

The Role of Electrostatics in Colicin Nuclease Domain Translocation into Bacterial Cells*

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The mechanism(s) by which nuclease colicins translocate distinct cytotoxic enzymes (DNases, rRNases, and tRNases) to the cytoplasm of *Escherichia coli* is unknown. Previous *in vitro* investigations on isolated colicin nuclease domains have shown that they have a strong propensity to associate with anionic phospholipid vesicles, implying that electrostatic interactions with biological membranes play a role in their import. In the present work we set out to test this hypothesis *in vivo*. We show that cell killing by the DNase toxin colicin E9 of *E. coli* HDL11, a strain in which the level of anionic phospholipid and hence inner membrane charge is regulated by isopropyl β -D-thiogalactopyranoside induction, is critically dependent on the level of inducer, whereas this is not the case for pore-forming colicins that take the same basic route into the periplasm. Moreover, there is a strong correlation between the level and rate of HDL11 cell killing and the net positive charge on a colicin DNase, with similar effects seen for wild type *E. coli* cells, data that are consistent with a direct, electrostatically mediated interaction between colicin nucleases and the bacterial inner membrane. We next sought to identify how membrane-associated colicin nucleases might be translocated into the cell. We show that neither the Sec or Tat systems are involved in nuclease colicin uptake but that nuclease colicin toxicity is instead dependent on functional FtsH, an inner membrane AAA⁺ ATPase and protease that dislocates misfolded membrane proteins to the cytoplasm for destruction.

Colicins are SOS-induced protein antibiotics that penetrate and kill cells in competing *Escherichia coli* populations (1, 2). Colicin entry is known to begin with binding to an extracellular receptor followed by translocation across the outer membrane (OM)³ by a mechanism that has similarities to that used by filamentous bacteriophages in the early stages of phage infection (3). Once in the periplasm the cytotoxic domains of colicins target the IM or, in the case of the nuclease colicins, are

translocated entirely across the IM to reach their cytoplasmic nucleic acid substrates (3, 4). There is currently no information available as to how this latter step is accomplished, although it is likely to require unfolding of the nuclease (5).

Passage across the OM for both pore-forming and nuclease type colicins is mediated by an OM receptor(s) and proteins of either the Tol (for Group A colicins such as A, N, and E1–E9) or Ton (for Group B colicins such as B, D, Ia, and Ib) complexes. Colicins are able to utilize a wide variety of OM proteins as their primary receptor, such as FepA, FhuA, Cir, BtuB, and OmpF, all involved in passive or active nutrient transport across the OM (3). In contrast, the translocation step is restricted to either the Ton or Tol proteins, with movement across the OM likely by a common mechanism because colicins can be engineered to take either route by the simple exchange of colicin domains or indeed the requisite phage domains (6, 7). The physiological role of the Ton (TonB·ExbB·ExbD) complex is in the energy-dependent transport of nutrients across the OM. The complex is coupled to the proton motive force across the IM and is transduced to OM receptors via TonB through so-called “TonB box” sequences, which stimulate the passage of nutrients across the OM (8). The Tol·Pal system (TolA·TolQ·TolR·TolB·Pal) is also a transmembrane assembly responsive to the proton-motive force. In contrast to the Ton system, however, the Tol·Pal complex is required for stability of the OM, with recent data suggesting that its physiological role is that of an energized tether that maintains the appropriate juxtaposition between the inner and outer membranes and newly formed peptidoglycan during cell division (9, 10).

Association with these energized periplasm-spanning systems bring the cytotoxic domains of pore-forming colicins to the periplasmic side of the IM into which they spontaneously insert forming voltage-dependent ion channels that depolarize the cytoplasmic membrane (11). In contrast, nuclease type colicins, or at least their cytotoxic domains, must pass into the cytoplasm where they act enzymatically on DNA (colicins E2, E7, E8, and E9), tRNA (E5 and D), or rRNA (E3, E4, and E6) (4, 12). From work on colicins D and E7 has come the suggestion that the nuclease domains are cleaved from the remainder of the toxin while in the periplasm and/or passage across the IM. In the case of colicin D, the protease has been identified as the signal peptidase LepB or a factor processed by LepB (13, 14). A key consideration for this group of cytotoxins is the absence of any sequence or structural similarity between the different nuclease domains or indeed between nucleases that import via the Tol or Ton pathways, emphasizing that whatever route(s)

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³ The abbreviations used are: OM, outer membrane; IM, inner membrane; IPTG, isopropyl β -D-thiogalactopyranoside; ER, endoplasmic reticulum; LPS, lipopolysaccharide.

