Title

Colicin E1 opens its hinge to plug TolC

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Abstract

The double membrane architecture of Gram-negative bacteria forms a barrier that is impermeable to most extracellular threats. Bacteriocin proteins evolved to exploit the accessible, surface-exposed proteins embedded in the outer membrane to deliver cytotoxic cargo. Colicin E1 is a bacteriocin produced by, and lethal to, *Escherichia coli* that hijacks the outer membrane proteins TolC and BtuB to enter the cell. Here we capture the colicin E1 translocation domain inside its membrane receptor, TolC, by high-resolution cryoEM to obtain the first reported structure of a bacteriocin bound to TolC. Colicin E1 binds stably to TolC as an open hinge through the TolC pore—an architectural rearrangement from colicin E1's unbound conformation.

35 This binding is stable in live *E. coli* cells as indicated by single-molecule fluorescence
36 microscopy. Finally, colicin E1 fragments binding to TolC plug the channel, inhibiting its native
37 efflux function as an antibiotic efflux pump and heightening susceptibility to three antibiotic
38 classes. In addition to demonstrating that these protein fragments are useful starting points for
39 developing novel antibiotic potentiators, this method could be expanded to other colicins to
40 inhibit other outer membrane protein functions.

41 42 **Introduction**

43
44 In Gram-negative bacteria, the outer membrane, cell wall, and cytoplasmic membrane
45 form concentric barriers that protect the cell from extracellular threats. Of these protective
46 structures, the outer membrane forms a particularly formidable obstacle (Alexander & Rietschel,
47 2001), owing to the impermeability of the lipopolysaccharide (LPS) layer that constitutes the
48 outer membrane (Kamio & Nikaido, 1976). The primary means by which external molecules can
49 gain access to the cell is through the ~100 variations of barrel-shaped proteins that are embedded
50 in each bacterium outer membrane (Freeman & Wimley, 2012) and whose diverse functions
51 include the transport of molecules across the membrane—specifically, the import of nutrients and
52 metabolites and the export of toxins and waste.

53 Because outer membrane proteins (OMPs) are accessible from outside the cell,
54 bacteriophage and bacterial toxins have evolved to exploit OMPs to initiate delivering cargo
55 across the outer membrane. Bacteriocins hijack the OMPs of a target bacterium to cross its
56 impermeable outer membrane and kill the bacterium. Colicins are *E. coli*-specific bacteriocins,
57 protein toxin systems through which bacteria engage in bacterial warfare with other, similar
58 bacteria. Colicins can be divided into groups A and B based on the requirement for which
59 periplasmic protein systems they rely on to enter cells. The cytotoxicity of group A colicins
60 requires the Tol system (Nagel de Zwaig & Luria, 1967) which is coupled to the proton motive
61 force for its role in maintaining membrane integrity (Lazzaroni *et al.*, 1989). Colicins differ in

62 their receptor targets and killing mechanisms. Most colicins share a common tri-domain
63 architecture, comprising the following components: (i) an N-terminal translocation (T) domain,
64 (ii) a receptor-binding (R) domain, and (iii) a C-terminal cytotoxic (C) domain (Figure 1A). Much
65 of what is known of group A colicin import has been determined through studies of the colicin E3
66 and E9 as reviewed by Cramer *et al.* (Cramer *et al.*, 2018). For colicins E3 and E9 import is
67 initialized by high affinity R domain binding to the vitamin B12 transporter, BtuB (Kurusu *et al.*,
68 2003, Housden *et al.*, 2005, Francis *et al.*, 2021); this binding localizes the colicin onto the outer
69 membrane. Once the colicin is tethered to the outer membrane surface, the T domain of ColE3
70 and ColE9 initiates translocation using the secondary OMP receptor/translocator OmpF to access
71 the Tol-Pal system in the periplasm. For most colicins, the T domain requires an OMP distinct
72 from the R domain target for colicin translocation (Cascales *et al.*, 2007). A handful of colicins
73 have been structurally characterized with their OMP counterparts (Kurusu *et al.*, 2003, Sharma *et*
74 *al.*, 2007, Buchanan *et al.*, 2007). Colicin E9 bound to both BtuB and OmpF was structurally
75 resolved as a complete translocon complex (Francis *et al.*, 2021). The structure of the full ColE9
76 translocon showed that T domain can fully penetrate deeply into lumen of the outer membrane
77 receptors while the R domain interact with the extracellular loop regions of its receptors. Studies
78 of the bacteriocin pyocin S5 from *Pseudomonas aeruginosa* suggest that bacteriocin architectures
79 and mechanisms may be conserved across all Gram-negative species (Behrens *et al.*, 2020).

80 Colicin E1 is a bacteriocin produced by *E. coli* that enters the periplasm of neighboring
81 cells and forms a pore on the cytoplasmic membrane leading to membrane depolarization and cell
82 death. Unlike any of the other E colicins, the T domain of Colicin E1 binds to TolC (Benedetti *et*
83 *al.*, 1991), the outer membrane component of the acridine efflux pump, and the R domain binds
84 BtuB (Benedetti *et al.*, 1989) the primary receptor shared by all E colicins. A high-resolution x-
85 ray structure exists of only the cytotoxic domain of colicin E1 (Elkins *et al.*, 1997) but not the T
86 or R domains, thus no mechanistic insights can be gained about initial steps of import. Domain

87 swapping experiments (Benedetti *et al.*, 1991) between colicin A and E1 identified regions of the
88 protein that interact with specific outer membrane proteins. Early studies of pore forming colicins,
89 including E1, showed that cytotoxic-domain-induced K⁺ efflux on the inner membrane can be
90 reversed after it has begun by subsequent addition of trypsin to cells. This, in addition to trypsin's
91 inability to cross the outer membrane, led to the belief that portions of colicin remain tethered to
92 their OMP partners as the cytotoxic domain depolarizes the cytoplasmic membrane.(Plate &
93 Luria, 1972, Dankert *et al.*, 1980, Dankert *et al.*, 1982, Benedetti *et al.*, 1992)

94 In order to understand the early stages of colicin E1 import, functional studies of
95 truncations of colicin E1 which lack the cytotoxic domain have been characterized *in vitro*.
96 Residues 100-120 of colicin E1 (termed the 'TolC box', Figure 1A) have been shown to be
97 necessary but not sufficient for binding TolC. Peptides that include the TolC box co-elute with
98 TolC (Zakharov *et al.*, 2016) and disrupt channel conductance in planar lipid bilayer
99 membranes(Zakharov *et al.*, 2004, Zakharov *et al.*, 2016). Moreover, *E. coli* exposure to TolC-
100 box-containing peptides can prevent subsequent binding and cytotoxicity of full-length colicin E1
101 (Jakes, 2017). Circular dichroism of the T domain indicates that it exists as a helical hairpin
102 (closed hinge) in solution similar to other colicin T domains (Wiener *et al.*, 1997, Housden *et al.*,
103 2021) and that the proline at the center of the TolC box forms its apex (Zakharov *et al.*, 2016,
104 Housden *et al.*, 2021). This measurement led to the proposal, known as the "pillar model,"
105 (Zakharov *et al.*, 2016) that the T domain inserts into the TolC barrel as a helical hairpin where
106 the N and C-termini are pointing to the cell exterior. According to this model, the hairpin stuck in
107 TolC acts as a buttress to facilitate the C terminal-first cytotoxic domain entry directly through the
108 membrane where it is possibly cleaved from the TR domain to reach its inner membrane target
109 similar to ColE7 (Shi *et al.*, 2005) and ColD (de Zamaroczy *et al.*, 2001).

110 A competing model for colicin E1 import, known as the "total thread" model (Cramer *et al.*
111 *et al.*, 2018, Zakharov *et al.*, 2016, Housden *et al.*, 2013, Francis *et al.*, 2021) posits that the protein

112 unfolds and passes through TolC N-terminus-first as an unstructured peptide and binds to TolA
113 (PilsI & Braun, 1995) in the periplasm. In this model the binding between the intrinsically
114 unstructured colicin N-termini and periplasmic proteins (Jakes, 2017, Housden *et al.*, 2013)
115 creates a pulling force that results in the translocation of the whole colicin.

116 Here we use cryogenic electron microscopy (cryoEM) to solve the high-resolution
117 structure of a bacteriocin, colicin E1, bound to TolC. We find that colicin E1 binds stably to TolC,
118 not as a helical hairpin but as a single-pass folded helix with the N-terminus inside the periplasm.
119 Additionally, we find that the ColE1 TR domain binds TolC *in vivo* as well. Using single-
120 molecule fluorescence microscopy, we find that ColE1-TR, lacking the cytotoxic domain,
121 remains stalled on the outer membrane and does not fully translocate into cells. Lastly, we
122 leverage this stalling of ColE1 to block the native TolC function as an antibiotic efflux pump.
123 Because they are accessible from outside the cell, OMPs are attractive targets for the development
124 of novel antibiotics, and research has begun to reveal the therapeutic potential of interfering with
125 OMP structure and function. (Storek *et al.*, 2018, Kaur *et al.*, 2021)

126 Here by determining the structure and mechanism of ColE1 insertion we establish an
127 alternative approach for targeting OMPs—the development of molecular plugs that block OMP
128 pores. Such plugs would allow for the manipulation of bacterial transport, providing a means of
129 either starving the bacterium by preventing the import of valuable nutrients or poisoning the
130 bacterium by preventing the export of toxins. Through real-time efflux assays and minimum
131 inhibitory concentration (MIC) experiments, we find that ColE1-TR and ColE1-T are able to
132 inhibit TolC-mediated efflux. We find that this plugging of TolC reduces the amount of
133 antibiotics required to inhibit the bacterial growth—indicating that this colicin E1 fragment
134 reduces the antibiotic resistance conferred by TolC.

