressed.

The ability of isolated T domain (T225) to compete with colicin Ia or either IaE3R or IaΔR and protect cells from killing was assayed in liquid culture experiments. At sufficiently high molar excess, the full-length T domain construct gave substantial protection from killing of wild-type *E. coli* by either colicin Ia or IaΔR (Table 1). Similarly, both wild-type and *btuB*⁻ indicator cells were protected by T225 from killing by IaE3R (Table 2 and Fig. 3). Despite the fact that ~6,500-fold more IaΔR than wild-type colicin Ia resulted in even less killing of wild-type cells (consistent with the spot test results shown in Fig. 2), protection of those cells from IaΔR by T225 was much more effective than protection from wild-type colicin Ia (Table 1). Thus, the T domain was much more effective at protecting cells from colicin than

cannot bind to its target via a receptor-binding domain. The same appears to be true in the case of IaE3R. Although 1,000-fold more IaE3R was used to kill *btuB*⁻ cells than wild-type *E. coli*, protection by the same amount of T225 was much more effective for *btuB*⁻ cells, to which IaE3R could not bind (Table 2, Fig. 3). Maximal protection was reached at around a molar excess of ~500 molecules of T225 domain per molecule of either IaE3R or IaΔR, when tested on cells without the appropriate receptor, or as much as 3×10^6 molecules of T225 per molecule of colicin, in cases where the colicin had a receptor-binding domain and the target cells had the receptor; these represent the maximum concentration of T225 that could be obtained in the assay, and protection appeared to level off at approximately 60% survivors, compared with a culture to which no colicin was added (Fig. 3). This concentration of T domain represents an excess of as much as 1.6×10^5 T domain per copy of Cir, based on an estimate of 5,000 receptors/cell (Konisky & Cowell, 1972). It is clear that the binding of T domain to Cir must be very weak, given the large excess needed for protection from killing.

When a molar equivalent amount of bovine serum albumin was added to mixtures of *E. coli* and the colicin Ia constructs as a control, no protection from killing resulted; if anything, killing was sometimes even enhanced (Tables 1, 2 and 3), a non-specific effect of protein addition that has sometimes been observed in the past without explanation (K.S. Jakes and N.D. Zinder, unpublished results).

Much shorter portions of the T domain of colicin E3 appear to be involved in that colicin's interaction with OmpF. Housden *et al.* (2005) showed that deletion of the first 60 residues of colicin E3 completely abolished the ability of the colicin to recruit OmpF in a complex of the colicin and BtuB. A peptide consisting just of the intrinsically disordered first 83 residues of colicin E3 (T83) occludes OmpF channels in planar lipid bilayer membranes, and that fragment appears to crystallize within the pore of OmpF (Yamashita *et al.*, 2008). In order to assess the ability of an analogous portion of the Ia T domain to protect cells from killing by colicin Ia constructs, we cloned and expressed the largely unstructured segment of colicin Ia, consisting of the first 64 residues of the colicin (T64) (Wiener *et al.*, 1997). Unlike the longer T225, however, T64 did not protect sensitive *E. coli* from killing by IaE3R or IaΔR, at a molar ratio at which nearly 60% protection was seen with T225 (Table 3 and data not shown).

The first 64 residues of T domain are necessary for binding to Cir, but the TonB box is not required

Although the unstructured N-terminal part of T domain alone did not protect cells from killing by colicin Ia, the first 64 residues are necessary for that protection by the longer T domain construct, T225. A T domain construct from which residues 2-64 were deleted (called TΔ2-64) afforded no protection to cells from killing by either IaE3R or IaΔR (Table 3). The TonB box, residues 23-27 of colicin Ia, is not involved in the binding of T domain to receptors, however. A T domain construct from which only residues 23-27 had been deleted (called TΔTonB box) protected cells from killing by either IaE3R or IaΔR about as efficiently as full-length T domain (Table 3).

Inhibition of killing at very high concentrations of colicin Ia

We have observed that when cells growing in liquid culture are treated with wild-type colicin Ia at high concentrations of the colicin, representing saturation of all of the Cir receptors, killing is actually inhibited. At a much lower colicin concentration, killing is very fast, as measured by the cessation of an increase in culture turbidity (Fig. 4). Thus, while cells treated with colicin Ia at 0.15 μg/ml stopped growing immediately, treatment with 15 μg/ml of colicin Ia allowed growth that was almost as robust as the untreated control

culture. These concentrations represent about 8,700 molecules of colicin per cell, or ~1.7 molecules of colicin per Cir receptor (based on the estimate of 5,000 Cir/cell (Konisky & Cowell, 1972)) at 0.15 $\mu\text{g/ml}$, and about 170 molecules of colicin Ia per receptor at 15 $\mu\text{g/ml}$. Thus, there is virtually no killing when every receptor is occupied, whereas killing is very efficient when not all receptors are occupied. The implications of this result are discussed in the following section.