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exist for entry to the cytosol they are insensitive to the structure of the nuclease.

Here, we provide the first *in vivo* evidence demonstrating the importance of nuclease domain charge on colicin translocation, which implies that prior to import to the cytoplasm there is an electrostatically driven association with the *E. coli* IM. We also identify a putative translocation route for membrane-associated colicin nucleases.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—The cells were grown in LB broth or on LB agar with kanamycin (50 $\mu\text{g ml}^{-1}$), tetracycline (5 $\mu\text{g ml}^{-1}$), chloramphenicol (34 $\mu\text{g ml}^{-1}$), or ampicillin (100 $\mu\text{g ml}^{-1}$) where required. AR3289 (W3110 *sfhC21 zad220::Tn10*) and AR3291 (W3110 $\Delta\text{ftsH3 sfhC21 zad220::Tn10}$) are described by Tatsuta *et al.* (15). JARV15 (MC4100 $\Delta\text{tatA } \Delta\text{tatE}$), BØD (MC4100 ΔtatB), and B1LK0 (ΔtatC) are described by Stanley *et al.* (16). GN15 (MC4100 *secY205 ompT::kan*), GN31 (*secY39 ompT::kan*), GN32 (*secY125 ompT::kan rpsE*), NH146 (MC4100 *secD1 ompT::kan*), and NH195 (*secE501*) are described by Matsumoto *et al.* (17). Strain HDL11 (*pgsA::kan* Φ *(lacOP-pgsA⁺)1 lacZ' lacY::Tn9 lpp2 zdg::Tn10*) is described by Kusters *et al.* (18), and strains SD12 and BW25113, which are wild type for phospholipid biosynthetic enzymes, are described by Shibuya *et al.* (19) and Baba *et al.* (20), respectively. The colicin-producing strains BZB2101 (colicin A), BZB2102 (colicin B), BZB2103 (colicin D), BZB2104 (colicin E1), BZB2108 (colicin E5), BZB2114 (colicin Ia), BZB2115 (colicin Ib), PAP1 (colicin M), and BZB2123 (colicin N) are described by Pugsley (21) and were obtained from the Institut Pasteur strain collection. pIFH108 encoding wild type FtsH and derived plasmids encoding the FtsH mutants K201N, F228R, E255Q, D304A, D307A, E418Q, and H421Y have been previously described (22–24). Mutations in the DNase domain of colicin E9 (K21E, K45E, K21E/K45E, S77K/S78R, and S77K/S78R/S80K; DNase numbering) were made by the Stratagene QuikChange method using the plasmid pRJ345 as the template. The NcoI-XhoI fragment of the mutagenized plasmid was ligated into the same sites of the plasmid pCS4.

Protein Purification—Nuclease colicins were used either as complexes with their immunity proteins (25) or as isolated toxins; cell killing kinetics are unaltered by the presence of the immunity protein (26, 27). The colicin E3·Im3 and colicin E9·Im9 (E2·Im2, E7·Im7, and E8·Im8) complexes and uncomplexed colicin E9 were purified as described previously (28). Colicin Ia and Ib were purified from strains BZB2114 and BZB2115, respectively (described below). The cells were grown in LB medium (1.6 liters) to an $A_{600} = 0.5$, mitomycin C was added to a final concentration of 0.6 $\mu\text{g ml}^{-1}$, and the cells were grown for a further 4 h all at 37 °C. The harvested cells were resuspended in 30 ml of 50 mM KP_i buffer, pH 7.0, and broken by passage through a French press. After removal of the cell debris by centrifugation, ammonium sulfate was added to a final concentration of 114 g liter⁻¹ at 4 °C and stirred for 1 h at this temperature. The precipitate was removed by centrifugation, and ammonium sulfate (193 g liter⁻¹) was added. After centrifugation the supernatant was discarded, and the pellet was resuspended in a small volume of 50 mM KP_i , pH 7.0, dia-

lyzed into 2 × 5 liters of the same buffer. The proteins were then applied to a MonoS column and eluted with a 0–300 mM NaCl gradient.