135

136 **Results**

137
138 **CryoEM structure of TolC embedded in nanodiscs**

139 To determine the structural details of colicin E1 binding to TolC, we solved the cryoEM
140 (cryogenic electron microscopy) structure of TolC embedded in nanodiscs with and without
141 added ColE1-T (PDB 6WXH and 6WXI, respectively). We recently reported a high-yield TolC
142 preparation by refolding from inclusion bodies (Budiardjo *et al.*, 2021) and inserted refolded TolC
143 into nanodiscs. The cryoEM structure of refolded TolC alone (Figure 1B) is similar to the
144 previously published crystal structures of natively derived TolC alone (Koronakis *et al.*, 2000) or
145 in complex with hexaminecobalt (Higgins *et al.*, 2004) but with more splayed loops at the
146 periplasmic opening (residues 165-175). There was local variation in resolution within the model
147 with lower resolution associated with the extracellular/periplasmic ends and the nanodisc scaffold
148 protein (Figure 1B). The lower residue resolution at these apertures may indicate dynamics not
149 captured in the x-ray crystal structures.

150 We next determined binding of colicin E1-T domain to capture the complex for structure
151 determination. Residues 1-190 (ColE1-T) which span the translocation domain are known to bind
152 to TolC (Figure 1A). We identified ColE1-T:TolC binding *in vitro* via co-elution by size
153 exclusion chromatography (SEC) as previously described (Zakharov *et al.*, 2016). When ColE1-T
154 and TolC were mixed, we observed a leftward shift in the TolC peak and a decrease in intensity
155 associated with the ColE1-T peak indicating that a subset of the population had migrated with
156 TolC (Figure 1C). We analyzed the peak (Figure 1C arrow) using SDS-PAGE and found the
157 presence of both ColE1-T and TolC. The unimodal shifted peak observed with ColE1-T:TolC
158 indicates that there is a single species of fully bound TolC. When resolved by cryoEM, addition
159 of ColE1-T to TolC breaks the three-fold channel symmetry and the additional protein is observed
160 running all the way through the TolC barrel as a single-pass, all- α -helical chain spanning more
161 than 130 Å (Figure 1D). The maps refined to nominal global resolutions of 2.84 Å and 3.09 Å for

162 the TolC and ColE1-T:TolC, respectively (Figure 1, source data 1).

Figure 1. Colicin E1-T binds within the lumen of TolC. (A) Architecture of full-length colicin E1 showing domains and their known binding partners. Three truncation constructs used in this study (B) CryoEM structure of TolC embedded in nanodiscs. Side, top, and bottom views are colored by local resolution, as computed in cryoSPARC from the final half-maps. The side view is cropped to display the particle interior. (C) SEC chromatogram of ColE1-T (red line) and TolC (purple line). The arrow indicates the co-elution (black line) fractions (eluted in buffer containing 40 mM NaCl) that were analyzed by SDS-PAGE. On the SDS-PAGE gel (bottom), red arrows indicate the presence of the colicin E1 construct that has co-eluted with TolC. SEC chromatogram of TolC purification (Figure 1 supplemental figure 1) and co-elution with 200 mM NaCl (Figure1 supplemental figure 2). (D) The CryoEM structure of ColE1-T bound to TolC and colored by local resolution as in (B). Cryo-EM data collection, refinement, and validation statistics in Figure 1, source data 1.

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The ColE1 T domain inserts into TolC with its amino terminus pointing inwards through the periplasmic iris, and 2D class-averages show that the carboxy terminus of the helix continues and projects out into the extracellular space (Figure 2A). 85 of the 190 ColE1-T residues could be modeled to this density (residues 46-131) (Figure 2B). No such regular protrusion was seen for the glycine-rich ColE1 amino-terminus, which we expect to be disordered in the periplasmic space (Zakharov *et al.*, 2016, Housden *et al.*, 2013, Housden *et al.*, 2018). The ColE1 chain binds TolC asymmetrically, primarily contacting only one of the three TolC chains.

Compared to the unbound state, asymmetric ColE1 binding dilates the periplasmic TolC aperture (Figure 2C) and, makes only minor adjustments to the loops on the extracellular aperture (Figure 2D) so that they can accommodate ColE1 in the absence of any other proteins or motive force.

In solution, unbound colicin E1 is a two-helix hairpin as indicated by far UV circular dichroism (CD) (Zakharov *et al.*, 2016). A structural model built using AlphaFold (Jumper *et al.*, 2021) also predicts the closed hinge conformation with proline 110 at the apex (Figure 2E top). However, our cryoEM reconstruction of colicin E1 in complex with TolC shows that ColE1-T hinges open at the TolC box to an extended conformation upon binding to TolC (Figure 2B, and 2E bottom). The ColE1-T helices have a short kink in the middle around proline 110, precisely at the proposed turn location in the hairpin model (Figure 2B inset). This area is also the center of the TolC box (Figure 1A) that has been known to be critical for the ColE1:TolC interaction (Jakes, 2017, Zakharov *et al.*, 2016).

Figure 2. Colicin E1 binds to TolC as a single-pass kinked helix. (A) 2D class averages of ColE1-T bound to TolC imbedded in nanodiscs (B) Cutaway side view of the TolC interior (gray). ColE1-T (red) binds asymmetrically in an open-hinge conformation. The hinge region of ColE1-T including P110 shown in stick representation (inset). (C and D) The apo cryoEM structure of TolC (light gray) compared to holo cryoEM structure (dark gray). (C) Periplasmic aperture. Two of the TolC helices in the bound structure (magenta) dilate outwards compared to those two helices in the unbound state (cyan) (D) The extracellular aperture remains more similar between the bound and unbound structures. (E) In the unbound state, ColE1-T (red cylinders) exists as a closed hinge. When bound, ColE1-T is in an open-hinge conformation through TolC with the N-terminus into the pore of TolC (grey). The side chain of the hinge proline is shown making a small pentagon in both the closed and open hinge states. The model of ColE1-T in its solution state was built using AlphaFold (Jumper *et al.*, 2021) and is consistent with previous experiments (Zakharov *et al.*, 2016).

190 **Colicin E1 makes residue-specific interactions with TolC**

191 TolC forms a large rigid conduit in the outer membrane and periplasm (Figure 3A) with a
192 water filled lumen. The hydrophilic nature of the channel (Dhar *et al.*, 2021) coupled with the
193 inflexibility of the outer-membrane-embedded barrel largely precludes the formation of any
194 hydrophobic interfaces that are typically the basis of protein-protein interfaces involving helical
195 peptides (Eisenberg *et al.*, 1982). Yet, we did observe specific polar contacts between colicin E1's
196 TolC box and the TolC barrel.

197 To obtain the most accurate atomic model for interpretation of atomic interactions
198 between peptides, we utilized map-restrained molecular dynamics in model refinement (Wu *et al.*,
199 2013). The refined model had improved chemical plausibility (for instance, the MolProbity
200 (Williams *et al.*, 2018) score improved from 1.25 to 0.50) and polar contacts were more easily
201 identified. Because this refinement improved the concordance between the map and model, we
202 conclude that the use of cryoEM density as a restraint was successful in preventing overfitting.
203 Specifically, the EMRinger (Barad *et al.*, 2015) score improved from 2.77 to 3.81 and the map-
204 model FSC=0.5 improved from 3.44Å to 3.37Å.

205 The atomic model showed significant polar interactions form between colicin E1 and
206 TolC at the apertures and around the TolC box. The acidic patch of colicin E1 near the
207 extracellular aperture contains two arginine residues (R129 and R131) that form hydrogen bond
208 networks with one TolC chain each (Figure 3B). In addition, colicin E1 residues T113, A116,
209 N119, N120 (all part of the TolC box) establish polar interactions with TolC residues G67, D69,
210 S90 and K305 on one TolC chain (Figure 3C, light red and light blue) while colicin H117
211 interacts with TolC F310 on a neighboring TolC chain (Figure 3C, light red and light gold). These
212 contacts are strong enough that even when the proline hinge of the TolC-box is mutated (P110A)
213 ColE1-T still binds TolC (Figure 3, supplementary figure 2).

214

Figure 3. Inter-chain contacts between ColE1 T and TolC. (A) Molecular dynamics simulation refined structure of the ColE1-T:TolC complex colored by chain (ColE1-T: light red, TolC: light blue, light green, light gold) (B-C) Polar interaction network of ColE1-T and TolC on the C-terminal side of the proline kink in the β -barrel region. Locations of previous mutagenesis data near the TolC box (figure 3 supplemental figure 1 and figure 3 – video 1). (B) Near the extracellular opening of TolC and (C) in the TolC barrel near the transition to the TolC periplasmic helical region. Residues involved in forming polar interactions and proline 110 are shown in sticks. These interactions support binding such that even when the hinge proline is mutated to alanine (P110A) binding is maintained (Figure 3 supplemental figure 2). (D) ColE1-T spanning the TolC helical barrel does not make full contact with the side of the TolC barrel. The cavity (black spheres) between colicin (light red) and one chain of TolC (light blue) was detected using GHECOM (Kawabata, 2010, 2019, Kawabata & Go, 2007).

