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Review

Mechanisms of colicin binding and transport through outer membrane porins

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Abstract

To kill *Escherichia coli*, toxic proteins, called colicins, pass through the permeability barrier created by the outer membrane (OM) of the bacterial cell envelope. We consider a variety of different colicins, including A, B, D, E1, E3, Ia, M and N, that penetrate through the porins OmpF, FepA, BtuB, Cir and FhuA, to subsequently interact with a few targets in the periplasm, including TolA, TolB, TolC and TonB. We review the mechanisms, demonstrated and postulated, by which such toxins enter bacterial cells, from the initial binding stage on the cell surface to the internalization reaction through the OM bilayer Our discussions endeavor to answer two main questions: what is the origin of colicin-binding affinity and specificity, and after adsorption to OM porins, do colicin polypeptides translocate through porin channels, or enter by another, currently unknown pathway? © 2002 Société française de biochimie et biologie moléculaire / Éditions scientifiques et médicales Elsevier SAS. All rights reserved.

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1. Colicin binding on the cell surface

The emergence of bacterial proteins as lethal weapons against other prokaryotic species was one of the very first examples of biological warfare, that was implemented on a molecular scale against an entire population of organisms. This evolutionary process resulted in mobile, plasmid-mediated toxic molecules that kill by several different mechanisms, including depolarization of membrane ion gradients, degradation of nucleic acids, in some cases leading to inhibition of protein synthesis, and degradation of cell walls. Such toxins, or *bacteriocins*, that are called *colicins* when they target *Escherichia coli*, likely predate the hemolysins and ADP-ribosylating exotoxins that act against eukaryotic cells. In a manner that likely emulated the origin of antibiotics, bacteriocins derived from cytoplasmic enzymes and membrane proteins of one prokaryotic genus that fortuitously interfered with the biochemistry of another genus, or even the same genus. Once accidentally recognized in this way, selection optimized the toxicity of the proteins, as well as their mechanisms of exportation from the host species, and most importantly here, their entry into target cells. Colicins pass through the *E. coli* outer membrane (OM), a formidable barrier that itself evolved to exclude entry of all shapes, sizes and types of noxious molecules. This primordial conflict for resources produced colicin molecules that join three diverse protein domains, which catalyze three sequential actions in the implementation of toxicity: binding, translocation and killing (Fig. 1). The structural organization of colicins constitutes just another example of tripartite architecture in the microbial world: numerous enzymes, and OM proteins in particular [1], form hetero- or homotrimers, which enhances or imparts their biochemical attributes.

The three different domains of colicins [2–4] apparently each perform different functions. The central regions form unique structures that bind to OM receptor proteins [3,5–9], and the N-termini [3,10] act in unknown ways during the transport of the toxin through the OM [11–13]: group A colicins [14] are Tol-system dependent and group B colicins [15] are TonB dependent. The C termini contain the toxic activities of colicins [3,16,17] that either create voltage-gated pores in the cytoplasmic membrane (colicin A: [18,19]; colicin E1: [20]; colicin Ia: [21]; colicin N: [22];

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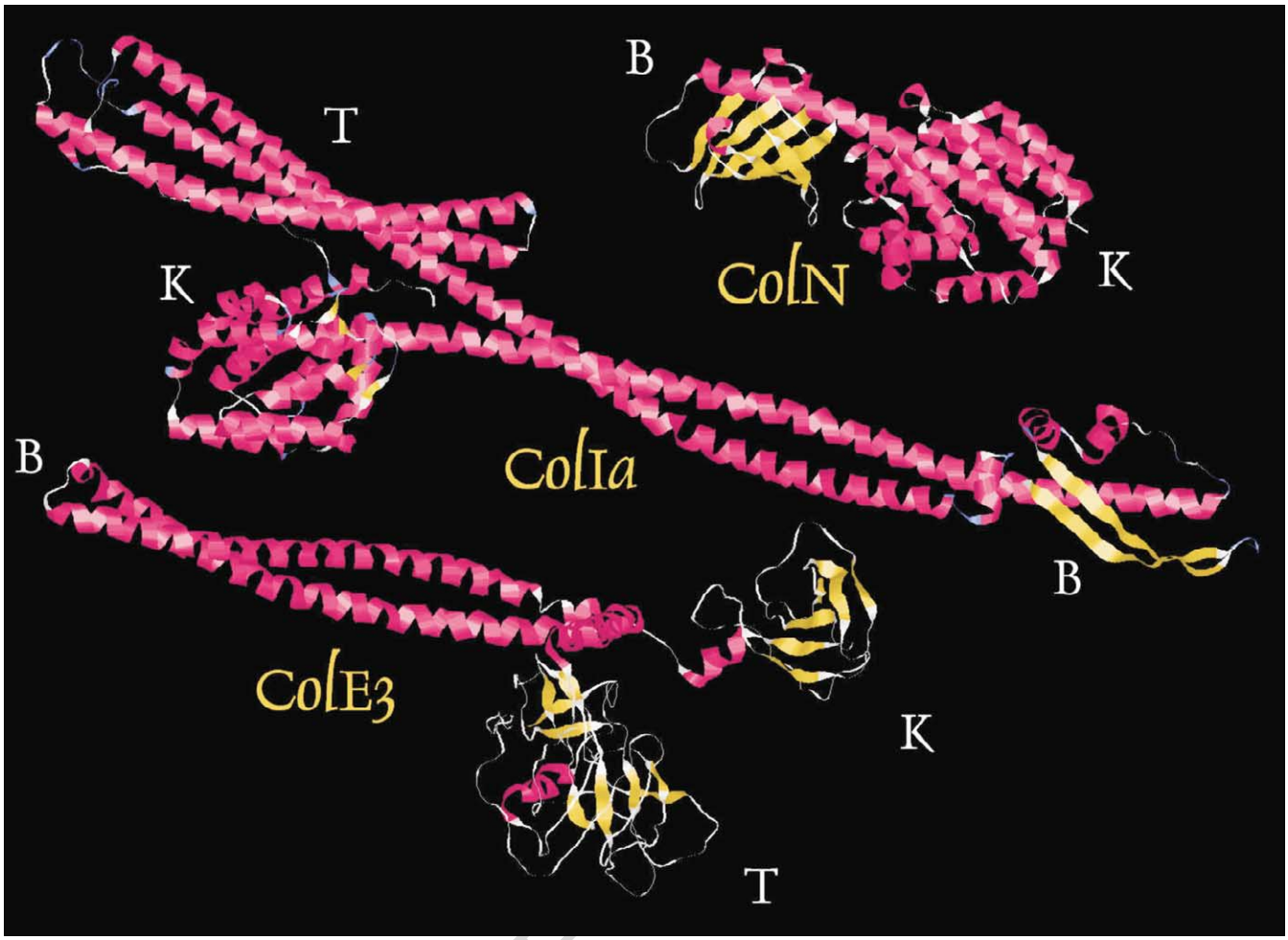