Liquid Culture Cell Death Assays—To measure cell death in liquid culture an overnight culture of *E. coli* HDL11 was diluted 1:100 into 50 ml of LB broth with or without IPTG (20–500 μM) and grown at 37 °C with shaking. After 180 min the appropriate purified colicin was added ($A_{600} = \sim 0.6$). The A_{600} of each culture was taken at 30-min intervals throughout the experiment. For spot test assays of purified colicin, LB agar plates were overlaid with 5 ml of molten 0.7% (w/v) non-nutrient agar, to which was added 100 μl of a culture of the appropriate indicator strain with an A_{600} of 0.6–0.8. For AR3291, 500 μl of culture was used, because this strain grows slowly on LB agar. Aliquots of 2 μl of 5-fold dilutions of colicin were spotted on the plates, which were then incubated at 37 °C overnight. To test the cytotoxicity of colicins without prior purification of the protein, colicin expressing cultures were stabbed into LB agar plates and incubated at 37 °C overnight. The plates were exposed to chloroform vapor for 15 min to break the cells and then overlaid with 5 ml of molten 0.7% (w/v) non-nutrient agar containing the indicator strain prepared as described above. The plates were incubated overnight at 37 °C and then inspected for a clear zone of growth inhibition around the test culture.

Lux Reporter Assay—All of the assays were done as described previously using the DPD1718, a strain housing an SOS-inducible *lux* reporter system (27). DPD1718 cells were grown to mid-log ($A \sim 0.35$ – 0.45), after which they were diluted 1:2 (100 μl total volume) into black 96-well plates with an optical bottom (Nunc), and wild type colicins or mutant proteins were added to a final concentration of 0.4 nM. Trypsin (final concentration, 0.05 mg/ml) was added at regular time intervals between 0 and 30 min after colicin addition. Induction of luminescence was followed over a period of up to 2 h with readings taken every 300 s. Gamma values (27) for the luminescence induction by each colicin were calculated, and the amount of protection offered by trypsin treatment was obtained by comparing the luminescence induced by colicin in the presence and absence of trypsin. All of the assays were performed at least twice, and error bars, where shown, represent the means \pm S.E. for at least two independent experiments.

Cell Survival Assay—HDL11 was grown in LB broth at 37 °C to mid-log phase ($A_{600} = \sim 0.6$) in the presence or absence of 100 μM of IPTG. Colicin at a final concentration of 80 nM was added to 1-ml aliquots of the cells with trypsin (1 mg ml⁻¹) added at defined time points after the addition of the colicin. Each sample was left to incubate at 37 °C for a further 30 min, after which cells were diluted in LB and plated onto LB agar with the appropriate antibiotics. After overnight growth at 37 °C, the cells were counted, and the percentage of survival was calculated relative to a control sample to which no colicin was added.

RESULTS

Colicin DNase Domain Charge Modulates Cell Killing Efficiency in *E. coli* Cells Depleted of Anionic Phospholipids—We have demonstrated previously that the colicin E3 rRNase and