DISCUSSION

We have identified Cir, the colicin Ia receptor, as both the outer membrane receptor and the second receptor, or translocon, by which colicin Ia can gain access to the periplasm of susceptible *E. coli*. (This is analogous to the situation with colicin E3, except that there the receptor, BtuB, is different from the translocon, OmpF.) By replacing the receptor-binding domain of colicin Ia with that of colicin E3, we have shown that killing of *E. coli* uses the colicin Ia receptor protein, Cir, for an additional step in the killing process. We propose that, in addition to using Cir as its high-affinity outer membrane receptor, colicin Ia, via its T domain, seeks out a second copy of Cir, which it then uses as its translocon and via which it actually enters the periplasm. The evidence supporting this model is as follows: First, a hybrid colicin, with the T domain and channel-forming (C) domain from colicin Ia and the receptor-binding (R) domain from colicin E3 (Fig. 1) requires **both** the colicin E3 receptor, BtuB, **and** the colicin Ia receptor, Cir, to kill *E. coli*. Second, despite having an altered R domain, the hybrid IaE3R retains an absolute requirement for the TonB protein for killing and does not need the TolA protein, which is used by colicin E3 but not by colicin Ia (Fig. 2). Despite the change in receptor specificity to BtuB, and the fact that colicins that use BtuB require TolA, but not TonB, the IaE3R retains a requirement for the Cir-TonB interaction. A deletion mutant of colicin Ia, lacking any receptor-binding domain at all retains measurable killing activity, provided that the treated cells have both Cir and TonB (Fig. 2). Thus, there is a step in the killing process after the initial binding to the outer membrane that requires both Cir and TonB. Third, an independent interaction of the colicin Ia translocation domain with Cir is supported by the fact that purified T domain can protect *E. coli* from killing by wild-type colicin Ia or by IaE3R or Ia Δ R (Tables 1 and 2). Finally, at high enough concentrations of colicin Ia in liquid cultures, representing full occupancy of all Cir receptors by tightly-bound colicin receptor domain, killing is actually inhibited (Fig. 4), presumably because there are no unoccupied copies of Cir through which the colicin can translocate into the periplasm.

(The inhibition of killing by high concentrations of colicin in liquid culture is sometimes manifested in spot test assays of colicin Ia, as well. We have occasionally observed strange spots, with turbid centers and a clear ring at the periphery, when very high concentrations of colicin were spotted on agar plates with lawns of sensitive cells (K.S. Jakes and S.L. Slatin, unpublished results). In light of our results in liquid culture, our interpretation of those halos is that at the center of the spot, where the colicin concentration is highest, all Cir receptors are occupied and there are none free to translocate a molecule of colicin into the cell. As the colicin diffuses on the plate, to the periphery of the spot, its concentration falls and reaches a level low enough that there are unoccupied Cir molecules through which the toxin can enter the cell, and cause a clear killing zone.)

A peptide consisting of the entire colicin Ia T domain, T225, gave nearly full protection from killing by IaE3R only when measured using a colicin E3-resistant *btuB*⁻ mutant as the target strain (Table 2). When assayed instead using the wild-type strain, which is much more sensitive to killing, T225 gave ~20-fold protection. This compares with >3,000-fold protection against the 100 times more colicin IaE3R used to kill the *btuB* mutant in the same experiment (Table 2). Presumably, since the IaE3R colicin can bind efficiently to BtuB on

the outer membrane of the wild-type cells, the colicin is thereby concentrated at the cell surface and can more easily “find” a copy of Cir to gain access to the cell. Once that happens, the cell is destined to die. In the case of the hybrid IaE3R killing the *btuB*[−] mutant, the colicin and the T225 are competing on a more equal playing field, each searching for Cir in three dimensional space. Even given the fact that presumably the T domains of the hybrid colicin and T225 have more or less equal affinities for Cir, a 500-fold molar excess of T225 was necessary to prevent killing of the target cells. Similar results were seen when T225 protection of wild-type *E. coli* from killing by wild-type Ia was compared with IaΔR (Table 1). These results are a powerful demonstration of the effect of concentrating colicin at the cell surface by tight binding to a receptor; killing is enhanced by several orders of magnitude over killing where the search for an entryway is done in three dimensions, rather than in two dimensions, at the cell surface.

Binding of colicin Ia T domain to Cir appears to be dependent on the first 64 residues, which are unstructured in the crystal structure (Wiener *et al.*, 1997), since deleting those residues abolished the ability of the T domain to protect cells from killing by the colicin (Table 3). This is consistent with experiments with the Tol-dependent colicins. Deletions encompassing some or the entire 83-residue unstructured N terminus of colicin E9 reduced or abolished recruitment of its translocon, OmpF, to a complex with BtuB and the colicin (Housden *et al.*, 2005; Bonsor *et al.*, 2008). The unstructured domain alone (T64), however, cannot bind Cir and protect cells from killing by colicin Ia (Table 3).

The initial interaction of T domain with Cir does not involve the TonB box. Deletion of residues 23-27 did not impair the ability of T domain to protect sensitive cells from killing by either IaE3R or IaΔR (Table 3). This result was not surprising, since the interaction of colicin with TonB occurs in the periplasm, presumably after the N terminus has traversed the outer membrane.

A cartoon of our model for how colicin Ia enters a susceptible cell is depicted in Fig. 5. Colicin Ia first binds with high affinity (reported K_{assoc} of $1 \times 10^{10} \text{ M}^{-1}$ (Konisky & Cowell, 1972)) to one copy of its receptor, the Cir protein, as shown in Fig. 5A. That binding has been shown not to displace the plug domain of Cir (Buchanan *et al.*, 2007). Extending at about a 45° angle away from the receptor, the extended shape of colicin Ia thereby positions the T domain at ~80 Å away from the receptor (Buchanan *et al.*, 2007) and enables it to search for a second copy of Cir nearby, from among the ~5,000 copies of Cir/cell (Konisky & Cowell, 1972). In fact, from this picture alone, it is difficult to see how a colicin Ia molecule could use the same copy of Cir for both the initial binding step and translocation. We speculate that the flexible, largely unstructured N-terminal portion of the T domain of Ia (Wiener *et al.*, 1997) interacts with a nearby second copy of Cir (Fig. 5A). We think that the interaction mimics binding of the natural micronutrient substrate to the receptor and initiates an interaction of the TonB box of that copy of Cir with a molecule of TonB (Pawelek *et al.*, 2006; Shultis *et al.*, 2006) (Fig. 5B). (An alternative possibility, consistent with the observations of Baboolal *et al.* with colicin N and its trimeric OmpF receptor/translocon (Baboolal *et al.*, 2008), is that the colicin somehow threads its way down the periphery of the second copy of Cir, at the protein/lipid interface. Such a model is difficult to reconcile with the requirements for a functional TonB box in both the receptor and the colicin, however.) By whatever mechanism rearranges or removes the plug during substrate transport, the plug then similarly rearranges to allow the unstructured N terminus of the colicin to thread through the passageway formed by the β-barrel of Cir. This allows the TonB box of the colicin to reach the periplasm, where it can bind to a second copy of the TonB protein (Fig. 5B), which can provide the necessary energy to begin unwinding the rest of the colicin molecule and pull it into the periplasm. (This model necessitates the dissociation of the R domain from the first copy of Cir, in order for the channel-forming