Fig. 1. Colicins E3, Ia and N. The crystal structures of the three colicins E3, Ia and N [21,22,98,99] show different tertiary forms in the binding (B), translocation (T) and killing (K) domains. For ColN, the T-domain is not yet crystallographically solved.

colicin B: [23,24]), or digest nucleic acids (DNA: [25–27]; RNA: [28–31]), or degrade peptidoglycan (Col M; [32–34]), or inhibit protein synthesis by nuclease activity (E3, D; Bowman, 1971; Tomita et al., 2000).

1.1. Structural mimicry

The binding of bacteriocins to the prokaryotic cell surface involves structural mimicry that was easily imagined [35], but difficult to demonstrate. For example, even crystallographic knowledge of colicin and porin structures did not resolve the identities of residues that participate in the initial ligand-receptor association reaction. Colicins are water-soluble, monomeric proteins [36]. Several of their different functional domains were cloned and purified [6,9,37], and their receptor-binding domains were often defined by a combination of sequence homology studies and activity mapping. Pre-crystallographic elucidation of colicins and colicin receptors, however, did not predict the unusual architecture of the former, the complexity of the surface topologies of the latter, or the individual nuances of either class of proteins (Figs. 1–3). Although OM proteins all contain a transmembrane β-barrel, their surface loops create structural and functional individuality. Bacteriophage and bacteriocins use the many OM transport proteins that Gram-negative bacteria synthesize as surface receptors. Most phage and colicin receptors were identified several decades ago [14,15,35,38–40] before their biochemical functions were known, but the list of opportunistic organisms still continues to expand in this millennium [41]. Noxious agents of this kind usually enter bacterial cells through nutritionally indispensable uptake pathways, and the selectivity of their ligand-receptor recognition reactions often involves a cell surface competition between the toxin and a small metal chelate (called a *siderophore* if it contains iron) for binding to the same OM protein. In spite of the general similarity of Omp architecture (Fig. 2), and the apparent similarity of many colicin-binding domains (Fig. 1), and the chemical similarities of metal chelate structures, many *E. coli* OM receptor proteins display specificity for a single solute and one or a few colicins. Thus the siderophore ferric enterobactin and the colicins B and D bind to and enter through FepA [35,42] and only FepA, whereas the siderophore ferrichrome and colicin M bind to and enter

