

Import of the Transfer RNase Colicin D Requires Site-Specific Interaction with the Energy-Transducing Protein TonB

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The transfer RNase colicin D and ionophoric colicin B appropriate the outer membrane iron siderophore receptor FepA and share a common translocation requirement for the TonB pathway to cross the outer membrane. Despite the almost identical sequences of the N-terminal domains required for the translocation of colicins D and B, two spontaneous *tonB* mutations (Arg158Ser and Pro161Leu) completely abolished colicin D toxicity but did not affect either the sensitivity to other colicins or the FepA-dependent siderophore uptake capacity. The sensitivity to colicin D of both *tonB* mutants was fully restored by specific suppressor mutations in the TonB box of colicin D, at Ser18(Thr) and Met19(Ile), respectively. This demonstrates that the interaction of colicin D with TonB is critically dependent on certain residues close to position 160 in TonB and on the side chains of certain residues in the TonB box of colicin D. The effect of introducing the TonB boxes from other TonB-dependent receptors and colicins into colicins D and B was studied. The results of these and other changes in the two TonB boxes show that the role of residues at positions 18 and 19 in colicin D is strongly modulated by other nearby and/or distant residues and that the overall function of colicin D is much more dependent on the interaction with TonB involving the TonB box than is the function of colicin B.

When the availability to the bacterial cell of iron is limited, efficient acquisition systems based on substrate-specific high-affinity receptors are expressed at the cell surface. Ferric siderophores, vitamin B₁₂, several antibiotics, phages, and colicins use an energy-coupled process to cross the outer membrane in gram-negative bacteria (10, 23). Colicins are plasmid-encoded cytotoxins of *Escherichia coli*, which are secreted into the medium and kill competing bacteria. As molecular parasites, they take advantage of target cell functions during their attack on sensitive cells. Despite the fact that they harbor dramatically different lethal activities, both colicin B and colicin D appropriate the outer membrane iron siderophore (ferric enterobactin) receptor, FepA, and need the TonB/ExbB-D energy transduction system to reach the periplasmic space (2, 23). The first 313 N-terminal amino acids of colicins B and D, required for binding to a cell surface receptor and for translocation across the outer membrane, are 96% identical (4, 24). Colicin D carries a C-terminal catalytic domain which acts in the cytoplasm to hydrolyze specifically the anticodon loop of all four tRNA isoacceptors of Arg (27), whereas the C-terminal part of colicin B exhibits an inner membrane pore-forming activity (14, 23). The 75-kDa nuclease colicin D forms a tight equimolar heterodimer complex with the cognate immunity protein (ImmD) (6).

During siderophore uptake from the external environment to the periplasm, the inner membrane protein TonB, extending into the periplasm, is converted to its energized conformation by ExbB-D (13). The TonB protein contacts outer membrane receptors, allowing energy due to the proton motive force of the cytoplasmic membrane to be transduced to the outer membrane. In vivo cross-linking experiments show an interaction

between TonB and FepA (26), and genetic studies confirm the importance of this interaction in ligand transport (1, 9, 25). Additional site-directed disulfide or formaldehyde cross-links led to similar conclusions in the case of the BtuB, FhuA, and FecA receptors and showed that their interaction with TonB is enhanced when they are loaded with substrate (3, 17, 20).

Point mutations that confer a TonB-uncoupled phenotype (7) were localized in a short conserved peptide motif (called the TonB box), located near the amino terminus of TonB gated transporters (cork domain). They abolish the TonB-dependent transport function but have no effect on ligand binding to the receptor or on TonB insertion and folding. TonB box-like sequences have been described at the N terminus of TonB-dependent colicins. Moreover, mutations affecting the Q160 residue of TonB, which render TonB box mutants of receptors active, also restore some sensitivity to inactive derivatives of colicins B and M carrying mutations in their TonB box-like sequence (16, 22).

Here we show that a site-specific interaction between the TonB box sequence of colicin D and TonB protein is essential during toxin penetration into target cells and is highly dependent on certain identified residues in each protein.