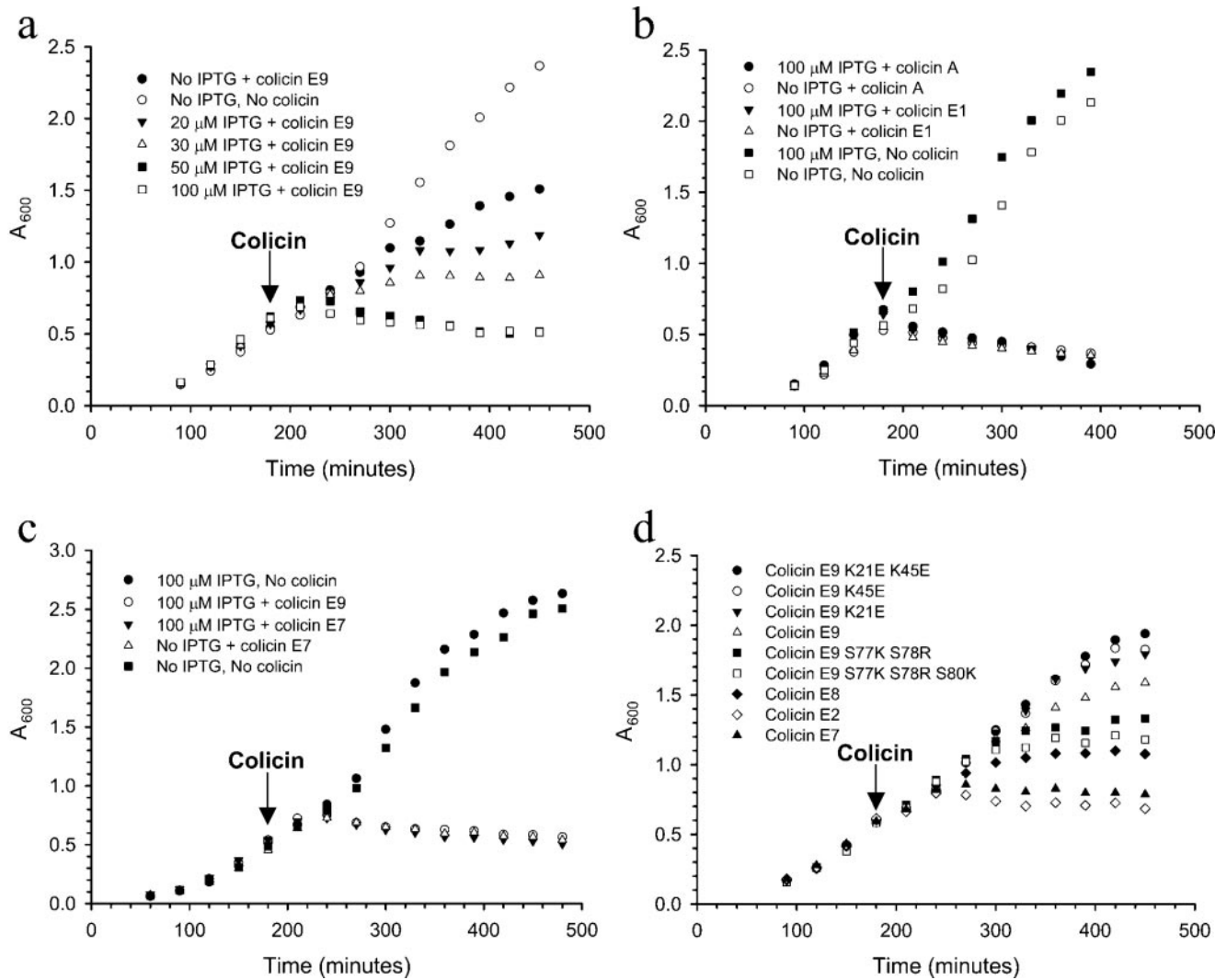


FIGURE 1. The efficiency of cell killing by DNase colicins is dependent on the level of anionic phospholipid in the target cell and the positive charge on the nuclease. *a*, colicin E9-induced cell killing of *E. coli* HDL11 in liquid culture in the presence and absence of IPTG. Colicin E9-Im9 complex ($5 \mu\text{g ml}^{-1}$) was added at the time indicated. Cell killing is more rapid with increasing IPTG concentrations up to $50 \mu\text{M}$ as indicated by a smaller increase in A_{600} after addition of the colicin. *b*, killing of HDL11 by colicins E1 and A ($5 \mu\text{g ml}^{-1}$), showing there is no dependence on IPTG concentration ($\pm 100 \mu\text{M}$). *c*, treatment of HDL11 cells with colicin E7-Im7 complex ($5 \mu\text{g ml}^{-1}$) showing cell killing is independent of IPTG concentration ($\pm 100 \mu\text{M}$) and very similar to that observed for colicin E9 in the presence of IPTG. *d*, susceptibility of strain HDL11 in the absence of IPTG to the naturally occurring colicins E2, E7, E8, E9, and colicin E9 mutants engineered for decreased (E9, K21E; E9, K21E/K45E) or increased (E9, S77K/S78R; E9, S77K/S78R/S80R) positive charge on the DNase domain.