216 By contrast with the β -barrel of TolC, in the interior of the periplasmic helical barrel of
217 TolC, colicin E1 does not make full contact with the α -helical barrel interior and there is a gap
218 between the two proteins (Figure 3D, Figure 3- video 1). This is in agreement with previous
219 studies that showed a colicin truncation (residues 1-100), that ends just before the beginning of
220 the TolC box, does not bind to TolC (Zakharov *et al.*, 2016) or prevent cytotoxicity of full-length
221 colicin in cells (Jakes, 2017) as this region of the colicin does not make strong interactions with
222 TolC (Figure 3D).

Moreover, this structure is consistent with reports that mutations at TolC sites G65 or S279 abrogate ColE1 activity (Masi *et al.*, 2007) as these residues are contact sites between TolC and ColE1 (Figure 3 figure, supplemental figure 1 and Figure 3- video 1). Conversely, the absence of any effect of mutating colicin R103 and R108 (Jakes, 2017), is consistent with interaction those residues have with the solvent in the barrel rather than with TolC. The newly solved structure, in combination with previous work (Jakes, 2017, Zakharov *et al.*, 2016), indicates the specificity of colicin E1 binding to TolC is encoded in the portion that binds to TolC β -barrel.

Membrane localization of ColE1 truncations

Since we find the ColE1 T and TR domains bind to TolC *in vitro*, we investigated if colicin E1 fragments bind to the native TolC in *E. coli* cells. Though ColE1 C domain activity—depolarizing the inner membrane—requires the C domain to pass through the outer membrane (Brunden *et al.*, 1984), it remains unclear if the ColE1-TR domains translocate as well. We first determined that TolC binds to the TR domain *in vitro* similarly to TolC binding the T domain. ColE1-TR includes residues 1-364 (ColE1-TR) contains the N-terminal portion that binds to TolC and the C-terminal portion that binds to BtuB. ColE1-TR aggregates at the salt concentration (40 mM) used for the co-elution experiment used for ColE1-T so we increased the NaCl concentration to 200 mM. ColE1-T still binds to TolC at 200 mM NaCl although to a much lesser degree (Figure 1 supplemental figure 1). Unlike ColE1-T (Figure 1C), ColE1-TR shows a bimodal distribution indicative of a mixed population of bound and unbound TolC (Figure 4A).

We assessed whether ColE1-T and ColE1-TR translocate into the cell or localize on the cell surface using an extracellular protease digestion assay (Besingi & Clark, 2015). Colicin fragment stalling on the outer membrane surface would render the colicin fragments susceptible to digestion when trypsin is in the extracellular environment. Conversely, if the colicin fragment

translocates into the intracellular environment, the outer membrane will shield the protein from digestion by trypsin.

Colicin fragment localization was probed through western blot analysis of specific colicin fragments with C-terminal polyhistidine-tags. Cells were incubated with these colicin fragments and subsequently exposed to two different concentrations of trypsin. If the protein enters the cell, the outer membrane shields it from trypsin digestion, and a corresponding band is present on the western blot. Periplasmic protein SurA was used as a membrane integrity control. SurA digestion indicates that the periplasm was accessible to trypsin and a SurA band indicates that the periplasm was shielded from trypsin. As a trypsin activity control, half of the cell sample was lysed (lysed cell condition) exposing all cellular compartments to trypsin.

When probing for the C-terminal epitope by immunoblotting, there was no detectible ColE1-T binding (Figure 4B, left), though there was detectible binding of ColE1-TR (Figure 4B, right). Moreover, we found that if ColE1-TR is incubated with cells and subsequently treated with two different trypsin concentrations, the ColE1-TR band disappeared, indicating that the colicin E1 fragment was localized to the outer membrane surface (Figure 4B, right). The periplasmic control SurA was not degraded at any trypsin concentration unless the cells were lysed before trypsin digestion (Besingi & Clark, 2015).

We further probed the interaction between the cell membrane and surface-localized ColE1-TR with single-molecule fluorescence microscopy using the fluorescent dye Cy3 (Tuson & Biteen, 2015, Al-Husini *et al.*, 2020). When ColE1-TR-Cy3 was added to the extracellular environment of WT BW25113 *E. coli* (containing TolC), distinct puncta (Figure 4C, right) formed on 94% of the cells (number of cells, n = 111) (Figure 4D); cells with observed puncta most often featured a single punctum, though a small fraction had 2 puncta. On average, the WT cells had 1.2 puncta. In a $\Delta tolC$ strain, puncta were observed on only 18% of cells (n = 99) (Figure 4C, right; Figure 4D); on average, the $\Delta tolC$ cells had 0.2 puncta. As a $\Delta btuB$ control

274 strain, we used BL21 (DE3) which is known to have a premature stop codon at BtuB residue 58
275 (Studier *et al.*, 2009). Puncta were observed on only 3% of cells lacking BtuB (n = 105) (Figure
276 4D and Figure 4 supplemental figure 2A); on average, the $\Delta btuB$ cells had 0 puncta.

277 The bright ColE1-TR-Cy3 puncta photobleach over time as they are observed in the
278 fluorescence microscope, ultimately leaving a single molecule fluorescing before complete
279 photobleaching. We therefore divided the brightness of each punctum by the brightness of that
280 single molecule to estimate the number of molecules in each cluster (Bakshi *et al.*, 2012). In WT
281 and $\Delta tolC$ cells that featured puncta, ColE1-TR form puncta consistent with ~20 molecules; we
282 never detect an isolated single ColE1-TR molecule on either WT or $\Delta tolC$ cells before
283 photobleaching. The observed size and number of molecules agree with previous studies of BtuB
284 clusters (Rassam *et al.*, 2015, Chavent *et al.*, 2018) and the punctum locations within the cell were
285 variable. To rule out punctum formation as an artifact of Cy3 conjugation, we found ColE1-TR-
286 GFP displayed the same cluster formation as ColE1-TR-Cy3 (Figure 4 supplemental figure 2B).

287 Because we usually only see one punctum per cell, we anticipate that some BtuB and
288 TolC may remain unbound because of the geometric constraints of punctum formation. ColE1
289 must engage both TolC and BtuB simultaneously. Therefore, both receptors must be in close
290 proximity, similar to the ~50 Å proximity of BtuB to OmpF when both bound to a another type A
291 colicin, ColE9 (Francis *et al.*, 2021). Given the two-dimensional surface of a membrane there are
292 restrictions to how many proteins can be within 50 Å of any other protein. For the clusters we see
293 to occur, when BtuB clusters together in groups of approximately 20 (Figure 4) TolC must also
294 cluster in groups of approximately 20, ultimately requiring approximately 40 proteins in a
295 relatively small area. The competition between the clustering of BtuB and the requirement for
296 proximity of BtuB and TolC for binding could lower the number of BtuB/TolC sites available for
297 the T and R domains of colicin E1 due to the relative TolC and BtuB geometries needed for
298 ColE1 binding.

Figure 4. Colicin E1-TR localizes on the outside of the cell. (A) SEC chromatogram of ColE1-TR (red line) and TolC (purple line). The arrow indicates the co-elution (black line) fractions that were analyzed by SDS-PAGE. On the SDS-PAGE gels (right), red arrow indicates the presence of colicin E1 construct that has co-eluted with TolC (purple arrow). (B) Extracellular protease digestion assay with two colicin E1 truncation constructs incubated with *E. coli*, each construct is labeled with a C-terminal His-Tag. Periplasmic SurA was used as a membrane integrity control. After incubation with colicin fragments, cells were left intact or lysed with lysozyme. Proteins degraded by trypsin in intact cells are not protected by the outer membrane. (C) Fluorescence image (right) of ColE1-TR-Cy3 overlaid on outlines of living *E. coli* cells from phase-contrast microscopy (left) for WT and $\Delta tolC$. Red arrow points to a punctum. A larger variety of images is also available (Figure 4 supplement 1). Similar localization was seen for ColE1-TR-GFP (Figure 4 supplemental figure 2) demonstrating that the effect was not caused by the fluorophore, and ColE1-TR Δ_{1-40} -Cy3 (Figure 4 supplemental figure 3, Figure 4, source data 1) demonstrating that the effect is not TolA dependent. The puncta were stable and were found to remain intact for more than five minutes. The punctum shown here remained intact for at least 30 seconds (Figure 4 - video 1). (D) Cell counts where ColE1-TR-Cy3 punctum formation was observed for WT, $\Delta tolC$, and $\Delta btuB$. Number of cells observed, $n = 111, 91, 105$,

Fluorescently labeled pyocins, the colicin analog in *Pseudomonas*, have previously been

used to detect translocation across the outer membrane of *Pseudomonas aeruginosa* (White *et al.*,

2017). Here we use an analogous experiment with fluorescently labeled ColE1 to determine

cellular localization in *E. coli*. In time courses, bound ColE1-TR puncta remained immobile for >

5 minutes (Figure 4 - video 1 corresponds to 30 seconds of data used to attain the WT image in

Figure 4C). This is consistent with continued association of ColE1-TR with membrane-embedded

305 BtuB, which has limited mobility (Kleanthous *et al.*, 2015). This result indicates that ColE1-TR
306 does not fully translocate (White *et al.*, 2017), because if ColE1-TR entered the periplasmic space
307 it would freely diffuse on these timescales.

308 Colicin constructs lacking the R domain (ColE1-T-Cy3) showed no detectable binding
309 either to WT or $\Delta tolC$ cells (Figure 4 supplemental figure 2C), indicating that the TolC-ColE1-T
310 interaction is weaker than the BtuB-ColE1-TR interaction, likely due to the absence of the R
311 domain-BtuB interaction.