MATERIALS AND METHODS

Localization of spontaneous *tonB* mutant D1. Spontaneous ColD^r ColB^s mutant strains were isolated by screening 8,000 spontaneous ColD^r mutants. After random insertion of Kn^r mini transposons from λNK1316 (11) into the ColD^r ColB^s mutant strain D1, three Kn^r derivatives (D117, D17, and D18) were selected on the basis of the high level of P1-mediated cotransduction of the transposon with the ColD^r phenotype: 74, 87, and 98%, respectively. Following digestion of chromosomal DNA from strain D1 by *Taq* I, inverse PCR was employed to amplify sequences adjacent to the mini transposons (5). Sequencing showed the mini transposons in mutants D117, D17, and D18 to be located in the *rssB*, *oppA*, and *kch* genes, respectively, and that the mutation conferring ColD^r to strain D1 was within the nearby *tonB* gene, 1.4 kb from *kch*. The *tonB* gene in strain D1 and other spontaneous mutants (Table 1) was found to carry in each case a single point mutation in the *tonB* reading frame.

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TABLE 1. Spontaneous ColD^r ColB^s mutant strains carrying a point mutation in the *tonB* gene

Strain	Mutated residue ^a	Sensitivity ^c to:							
		ColD	ColB	+pTonB ^d		ColM	ColIa	ColE2	φ80
				ColD	ColB				
WT^e	RALS <u>SRNQ</u> PQY PARA	5	5	ND	ND	4	5	5	6
D1	S	R	4–5	4–5	5	4	5	5	6
E	C	3	4–5	5	5	4	5	5	6
G	T	3	4–5	5	5	4	5	5	6
K19^b	L	R	4	4	5	4	5	5	6

^a Strain names and spontaneous substitutions affecting the central part of the *E. coli* chromosomal *tonB* gene are indicated in bold and placed below the wild-type peptide sequence. Mutations occurred at positions 158, 161, and 164.

^b Two other strains (M78 and P64) carrying the same Pro161Leu mutation were isolated during independent selections.

^c The cytotoxicity of the native, wild-type colicins (ColD, ColB, ColM, ColIa, or ColE2) was tested in vivo by zone of inhibition (halo) assay. Undiluted colicins and 10-fold serial dilutions were spotted onto a lawn of sensitive cells and incubated overnight. Values indicate the last serial 10-fold colicin dilution (scored in three independent assays) that resulted in a clear zone of growth inhibition (R, resistance to undiluted colicin preparation). φ80 phage sensitivity was determined similarly by the detection of clear lysis plaques. ND, not determined.

^d Plasmid pTonB, the wild-type *tonB* gene from *E. coli*, as a SacI and HindIII fragment, was inserted into MCS of pUC18 (21). +pTonB indicates that the spontaneous, chromosomal *tonB* mutant strains were complemented by plasmid-borne *tonB* gene.

^e *E. coli* strains D10 and XAC were used as wild-type (WT) sensitive strains.

Purification of colicins and cloning of pColD1. Colicin D complexed with its immunity protein and colicin B were purified as previously described (5, 6). Plasmid pColD1 carrying the ColD operon and ampicillin resistance marker was constructed by amplifying the *bla* (*Amp^r*) gene and the replication origin region from pUC18 template DNA with primers introducing EcoRI and BamHI restriction sites upstream and downstream of the amplified region (underlined in the sequences listed below). In addition, the primers carried 50-nucleotide sequences preceding the *cd*a promoter region and following the *cd*l stop codon, respectively (shown in italics), flanking the restriction sites. The primer sequences are as follows: 5'-*GTGGCGGGTCTTCGTC**CGATCAGAA**GCTTCGACCTCTTCTAATCGGCGAATTCAAGCTTTTAGACGTCAGGTG*GC^{ACTT}; 5'-*ACCCGGAGACCA**CCATGAAACGCCATAGACTGACCGAAG**CCGAAGAGCGCAAGCTTGGATCCGCGGTAATACGGTTATCCACAG*.

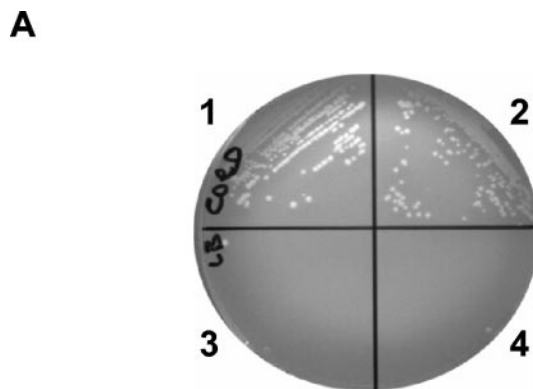
After removal of template DNA by DpnI hydrolysis, wild-type pColD-CA23 plasmid (4, 24) and the linear amplified DNA fragment, carrying the short homologous arms, were coelectroporated into NC397 (DY329) cells (28), which carry the recombination genes *redα*(*exo*)*β*(*bet*)*γ*(*gam*). The 1.9-kb linear amplified fragment was recombined into pColD-CA23 by ET cloning (18), allowing deletion of part of the target plasmid, which is outside the colicin D operon, including its SOS promoter region. The recombinant plasmid pColD1(*Amp^r*) had the expected size of 4.8 kb, including the 3-kb ColD operon.