E9 DNase domains (12 and 15 kDa, respectively) interact with anionic but not neutral phospholipid vesicles, with these electrostatically driven associations causing the domains to become destabilized (29, 30). Colicin DNases also have the ability to form voltage-independent ion channels in planar lipid bilayers (5). Considering that the *E. coli* IM is normally composed of 70–80% neutral phospholipids and 20–30% anionic phospholipids (31), these experiments pointed to the possibility that colicin nucleases, which are positively charged domains, might interact directly with one or both of the membrane systems of *E. coli* en route to the cytoplasm. The present work set out to test this hypothesis.

In addressing the importance of electrostatically driven protein-lipid interactions in the translocation of the E9 DNase domain, we used an *E. coli* strain depleted in anionic phospholipids. The strain HDL11 contains a single copy of the *pgsA* gene under the control of the *lac* operon and so is IPTG-inducible

(18). The product of the *pgsA* gene is responsible for the production of the major anionic phospholipid phosphatidylglycerol. In the absence of IPTG, HDL11 produces little phosphatidylglycerol (2%) or cardiolipin (1%) but does contain phosphatidic acid (6%) and so has a total content of anionic lipid of around 10% (18). This is increased to $\sim 20\%$ in the presence of $50 \mu\text{M}$ IPTG, with phosphatidylglycerol accounting for the majority (15%) as is the case in wild type K-12 strains (18). The addition of concentrations of IPTG in excess of this does not increase the proportion of anionic phospholipid in the IM. We found that killing of strain HDL11 by an excess of colicin E9 ($5 \mu\text{g ml}^{-1}$), where cell death was monitored by measurement of the optical density of the growing culture, was dependent on IPTG concentration (Fig. 1*a*). In the absence of IPTG, cell growth continued for several hours after the addition of the colicin, although cell death eventually ensues. In the presence of $50 \mu\text{M}$ IPTG, however, the rate of cell killing was restored to a level similar to that observed in

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wild type K-12 strains (data not shown). The addition of higher IPTG concentrations (100 or 500 μM) did not increase the level of cell killing further; with 20 or 30 μM IPTG, the level was intermediate between 0 and 50 μM (Fig. 1*a*). The data show that although HDL11 is not resistant to colicin E9, the kinetics of cell killing are strongly dependent on the level of anionic phospholipid expressed by the bacterium.

The phospholipid composition of the IM bilayer and the inner leaflet of the OM is affected in the HDL11 strain. Consequently, the effect of IPTG concentration on colicin E9 toxicity could reflect changes at either surface. The outer leaflet of the OM is comprised predominantly of LPS and so is unlikely to be grossly affected in HDL11. Nevertheless, the phospholipid:LPS ratio of the OM would be expected to change, and this could affect early steps in colicin import involving receptor binding and translocation across the OM. To help address this issue, we analyzed the susceptibility of HDL11 to the group A pore-forming colicins E1 and A, which translocate across the OM by the same basic mechanism as nuclease colicins; both colicins parasitize BtuB as their primary receptor and then recruit OM translocators (TolC and OmpF, respectively) followed by translocation to the periplasm through binding of the Tol-Pal complex. We found that both pore-forming colicins killed HDL11 with identical killing profiles irrespective of whether IPTG was added to the culture (Fig. 1*b*), suggesting that the machinery for translocating colicins across the OM has remained largely unaltered. We conclude therefore that the effect of reduced anionic phospholipid content on colicin E9 toxicity most likely concerns effects at the IM.