domain to enter the periplasm via the second Cir.) Once it reaches the periplasm, the channel-forming domain can spontaneously insert in the inner membrane, form a voltage-dependent channel, and kill the cell (reviewed in Jakes *et al.*) (Jakes *et al.*, 1999). Involvement of a second copy of TonB is consistent with data demonstrating *in vivo* dimerization of TonB (Sauter *et al.*, 2003), and increased recruitment of a second TonB to the complex with receptor and substrate (Khursigara *et al.*, 2004; Khursigara *et al.*, 2005). We speculate that the second and third steps of this model are still operating under the conditions of some of the experiments described in this paper, namely, that even in the absence of the initial binding of the R domain to the first copy of Cir, the T domain of either IaE3R or IaΔR binds to a copy of Cir and triggers the TonB-dependent processes necessary to translocate the toxin into the periplasm. The model also accounts for the experimental evidence demonstrating that a functional TonB box is required in **both** the receptor **and** the colicin in order for killing to proceed efficiently (Bell *et al.*, 1990; Buchanan *et al.*, 2007). Thus, as originally suggested by Braun *et al.* (Braun *et al.*, 2002) for colicins B and M, transport across the outer membrane requires two distinct interactions with the receptor.

Recruitment of a second Ton B protein by the natively disordered N-terminus of colicin Ia may be another example of the much better characterized mechanism of “molecular mimicry” that operates to bring E colicins through the outer membrane. Extensive structural and binding experiments with the E9 natively disordered N-terminus have demonstrated that the colicin domain efficiently displaces Pal (peptidoglycan-associated lipoprotein) from its binding pocket in TolB, by “mimicking” the structure of Pal, and thereby recruits TolA to the complex (Bonsor *et al.*, 2007; Bonsor *et al.*, 2009). Presumably, the formation of this complex thus links the outer membrane to the energized inner membrane complex of TolQRA, providing the necessary energy to drive colicin entry through OmpF.

Recent work from two groups investigated whether treatment with colicin B, another TonB-dependent colicin, results in movement of the N-terminal plug domain of its receptor, FepA, into the periplasm, where it becomes accessible to a labeling reagent (Devanathan & Postle, 2007; Smallwood *et al.*, 2009). These investigators, using different labeling reagents, different bacterial colicin-sensitive strains, different colicin concentrations and treatment conditions, and different fractionation and detection procedures, arrived at opposite conclusions. Devanathan and Postle (2007) saw as much as a 25-fold increase in the labeling of several residues of the first 51 amino acids of the N-terminal FepA plug, with much smaller (2 to 4-fold) increases in labeling in the C-terminal portion of the plug region, upon treatment of cells with colicin B. The changes they observed were TonB-dependent, supporting the argument that the increased accessibility of the plug domain that resulted from colicin B treatment meant that the plug domain exits the FepA barrel upon colicin B treatment, and that these measurements were biologically relevant. By contrast, Smallwood *et al.* (2009), using a different label and conditions, observed no change in accessibility of FepA plug domain residues to the periplasmic label upon treatment of cells with colicin B, whether or not the treated cells had a functional TonB protein. Because of the differences in the way these experiments were performed, we believe that it is not yet possible to answer the question of whether the plug domain of the colicin B TonB-dependent receptor, FepA, exits the barrel and becomes accessible to the periplasm upon colicin treatment. Nevertheless, it is clear from our experiments that, at least for the case of colicin Ia, its receptor serves two roles in its uptake—both as the high affinity cell surface receptor and then as the translocon, through which the colicin gains access to the periplasm.

EXPERIMENTAL PROCEDURES

Bacterial strains

The wild-type indicator for assaying colicin killing was K361, which is W3110 *rpsL* (our lab collection); the *btuB*:Tn10 mutant was K1022 (this work), made by P1 transduction (Miller, 1972) of W3110 *rpsL* with a lysate from CGSC#6405 (Hufton *et al.*, 1995) from the Yale Coli Genetic Stock Center; *tolA* was Luria strain A592, obtained as CGSC#4923 from Yale; Δ *tonB760::kan* was CGSC #11229 from Yale; Δ *tonB* OKN1 (Ma *et al.*, 2007) was a gift from P. Klebba (U. of Oklahoma); *cirA51* is CGSC #5814. It should be noted that CGSC #5814 is strain ASH102 (Buxton & Holland, 1973), which was originally characterized as being tolerant to (not killed by) colicin E2. We are not aware that that marker, *cet*, was ever further characterized, but it maps far from *cirA* on the *E. coli* chromosome (Cardelli & Konisky, 1974).

Sensitivity to colicins was measured by spotting 20 μ l of 10-fold serial dilutions of 1 mg/ml solutions of colicin or colicin constructs on lawns of indicator strains spread in melted soft agar on Petri dishes. The inverse of the highest dilution at which inhibition of growth of the indicator lawn is seen is taken as killing units/mg.