Construction of colicin D and B molecules mutated at the TonB box. Using plasmid pColD1 or wild-type pColB as the template, 28 point mutations switching amino acids were generated by QuikChange site-directed mutagenesis (Stratagene) in the *cd*a or *cd*b gene, between positions 48 and 69, affecting the TonB boxes of colicins D and B, respectively. Two oligonucleotide primers containing the desired mutation were used, with each complementary to opposite strands of the plasmid, to generate the mutant derivatives. These were introduced into XL1-Blue cells, prepared in the presence of 10 mM RbCl. The mutated colicins were obtained after concentration by ultrafiltration (Amicon 10k; Millipore) from the supernatant of a culture induced by mitomycin C (200 μg/liter) of the C600 strain carrying the mutated pCol derivatives.

RESULTS

Spontaneous mutations in *tonB* confer resistance to colicin D but not to colicin B. The majority of colicin D-resistant mutants so far identified were found also to be resistant to colicin B, since the mutations mainly affect components of the common FepA/TonB/ExxB-D import pathway (5). To identify cell functions that may be used in later steps of invasion by colicin D, we sought to isolate mutants of *E. coli* resistant to colicin D but sensitive to colicin B, like the previously characterized A38 mutation in *lepB*, encoding leader peptidase, that plays a specific and essential role in cell killing by colicin D (5). A new spontaneous ColD^r ColB^s mutant (D1) of this type was

isolated that was resistant to undiluted purified colicin D solution, whereas colicin D diluted 10⁵-fold yielded a clear zone of growth inhibition with the wild-type D10 strain (Fig. 1 and Table 1). Genetic mapping experiments (see Materials and Methods) suggested that the mutation was located in the *tonB* gene, and plasmid pTonB, carrying this gene, was found to restore sensitivity to colicin D to the D1 mutant (Fig. 1B).



B

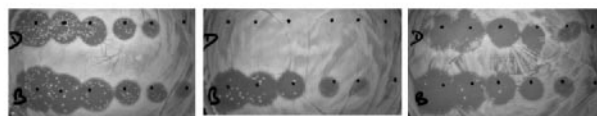


FIG. 1. In vivo phenotype of the spontaneous *tonB* mutant strains isolated after colicin D treatment of *E. coli* strains D10 or XAC. (A) Growth of mutant (D1 [1] and K19 [2]) and wild-type (D10 [3] and XAC [4]) strains at 37°C in the presence of purified colicin D (~3 μg) spread onto LB plates. (B) Complementation of mutant D1 by the wild-type *tonB* gene was analyzed by inhibition assay (cytotoxicity test) with a series of 10-fold dilutions of colicin D or colicin B (10⁻¹ to 10⁻⁵), spotted (3 μl) onto a lawn of sensitive wild-type D10 (left), mutant D1 (middle), and mutant D1(pTonB) (right) cells. The clear zone of inhibition on the lawn appears as a dark halo.

Transduction into the D1 strain of the *pcnB::Kn* allele, which drastically lowers the copy number of ColE1-like plasmids (15), confirmed that the observed complementation was not due to artifacts related to the overexpression of *tonB* (12) (data not shown). DNA sequence analysis identified a point mutation in the *tonB* gene of D1 strain, changing Arg158 to Ser (Table 1). A normal level of sensitivity to colicin D of five further independent ColD^r ColB^s mutants was also restored by plasmid pTonB (Table 1). DNA sequencing revealed that the first three of these mutants (K19, M78, and P64) all carry the same C→T substitution that provokes a Pro161Leu change in TonB. A hundredfold reduction in sensitivity to colicin D shown by mutants E and G was due to mutations provoking Arg158Cys and Pro164Thr replacements in TonB, respectively (Table 1).