We also investigated other naturally occurring DNase type E colicins, E2, E7, and E8, which have been characterized extensively (32, 33). DNase colicins are 60-kDa toxins that share a high degree of sequence identity in their receptor-binding and translocation domains (>90%) but are less conserved in their respective 15-kDa DNases (~65%), and although all are basic domains ($\text{pI} > 9.6$), they differ in the number and type of charged residues. In particular, the net positive charge for all four DNase domains varies considerably, with E7, E2, E8, and E9 having +13, +11, +9, and +7 charges, respectively. We found that in contrast to colicin E9, where killing of *E. coli* HDL11 was dependent on the IPTG concentration, colicins E7 (Fig. 1*c*) or E2 (Fig. 1*d*) were equally cytotoxic against HDL11 regardless of whether IPTG was added to the culture (only data in the absence of IPTG are shown in Fig. 1*d*). Thus, for the most positively charged variants reducing the proportion of negatively charged lipid does not retard the level of cell killing. With colicin E8 (+9) (data not shown) the absence of IPTG had some effect on cell killing, but not to the extent observed with colicin E9 (+7) (Fig. 1*c*).

DNase E colicins are essentially identical in the sequences of their receptor-binding and translocation domains, and so these are unlikely to explain the differing behavior of these toxins on HDL11. Two effects could reasonably account for the influence of colicin DNase charge and inducing agent on cell killing: (i) The endonucleolytic activities and hence cytotoxicities of the most positively charged variants (E2 and E7) are significantly greater than those of E8 and E9. This can be discounted because a comparison of the enzymatic activities of all DNase colicins

shows no such trend. Indeed, colicin E8 has the greatest relative activity *in vitro* in both plasmid-nicking and spectrophotometric assays, with E2, E7, and E9 having approximately equivalent activities (33). (ii) The differing net positive charge of the DNases affects their ability to associate with the IM of HDL11. If this explanation is correct, then we rationalized it should be possible to engineer enhanced or diminished cell killing activity merely by increasing or decreasing, respectively, the positive charge on a single colicin nuclease domain. This would also discount the possibility that the different levels of activity against HDL11 was due to subtle structural differences between the enzymes of the DNase colicin family and so unrelated to the amount of positive charge.

To test this hypothesis we engineered charge variants in the DNase of colicin E9, both increasing and decreasing the net positive charge. Positions outside of the enzyme active site were chosen that are not involved in catalysis or DNA binding (34). Single and double Lys-to-Glu substitutions (at Lys²¹ and Lys⁴⁵; numbering for the isolated domain) were engineered to reduce the amount of positive charge to +5 and +3, respectively. Both mutants showed reduced killing against HDL11 relative to the wild type colicin E9, with the double mutant more strongly impaired (Fig. 1*d*). In contrast, the introduction of additional positive charges into the DNase domain of colicin E9 (S77K/S78R and S77K/S78R/S80K) showed enhanced cell killing of HDL11 relative to wild type colicin E9 (Fig. 1*d*). We tested the enzymatic activities of all the engineered charge variants and found no correlation with their biological activity; indeed, both sets of mutants had slightly reduced plasmid DNA nicking activities relative to wild type colicin E9 (data not shown). Our data demonstrate that the cell killing efficiency of a colicin DNase against *E. coli* HDL11 with reduced anionic phospholipid content can be enhanced or reduced merely by changing the number of net positive charges and that this is not due to differences in enzymatic activity between the colicin DNases.

To ascertain whether there is a quantitative relationship between cell killing ability and colicin DNase charge, we plotted all of the HDL11 IPTG cell killing data (quantified as the change in A_{600} following the addition of colicin) as a function of the positive charge of the domain. From the resulting plot it is clear that there is a strong correlation between the net positive charge on a colicin DNase domain and its cytotoxicity activity against HDL11 (Fig. 2). We also looked for systematic variations in toxicity when HDL11 was induced with 100 μM IPTG and compared this to a wild type strain of *E. coli* K-12 (SD12). In both cases a weak correlation with enzyme charge was apparent (data not shown) but was less clear-cut; the most-to-least positively charged variants displayed only small differences in optical density (<0.3) compared with HDL11-IPTG. Hence, the correlation between enzyme charge and cytotoxicity, at least as measured by changes in culture optical density, is masked when the IM carries wild type negative charge and only becomes readily apparent in *E. coli* that is depleted of anionic phospholipids.