312 TolA is known to be required for ColE1 cytotoxicity (Nagel de Zwaig & Luria, 1967, Pilsl
313 & Braun, 1995). ColE1 lacking the TolA box at the N-terminal T-domain would likely be
314 translocation incompetent due to uncoupling from TolA. Similarly, there is a loss of biological
315 activity due to mutations in ColE9 N-terminal region that indirectly interacts with TolA (Loftus *et*
316 *al.*, 2006).(Schneider *et al.*, 1997) Biological inactivation has also been caused by mutation to the
317 Tol-Pal interacting regions of ColE3 (Escuyer & Mock, 1987) and ColA (Pilsl & Braun, 1995).
318 We found that ColE1-TR $_{\Delta 1-40}$ binds to the cell surface of WT, $\Delta tolC$, and $\Delta btuB$ in a similar
319 manner as ColE1-TR (Figure 4 supplementary figure 3, Figure 4, source data 1). Cell surface
320 localization of ColE1-TR matching that of translocation deficient ColE1-TR $_{\Delta 1-40}$ suggests that
321 ColE1-TR puncta are not within the cell and likely stalled on the outer membrane receptor. The
322 requirement for TolA may serve an alternate role in translocation as we consider in the Discussion
323 section.

324

325 **ColE1-TR inhibits efflux and makes *E. coli* more susceptible to antibiotics**

326 Since the colicin truncations are stalled on their respective OMP receptors, we
327 investigated if this stalling could disrupt native TolC efflux. Real-time efflux inhibition by colicin
328 E1 fragments was assessed using a live-cell assay with N-(2-naphthyl)-1-naphthylamine (NNN)-
329 dye, which passively diffuses into cells and is effluxed by the AcrAB-TolC efflux pump. NNN

fluoresces when it is localized in the hydrophobic interior of one of the membranes (Bohnert *et al.*, 2011) thereby acting as a reporter for the relative rates of diffusion and efflux. NNN efflux can be turned off by the protonophore CCCP, which neutralizes the proton motive force and allows NNN to accumulate within the cell. Active efflux can then be monitored by the fluorescence decay once proton motive force is reenergized by glucose addition (Bohnert *et al.*, 2010, Seeger *et al.*, 2008, Bohnert *et al.*, 2011, Iyer *et al.*, 2015, Misra *et al.*, 2015).

We assessed the ability of colicin E1 truncations to plug TolC by monitoring real-time NNN efflux. Cells exposed to ColE1-T₁₀₀₋₁₄₃ did not show lower real-time efflux than untreated cells (Figure 5A). This observation is notable in light of the fact that, in previous conductance studies, similar peptides were shown to bind TolC (Zakharov *et al.*, 2016, Jakes, 2017) and to occlude the channel (Zakharov *et al.*, 2016, Zakharov *et al.*, 2004).

Cells exposed to high concentrations of ColE1-T showed less decay in final fluorescence than untreated cells, indicating that ColE1-T partially inhibits efflux (Figure 5B). Finally, exposure to high concentrations of ColE1-TR produced no decrease in fluorescence, showing full inhibition of efflux (Figure 5C).

Because ColE1-TR completely inhibits NNN efflux, we evaluated the capacity of ColE1-TR to potentiate antibiotics representing three different classes that are known TolC substrates: ciprofloxacin, erythromycin, and benzalkonium chloride (from fluoroquinolones, macrolides, and quaternary ammonium compounds, respectively). An effective TolC plug will reduce the concentration required to inhibit growth as antibiotics remain trapped within the cell. WT *E. coli* cells that were exposed to 100 μ M ColE1-TR with each of these antibiotics showed significantly lower minimum inhibitory concentrations (MICs), the lowest concentration of antibiotic that inhibits visible growth, than cells exposed to the antibiotics alone (Figure 5D): exposure to 100 μ M ColE1-TR made WT *E. coli* ~2 to 7-fold more susceptible to these antibiotics (Figure 5,

354 source data 1). Exposure of WT cells to 10μM ColE1-TR was sufficient to potentiate
355 erythromycin.

356

Figure 5. Colicin E1 fragments inhibit efflux and potentiate antibiotics. (A-C) Effect of colicin E1 fragments on efflux: WT with no protein (green), $\Delta tolC$ (red), WT + 10 μ M (orange), WT + 100 μ M protein (blue). (A) ColE1-T₁₀₀₋₁₄₃, (B) ColE1-T, (C) ColE1-TR. All efflux experiments used 10 μ M of fluorescent reporter dye NNN. (D) Antibiotic susceptibilities in the absence (green) and presence of ColE1-TR added to WT at 10 μ M (orange) and 100 μ M (blue). MICs for $\Delta tolC$ (red) are included as a reference. Data shown from 3 biological replicates reported as the mean and individual data points. Bars indicate standard error of the mean. Data values shown in Figure 5, source data 1.

TolA is normally involved in outer membrane invagination during cell division (Gerding *et al.*, 2007). Like Colicin E1, Colicin E9 contains an intrinsically unstructured N-terminus that engages the Tol-Pal system once it penetrates into the periplasm (Loftus *et al.*, 2006). Engagement of the Tol-Pal system by the N-terminal region of colicin E9 disrupts native Tol-Pal system function leading to outer membrane defects (Rassam *et al.*, 2018). Outer membrane defects may lead to enhanced antibiotic susceptibility. To rule out outer membrane-mediated defects as the main contributor to the reduction in MICs seen in Figure 5, we tested the effect of using a truncation of ColE1-TR (ColE1-TR Δ 1-40) lacking the N-terminal 40 residues including the TolA box. The MIC of ColE1-TR and ColE1-TR Δ 1-40 are identical (Figure 5 supplemental figure 1) indicating that ColE1-TR engagement of TolA does not contribute to the observed antibiotic susceptibility.

Discussion

There are two conflicting models of ColE1 translocation: 1) the total thread model in which the entire colicin unfolds and threads through TolC (Cramer *et al.*, 2018, Jakes, 2017, Francis *et al.*, 2021) and 2) the pillar model, in which colicin E1 inserts into TolC as a helical hairpin, facilitating LPS-mediated, self-translocation of the colicin cytotoxic domain (Zakharov *et al.*, 2016). Our data support aspects of both models. The belief in a closed hinge conformation of bound ColE1 prompted two arguments against the total thread mechanism: 1) the ColE1-T closed hinge conformation is too wide to fit through TolC and 2) both ends would face the extracellular milieu (Zakharov *et al.*, 2016, Cramer *et al.*, 2018). The ColE1-T hinge opening in the bound state—which was unanticipated by the pillar model—resolves these objections: colicin threads into the TolC barrel with the amino terminus pointing into the periplasm, as the total thread model hypothesized. Our trypsin digests show ColE1-TR stalls at the outer membrane of *E. coli* cells and the single-molecule fluorescence images confirm that ColE1-TR remains clustered and does not diffuse as would be expected if entering the periplasm or cytoplasm. These puncta form regardless of the presence or absence of the TolA binding region on ColE1-TR.

Though we cannot exclude the possibility that ColE1 T and R domain translocation depends on the C domain, we observe—as hypothesized by the pillar model—that colicin sits inside TolC but does not translocate. These results are in agreement with early studies of pore-forming colicins in which trypsin added to the extracellular environment can reverse colicin activity (Plate & Luria, 1972, Dankert *et al.*, 1980, Dankert *et al.*, 1982, Benedetti *et al.*, 1992, Duche, 2007). We anticipate that the stable ColE-TR:TolC complex forms regardless of the presence of the C domain.

If the C domain does translocate while the T and R domains remain bound, we anticipate that the requirement for TolA is due to its role in membrane maintenance. Recruitment of the Tol-

397 Pal system by a different group A colicin (ColE9) causes membrane defects similar to those in a
 398 $\Delta tolA$ strain (Rassam et al., 2018). Therefore, in the pillar model, colicin binding of TolA may
 399 facilitate C domain insertion through the defective membrane, whereas in the total thread model
 400 binding TolA transduces the proton motive force to unfold ColE1 and pull it into the periplasm
 401 (Figure 6, supplemental figure 1). Because we did not use the toxic C domain, our data cannot
 402 clarify what occurs after the T and R domain of full length colicin bind the outer membrane
 403 proteins. Although our studies find that ColE1-TR remains on the surface of the bacteria, it is
 404 certain that the C domain, when present, translocates across the membrane into the target bacteria
 405 to kill the cell. Our data does not exclude the possibility that the translocation of the T and R
 406 domains occurs along with the C domain in full length colicin, nor does it support T or R domain
 407 translocation. Thus, though our studies give a more complete view of the early stages of ColE1
 408 binding the surface of its target, we cannot determine if insertion of the C domain occurs via the
 409 total thread model or via a modified pillar model.

410 Shortly after this work was made available, a similar structure demonstrated that KlebC,
 411 an rRNase bacteriocin from *Klebsiella pneumoniae*, binds TolC from *Klebsiella*
 412 *quasipneumoniae* with a binding mode similar to that of ColE1 binding TolC.(Housden *et al.*,

Figure 6. Model of colE1-TR inhibition of efflux. The R domain of ColE1-TR (orange) binds to BtuB (cyan) with high affinity and anchors colE1-TR to the surface of the cell. The T domain (red) then inserts into TolC (lavender), plugging the channel and blocking exit of antibiotics (*spheres*). We only see this interaction when ~20 colE1-TR bind in the same cluster though for simplicity only 12 BtuB and 12 TolC proteins are pictured here. (A) Before binding, antibiotics are effluxed (B) After binding the colicin E1 fragments prevent some antibiotic efflux.