tonB mutants D1 and K19 and the wild-type strain D10 grew similarly on plates containing 0.2 mM 2,2'-dipyridyl, chelating the iron in the medium, in contrast to a colicin D-resistant *tonB*-null strain, which did not grow. Experiments employing siderophore nutrition tests with FepA (19) have also been performed and support our contention that the FepA-dependent iron uptake, requiring the recognition of the FepA TonB box by the mutated TonB proteins, is sufficiently functional to allow growth similar to that observed with wild-type TonB protein (data not shown). The sensitivity to phage φ80 and colicins M, Ia, and E2 was unaltered in all of the *tonB* mutants (Table 1), indicating a wild-type level of TonB-mediated uptake of these substrates (except for the TonB-independent colicin E2) by FhuA, Cir, and BtuB outer membrane receptors, respectively. Thus, mutants D1 and K19 specifically exhibit a TonB-uncoupled phenotype, presumably with respect to the colicin D import, whereas other TonB-dependent transport activities were not apparently affected. The level of TonB in the *tonB* mutants D1 and K19, measured in crude extracts by Western immunoblot analysis, decreased by 30 to 50%, respectively (data not shown). This is probably an insignificant decrease with respect to colicin D sensitivity, since a ninefold reduction in the cellular level of TonB resulted in a reduced sensitivity of only fivefold toward colicins B, D, Ia, and M (12).

The sequences of the TonB boxes, located close to the N termini of TonB-gated receptors and colicins are summarized in Fig. 2 (3, 16, 22). The Val residue in the fifth position is the most conserved residue of the consensus sequence (D/E)TX(V/T)V. A Met residue at the third position is found more generally among colicins. It is frequently replaced by Ile or Leu in receptor TonB boxes. The FepA and colicin B TonB boxes fit best with their respective consensus sequences (Fig. 2). In contrast, the colicin D TonB box exhibits two major differences: the His and the following Ser residues. Several mutations affecting the TonB box of both colicins D and B were constructed (Table 2) to study their toxicity in the *tonB* mutant strains. For colicin D mutagenesis, the whole ColD operon of *E. coli* was cloned into the pUC18 vector by using a recombination strategy, yielding a pColD1 derivative.

Specific changes in TonB box reverse ColD^r phenotype due to *tonB* mutations Arg158Ser or Pro161Leu. Changes Met19Pro, Val21Glu, and Trp22Glu (Table 2) resulted in inactive colicin D derivatives, as in colicin B (16). Changes of Met19 to Ile, Val, or Leu did not affect the colicin D sensitivity of the wild-type strain, but the Met19Cys mutation diminished

FepA	TPVSHD	<u>DTIVVTAA</u>	EQNLQA
FhuA	AVEPKE	<u>DTITVTAA</u>	PAPQES
BtuB	AQDTSP	<u>DTLVVTAN</u>	RFEQPR
FecA	APAPKE	<u>DALTVVGD</u>	WLG DAR
Cir	AVDDDG	<u>ETMVTAS</u>	SVEQNL
FyuA	QTSQQD	<u>ESTLVVTA</u>	SKQSSR

(D/E) T (I/L) (V/T) V (T/V) AA consensus

ColB	EGIDYG	<u>DTMVVWPS</u>	TGRIPG (FepA)
ColD	EGIDYG	<u>HSMVVWPS</u>	TGLISG (FepA)
ColM	M	<u>ETLTVHAP</u>	SPSTNL (FhuA)
ColIa	YDSGDH	<u>EIMAVDIY</u>	VNPPRV (Cir)
Col5	VGVDTG	<u>DTITATLA</u>	TGTENV (Tsx)
Pesticin	MS	<u>DTMVVNGS</u>	GGVPAF (FyuA)

G (D/E) **TM** (V/T) VXXSTG consensus

FIG. 2. Consensus sequences at the TonB box of TonB-dependent receptors and colicins. TonB boxes are underlined. The most frequent residues at each position are shown in boldface type. The most conserved Val in the fifth position of the consensus sequence is underlined twice. Changes between colicin B and colicin D sequence motifs are indicated in italics in the latter. The names of the two most stringent TonB boxes (FepA and ColB) with regard to the consensus sequences are indicated in boldface type.

the cytotoxicity by a factor of 100. All other changes in the colicin D TonB box resulted in fully toxic colicin D derivatives, indicating that the protein stability of these mutated derivatives was not significantly reduced. The entire pentapeptide of the colicin D TonB box could be functionally replaced by other TonB box motifs, like that from colicin B (but only partially from colicins M and Ia) or from the FepA, FhuA, BtuB, and Cir receptors (Table 2). It seems likely that the TonB box pentapeptides tested here could alone be sufficient for the interaction with TonB to promote colicin D uptake, suggesting that different sets of functional contacts exist between the protagonists: colicins (or receptors) and TonB. Similarly, FepA, Cir, and colicin D type pentapeptides can efficiently replace the TonB box motif in colicin B.