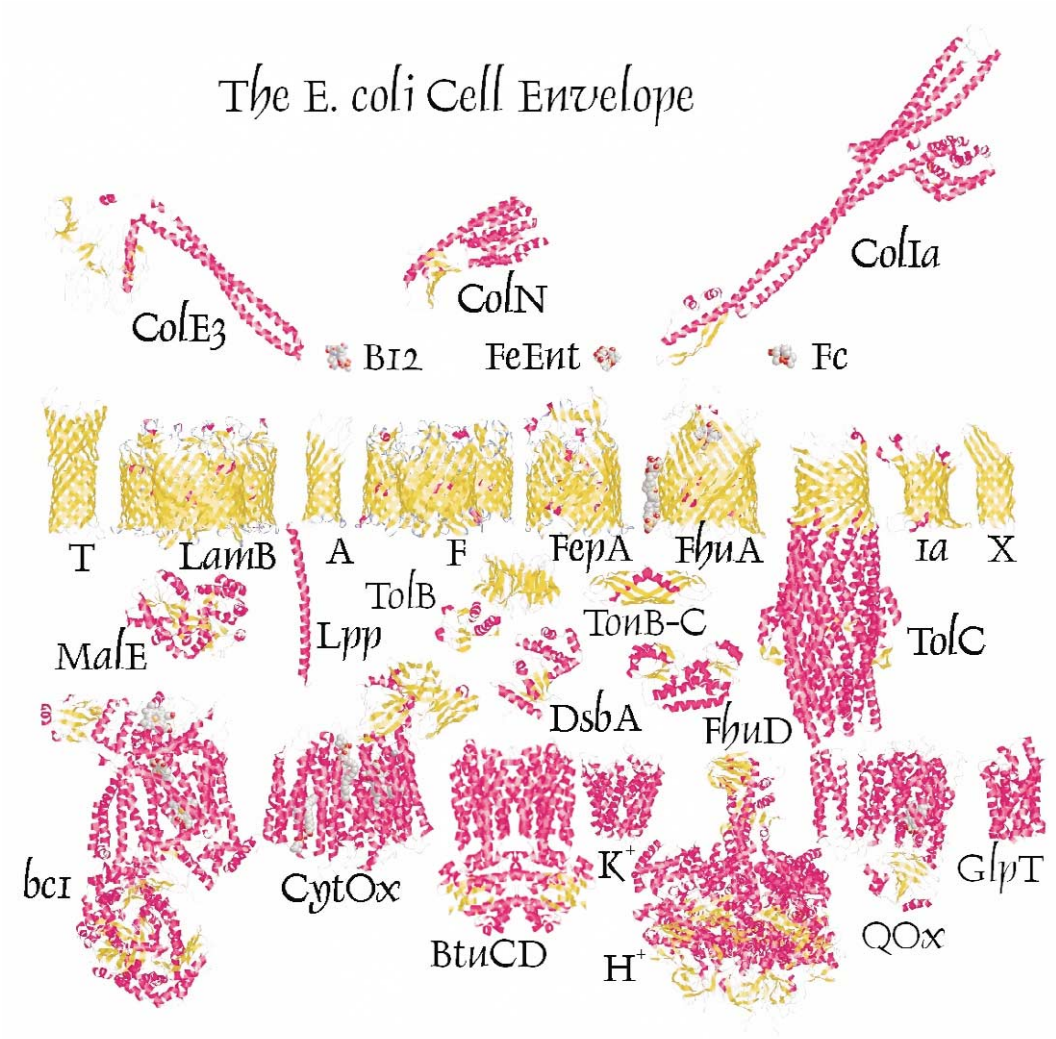

Fig. 2. The *E. coli* cell envelope. Crystallographic data from the following soluble and membrane proteins now generally depict the structural organization of trilaminar bacterial cell envelope: *extracellular toxins and metal complexes*, colicins E3, N, and Ia, vitamin B12, the siderophores ferric enterobactin and ferrichrome: the *OM proteins* OmpT [100], LamB [101], lipoprotein [102], OmpA, OmpF [103], FepA [104], FhuA [55,56], TolC [105], Omp1a [106], and OmpX [107]; the *periplasmic proteins* MalE [108], DsbA [109], TolB [90], the TonB C-terminus [110], and FhuD [111]; the *IM proteins* cytochrome BC1 (from yeast: [112]), cytochrome oxidase (from *Paracoccus*: [113]), BtuCD [89], proton ATPase [114], K+ channel [115], Ubiquinone oxidase, and GlpT [116].

through FhuA [35] and only FhuA, and vitamin B12 and the colicins E1 and E3 bind to BtuB and only BtuB [39,43,44]. None of these metal chelates or protein toxins bind OmpF, but two other colicins, A and N, utilize OmpF as their receptor [45–51]. No colicins are known to interact with the other OM proteins OmpA, OmpT, LamB or OmpX.

The presence of characteristic antiparallel β-sheets within otherwise completely α-helical structures was one of the surprising features of the ColN and ColIa binding domains [21,22]. But the significance of the sheets is not known, nor is it certain that they play a unique role in the recognition reaction by OM receptors. Apparently they do not, because ColE3 lacks β-structure in its binding tip. Nevertheless, the information for recognition of an OM receptor protein resides in the primary structure of the toxin binding domain, and these regions were designated as "R" (receptor) domains. The information they contain is precise: although the surfaces of bacterial porins are distinct in amino acid composition, loop topology and vestibule shape, they are also fundamentally similar in terms of residue hydrophilicity, in the approximate size of their component loops, and in the types of attractive forces of which they are capable (hydrogen and hydrophobic bonds, ionic attractions, and van der Waals forces). These considerations suggest that colicin molecules recognize receptor proteins by rigorous alignment of residues on their binding tips and residues within the vestibules of OM porins; crystallographic images (Figs. 3 and 4) support this conclusion. In the ColN–OmpF interaction, properly aligned opposite charges surround the toxin binding domain (basic side chains), and the OmpF outer vestibule (acidic side chains). But exactly how ColN adsorbs to OmpF remains unknown. The toxin may bind to monomers within the porin trimer, or to a single determinant formed by the coalescence of

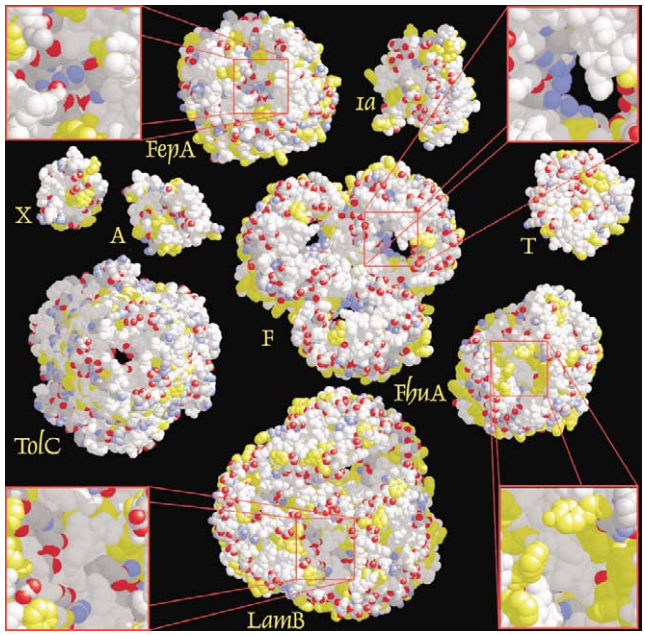

Fig. 3. Surface structures of OM proteins. From the perspective of the cell exterior, OM proteins (as referenced in the figure) project unique surface binding determinants, that create a landscape of protuberances, cavities and pores, that are populated by different types of amino acids that may impart ionic character, hydrophobicity, aromaticity, or distinct structural forms. Acidic and basic residues appear in CPK colors; aromatic amino acids are yellow.