Plasmid construction

IaE3R—The 135-residue colicin E3 receptor (R) domain was substituted for the colicin Ia R domain as follows: A *NheI* restriction site overlapping residues 250 and 251 of colicin Ia and a *MluI* site overlapping residues 407 and 408 (Mankovich *et al.*, 1986) were created by two simultaneous site-directed mutagenesis reactions, using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies) on pKSJ340. pKSJ340 has the colicin Ia operon (promoter region, colicin gene and immunity protein gene, from residues 711 to 3,347 of the ColIa sequence (Mankovich *et al.*, 1986)) cloned between the *EcoRI* and *BamHI* sites of pUC19. The resulting plasmid with restriction sites flanking the Ia R domain was digested with *NheI* and *MluI* and the vector-containing fragment with the remainder of the colicin Ia operon was purified.

The E3 R domain of the colicin E3 gene was made by amplification, by PCR, from pKSJ28 (Soelaiman *et al.*, 2001) of sequences encoding residues 312–447 (Masaki & Ohta, 1985), with *NheI* and *MluI* restriction sites flanking the colicin sequences. This fragment was ligated with the cut pKSJ340 from which the colicin Ia R domain had been removed. The resulting plasmid, pKSJ338, encodes a protein with colicin Ia residues 1–249, followed by alanine and serine, residues 312–447 of colicin E3, threonine and arginine, followed by residues 409–626 of colicin Ia. The amino acid residues flanking the E3 R domain result from the restriction sites introduced to effect the splicing of the two proteins. The resulting 603-residue protein, IaE3R, has a molecular weight of 66,964.

Ia Δ R—In order to delete the Ia R domain, complementary mutagenic oligonucleotides were used, with sequences upstream of and including those encoding residue 250, and downstream of and including residue 409. Those oligonucleotides were used to delete residues 251–408 from pKSJ340, using Agilent's QuikChange Site-directed Mutagenesis kit, resulting in pKSJ345. The resulting protein has a MW of 51,938.

Colicin Ia C-terminal channel-forming domain—The channel-forming domain of colicin Ia, consisting of residues 438–626 of the colicin, was CT-M, as described in Kienker *et al.* (Kienker *et al.*, 2000). CT-M was stored at -80°C in 20 mM Tris-Cl, pH 8.4, 150 mM NaCl, at 4.8 mg/ml. This protein was shown to make channels comparable to those made by full-length, wild-type colicin Ia in planar lipid bilayer membranes (Kienker *et al.*, 2000).

T225—Colicin Ia translocation domain with a C-terminal thrombin-cleavable histidine₁₀-tag was cloned and expressed from pET-52b(+) (EMD Biosciences). DNA encoding amino acids 1—225 of colicin Ia was amplified by PCR from pKSJ340, with a 5′-terminal NcoI site and a 3′-terminal SacI site. The T domain-encoding fragment was inserted into pET-52b(+) that had been digested with the same enzymes. The resulting construct had a serine (TCT) instead of alanine (GCT) as its second residue, as a result of ligation within the NcoI site; that was reverted by site-directed mutagenesis, thus destroying the NcoI site in the final construct, but yielding the native sequence of the colicin Ia T domain, in pKSJ344. The resulting protein has a C-terminal, cleavable histidine tag. Protein was expressed from pKSJ344 in BL21(DE) as follows. A single colony was used to inoculate 500 ml Overnight Express Instant TB Medium (EMD Biosciences) containing ampicillin (100 µg/ml). Cells were grown at 37°C. overnight and harvested. Protein was purified essentially as described in Wu *et al.*, (Wu *et al.*, 2006) except that a 5-ml His Trap HP column (GE Healthcare) was used. The yield from 500 ml culture was ~150 mg protein. Protein was dialyzed into 20 mM Tris-Cl, pH 8.4, 150 mM NaCl and aliquots were stored at −80°C.; working stocks were stored at −20°C.

T64—A shorter, 64-residue T domain was cloned and expressed in a similar manner. DNA encoding the first 64 residues of colicin Ia was amplified by PCR from pKSJ340, with an NdeI site at the 5′-end and XhoI site at the 3′-end. The fragment was inserted between the NdeI and XhoI sites of pET-22b, which creates a C-terminal non-cleavable His₆-tag. Protein was expressed and purified from the resulting plasmid, pKSJ341, as described above for the longer T domain construct. The yield was about 50 mg/500 ml culture.

TΔ2-64—Residues 2 to 64 were deleted from full-length T domain (T225) in pKSJ344. Mutagenic oligonucleotides complementary to sequences upstream of residue 2 and downstream of residue 64 were made and the deletion mutagenesis was performed using the Stratagene QuikChange Site-directed Mutagenesis kit (Agilent Technologies). Protein was expressed from the resulting plasmid, pKSJ346, and purified as described above for full-length T domain. The yield was ~110 mg protein from 500 ml culture. The protein has a calculated MW of 20,998.

TΔTonB box—The TonB box sequence, EIMAV, residues 23-27, was deleted from full-length T domain in pKSJ344 by site-directed mutagenesis. The resulting plasmid, pKSJ347, was used for expression and purification of protein, as described for the other T domain constructs. The yield of protein from 500 ml culture was ~140 mg. This protein has a MW of 27,311.

Colicin purification

Colicin Ia and E3 were purified as described previously (Jakes & Zinder, 1974; Kienker *et al.*, 2008). IaE3R and IaΔR were grown and purified as for colicin Ia. The yield of IaE3R was ~25 mg/l of culture; the yield of IaΔR was ~10 mg/l of culture. Proteins were stored long term at −80° C. in 50 mM boric acid, pH 9.0 (NaOH), 2 mM EDTA, 300 mM NaCl; working solutions were stored at −20° C.