Among the 22 TonB box derivatives constructed here, only one, the Ser18Thr mutant, appeared to be able to reverse the colicin D resistance of the D1 mutant (Table 2). The His17Asp change, alone or accompanied by Ser18Thr (thus generating a complete colicin B type TonB box), failed to restore the colicin D sensitivity (Table 2). The presence of His17 in colicin D is therefore required for the restoration of the colicin D toxicity of *tonB* mutants.

The reversal of the phenotype in the D1 mutant by a second site-specific mutation in colicin D suggests an interaction between both proteins at the mutated sites, presumably required for the import of the wild-type colicin D into sensitive cells. Less direct effects of residues at these particular positions, modulating the conformation of the sequences involved in the interaction cannot be excluded. It is worth noting that the permutation of the two residues, as found in the wild-type TonB and colicin D (Arg158 and Ser18, respectively) did not lead to a functional restoration of the colicin D-TonB interac-

TABLE 2. Sensitivity of spontaneous TonB mutants to colicins D and B and variants mutated at their TonB box

Colicin mutant	TonB box ^a	Sensitivity ^c to:		
		WT	D1 (R158S)	K19 (P161L)
WT ColD	<u>GHS</u>MVVWPSTG	4	R	R
H17D	D	4	R	R
S18T	T	4	2–3	2
S18A	A	4	R	R
S18L	L	4	R	R
S18I	I	4	R	R
S18C	C	4	R	R
S18R	R	4	R	R
M19I	I	4	R	3–4
M19V	V	4	R	2–3
M19L	L	4	R	2
M19P	P	R	R	R
M19C	C	2	R	R
V20E	E	4	R	R
V21E	E	R	R	R
W22E	E	R	R	R
ColB ^b	DT	4	[0–1]	[0–1]
FepA^b	DTI	4	R	3–4
FhuA ^b	DTIT	4	R	R
BtuB^b	DTL	4	R	2
Cir ^b	ET	4	R	R
ColM ^b	ETLT	3	R	R
ColIa ^b	EI A	2–3	R	R
WT ColB	<u>GDT</u>MVVWPSTG	4	3–4	3–4
D17H	H	4	3–4	3–4
T18S	S	4	2	2
T18R	R	4	3–4	3–4
ColD ^b	HS	4	3	2–3
FepA ^b	I	3–4	3	3–4
Cir ^b	E	3–4	2	2

^a Positions of the site-directed mutation(s) introduced in the colicin (D or B) TonB box (underlined) are shown. The leftmost position in the sequence is position 16.

^b Changes necessary to introduce different wild-type TonB box motifs are given.

^c The cytotoxicity of the wild-type (WT) or mutant colicins D and B was tested with wild-type and TonB mutant strains as described for Table 1. Values indicate the last serial 10-fold colicin dilution that resulted in a clear zone of growth inhibition. R indicates resistance to undiluted colicin preparation (numbered as 0). Values in brackets indicate the dilution corresponding to the turbid zone of marginal growth inhibition. Functionally important substitutions are indicated in bold.

tion. Thus, the effect of the residues at the mutated positions on colicin D uptake seems to be specific and dependent on the identity of flanking residues in each protein.

The mutation Asp17His, as well as the whole reconstitution of a colicin D-like TonB box in colicin B, did not greatly affect the sensitivity of strains D1 and K19. In contrast, the Thr18Ser mutation impaired the toxicity by 2 orders of magnitude (Table 2). Thus, the D1 and K19 strains are quite unable to import wild-type colicin D with Ser18, but a Ser18 residue in colicin B, though provoking a diminution in sensitivity of 100-fold, retains significant toxicity.