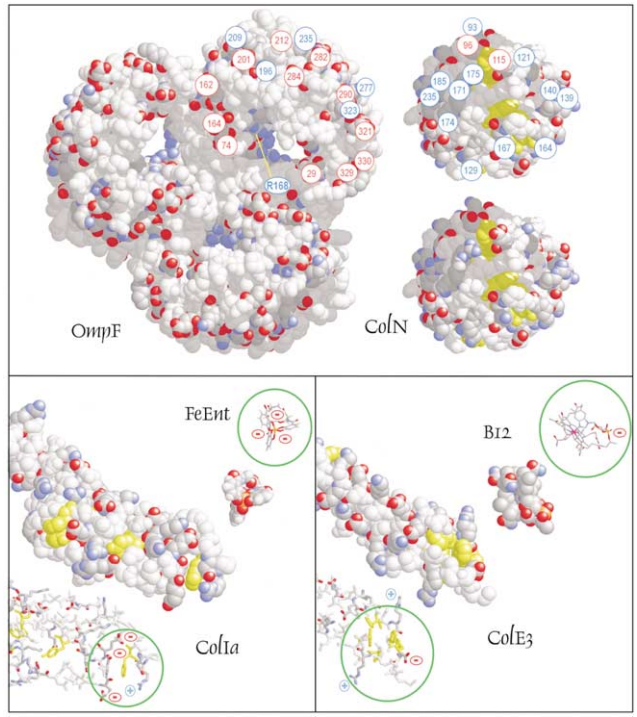

Fig. 4. Receptor-binding and structural mimicry by colicins. (Top) The surfaces of ColN and OmpF suggest charge interactions as their major determinants of binding affinity: basic residues on the binding domain of ColN (enumerated in blue) presumably align with acidic residues in the loops of OmpF (enumerated in red). For two colicins that parasitize metal chelate receptors, on the other hand (Bottom), the binding domains of ColIa (left) and ColE3 (right) are narrowed, elongated, and structurally adapted to mimic the shape and charge of a catecholate metal complex (FeEnt; left) and vitamin B12 (right), respectively. Individual residues of the OM protein and the colicin are colored as in Fig. 3, except that aromatic residues are not highlighted in OmpF.

monomers to create a trimer. The binding tips of colicins Ia and E3, on the other hand, probably mimic the size, shape and chemistry of a catecholate iron complex, and cyanocobalamin, respectively. In the former case, besides a general shape similarity, the overall negative charge of the Ia binding tip appropriately corresponds to the negative charge of a catecholate iron complex. In the latter case, a striking shape similarity accompanies the distinctive placement of negative charge at the extremity of a network of aromaticity. Hence, not only did evolution join disparate specificity and killing domains, but it also adapted the former to resemble the basic chemistry of minuscule metal complexes.

1.2. Cell surface competition phenomena

The competition between ferric enterobactin and colicins B and D for adsorption to FepA illustrates many of the salient features of such systems. The binding of vitamin B_{12}, the E colicins, and phage BF23 to BtuB was the paradigm for bacterial cell surface competition phenomena [39], but iron transport systems are presently better understood, as a result of biochemical, crystallographic and mutagenesis data. The ligands that utilize FepA exclude other OM proteins as adsorption sites, and size is the first potential consideration for this specificity. Among structurally defined porins (Figs. 2 and 3), OmpA, OmpX and Omp1a possess vestibules that appear too small to accommodate even the smallest known colicin-binding domain of ColE3. This conclusion assumes, perhaps incorrectly, that the protein structures are static and rigid. Nevertheless, small size likely prohibits certain OM proteins from activity as colicin receptors. On the other hand, a variety of other OM proteins that do not bind ColB, ColD, ColIa and ColE3, including OmpF, LamB, FhuA and TolC, contain larger vestibules, big enough to accept the size and elongated shape of toxins like ColIa or ColE3 (Figs. 1–4).

These and other observations suggest that lock-and-key interactions between surface residues of the two proteins underlie their binding equilibria. FepA and FhuA, that serve as receptors for FeEnt/col B and Fc/colM, respectively, show potentially explicatory differences in amino acid composition *within* their vestibular entrances. The former protein, that binds an acidic solute, contains numerous basic residues in the pore entrance, while the latter, that recognizes a neutral solute, is devoid of charged amino acids in the vestibule opening, and predominantly contains aromatic residues instead. Besides the size similarity discussed above, acidic residues at the extremity of the ColIa binding tip recapitulate the negative charge of catecholate siderophores (Fig. 4). The question arises, though, why do not colicins Ia, B and D enter and bind within the OmpF vestibule, that also contains basic residues in potentially

appropriate positions? The likely explanation is that although opposite charges on receptor and ligand provide determinants of adsorption affinity (and hence, specificity), in order to create high affinity binding, they must be *exactly* configured. Biochemical data support this inference. In FepA, substitution of Ala for Arg 316, deep within the vestibule, and for K483, closer to the entrance, decreases affinity for ligands by 100- and 10-fold, respectively (Table 1; [52,53]). Furthermore, among the non-covalent forces that may adhere proteins in complex, ionic interactions are the strongest, but hydrophobic and hydrogen bonds may optimize these interactions. Again for FepA, the slower adsorption rates of FeEnt to site-directed Tyr→Ala substitution mutants [54] intimate that hydrophobic interactions initiate the ligand binding reaction. Existing data partially substantiate some of the many conceivable variations on this theme. For FhuA, the predominantly aromatic nature of the opening to its antechamber (Fig. 3) reiterates that hydrophobicity provides the initial attractive force for ligand adsorption. Anyone who has spent time extracting siderophores from culture media immersed in the aromas of chloroform, benzyl alcohol, or ethyl acetate will appreciate the obviousness of this postulate. Once adsorbed from solution, ferrichrome progresses to the interior of FhuA, where it ultimately sits poised above the N-terminal domain, bound by a network of hydrogen bonds [55,56]. In summary, size, charge, hydrophobicity and hydrogen bonding contribute to the recognition and binding of solutes and colicins to OM proteins, but the exact magnitudes and relative importance of these forces await precise definition.