2021) KlebC is closed in the unbound state as confirmed by the high-resolution x-ray crystal structure, which supports our proposed conformation (Figure 2E), and opens into a single-pass, kinked-helix to bind to TolC. Taken together, these findings suggest a conserved mechanism for bacteriocins that hijack trimeric efflux pump proteins.

In addition to demonstrating a colicin insertion mechanism, our observations form the basis of a means to manipulate bacterial import and efflux (Figure 6). Though colicin E1 confers some level of antibiotic potentiation, the relatively high concentration of ColE1-TR needed to inhibit efflux may be explained by a combination of the geometric constraints of creating large clusters of BtuB and TolC, and residually unblocked pore, even in the bound state as the large internal volume of TolC is not fully occupied by ColE1. Because of their limited potency, our ColE1-T and ColE1-TR fragments are not likely to be practical in direct applications of antibiotic potentiation. However, our proof-of-concept findings offer a potential roadmap for further development. A more potent TolC binder would not need the R domain anchor for affinity. Such a binder could be designed using the atomic details of the ColE1-T:TolC structure as a basis. Moreover, this scaffold can be used for efflux pump inhibitors for at least five other bacterial organisms. Each of these organisms has a structurally characterized outer membrane efflux pump that is homologous and structurally similar to TolC (Franklin *et al.*, 2018).

More broadly, there are at least nine known outer membrane protein receptors for bacteriocins. These outer membrane proteins have a variety of functions including adhesion, iron transport, and general import (Cascales *et al.*, 2007, Kleanthous, 2010). Using this same strategy with fragments derived from other bacteriocins may additionally allow for controlled inhibition of other bacterial functions.

Materials and Methods

Key Resources Table

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Antibody	THE HisTag mAb Mouse (mouse monoclonal)	GenScript	Cat#A00186-100; RRID:19J001957	(1:2,000)
Antibody	anti-SurA Rabbit polyclonal (rabbit polyclonal)	Cusabio	Cat#CSB-PA359693HA01EN V; RRID:E0109A	(1:2,500)
Antibody	IRDye 800CW goat anti-mouse IgG secondary (goat polyclonal)	LI-COR	Cat#925-32210; RRID:C81106-01	(1:10,000)
Antibody	IRDye 800CW Donkey anti-Rabbit (donkey monoclonal)	LI-COR	Cat#925-32213; RRID:C80829-10	(1:10,000)
Cell line (<i>E. coli</i>)	BW25113	Coli Genetic Stock Center	Cat#7636	
Cell line (<i>E. coli</i>)	JW5503-1	Coli Genetic Stock Center	Cat#11430	
Cell line (<i>E. coli</i>)	BL21(DE3)	Coli Genetic Stock Center	Cat#12504	
Recombinant DNA reagent	pTrc99a-NoSS-TolC	This paper	Addgene: 155179	Addgene: 155179
Recombinant DNA reagent	pET303-colE1-T	This paper	Addgene: 155180	Addgene: 155180
Recombinant DNA reagent	pET303-colE1-TR	This paper	Addgene: 155181	Addgene: 155181
Recombinant DNA reagent	pET303-colE1-T-E192C	This paper	Addgene: 180233	Addgene: 180233
Recombinant DNA reagent	pET303-colE1-TR-E366C	This paper	Addgene: 180234	Addgene: 180234
Recombinant DNA reagent	pET303-colE1-TR-E366C-Δ1-40	This paper	Addgene: 180235	Addgene: 180235
Recombinant DNA reagent	pET303-colE1-TR-Δ1-40	This paper	Addgene: 180236	Addgene: 180236
Recombinant DNA reagent	pET303-colE1-T-P110A	This paper	Addgene: 180237	Addgene: 180237
Recombinant DNA reagent	MSP1D1	Addgene	Addgene: 20061	
Software, Algorithm	cryoSPARC 2 v2.12	Punjani, Rubinstein et al. 2017	https://cryosparc.com/	

Software, Algorithm	phenix v1.14-3260	Afonine, Poon et al. 2018	https://www.phenix-online.org/	
Software, Algorithm	serialEM v3.7	Mastronarde 2018	https://bio3d.colorado.edu/SerialEM/	
Software, Algorithm	coot v0.8.9.2-re 7766	Emsley, Lohkamp et al. 2010	https://www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot/	
Software, Algorithm	ISOLDE v1.0b4.dev1	Croll 2018	https://isolde.cimr.cam.ac.uk/static/isolde/doc/tools/ISOLDE.html	
Software, Algorithm	UCSF ChimeraX	Goddard, Huang et al. 2018	https://www.cgl.ucsf.edu/chimerax/	
Software, Algorithm	PyMOL	Schrödinger , LLC	https://pymol.org/2/	

***E. coli* strains.** *E. coli* strains BW25113 and JW5503-1 were purchased from the Coli Genetic Stock Center (CGSC). JW5503-1 is a tolC732(del)::kan from the parent strain BW25113. BL21(DE3) were used for production of the colicin constructs and TolC. BL21(DE3) has a premature stop codon at residue 58 of the *btuB* gene and therefore we used it as a $\Delta btuB$ strain for microscopy.

Expression and Purification. The gene for ColE1-TR was synthesized as a gBlock (Integrated DNA Technologies) and cloned into pET303. Inverse PCR was used to delete the R domain and produce colicin E1-T. Colicin E1-TR-GFP was produced by inserting GFP at the C terminus of ColE1-TR. A gBlock (Integrated DNA Technologies) was synthesized for GFP containing complimentary flanking sequences to the plasmid with ColE1-TR and inserted with the omega-PCR technique. ColE1-TR $_{\Delta 1-40}$ was produced by inverse PCR to delete residues corresponding to 1 through 40. All point mutants (ColE1-T-E192C, ColE1-TR-E366C, ColE1-TR-E366C $_{\Delta 1-40}$, and ColE1-T-P110A) were generated by inverse PCR with mutagenic primers.

Plasmids were transformed into *E. coli* BL21(DE3) cells and plated on LB + agar + 100 µg/mL carbenicillin. Single colonies were inoculated into 50 mL LB broth with 100 µg/mL carbenicillin and grown overnight at 37 °C with shaking at 250 r.p.m. Proteins were produced by inoculating 1L of TB supplemented with 0.4% glycerol, 10 mM MgCl₂ and 100 µg/mL carbenicillin with 20 mL of the overnight culture. The culture was grown at 37 °C to an OD₆₀₀ of 2.0 and induced with 1 mM IPTG. Expression cultures were then grown at 15 °C for 24 hours and harvested at 4,000 g for 30 minutes at 4 °C. Cell pellets were resuspended at 3 mL/g of cell pellet in lysis buffer (TBS, 5 mM MgCl₂, 10 mM imidazole, 1mM PMSF, 10 µg/mL DNase, 0.25 mg/mL lysozyme) and lysed via sonication (2 minutes, 2s on, 8s off, 40% amplitude, QSonica Q500 with 12.7 mm probe) in an ice bath. Lysates were centrifuged at 4,000 g for 10 minutes to remove un-lysed cells and debris. The supernatant was centrifuged again at 50,400 g in a Beckman Coulter J2-21 for 1 hour at 4 °C. Clarified lysates were applied to a 5 mL HisTrap FF column and purified using an ÄKTA FPLC system with a 20-column volume wash step with binding buffer (TBS, 25 mM imidazole) and eluted using a linear gradient from 0-50% elution buffer (TBS, 500 mM imidazole) in 10 column volumes. Concentrated proteins were loaded onto a HiLoad Superdex 16/60 200 pg gel filtration column and eluted into phosphate buffered saline (PBS) pH 7.4.

TolC production and purification was conducted in the same manner for preparation for cryoEM and for SEC. The gene for full-length TolC (a generous gift from R. Misra) was cloned into pTrc99a with the signal sequence deleted for expression into inclusion bodies. Plasmids were transformed into BL21(DE3) and plated on LB + agar + 100 µg/mL carbenicillin. A single colony was picked and grown in LB at 37 °C with shaking at 250 r.p.m. overnight. In the morning, 1L of LB was inoculated with 20 mL of the overnight culture and grown at 37 °C with shaking at 250 r.p.m. until the culture reached an OD₆₀₀ of 0.6, at which point protein expression was induced with 1mM IPTG for an additional 4 hours. Cells were then harvested at 4,000g for 30 minutes at 4

481 °C. Cell pellets were resuspended in mL of lysis buffer (TBS, 5 mM MgCl₂, 0.2 mg/mL
482 lysozyme, 5 µg/mL DNase, 1mM PMSF) at 3 mL/1g of cell pellet and lysed via sonication (4
483 minutes, 2s on, 8s off, 40% amplitude, QSonica Q500 with 12.7 mm probe) in an ice bath. Cell
484 lysates were centrifuged at 4,000 g for 30 minutes at 4 °C to harvest inclusion bodies. Inclusion
485 body pellets were resuspended in inclusion body wash buffer (20 mM Tris pH 8.0, 0.5 mM
486 EDTA, 1% Triton X-100) and centrifuged at 4,000g for 30 minutes at 4 °C. The inclusion body
487 wash was repeated two more times. A final wash was done in 20 mM Tris pH 8.0 and inclusion
488 bodies were stored at -20 °C. Inclusion bodies were solubilized in 20 mM Tris pH 8.0, 8M urea at
489 500 µM. N-octylpolyoxyethylene was added to 5 mL of solubilized TolC to a final concentration
490 of 10% detergent and pipetted into a Slide-A-Lyzer G2 dialysis cassette with a 10,000 molecular
491 weight cut off. Refolding was initiated by dialysis in 5L of 20 mM Tris pH 8.0, 100 mM NaCl at
492 4 °C with stirring overnight. TolC was centrifuged at 15,200 g for 10 minutes at 4 °C to remove
493 aggregates. The supernatant was filtered through a 0.22 µm filter, concentrated to 2 mL, applied
494 onto a HiLoad 16/60 Superdex 200 pg column on an ÄKTA Pure FPLC system, and eluted with
495 1.5 column volumes in 20 mM Tris pH 8.0, 100 mM NaCl, 0.05% n-dodecyl-β-D-maltoside.
496 TolC containing fractions were pooled and concentrated to 300 µM in Amicon centrifugal filters
497 with molecular weight cutoff of 30kDa.