Killing assays in liquid culture and protection assays with T domain

Bacteria, either K361 or K1022 (to be tested for killing by colicin or by IaE3R or IaΔR or for protection from killing by T domain), were grown to OD₆₆₀ = 0.4 (~1.5—2 × 10⁸/ml) in tryptone broth. In a typical experiment, 100 µl of cells were added to 100 µl of broth, or broth plus T domain buffer, or broth plus T domain or broth plus bovine serum albumin at equivalent molar concentration as T domain. The mixture was incubated with shaking at 37°C. for 2 to 10 minutes, and then colicin or colicin dilution buffer was added, and

incubation was continued with shaking. At the end of the killing period (generally 15 to 30 min.) an aliquot was immediately diluted 100-fold into sterile 6 mM CaCl₂-410 mM NaCl to stop cell growth. Aliquots of appropriate serial dilutions were spread on tryptone-agar plates, incubated overnight at 37°C., and surviving colonies were counted.

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References

- Baboolal TG, Conroy MJ, Gill K, Ridley H, Visudtiphole V, Bullough PA, Lakey JH. Colicin N binds to the periphery of its receptor and translocator, outer membrane protein F. *Structure (Camb)*. 2008; 16:371–379. [PubMed: 18334212]
- Bell PE, Nau CD, Brown JT, Konisky J, Kadner RJ. Genetic suppression demonstrates interaction of TonB protein with outer membrane transport proteins in *Escherichia coli*. *Journal of Bacteriology*. 1990; 172:3826–3829. [PubMed: 2193917]
- Benedetti H, Frenette M, Baty D, Lloubes R, Geli V, Lazdunski C. Comparison of the uptake systems for the entry of various BtuB group colicins into *Escherichia coli*. *J Gen Microbiol*. 1989; 135:3413–3420. [PubMed: 2699893]
- Bonsor DA, Grishkovskaya I, Dodson EJ, Kleanthous C. Molecular mimicry enables competitive recruitment by a natively disordered protein. *J Am Chem Soc*. 2007; 129:4800–4807. [PubMed: 17375930]
- Bonsor DA, Hecht O, Vankemmelbeke M, Sharma A, Krachler AM, Housden NG, Lilly KJ, James R, Moore GR, Kleanthous C. Allosteric beta-propeller signalling in TolB and its manipulation by translocating colicins. *EMBO J*. 2009; 28:2846–2857. [PubMed: 19696740]
- Bonsor DA, Meenan NA, Kleanthous C. Colicins exploit native disorder to gain cell entry: a hitchhiker's guide to translocation. *Biochem Soc Trans*. 2008; 36:1409–1413. [PubMed: 19021565]
- Bouveret E, Rigal A, Lazdunski C, Benedetti H. The N-terminal domain of colicin E3 interacts with TolB which is involved in the colicin translocation step. *Mol Microbiol*. 1997; 23:909–920. [PubMed: 9076728]
- Bouveret E, Rigal A, Lazdunski C, Benedetti H. Distinct regions of the colicin A translocation domain are involved in the interaction with TolA and TolB proteins upon import into *Escherichia coli*. *Molecular Microbiology*. 1998; 27:143–157. [PubMed: 9466263]
- Braun V, Patzer SI, Hantke K. Ton-dependent colicins and microcins: modular design and evolution. *Biochimie*. 2002; 84:365–380. [PubMed: 12423780]
- Braun V, Pilsl H, Gross P. Colicins: structures, modes of action, transfer through membranes, and evolution. *Arch Microbiol*. 1994; 161:199–206. [PubMed: 8161282]
- Buchanan SK. Bacterial metal detectors. *Molecular Microbiology*. 2005; 58:1205–1209. [PubMed: 16313609]
- Buchanan SK, Lukacik P, Grizot S, Ghirlando R, Ali MMU, Barnard TJ, Jakes KS, Kienker PK, Esser L. Structure of colicin I receptor bound to the R-domain of colicin Ia: implications for protein import. *Embo Journal*. 2007; 26:2594–2604. [PubMed: 17464289]
- Buxton RS, Holland IB. Genetic studies of tolerance to colicin E2 in *Escherichia coli* K-12. I. Relocation and dominance relationships of cet mutations. *Mol Gen Genet*. 1973; 127:69–88. [PubMed: 4589345]
- Cao Z, Klebba PE. Mechanisms of colicin binding and transport through outer membrane porins. *Biochimie*. 2002; 84:399–412. [PubMed: 12423783]
- Cardelli J, Konisky J. Isolation and characterization of an *Escherichia coli* mutant tolerant to colicins Ia and Ib. *J Bacteriol*. 1974; 119:379–385. [PubMed: 4604639]