1.3. Colicins B and D, FeEnt and FepA

Several types of mutagenesis experiments helped delineate the interaction between colicins B and D and FepA. A series of sequential loop deletions created three classes of mutant phenotypes (Table1; [57]). Among FepA's 11 surface loops, deletions of L3 and L4 retained wild-type susceptibility to ColB (class 1); deletions of loops 2, 5, 9, 10 and 11 reduced in ColB binding affinity up to 40-fold, and decreased killing efficiency up to 500-fold (class 2). Only deletions of L7 or L8 (class 3) showed total loss of colicin susceptibility. The recent finding of dramatic conformational motion in loop 7 of FepA [53] and the structurally related siderophore receptor FecA [58] imparts new significance to the class 3 mutants. Unexpectedly, the complete or partial removal of their N-terminal globular domains did not significantly decrease the ability of FepA [59] or FhuA [59,60] to serve as receptors for ColB and M, respectively. Furthermore, genetic exchange of FepA and FhuA N-termini did not change the specificities of the two OM proteins [59]. These data confirm the ideas developed above, that the selectivity of colicin-binding interactions resides predominantly in the loops of the β-barrel domain.

For colicins that use BtuB [38,44,61], the cell surface colicin-binding region is not as well studied, in large part because the structure of the receptor is unknown. Nevertheless, ColE1 reportedly binds to loops 1 and 2, whereas colicins E2 and E3 adsorb to a different region, loops 5–7 [62]. A 76-residue fragment of ColE9 (residues 343–418) retained the ability to bind to BtuB [9], and the same fragment eliminated vitamin B12-dependent growth.

Substitution mutagenesis experiments demonstrated that multideterminant interactions occur between colicins and their receptors. OM receptors avidly bind their ligands [52,54,57,59,63,64], and the alteration of a single residue does not often dramatically change the interaction (Table 1; [52,54]). Significant changes in the ligand-receptor interaction require simultaneous substitutions of two or more residues [52,54]. The colicins that use FepA exhibit avid binding ($K_d = 10^{-7}$ M), but the affinity of ColB or ColD for FepA is considerably less than that of its siderophore counterpart ($K_d = 10^{-10}$ M; [54,57,59,63]). The weaker interaction most likely occurs because the colicin does not complement the shape of the receptor's binding cavity as well as the ferric siderophore. From an evolutionary standpoint, the ferric siderophore-receptor interaction requires higher affinity because bacteria absorb iron in environments where the concentration of free iron is extremely low [65]. However, colicinogenic bacteria may produce colicins at high local concentrations without compromising their own survival, which is protected by immunity proteins expressed from their colicin plasmids. This concentration difference between colicins and siderophores indicates that the lower binding affinity of the toxins is still sufficient to accomplish their adsorption *in the absence of the ferric siderophore*. These relative affinities also explain the ability of ferric siderophores to competitively protect bacteria against colicin killing.

The negative charge and aromaticity of FeEnt suggested that basic and aromatic amino acids in FepA are important for siderophore adsorption [66], and mutagenesis experiments corroborated this assumption [52,54]. Both ColB and FeEnt adsorb to FepA with biphasic kinetics [63], defining two binding sites or conformations (named B1 and B2; [54]). However, among single substitutions, mutations to only one residue (R316), in site B2, had effects on ColB and ColD killing [54]. Other single mutants in B1 and B2 showed wild-type susceptibility to colicins, even though their siderophore binding and uptake activities were significantly reduced.

Colicins and their corresponding siderophore or vitamin ligands compete for the same subdomains on OM receptors [52,67], but other specific interactions with the larger colicin molecules are clearly different. For instance, the hydrophobic surfaces created by aromatic residues in OM proteins are important in siderophore-receptor interactions [54], even for iron chelates (e.g., ferrichrome) that are not aromatic [55,56,68]. However, few aromatic residues studied so far affect the colicin-receptor interaction. With regard to ionic interaction, on the other hand, colicin Ia crystal structure [21] showed that the Cir binding domain of Ia

resides in the central region (residues 282–385), which displays a high density of charged residues near its terminal loop where the receptor-binding domain-receptor interaction most likely occurs. In FepA, basic residues R316 [54] and K483 [53] were important to all FepA ligands, whereas E319 [54] affected only FeEnt binding and transport, and G549R blocked only colicin killing through FepA [69]. Although the overall negative charge of FeEnt rationalized the inhibition engendered by R316A, deletion of the entire loop 4, where R316 resides, did not have much effect on colicin activity. K483 exists on the cell surface in FepA L7, and like R316, its positive charge may provide a determinant for interaction with the acidic siderophore, and colicins B and D. The negative charge of E319, centrally localized within the FepA vestibule, intimates an unexpected complexity of charge–charge interactions among the residues that comprise the FepA ligand binding site, during the FeEnt binding/transport process. The impact of G549R, a mutation in L8, may result from either the direct involvement of residue G549 in colicin binding or killing, or to the introduction of charge in the interaction, or to a conformational change from the introduction of the charged residue. L7 in FepA [53], and loops 7 and 8 in another FeEnt transporter, FecA [58], change conformation in response to ligand binding. In summary, the mutagenesis results confirm that certain charged side chains of FepA are crucial to reception and uptake of both the colicins and the ferric siderophore, while others are important to one or the other class of ligand.