498 Membrane scaffold protein MSP1D1. MSP1D1 in pET28a was purchased from Addgene
499 and was expressed and purified as previously described(Hagn *et al.*, 2018).

500

501 **Peptide Synthesis.** Cole1-T100-143 was synthesized using standard Fmoc chemistry with a
502 CEM liberty blue microwave peptide synthesizer. The peptides were cleaved using a solution of
503 92.5:2.5:2.5:2.5 TFA:TIPS:H₂O:DoDt and the crude peptides were purified using HPLC.
504 Analytical HPLC traces were acquired using an Agilent 1100 quaternary pump and a Hamilton
505 PRP-1 (polystyrene-divinylbenzene) reverse phase analytical column (7 µm particle size, 4 mm x

25 cm) with UV detection at 215 nm. The eluents were heated to 45 °C to reduce separation of rotational isomers, and elution was achieved with gradients of water/ acetonitrile (90:10 to 0:100 containing 0.1% TFA) over 20 min. Low-resolution mass spectra (LRMS) were obtained using a Waters Micromass ZQ 4000 instrument with ESI+ ionization.

Extracellular Protease Digestion.

Growth, incubation with protein, and washing: 5 mL overnight cultures of *E. coli* (BW25113) were grown in LB media at 37 °C with shaking (250 r.p.m.) overnight. A 50 mL culture of LB was inoculated with 1 mL of the overnight culture and grown at 37 °C with shaking (250 r.p.m.) until an OD600 of 0.6 was reached. The cultures were centrifuged at 4,000 g at 4°C for 10 minutes. For the exogenous addition of protein, cell pellets were resuspended in cold PBS (Bio-Rad) containing 10 µM of either ColE1-T or ColE1-TR protein. Cells were incubated with protein with gentle rocking for 1 hour at 37 °C. After incubation with protein, the cells were collected at 4,000 g at 4 °C for 10 minutes and resuspended in cold PBS without protein. The centrifugation and wash were then repeated a second time. The PBS washes are designed to remove unbound protein.

Protease digestion of intact cells and lysed cells: Each sample is split into two-1 mL aliquots. The first aliquot is the intact cell condition which is placed on ice. The second aliquot is the lysed cells control for protease activity. This aliquot was lysed by adding 0.25 mg/mL lysozyme (10 µL of a 25 mg/mL in 10 mM Tris pH 8.0, 50% (v/v) glycerol) and incubated at room temperature for 15 minutes followed by five freeze-thaw cycles in liquid nitrogen. The intact cells condition and lysed condition were both split into three 270 µL aliquots. For each triplet, trypsin was added at 5 and 20 µg/mLs to digest any extracellularly exposed protein. 30 µL of PBS was added to the third aliquot of each triplet for a no trypsin sample. The three aliquots were incubated at room temperature to allow for trypsin digestion. After 30 minutes, 20 µL of 100

531 mM PMSF was added to terminate the trypsin digestion reaction. Then the samples associated
532 with the intact cells condition were lysed. Lysozyme was added to a final concentration of 0.25
533 mg/mL and incubated at room temperature for 15 minutes followed by five freeze-thaw cycles in
534 liquid nitrogen. All samples were stored at -20 °C until ready for western-blot analysis.

535 *Western blot:* Samples were separated by SDS-PAGE. Colicin proteins were probed with
536 THE HisTag mAb mouse (GenScript). SurA was probed with anti-SurA Rabbit polyclonal
537 (Cusabio). Western blots were imaged by fluorescence using a LI-COR Odyssey imager with
538 secondary antibodies IRDye 800CW goat anti-mouse IgG and IRDye 800CW Donkey anti-Rabbit
539 for Colicins and SurA, respectively.

540

541 **Single-Molecule Microscopy.** Cysteine mutations were introduced at the C-terminus before the
542 histidine tag for fluorophore conjugation. These constructs were purified as described in the
543 Expression and Purification section with the addition of 1 mM TCEP in all buffers. All
544 subsequent steps were performed with limited exposure to light and in amber tubes. Cyanine3
545 (Cy3) maleimide (Lumiprobe) was reconstituted in DMSO. Fluorophore labeling was achieved by
546 mixing a 20-fold molar excess of Cy3 maleimide to protein and incubating overnight at 4 °C. Free
547 dye was removed by gel filtration on a Sephadex NAP-10 G-25 column. Simultaneously to the
548 dye removal, the sample was buffer exchanged into storage buffer (PBS pH 7.4, 1 mM DTT, 1
549 mM EDTA). The degree of labeling was determined spectrophotometrically from the
550 concentrations of the dye and protein solutions using their respective extinction coefficients, ϵ , as
551 described by their manufacturers or for the proteins as estimated by Expasy ProtParam (Cy3
552 $\epsilon_{548\text{nm}} = 162,000 \text{ L mol}^{-1} \text{ cm}^{-1}$; ColE1-T-E192C $\epsilon_{280\text{nm}} = 9,970 \text{ L mol}^{-1} \text{ cm}^{-1}$; ColE1-TR-E366C
553 $\epsilon_{280\text{nm}} = 14,440 \text{ L mol}^{-1} \text{ cm}^{-1}$). Labeling efficiencies were ~75% and ~85% for ColE1-T-E192C
554 and ColE1-TR-E366C, respectively. Protein concentrations were adjusted according to the
555 percentage of labeled protein.

Cultures of *E. coli* (WT, $\Delta tolC$, or BL21(DE3)) were grown in LB medium at 37 °C with shaking (180 r.p.m.) overnight, then transferred to MOPS minimal medium (Teknova) with 0.2% glycerol and 1.32 mM K_2HPO_4 and grown at 37 °C for 13 h. The sample was transferred to MOPS medium and grown to turbidity at 37 °C overnight. A 1-mL aliquot of culture was centrifuged for 2 min at 4,850 g to pellet the cells. The pellet was washed in 1 mL MOPS and centrifuged a second time. The supernatant was then removed, and the cell pellet was resuspended in 500 μ L MOPS. A 1.0 μ L droplet of concentrated cells was placed onto a glass slide. Then, a 1.0 μ L droplet of 1 μ g/mL colicin E1 protein construct stock was added to the cells. The droplet was covered by an agarose pad (1% agarose in MOPS media) and a second coverslip. Samples were imaged at room temperature using wide-field epifluorescence microscopy with sensitivity to detect single dye molecules as described previously (Tuson & Biteen, 2015). Briefly, fluorescence was excited by a 561-nm laser (Coherent Sapphire 560-50) for Cy3 or a 488-nm laser (Coherent Sapphire 488-50) for GFP. The lasers were operated at low power densities (1 – 2 W/cm²), and fluorescence was imaged with an Olympus IX71 inverted microscope with a 100x, 1.40-NA phase-contrast oil-immersion objective and appropriate excitation, emission, and dichroic filters. A Photometrics Evolve electron multiplying charge-coupled device (EMCCD) camera with > 90% quantum efficiency captured the images at a rate of 20 frames per second. Each detector pixel corresponds to a 49 nm $\times$ 49 nm area of the sample. *E. coli* cells were identified and fluorescent signals were detected using the Single-Molecule Localization by Local Background Subtraction (SMALL-LABS) algorithm. (Isaacoff *et al.*, 2019)

Co-elution. The interaction of TolC and colicin E1-T or -TR were determined by co-elution on an SEC column. Purified TolC and colicin E1-T or -TR were mixed at a 1:2 molar ratio to ensure an excess of colicin to saturate TolC binding sites and incubated at room temperature for 1 hour before loading onto a Superdex 200 Increase 10/300 GL column (GE Healthcare). The protein

581 was eluted with 1.5 column volumes into 20 mM Tris pH 8.0, 40 mM NaCl, 0.05% n-dodecyl- β -
582 D-maltoside for ColE1-T. For ColE1-TR the NaCl concentration was increased to 200 mM to
583 prevent precipitation. Elution fractions were collected every 0.5 mL. Peak fractions were
584 concentrated to 20 μ L and analyzed by 4-20% SDS-PAGE.