The replacement of Met19 in colicin D with Ile fully restored colicin D sensitivity to the K19(Pro161Leu) mutant strain (Table 2). The restoration of toxicity appeared to be site specific, since the suppressor mutation did not reverse the resistance of mutant D1. In contrast to colicin D, the Met19 residue present in the wild-type colicin B TonB box did not preclude a functional interaction with TonB (Pro161Leu). The restoration of

K19 sensitivity found upon introduction of the FepA or BtuB type of TonB box seems to be due specifically to the changes Met19Ile and Met19Leu rather than to the simultaneous introduction of the His17Asp change. Colicin D sensitivity was also partially restored to the K19 mutant by a single Ser18Thr change.

DISCUSSION

The large N-terminal part of colicin molecules is involved in a series of specialized interactions necessary for target cell recognition and penetration of the outer cell membrane (14). Here we present evidence that the interaction with TonB, needed for the import of cytotoxic colicin D into bacteria, requires a functional interaction involving several particular amino acids localized in the central part of TonB and in the TonB box of colicin D. The dependence of colicin D import on TonB is shown by the complete resistance of strains with Arg158Ser or Pro161Leu mutations in TonB, and the specific reversal of this phenotype by suppressor mutations affecting Ser18 and Met19 of colicin D. The sensitivity to several other colicins was unaffected by the TonB mutations, and the normal function of TonB in iron uptake is not apparently impaired. Residues R158 and P161 in TonB, required for a functional interaction with wild-type colicin D, have already been studied in connection with interactions concerning other TonB boxes but have not previously been found to play any major or specific functional role. The results were unexpected, since not merely the rather conserved TonB box but indeed the whole 313-amino-acid-long N terminus of colicin D is 96% identical to that of colicin B. As well as a variety of point mutations, seven different wild-type TonB box pentapeptides were introduced in place of the colicin D sequence and were all shown to lead to highly active protein within the context of the colicin D N-terminal sequence, as previously shown in their original context. A similar conclusion was drawn from complete TonB box changes made in colicin B (Table 2).

Previous studies have shown that suppressor mutations at or around position 160 in TonB restore several transporter functions when an energy uncoupling mutation is present in the TonB box sequence (3, 8). Such suppressor mutations partially restore toxicity to the inactive colicin B and M derivatives affected, as in the case of BtuB (3), at the most conserved Val residue of the TonB box (16, 22). The interaction of Gln160 with this Val residue in a large number of TonB boxes appears to be of general importance to TonB-dependent transport processes.

It is interesting that quite small changes to residues in colicin D or in TonB can provoke large changes in resistance or sensitivity toward colicin D. Thus, the change Ser18Thr in colicin D, restoring sensitivity in both Arg158Ser and Pro161Leu TonB mutants, corresponds merely to the addition of a methyl group to residue 18. In the context of the Pro161Leu TonB mutant, the suppression of resistance by the mutation Met19Ile is again a minor change. Mutant E (Arg158Cys in TonB) provides a further example: compared to strain D1 (Arg158Ser), a difference of at least 10³ in sensitivity toward colicin D is associated with the small change OH to SH in the side chain of residue 158 in TonB.

The initial strategy for the isolation of colicin D-resistant

host mutants involved screening for host mutants that remained sensitive to colicin B. Our results show in a rather striking manner that not merely are the TonB mutants isolated sensitive to wild-type colicin B but all of the colicin B variants constructed remain active in the TonB mutant strains. Thus, a general conclusion may be drawn from comparing the variants of colicin D and colicin B that we have constructed, namely, that colicin B appears to be considerably more tolerant of changes affecting either the TonB box or the corresponding region in TonB around residue 160. In other words, the role of the interaction between these two domains appears to be much more specific to the function of colicin D than to that of colicin B or, in all probability, to that of most other colicins. Why this is remains to be determined. It may be associated with the very different nature of the toxicity of colicin B and colicin D and thereby to their quite different C-terminal domains, or it may be related to the other differences between the molecules, which include the presence in colicin D of an additional unique region of unknown function between the translocation domain and the RNase domain (4), or to the relatively small number of differences in the long N-terminal domain. It is possible that translocation is a more limiting step in the overall function of colicin D than of colicin B, perhaps because more molecules of colicin D than of colicin B may need to be imported to kill the cell. In summary, certain variations in the TonB boxes of colicins D and B are critical to the site-specific functional interactions between the two colicins and TonB, but these residue-specific effects remain largely dependent on other residues, located outside the TonB boxes.

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