2. Colicin transit through the OM

After encountering the bacterial cell surface, colicins presumably associate with OM porins such that the binding domains of the former intercalate into the surface loops of the latter. The rod-like binding domains of native colicins intimate that their narrowed tips may enter the surface vestibules of porin proteins (Figs. 1, 2 and 4), but this configuration raises a still puzzling question about colicin translocation: once bound on the cell surface by such a lock-and-key interaction, how do the other domains of the toxin, located as much as 200 Å away, cross the OM the bilayer, move through the periplasm, and penetrate the inner membrane (IM) prior to killing of the bacterium? Two alternative routes exist: passage through the receptor porin's β-barrel, or entry at a second site, either another OM protein or the bulk phase of the OM lipids.

2.1. The trans-porin "Nail" hypothesis

Directional movement of a globular, multidomain protein molecule through a transmembrane channel, is hard to imagine, but Cramer suggested a model in which the binding tip and shaft of a colicin begin transit through the cell envelope by penetrating a transmembrane β-barrel (i.e., an E colicin through BtuB). This so-called "nail" hypothesis of colicin entry is attractive in the sense that the size and shape of colicin molecules are compatible with the size and shape of porin channels, and because in at least the case of Ia, the enormous length of its helices are ostensibly capable of spanning the periplasm. However, the "nail" hypothesis faces several objections.

1. If the elongated tip of a colicin penetrates a bacterial cell as a nail drives through a surface, then what is the hammer? Bacteria have no known source of utilizable energy in the OM bilayer that colicins might parasitize to power such a motion, so the driving force for colicin uptake either originates in the structure of the colicin itself, or comes from the cell interior. The possibility exists, although again it is difficult to presage from crystallographic data, that the unusual architecture of the toxin creates potential energy that ligand binding releases, forcing the colicin through the OM bilayer. Such an event may resemble the injection of bacteriophage DNA through the cell envelope, mediated by a contractile tail spike or fiber that forcefully breaches the OM permeability barrier. On the other hand, the notion that a colicin may acquire energy from within also strikes a harmonic chord, at least for TonB-dependent toxins. At present, the energetics of not only colicin translocation, but also ferric siderophore uptake, are almost a total mystery. Since the original demonstration of the energy and TonB-dependence of ferric siderophore uptake by cell envelope proteins [70–72], little mechanistic insight has arisen. The structural mimicry that bacteriocins adopt at the adsorption stage may further extend to the transport reaction, in that TonB-dependent colicins may impersonate metal complexes and thereby utilize the same energetic machinery that catalyzes ferric siderophore internalization, ultimately duping the cell into its last act: uptake of a lethal toxin.
2. Are the diameters of porin channels sufficient to permit transit of a colicin, in either the folded or unfolded state? Porins have two pertinent dimensions, the diameter of their β-barrel, and the diameter of their pore opening. Within OmpF, these distances are 25 and 10 Å; for FepA and FhuA, on the other hand, they are 45 Å and 0. The discrepancy derives from a peculiarity of porin structure. In general and specific porins, the transverse loop (L3) creates an internal constriction zone that narrows the channel. In ligand-gated porins, the N-terminal approximately 150 amino acids completely fills and closes the channel. With regard to ColN, the 20 Å diameter of the folded toxin prohibits its penetration of OmpF channel. If the colicin dislodges or distorts L3 during transit, then its uptake is conceivable, but dubious, because the shape of the OmpF vestibule appears incompatible with colicin entry (Fig. 3). Thus the only possible mechanism of polypeptide uptake through OmpF

involves denaturation and threading of the linearized polypeptide through the porin. To accomplish transport of any ligand, FepA and FhuA must undergo dramatic changes that either create a pore within their N-terminal globular domain, or completely expel it from their channels. On the other hand, if their N-termini fully dislodge from their channels, then their approximately 20 Å surface openings expand to a viable 40 Å diameter within the OM bilayer. Pores of this magnitude are sufficiently large to accommodate passage of the binding tip and coiled helices of ColIa.

3. Even if one accepts the hypothesis that the binding domains of certain colicins adsorb in the surface loops and enter the transmembrane channels of certain OM proteins, the mystery of their transport remains, because penetration through porins in this manner ultimately strands the translocation and killing regions on the cell surface: their size precludes passage through any known OM channels. The conservation of structure and functionality in the killing domains of several different classes of bacteriocins suggests the universality of this conclusion. However, either of two alternative modes of transport may circumvent the dilemma: denaturation of the toxin to a linear form that fits through a pore, or passage through the OM bilayer at a site removed from the porin itself. Surprisingly strong evidence [37] supports the former "noodle" hypothesis (see below), but the spontaneous passage of a denatured polypeptide through a proteinaceous membrane channel is also presently unexplainable.

2.2. Translocation at a distant site

Another view of the solved structures of bacteriocins is that their elongated shapes evolved to separate their binding domains from their translocation and killing regions. It is conceivable that translocation of the killing domain occurs at a second site that is distinct and distant from the initial binding site. It is noteworthy in this regard, however, that ColN does not exhibit the same exaggerated length as Ia and E3. If translocation occurs away from the initial colicin-binding site, then only a few options exist for the identity and composition of the entry site: other proteins, lipids, or an uncharacterized "island" in the OM.

2.2.1. Other proteins

Reception of E-colicins reportedly requires the presence of both BtuB and OmpF. On the other hand, fragments of ColE1 [5] and ColE9 [9] and intact colE3 [73], all bind to BtuB. Therefore, the possibility exists that after the initial association occurs between ColE3 and BtuB, the toxin's killing domain binds to and translocates through OmpF. The pore-forming killing domains of ColN and ColB, but not the nuclease killing domain of ColE9, bind to trimeric OmpF [74]. However, the formation of these complexes depends on the presence of detergent or lipopolysaccharide (LPS), and their relevance to in vivo events is unknown. Furthermore, from a mechanistic standpoint, association of the killing domain with the surface of another OM protein does not circumvent any of the problems discussed above: in the native state, it is too big to pass through the OmpF vestibule or channel, and no obvious energy source exists to drive the transport reaction.