585

586 **Real-Time Efflux.** Real-time efflux activity in the presence of ColE1-TR was determined as
587 previously described with some modifications (Bohnert *et al.*, 2010, Bohnert *et al.*, 2011). Cells
588 were resuspended to OD600 1.5 in cold PBS with and without 10-100 μ M colicin proteins and
589 incubated for 15 minutes on ice. To turn off efflux, 100 μ M carbonyl cyanide m-chlorophenyl
590 hydrazone (CCCP) was added. After an additional 15 minutes the efflux dye NNN was added to
591 the cells to 10 μ M. To determine cell viability after treatment with CCCP and NNN, we counted
592 the colony-forming units of treated and untreated cells. Treated cells had 93% viability compared
593 to untreated cells as determined by colony-forming units (treated cells had 4.4×10^9 CFU/mL,
594 untreated cells had 4.7×10^9 CFU/mL). The cells were incubated at 25 °C with shaking at 140
595 r.p.m. for 2 hours. Cells were harvested at 3,500 g for 5 minutes and washed once in 20 mM
596 potassium phosphate buffer pH 7 with 1 mM $MgCl_2$. Cell concentrations were adjusted to OD600
597 1.0 and placed on ice. Then, 2 mL of the cell suspension was loaded into a quartz cuvette (Agilent
598 Technologies). Fluorescence was measured with an Agilent Cary Eclipse fluorescence
599 spectrophotometer with slit widths at 5 and 10 nm for excitation wavelength of 370 nm and
600 emission wavelength of 415 nm. Fluorescence measurements were taken every 1 second. After
601 100 seconds, 50 mM glucose was added to re-energize the cells and initiate efflux, and
602 fluorescence data were collected for an additional 600 seconds. Figure 5A-C, reflects time after
603 glucose addition.

604

605 **Minimum Inhibitory Concentrations (MICs).** MICs were determined using the broth dilution
606 method (Wiegand *et al.*, 2008) with some modifications in 96 well plate format using LB media
607 in 100 μ L well volumes. Cultures were grown at 37 °C with shaking at 250 r.p.m. and OD600
608 was read on a Biotek plate reader after 20 hours. MICs are defined by the lowest concentration
609 that prevents visible growth. We chose an OD600 of >0.1 as the cutoff for growth. We report
610 MICs as the mean of 3 biological replicates with each replicate plotted (Figure 5D, Figure 5,
611 source data 1). Due to the 2-fold discretized nature of concentration ranges used to determine
612 MICs we do not report statistical significance values as is typical of MIC reporting.

613
614 **Reconstitution of TolC into Amphipol.** 1 mL of TolC at 0.5 mg/mL was mixed with 0.75 mL of
615 A8-35 (Anatrace) amphipol at 33 mg/mL for a 50-fold weight excess and incubated at room
616 temperature for 30 minutes. Bio-Beads SM-2 resin that was washed in methanol and equilibrated
617 with 20 mM Tris, 40 mM NaCl was added to the reaction mixture at 0.5 g/mL to initiate detergent
618 exchange for A8-35 and incubated with rotation at 4 °C overnight. The mixture was transferred to
619 a tube with fresh Bio-Beads and incubated at 4 °C for an additional 4 hours. The reaction mixture
620 was loaded onto a HiLoad 16/60 Superdex 200 pg column on an ÄKTA Pure FPLC system and
621 eluted with 1.5 column volumes in 20 mM Tris pH 8.0, 40 mM NaCl to remove free A8-35 and
622 detergent. For colicin-bound TolC in A8-35, colicin E1-T was added to the reaction mixture at a
623 >2 molar excess prior to gel filtration. TolC or colicin-bound TolC in A8-35 was concentrated to
624 2-4 mg/mL for cryoEM.

625
626 **Reconstitution of TolC into lipid nanodiscs.** POPC (Avanti Polar Lipids) in chloroform was
627 dried under a stream of nitrogen and freeze dried to remove residual chloroform. Lipids were
628 reconstituted to 50 mM in cholate buffer (20 mM Tris pH 8.0, 100 mM NaCl, 0.5 mM EDTA,
629 100 mM cholate) in an amber glass vial. The vial was submerged under a stream of warm water

630 until the solution became clear. Lipids, membrane scaffold protein, and TolC were mixed in a
631 36:1:0.4 ratio as previously described (Daury *et al.*, 2016). Final concentrations were 4.5 mM
632 POPC, 125 μ M MSP1D1, 50 μ M TolC in a 2 mL reaction with cholate brought up to 20 mM and
633 dodecyl-maltoside up to 0.1%. The reaction mixture was incubated on ice for 1 hour. Bio-Beads
634 SM-2 resin that was washed in methanol and equilibrated with 20 mM Tris, 40 mM NaCl were
635 added to the reaction mixture at 0.5 g/mL to initiate nanodisc formation and incubated with
636 rotation at 4°C overnight. The mixture was transferred to a tube with fresh Bio-Beads and
637 incubated at 4 °C for an additional 4 hours. The reaction mixture was loaded onto a HiLoad 16/60
638 Superdex 200 pg column on an ÄKTA Pure FPLC system and eluted with 1.5 column volumes in
639 20 mM Tris pH 8.0, 40 mM NaCl to separate TolC inserted into nanodiscs from empty nanodiscs.
640 For colicin-bound TolC in nanodiscs, colicin E1-T was added to the reaction mixture at a >2
641 molar excess prior to gel filtration. TolC or colicin-bound TolC in nanodiscs was concentrated to
642 2-4 mg/mL for cryoEM.

643
644 **Cryoelectron microscopy.** 3 μ L of protein solution (TolC/ColE1-T in amphipol or TolC/ColE1-
645 T in nanodiscs) was diluted to approximately 1.05 mg/mL concentration, applied to a glow-
646 discharged TEM grid, and plunge-frozen in ethane using a Vitrobot Mark IV (FEI Company) with
647 grade 595 filter paper (Ted Pella). Glow discharge was performed in ambient atmosphere at 0.39
648 mBar pressure. Imaging was performed using a Talos Arctica (FEI Company) operated at 200 kV
649 with energy-filter and K2 Summit (Gatan, Inc.) for detection. To collect multiple images per hole
650 while maintaining parallel illumination conditions, a nonstandard 20 μ m condenser aperture was
651 used to image TolC-ColE1-T in nanodiscs. At nominal magnification of 205,000 $\times$, images were
652 acquired in counting mode with a calibrated pixel size of 0.6578 Å. Fresnel fringes attributable to
653 the beam interaction with the aperture were often seen in images. Some investigators have moved
654 the microscope stage and altered the nominal objective lens true focus point to generate a fringe-

free condition (Weis & Hagen, 2020). In this study, imaging at 205,000× with a 20 μm aperture yielded better results than imaging at 130,000× with a 50 μm aperture; at 130,000× with a 20 μm aperture the fringes were extremely severe due to the larger field of view, so a full dataset was not collected with those conditions. TolC in nanodiscs (without ColE1-T) was imaged at 130,000× with a 50μm condenser aperture (Figure 1, source data 1).

Micrographs were collected with SerialEM (Mastronarde, 2018) using in-house modifications of the scripts of Chen Xu (sphinx-emdocs.readthedocs.io). Briefly, multishot imaging was configured with 4 images per hole for each of 16 holes; intermediate-magnification montages of grid squares were acquired; points were selected manually for collection of 64 images per point; images were acquired using coma-compensated image shift as gain- and dark-corrected LZW-compressed TIFs. Side, top, and oblique views were seen in areas of thin ice. During screening, ice thickness was estimated at 17-30nm by the method of I_0/I_{ZLP} (Rice *et al.*, 2018).

The collection of micrographs of TolC without colicin at 130,000× magnification has been previously described (Budiardjo *et al.*, 2021).

3D reconstruction and modeling. Final reconstructions were obtained using cryoSPARC 2 (Punjani *et al.*, 2017). 1,018 micrographs were collected of amphipol-embedded TolC/ColE1-T. Micrographs were motion-corrected using RELION 3 (Zivanov *et al.*, 2018). CTF parameters were determined by means of *ctffind* (Rohou & Grigorieff, 2015). 115,362 particles were selected with crYOLO (Wagner *et al.*, 2019). 2D classification revealed that many particles had aberrant morphology and only 24,624 (21%) were selected for 3D reconstruction. *Ab initio* reconstruction in cryoSPARC 2 (Punjani *et al.*, 2017) was effective at recovering a map whose shape was similar to that of previously described TolC trimers. However, the data could only be refined to a nominal global resolution of 6.0 Å, and luminal density was insufficiently resolved. 4,492 micrographs were then collected of nanodisc-embedded TolC/ColE1-T and processed similarly.

680 Of the 339,779 particles detected by cryoSPARC Live, 179,834 (53%) were in good classes.
681 Although there were slightly fewer particles per micrograph in the nanodisc dataset, more
682 particles per micrograph were usable. Beginning with the *ab initio* model and mask derived from
683 the amphipol data, this particle set was refined by cryoSPARC 2 non-uniform refinement with or
684 without imposed C₃ symmetry. The maps refined to nominal global resolutions of 2.81 Å and
685 3.09 Å for the symmetrized and asymmetric maps, respectively. There was local variation in
686 resolution within the map, with consistent, high resolution in the middle of TolC and lower
687 resolution at the lids and for ColE1-T. Local resolution was computed in cryoSPARC by the
688 locally windowed FSC method (Cardone *et al.*, 2013) and rendered with UCSF Chimera. To
689 reduce the voxel-based values to averages for four regions of the complex, the local resolution
690 map was masked to include only voxels within 3 Å of a modeled atom and then the median value
691 was calculated for those voxels closest to ColE1, closest to TolC residues 168-172 and 386-390,
692 closest to TolC residues 285-301 and 76-82, or closest to other TolC residues. Furthermore, it is
693 notable that while the nanodisc appears as a double-belt in the symmetrized map, in the
694 asymmetric map the nanodisc protein mostly appears on the side of TolC that is bound to ColE1-
695 T. One possible explanation is that, despite masking, nanodisc asymmetry is a confounder of the
696 asymmetric refinement and is one source of heterogeneity in the data. Another possibility is that
697 the C-terminus of ColE1-T forms an interaction with the nanodisc, causing preferential alignment
698 of the nanodisc with respect to the TolC/ColE1-T complex.