- Cascales E, Buchanan SK, Duche D, Kleanthous C, Lloubes R, Postle K, Riley M, Slatin S, Cavard D. Colicin biology. *Microbiology and Molecular Biology Reviews*. 2007; 71:158–229. [PubMed: 17347522]
- Davies JK, Reeves P. Genetics of resistance to colicins in *Escherichia coli* K-12: cross-resistance among colicins of group A. *Journal of Bacteriology*. 1975; 123:102–117. [PubMed: 1095546]
- Devanathan S, Postle K. Studies on colicin B translocation: FepA is gated by TonB. *Mol Microbiol*. 2007; 65:441–453. [PubMed: 17578453]
- Gokce I, Raggett EM, Hong Q, Virden R, Cooper A, Lakey JH. The TolA-recognition site of colicin N. ITC, SPR and stopped-flow fluorescence define a crucial 27-residue segment. *J Mol Biol*. 2000; 304:621–632. [PubMed: 11099384]
- Hands SL, Holland LE, Vankemmelbeke M, Fraser L, Macdonald CJ, Moore GR, James R, Penfold CN. Interactions of TolB with the translocation domain of colicin E9 require an extended TolB box. *J Bacteriol*. 2005; 187:6733–6741. [PubMed: 16166536]
- Heller KJ, Kadner RJ, Gunther K. Suppression of the *btuB451* mutation by mutations in the *tonB* gene suggests a direct interaction between TonB and TonB-dependent receptor proteins in the outer membrane of *Escherichia coli*. *Gene*. 1988; 64:147–153. [PubMed: 3294107]
- Housden NG, Loftus SR, Moore GR, James R, Kleanthous C, Housden NG, Loftus SR, Moore GR, James R, Kleanthous C. Cell entry mechanism of enzymatic bacterial colicins: porin recruitment and the thermodynamics of receptor binding. *Proc Natl Acad Sci U S A*. 2005; 102:13849–13854. [PubMed: 16166265]
- Hufton SE, Ward RJ, Bunce NA, Armstrong JT, Fletcher AJ, Glass RE. Structure-function analysis of the vitamin B12 receptor of *Escherichia coli* by means of informational suppression. *Molecular Microbiology*. 1995; 15:381–393. [PubMed: 7746157]
- Jakes KS, Kienker PK, Finkelstein A. Channel-forming colicins: translocation (and other deviant behaviour) associated with colicin Ia channel gating. *Q Rev Biophys*. 1999; 32:189–205. [PubMed: 10845238]
- Jakes KS, Zinder ND. Highly purified colicin E3 contains immunity protein. *Proc Natl Acad Sci U S A*. 1974; 71:3380–3384. [PubMed: 4215078]
- Journet L, Bouveret E, Rigal A, Lloubes R, Lazdunski C, Benedetti H. Import of colicins across the outer membrane of *Escherichia coli* involves multiple protein interactions in the periplasm. *Molecular Microbiology*. 2001; 42:331–344. [PubMed: 11703658]
- Khursigara CM, De Crescenzo G, Pawelek PD, Coulton JW. Enhanced binding of TonB to a ligand-loaded outer membrane receptor - Role of the oligomeric state of TonB in formation of a functional FhuA center dot TonB complex. *Journal of Biological Chemistry*. 2004; 279:7405–7412. [PubMed: 14668326]
- Khursigara CM, De Crescenzo G, Pawelek PD, Coulton JW. Kinetic analyses reveal multiple steps in forming TonB-FhuA complexes from *Escherichia coli*. *Biochemistry*. 2005; 44:3441–3453. [PubMed: 15736954]
- Kienker PK, Jakes KS, Finkelstein A. Protein translocation across planar bilayers by the colicin Ia channel-forming domain: where will it end? *J Gen Physiol*. 2000; 116:587–598. [PubMed: 11004207]
- Kienker PK, Jakes KS, Finkelstein A. Identification of channel-lining amino acid residues in the hydrophobic segment of colicin Ia. *J Gen Physiol*. 2008; 132:693–707. [PubMed: 19029376]
- Konisky J, Cowell BS. Interaction of colicin Ia with bacterial cells. Direct measurement of Ia-receptor interaction. *J Biol Chem*. 1972; 247:6524–6529. [PubMed: 4561939]
- Kurisu G, Zakharov SD, Zhelnina MV, Bano S, Eroukova VY, Rokitskaya TI, Antonenko YN, Wiener MC, Cramer WA. The structure of BtuB with bound colicin E3 R-domain implies a translocon. *Nature Structural Biology*. 2003; 10:948–954.
- Lazdunski C, Bouveret E, Rigal A, Journet L, Lloubes R, Benedetti H. Colicin import into *Escherichia coli* cells. *Journal of Bacteriology*. 1998; 180:4993–5002. [PubMed: 9748429]
- Loftus SR, Walker D, Mate MJ, Bonsor DA, James R, Moore GR, Kleanthous C. Competitive recruitment of the periplasmic translocation portal TolB by a natively disordered domain of colicin E9. *Proc Natl Acad Sci U S A*. 2006; 103:12353–12358. [PubMed: 16894158]