2.2.2. Lipids as a translocation site

The most distinguishing feature of the Gram-negative bacterial OM, aside from the unique structure of the transport proteins it contains, is the LPS in the outer leaflet of the bilayer. LPS creates the permeability barrier that excludes undesirable molecules from entering the cell envelope [49]. It is particularly important to the survival of *E. coli* and is relative that the hydrophilicity of the LPS core and O-antigen sugars excludes detergents, like bile salts from the gut, because if such agents penetrate the OM in high concentrations, they will solubilize the IM. The LPS barrier also prevents the dissolution of hydrophobic antibiotics in the OM, and presumably excludes toxic proteins like bacteriocins from entry. But given what is already known about the remarkable abilities of colicins, no compelling a priori arguments exist to refute the idea that colicin killing domains gain access to the cell interior by passage through the bulk phase of OM lipids. If such an ability does exist, it likely derives from the translocation domains of colicins, which show structural conservation among colicins that target the same kinds of OM receptor proteins (i.e., TonB- vs. Tol-dependent).

2.2.3. The "Membrane Island" hypothesis

The notion that the Gram-negative bacterial cell envelope contains regions with an atypical protein or lipid composition, that may function to some advantage for one or more physiological processes, appeared several times in the past half century, beginning with the postulate of cell envelope DNA binding proteins that manipulate the bacterial chromosome during cell division [75–81], and including the idea that zones of adhesion between the inner and outer membranes contain a special complement of biosynthetic enzymes (for LPS and membrane protein secretion) and receptor proteins [82–84]. At a minimum, cell envelope "islands" may contain proteins of the *sec* system, chaperones, sugar and peptidyl transferases for synthesis of peptidoglycan and LPS, receptor proteins, and perhaps even the accessory proteins TonB and TolC, that function in such multicomponent transport systems. The biochemical uniqueness of such membrane islands, with potentially unusual protein and lipid compositions, may create different physical properties that distinguish them as preferred target sites for perforation of the OM barrier by colicins. Membrane islands may constitute a weak link in the cell's permeability defense system.

2.3. Interpretation of existing transport data

The existence of many different bacteriocins that recognize and enter the cell through many different OM proteins, with facilitation by two or more different types of translocation machinery (Tol and Ton), has enriched and broadened the understanding of colicin transport. On the other hand, the study of interactions between multiple toxins and receptor proteins by independent groups utilizing specialized methodologies has also increased the complexity of data interpretation. For example, in spite of the general structural organization of colicins into distinct binding, translocation and killing domains, it is still unknown whether all colicins penetrate the cell envelope by a common mechanism. Individual toxins within either of the A or B groups may enter bacteria by currently unrecognized adaptations to any general scheme that may exist. Hence, generalizations about transport mechanisms from studies of single colicins may be misleading. With that caveat, let us consider some selected data on bacteriocin transport, in relation to the theories discussed above.

2.3.1. The initial step of colicin uptake: denaturation on the cell surface

Although they preceded the focal time frame of this review, three papers from Latellier's laboratory [85–87] remain relevant because they elegantly delineated the events in colicin A transport that immediately follow its binding to the OM protein, OmpF. Unexpectedly, denaturation by urea increased the rate of colA uptake by bacteria, and binding of colA to OmpF induced an immediate conformational change in the toxin that mimics its unfolding by urea. In both studies, the experiments monitored the kinetics of IM depolarization (K+ efflux) in response to the insertion of the C-terminal channel-forming domain of ColA. In the former case, native ColA added to live bacteria only released K+ after a delay of approximately 1 min, which presumably represents the time required for transit of the killing domain through OM and into the IM. The lag-time for urea-denatured ColA, however, decreased 2–4-fold relative to the native toxin. In the latter experiments, the authors showed that reducing agents more rapidly disrupted disulfide bonds in the killing domain of urea-denatured ColA than in native ColA, and that binding of native ColA to the bacterial cell surface comparably increased the susceptibility of the site-directed disulfides to DTT. Together these data indicate that colicins do not breach the OM barrier in the native conformation that was visualized by crystallography. After binding to its OM receptor, ColA immediately unfolds to another structural form that promotes its movement into the cell. One of the novel inferences of these experiments is that the extended structures of colicins, that at first glance appear adapted to penetrate a membrane like a needle or nail, may in fact function toward a different end: creation of a "spring loaded" conformation that triggers a colicin's unfolding upon contact of its binding domain with a receptor site.

2.3.2. Colicin "threading" through the pore

Genetically engineered disulfide bonds were also used to address the question of whether colicins A and N pass through the OmpF channel. In this study, the transverse, L3 loop of OmpF was tethered at several different positions to the barrel wall by disulfide bonds, and bacteria expressing the mutant, disulfide-bonded porins were tested for susceptibility to colicins A and N. Using K+ efflux and fluorescence depolarization assays, the authors showed that among five different site-directed Cys pairs, only E117C–D312C manifested an increased resistance to ColN, but not to ColA. The other combinations, V105C–F129C, E117C–A333C and Y24C–D127C, showed normal susceptibility to colicins A and N; D107C–S177C was resistant to colicin A, but this solely derived from the deleterious effect of D107C. Although the authors concluded that their results refuted the idea that colicins translocate through the OmpF channel, we interpret their data with less certainty. First, the conformational flexibility of the L3 loop within the OmpF pore is unknown, but rigidity is not expected in solution. Similarly, rigidity is not expected in an unfolded colicin polypeptide (presumably the transported form). Next, because of our only rudimentary understanding of both the energetics of toxin uptake and the presumed subsequent interactions between colicins A and N and the Tol proteins, the forces involved in internalizing a polypeptide are impossible to estimate. Finally, from the locations of the disulfide bonds that they engineered, E117C–D312C and E117C–A333C were most likely to impair movement of a molecule through the channel. The former significantly retarded ColN uptake, and although the latter did not, it was only partially oxidized to disulfide form, which poses a problem with 10^5 OmpF proteins per cell, and a colicin multiplicity of 100.