699 196,158 particles of TolC without colicin were obtained as previously described
700 (Budiardjo *et al.*, 2021). Homogeneous refinement yielded a structure at 2.89 Å; local motion
701 correction and global CTF refinement yielded a final map at 2.84 Å.

702 Modeling was initiated by rigidly docking a crystal structure of TolC in complex with
703 hexamminecobalt (1TQQ) (Higgins *et al.*, 2004) into the symmetrized map density. Automated,
704 semi-automated, and manual real-space refinement was performed using phenix (Afonine *et al.*,

2018), ISOLDE (Croll, 2018), and coot (Emsley *et al.*, 2010). For TolC with ColE1-T, additional refinement was performed in AMBER using the cryoEM density map as a restraint. Although additional residues are present at the TolC C-terminus, these were not modeled because the density becomes unsharp after residue 428. Blurred density in the map suggests that the C-terminus follows helix 3 towards the periplasmic end of the molecule. ColE1-T was modeled *ab initio*. The 3-fold symmetrized map contains density at $\sim 1/3^{\text{rd}}$ occupancy for ColE1-T and this density contains some high-resolution information not present in the asymmetric map, except near the TolC lid regions where symmetrization overlays ColE1-T density with TolC density at a threefold-related position. First, polyalanine helices were placed in the helical density in coot. Cross-correlation coefficients for both helices are higher with the N-terminus oriented towards the periplasm. Moreover, the side chains angle slightly towards the N-terminus like the fronds of an evergreen angle towards the ground in typical “Christmas tree” conformation, indicating that this is the correct chain orientation. An estimate of the register was made by visual inspection of potential anchor residues. Finally, the hinge region was filled in using phenix and coot. This completed chain was refined against the asymmetric map in ISOLDE. Iteration between phenix, coot, and ISOLDE was continued until acceptable fit to density was achieved. In the case of TolC with ColE1-T, the map was further improved by combining map information with molecular dynamics force fields (Wu *et al.*, 2013). Briefly, starting with the phenix/coot/ISOLDE-refined model, we performed restrained simulated annealing in AMBER, heating from 0K to 300K for 0.2 nsec, holding constant temperature for 1 nsec, and then cooling to 0K over 0.2 nsec. The cryoEM density map is utilized as a restraint potential in the annealing so that both map information and AMBER force field information are simultaneously utilized to obtain an optimum model consistent with the data (Wu *et al.*, 2013). The protein force field used the ff14SB force field (Maier *et al.*, 2015) and a generalized Born implicit solvent model with *igb*=8 (Nguyen *et al.*, 2013), and a nonbonded cutoff of 20 Å. The relative weight of real-space map-based

restraints and the force field was fixed using $fcons=0.02$. For ColE1-T, information from the symmetrized map was integrated into the modeling procedure during manual remodeling in coot, but map-based refinement in phenix, ISOLDE, and AMBER were against the asymmetric map. TolC without ColE1-T was modeled similarly but using the TolC-ColE1-T structure as a starting point instead of 1TQQ, and without final AMBER refinement. Molecular representations were generated with Chimera, ChimeraX (Goddard *et al.*, 2018) or PyMOL (Schrödinger, LLC).

AlphaFold model of ColE1

AlphaFold (Jumper *et al.*, 2021) was used to generate a predicted structural model for full-length ColE1. The amino acid sequence for residues 1-522 of ColE1 was submitted to the AlphaFold Colab (<https://colab.research.google.com/github/deepmind/alphafold/blob/main/notebooks/AlphaFold.ipynb>) with the ‘prokaryotic’ parameter set as ‘True’.

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Supplementary Materials for Colicin E1 opens its hinge to plug TolC

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939 **Figure 1 supplement 1**

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942 **Figure 1, supplemental figure 1: Sample SEC chromatogram of TolC purification after**
943 **refolding.** Elution volumes 57 to 68 mL (shaded) were pooled for further experimentation with
944 TolC. Refolded TolC applied onto a HiLoad 16/60 Superdex 200 pg column on an ÄKTA Pure
945 FPLC system, and eluted with 1.5 column volumes in 20 mM Tris pH 8.0, 100 mM NaCl, 0.05%
946 n-dodecyl-β-D-maltoside

947

Figure 1 supplemental figure 2. Co-elution of ColE1-T with TolC at 200 mM NaCl (black). Under this higher salt concentration, when TolC (purple) and ColE1-T (red) are mixed, there is a smaller peak shift than that seen with ColE1-TR and the presence of a shoulder (black arrow). SDS-PAGE of the shoulder shows presence of both TolC (purple arrow) and ColE1-T (red arrow). Although some binding was detected, this higher salt concentration prevents full binding as indicated by a much fainter band for ColE1-T than seen at the lower salt concentration (Figure 4A).

Figure 1, source data 1.

Cryo-EM data collection, refinement, and validation statistics

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Figure 3, figure supplemental figure 1. Cut away side-view of TolC:ColE1-T complex. Mutations at positions G43 and S257 (spheres) in the TolC barrel (gray) make direct contact with ColE1-T (pink). TolC Mutations to bulkier side chains G43D and S257P were previously shown to abolish WT ColE1 function (Masi *et al.*, 2007) likely due to the introduction of steric clashes that prevent binding. Mutations to ColE1 side chains R108 and R103 (spheres) to glutamine do not abrogate its activity (Jakes, 2017).

Figure 3- video 1. Pan through the atomic model of the TolC/colE1-T complex, from the extracellular aperture to the periplasmic aperture. Carbon atoms of the three TolC polypeptides are colored three different shades of gray and carbon atoms of the colE1-T polypeptide are colored maroon, colE1-T residues of note are colored purple and labeled; field-standard coloring is used for heteroatoms.

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976 **Figure 3 supplemental figure 2. ColE1-TP110A retains binding to TolC.** SEC chromatogram
977 of ColE1-T_{P110A} (red line) and TolC (purple line). The arrow indicates the co-elution (black line)
978 fractions (eluted in buffer containing 40 mM NaCl)
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Figure 4 supplemental figure 1: Additional examples of cells observed during imaging described in Figure 4C and D. Brightfield phase-contrast images of living *E. coli* cells (left) and fluorescence images (right) of ColE1-TR-Cy3 for WT, $\Delta tolC$, and $\Delta btuB$. Scale bar: 2 μ m.

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Figure 4 supplemental figure 2: Single-molecule microscopy. Fluorescence images overlaid on outlines of living *E. coli* cells from phase-contrast microscopy for WT and *ΔtolC* for ColE1-TR-GFP and counts of cells where ColE1-TR-GFP punctum formation was observed. (A) 97% of cells showed no binding of Cy3-labeled ColE1-TR to *ΔbtuB* (B) ColE1-TR-GFP forms similar puncta as Cy3-labeled ColE1-TR (main text Figure 4C). (C) No binding of Cy3-labeled ColE1-T to WT or *ΔtolC* cells was detected with Cy3-labeled ColE1-T.

Figure 4 supplemental figure 3: Examples of cells observed with ColE1-TR Δ_{1-40} -Cy3. Brightfield phase-contrast images of living *E. coli* cells (left) and fluorescence images of ColE1-TR-Cy3 for WT, $\Delta tolC$, and $\Delta btuB$. Scale bar: 2 μ m.

Figure 4, source data 1: Comparison of percent cells with puncta and average number of puncta per cell between ColE1-TR-Cy3 and ColE1-TR Δ_{1-40} -Cy3 with WT $\Delta tolC$ and $\Delta btuB$.

Figure 5 supplemental figure 1. Minimum Inhibitory Concentration of Erythromycin with ColE1-TR TolA box deletion construct. The MIC for erythromycin with 100 µM ColE1-TR or ColE1-TR Δ 1-40 is identical indicating that TolA engagement by ColE1-TR does not contribute to antibiotic susceptibility.

Figure 5, source data 1

Mean minimum inhibitory concentrations (MICs) of antimicrobials in the presence of ColE1-TR.

Figure 6, supplemental figure 1. ColE1 TR sticks to the surface of *E. coli* but the mechanism of C domain insertion is unclear. The binding between ColE1-R to BtuB and ColE1-T to TolC are likely similar regardless of the presence of the ColE1 C domain. Our work excludes the possibility of the original pillar model. However, because our work did not utilize the C domain, we cannot distinguish between a modified version of the pillar model and the total thread model.

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Figure 4 - video 1.

A representative 30-second movie of ColE1-TR bound to a live cell. ColE1-TR localizes on, and remains bound to, the extracellular surface of *E. coli*. Fluorescence movie of Cy3-labeled ColE1-TR on living WT *E. coli* overlaid on outline of the *E. coli* cell from phase-contrast microscopy. Continuous imaging at 25 frames per second; the movie plays in real time at the same speed. This movie corresponds to the first 30 seconds of data used to attain the WT image in Figure 4C. Scale bar: 2 μ m.