- Ma L, Kaserer W, Annamalai R, Scott DC, Jin B, Jiang X, Xiao Q, Maymani H, Massis LM, Ferreira LC, Newton SM, Klebba PE. Evidence of ball-and-chain transport of ferric enterobactin through FepA. *J Biol Chem*. 2007; 282:397–406. [PubMed: 17056600]
- Mankovich JA, Hsu CH, Konisky J. DNA and amino acid sequence analysis of structural and immunity genes of colicin Ia and colicin Ib. *Journal of Bacteriology*. 1986; 168:228–236. [PubMed: 3531169]
- Masaki H, Ohta T. Colicin-E3 and Its Immunity Genes. *Journal of Molecular Biology*. 1985; 182:217–227. [PubMed: 3889348]
- Masi M, Vuong P, Humbard M, Malone K, Misra R. Initial steps of colicin E1 import across the outer membrane of *Escherichia coli*. *Journal of Bacteriology*. 2007; 189:2667–2676. [PubMed: 17277071]
- Mende J, Braun V. Import-Defective Colicin-B Derivatives Mutated in the TonB Box. *Molecular Microbiology*. 1990; 4:1523–1533. [PubMed: 2287276]
- Miller, JH. Experiments in molecular genetics. Cold Spring Harbor Laboratory; Cold Spring Harbor, N.Y: 1972. p. xvip. 466
- Pawelek PD, Croteau N, Ng-Thow-Hing C, Khursigara CM, Moiseeva N, Allaire M, Coulton JW. Structure of TonB in complex with FhuA, *E*-coli outer membrane receptor. *Science*. 2006; 312:1399–1402. [PubMed: 16741125]
- Penfold CN, Garinot-Schneider C, Hemmings AM, Moore GR, Kleanthous C, James R. A 76-residue polypeptide of colicin E9 confers receptor specificity and inhibits the growth of vitamin B12-dependent *Escherichia coli* 113/3 cells. *Molecular Microbiology*. 2000; 38:639–649. [PubMed: 11069686]
- Raggett EM, Bainbridge G, Evans LJ, Cooper A, Lakey JH. Discovery of critical Tol A-binding residues in the bactericidal toxin colicin N: a biophysical approach. *Mol Microbiol*. 1998; 28:1335–1343. [PubMed: 9680221]
- Sabet SF, Schnaitman CA. Purification and properties of the colicin E3 receptor of *Escherichia coli*. *J Biol Chem*. 1973; 248:1797–1806. [PubMed: 4348549]
- Sauter A, Howard SP, Braun V. In vivo evidence for TonB dimerization. *Journal of Bacteriology*. 2003; 185:5747–5754. [PubMed: 13129945]
- Schoffler H, Braun V. Transport across the outer membrane of *Escherichia coli* K12 via the FhuA receptor is regulated by the TonB protein of the cytoplasmic membrane. *Mol Gen Genet*. 1989; 217:378–383. [PubMed: 2549378]
- Sharma O, Yamashita E, Zhalnina MV, Zakharov SD, Datsenko KA, Wanner BL, Cramer WA. Structure of the complex of the colicin E2 R-domain and its BtuB receptor - The outer membrane colicin translocon. *Journal of Biological Chemistry*. 2007; 282:23163–23170. [PubMed: 17548346]
- Shultis DD, Purdy MD, Banchs CN, Wiener MC. Outer membrane active transport: Structure of the BtuB: TonB complex. *Science*. 2006; 312:1396–1399. [PubMed: 16741124]
- Smallwood CR, Marco AG, Xiao Q, Trinh V, Newton SM, Klebba PE. Fluoresceination of FepA during colicin B killing: effects of temperature, toxin and TonB. *Mol Microbiol*. 2009; 72:1171–1180. [PubMed: 19432807]
- Soelaiman S, Jakes K, Wu N, Li CM, Shoham M. Crystal structure of colicin E3: Implications for cell entry and ribosome inactivation. *Molecular Cell*. 2001; 8:1053–1062. [PubMed: 11741540]
- Whitney EN. The *tolC* locus in *Escherichia coli* K12. *Genetics*. 1971; 67:39–53. [PubMed: 4933702]
- Wiener M, Freymann D, Ghosh P, Stroud RM. Crystal structure of colicin Ia. *Nature*. 1997; 385:461–464. [PubMed: 9009197]
- Wu Z, Jakes KS, Samelson-Jones BS, Lai B, Zhao G, London E, Finkelstein A. Protein translocation by bacterial toxin channels: a comparison of diphtheria toxin and colicin Ia. *Biophys J*. 2006; 91:3249–3256. [PubMed: 16905612]
- Yamashita E, Zhalnina MV, Zakharov SD, Sharma O, Cramer WA. Crystal structures of the OmpF porin: function in a colicin translocon. *Embo Journal*. 2008; 27:2171–2180. [PubMed: 18636093]
- Zakharov SD, Eroukova VY, Rokitskaya TI, Zhalnina MV, Sharma O, Loll PJ, Zgurskaya HI, Antonenko YN, Cramer WA. Colicin occlusion of OmpF and TolC channels: Outer membrane translocons for colicin import. *Biophysical Journal*. 2004; 87:3901–3911. [PubMed: 15465872]

Zakharov SD, Zhalnina MV, Sharma O, Cramer WA. The colicin E3 outer membrane translocon: Immunity protein release allows interaction of the cytotoxic domain with OmpF porin. *Biochemistry*. 2006; 45:10199–10207. [PubMed: 16922495]

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Fig. 1.

Schematic diagram of domain structure of colicins Ia and E3, the chimeric construct IaE3R, the R domain deletion of colicin Ia, IaΔR, and the C-terminal channel-forming domain of colicin Ia, CT-M. Crystal structures of colicin Ia (Wiener *et al.*, 1997) and colicin E3 (Soelaiman *et al.*, 2001) are also shown, with the domains colored as in the schematic diagrams. The R domain of colicin Ia that was replaced or deleted comprises residues 251-407; the R domain of colicin E3 that replaced the Ia R domain included residues 312 to 447 of colicin E3. Ia CT-M is the last 189 residues of colicin Ia, from 438 to 626.

Fig. 2.

Specific activity of colicins Ia and E3 and constructs on various indicator lawns. Results of spots tests are displayed as killing units per mg. The killing unit is defined as the highest dilution of a 1 mg/ml colicin solution at which a reduction in turbidity of the lawn is observed. Arrows mean that no spots were observed at even the highest concentration of colicin tested, usually 1 mg/ml. CT-M, the isolated channel-forming domain of colicin Ia, was only tested on the wild-type indicator lawn, at a maximum concentration of 4.8 mg/ml. (The reduced sensitivity of the *cirA* mutant to colicin E3 may be related to the fact that this strain was originally described as being tolerant to (i.e., not killed by) colicin E2 (Buxton & Holland, 1973), and so may also be less sensitive to colicin E3. The *cirA* mutation is unrelated to, and maps far from, *cet*, the colicin E2-tolerant mutation. We have no explanation for the reduced sensitivity of the *tonB* deletion mutant to colicin E3, other than that pleiotropic effects of the mutation on other membrane proteins may be responsible.)

Fig. 3.

Titration of protection by T domain (T225) of *btuB*⁻ cells from killing by IaE3R. Cells at mid-log growth ($OD_{660} = 0.4$) were preincubated, for 2 min. at 37°C. with shaking, with increasing amounts of purified T225, in a total volume of 0.2 ml. Then 36 pmol of IaE3R were added and incubation was continued for 30 minutes. Dilutions were spread on nutrient agar plates and surviving colonies were counted. Per cent survivors were calculated based on a control culture to which no colicin was added.