It is remarkable that two of the mutations in OmpF that inhibit ColA and ColN translocation, D117C–E312C and D107C, respectively, exist deep within the pore, well beyond the possible reach of at least the ColN binding domain, which is too large to enter individual porin channels (Fig. 5). In addition, the impaired transport phenotypes of these mutants are distinct from that of another substitution, R168C, which abrogates colicin binding of ColA. Thus the proposal of Bainbridge et al. [88] that D117C–E312C and D107C primarily interfere with colicin binding is debatable. It is also conceivable that both mutations impair colicin killing by retarding the rate of colicin "threading" through OmpF. The fact that D107 resides beneath the bulk of L3, and can only directly contact the colicin after its passage beyond the channel constriction, supports this notion.

2.3.3. Involvement of Tol or TonB proteins in the colicin transport process

Killing by A group colicins depends on up to five Tol proteins, TolA, B, C, Q and R. The lack of complete information on the structures and interactions of these periplasmic and IM proteins constitutes a problem in the

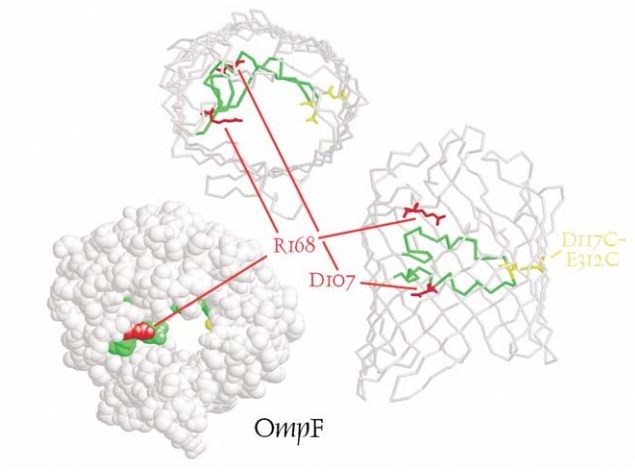

Fig. 5. Location of residues involved in colicin uptake through OmpF. A genetically engineered disulfide bond between D117 (in the L3 loop, colored green) and E312 (on the barrel wall) inhibited ColN uptake through OmpF. The single substitution D107C, located beneath the transverse loop, significantly decreased the rate of OmpF-mediated ColA killing. The figure also depicts the location of R168C, a mutation that blocks ColN binding and completely abrogates killing.

interpretation of Tol protein function in colicin susceptibility. In addition, the tendency of workers in the field to adopt sequence-derived predictions of (especially IM protein) structure as dogma imparts a false sense of understanding to a subject that is at present obscure. The unexpected crystal structure of the histidine permease (HisP; [89]), that was much different from what was predicted by such methods, illustrates the limitations of this approach. Nevertheless, in the last few years, crystallographic data on TolB [90], and biophysical measurements of the binding interaction between the N-terminal translocation domain of ColN and the TolA protein [91,92], significantly advanced this field. The latter experiments defined the affinity of the interaction between ColN and TolA ($K_d = 10^{-6}$ M), and identified a 27-residue segment of ColN that is crucial to the reaction. These data, which, for the first time, biochemically established the validity of prior genetic postulates, intimate that the third stage of colicin translocation involves specific interactions with one or more proteins that reside in the periplasm. Similar data do not exist for Group B, TonB-dependent colicins, but the findings for Group A raise the possibility of their similar third stage of transport through the cell envelope.

3. Summary and conclusions

Regarding the OM penetration process of colicins, we reiterate that evidence exists for three stages of transport of some colicins: (i) binding-stimulated denaturation on the bacterial cell surface, (ii) transit of colicin polypeptides through, or at least deep penetration into, porin channels, and (iii) subsequent interaction with target proteins in the periplasm. It seems furthermore conceivable, based on the low concentrations of most of the common periplasmic elements, that during penetration colicins may target specialized zones in the cell envelope, of currently unknown composition and architecture, containing such entities as the TonB and Tol proteins. From these inferences, we suggest two alternative potential mechanisms of entry. In the first postulate, once a portion of a colicin molecule breaches the OM bilayer, presumably through a porin channel, and binds to a target protein in the periplasm, the biochemical activity of the periplasmic target system accomplishes the further internalization of the remainder of the colicin polypeptide, ultimately dragging the C-terminal killing domain into the cell, where it exerts its toxic activity. Thus, in this case, the internal cellular process provides the energy for translocation of the full colicin polypeptide through the OM bilayer. In the second postulate, the initial porin receptor protein merely provides a docking site, to which the colicin receptor domain binds, affording its translocation and killing domains the opportunity to penetrate the OM at other sites of unique composition. If the secondary targets, accessory proteins like TonB and Tol, congregate at specialized regions of the cell envelope dedicated to, e.g., biosynthesis or transport, that may contain areas of adhesion or fusion between the IM and OM, then at such locations, the killing domains of colicins may acquire direct access to the cytoplasm through a single membrane bilayer. As is known from the elegant studies of colicin E1 [93–97] and other pore-forming bacteriocins, the C-terminal portion of such toxin polypeptides has the ability to enter and form channels within membrane bilayers.

Our inability to solve the physiological riddles of TonB and Tol now stands as the principal stumbling block to the delineation of colicin uptake processes. Unfortunately, the exact functions that TonB and the Tol proteins perform in the cell envelope, and the mechanisms by which they accomplish them, remain elusive.

. Manque renvois de biblio

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