Fig. 4. Inhibition of killing by high concentrations of colicin Ia in liquid culture. K361 was grown in Luria broth with shaking at 37° C. to $OD_{660} = 0.3$. The culture was then divided into three separate flasks, and colicin Ia was added to one flask to a concentration of 0.15 µg/ml (◆) and to a second flask to a concentration of 15 µg/ml (■). Growth was continued with shaking at 37° C. and the OD of the untreated (●) and colicin-treated cultures was monitored at 15- to 20-minute intervals.

Fig. 5.

Model of how colicin Ia uses one copy of Cir as its high affinity outer membrane receptor and a second copy of Cir as its translocon. This cartoon is not drawn to scale. The β -barrel of Cir is drawn as a light blue cylinder, with its N-terminal plug shown as a purple squiggle. In A., colicin Ia, with its channel-forming domain shown in yellow and its T domain in red, binds, via its receptor-binding domain (green) to a copy of Cir. The unstructured 64-residue N terminus of colicin Ia (solid red line) has found a second copy of Cir nearby (from the ~5,000 copies/cell (Konisky & Cowell, 1972)). The TonB boxes of both the colicin and Cir are shown as blue rectangles, though their sequences are not identical. In B., the binding of T domain to the second copy of Cir has triggered two events: (1) binding of TonB protein to the TonB box of the plug domain of Cir, which caused rearrangement of the plug to allow passage of the N terminus of the colicin through the barrel, where (2) its TonB box (at residues 23-27) binds a second copy of TonB. Ton B is depicted with its TonB box-binding domain as an orange oval. The yellow lightening bolts signify that TonB transduces energy from the inner membrane to drive its interactions in the periplasm. Not shown are the inner membrane or the inner membrane binding partners of TonB, ExbB and ExbD.

Table 1

Translocation domain (T225) protects wild-type *E. coli* from killing by either wild-type colicin Ia or IaΔR.

Addition to cells	Colicin	% surviving colonies
Buffer only	Wt. Ia (3.5 fmol)	0.02
T225 (1.7 nmol)	Wt. Ia (3.5 fmol)	0.23
T225 (5.7 nmol)	Wt. Ia (3.5 fmol)	2.7
T225 (11 nmol)	Wt. Ia (3.5 fmol)	5.6
BSA (17 nmol)	Wt. Ia (3.5 fmol)	0.01
Buffer only	IaΔR (23 pmol)	0.2
T225 (1.7 nmol)	IaΔR (23 pmol)	18.0
T225 (5.7 nmol)	IaΔR (23 pmol)	39.0
T225 (17 nmol)	IaΔR (23 pmol)	47.0
BSA (17 nmol)	IaΔR (23 pmol)	0.1

Wild-type indicator cells were incubated for 5 min. at 37° C. with shaking, with the additions shown, in a total volume of 200 μl. ("Buffer only" is 20 mM Tris-Cl, pH 8.4, 150 mM NaCl, the buffer in which both T225 and BSA were dissolved.) Colicin (either wild-type Ia or IaΔR) was added and incubation was continued for 25 min. at 37° C. with shaking. Cells were diluted, plated, and the percentage of surviving colonies was determined, based on a control culture to which no colicin had been added.

Table 2

Translocation domain (T225) protects wild-type or *btuB*[−] *E. coli* from killing by IaE3R

Indicator Strain	Colicin IaE3R	Addition to cells	% surviving colonies
Wild-type	0.36 pmol	None	0.1
Wild-type	0.36 pmol	T225	2.1
Wild-type	0.36 pmol	BSA	0.01
<i>btuB</i> [−]	36 pmol	None	0.02
<i>btuB</i> [−]	36 pmol	T225	60.4
<i>btuB</i> [−]	36 pmol	BSA	0.04

Cells, either wild-type or a *btuB*[−] mutant strain, were incubated for 5 min. at 37° C. with either buffer (None), 450 μg T225 (17 nmol), or with a molar equivalent amount of BSA. Then IaE3R was added and incubation was continued for 20 min. at 37° C. Cells were diluted, plated, and the percentage of surviving colonies was determined, based on control cultures to which no colicin had been added.

Table 3

Protection from colicin killing by translocation domain requires the first 64 residues but not the TonB box of T domain

Addition to cells	Indicator cells	Colicin	% Surviving colonies
Buffer only	<i>btuB</i> ⁻	IaE3R	0.05
T225	<i>btuB</i> ⁻	IaE3R	58.
TΔTonB box	<i>btuB</i> ⁻	IaE3R	42.
T62-64	<i>btuB</i> ⁻	IaE3R	0.04
T64	<i>btuB</i> ⁻	IaE3R	0.01
BSA	<i>btuB</i> ⁻	IaE3R	0.03
Buffer only	Wild-type	IaΔR	4.6
T225	Wild-type	IaΔR	59.
T6TonB box	Wild-type	IaΔR	47.
TΔ2-64	Wild-type	IaΔR	1.1
BSA	Wild-type	IaΔR	0.6

Results of two separate experiments are shown. Indicator cells were preincubated for 5 min. at 37°C. with shaking, with the additions shown, in a total volume of 200 μl. In the experiment with IaE3R, 11.5 nmol of each T domain construct were added; in the experiment with IaΔR, 11 nmol of the T domain constructs were added. ("Buffer only" is 20 mM Tris-Cl, pH 8.4, 150 mM NaCl, the buffer in which the T domain constructs and BSA were dissolved.) After colicin addition (18 pmol of IaE3R or 23 pmol of IaΔR), incubation was continued for 25 min. at 37°C. with shaking. Cells were immediately diluted and plated, and the percentage of surviving colonies was determined, based on control cultures to which no colicin had been added. (Note that, like BSA, the inactive T domain constructs actually appear to enhance killing activity. We believe this is a non-specific effect of protein addition, as noted in Results.)