Electrostatic Interactions Gate Entry of DNase Colicins into Wild Type E. coli Cells—To probe further the involvement of electrostatic interactions in colicin entry, particularly in wild type strains, and to provide quantitative data on cell entry

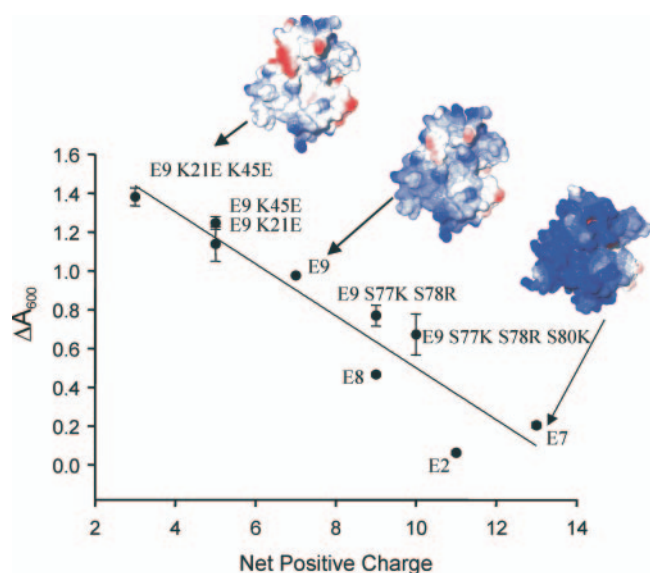


FIGURE 2. Correlation between the net positive charge on a colicin DNase and the death of *E. coli* HDL11 in the absence of IPTG. The figure, which includes the naturally occurring variants E2, E7, E8, and E9 as well as charge mutants of colicin E9, shows the relationship between nuclease charge and cell killing efficiency, as deduced from change in A_{600} between the addition of the colicin after 180 min and the end of the experiment (450 min, see Fig. 1). The line of best fit is shown ($r^2 = 0.89$). All of the experiments were performed in duplicate. The calculated electrostatic surface distributions of colicin E9 K21E/K45E (+3), E9 (+7), and E7 (+13) DNase domains are also shown. Structures for colicins E7 and E9 are taken from their respective Protein Data Bank codes (7CEI and 1EMV, respectively).

kinetics, we resorted to trypsin-protection assays of colicin toxicity. Trypsin rapidly degrades colicins, rendering them inactive, and has been used extensively to investigate colicin entry kinetics (35, 36). Trypsinolysis of colicin-treated cells was used by Benedetti *et al.* (36) to show that the pore-forming toxin colicin A is unfolded and exposed to the extracellular environment even when its cytotoxic domain is depolarizing the IM. Similar experiments have been reported recently by Duché (37) for the DNase colicin E2, demonstrating that the toxin remains attached to its OM receptor while contacting the Tol proteins in the periplasm. Colicins must span large distances (100–200 Å) to simultaneously contact one or more proteins in the OM and periplasm, and this likely explains why colicins such as E3 and Ia have elongated hairpin structures with long coiled-coil regions connecting their cytotoxic and translocation domains (38, 39). It is thought the coiled-coil opens or unfurls like a penknife during translocation, and this is consistent with the inhibitory effect of a disulfide bond engineered across the coiled-coil, with full activity restored on reduction of the disulfide with dithiothreitol (40, 41).

In the context of our experiments on HDL11 and previous work on colicin translocation (35–41), we reasoned that electrostatic interactions between the positively charged enzymatic domains and the anionic phospholipid head groups of the IM might gate entry of the nuclease to the cytoplasm and so regulate the overall rate of cell killing. To test this hypothesis we conducted trypsin protection experiments on colicin-treated wild type *E. coli* K-12 cells using DNase colicins of differing overall charge. The rate of cell entry was estimated initially by the extent of DNA damage inflicted on the cell by the translo-

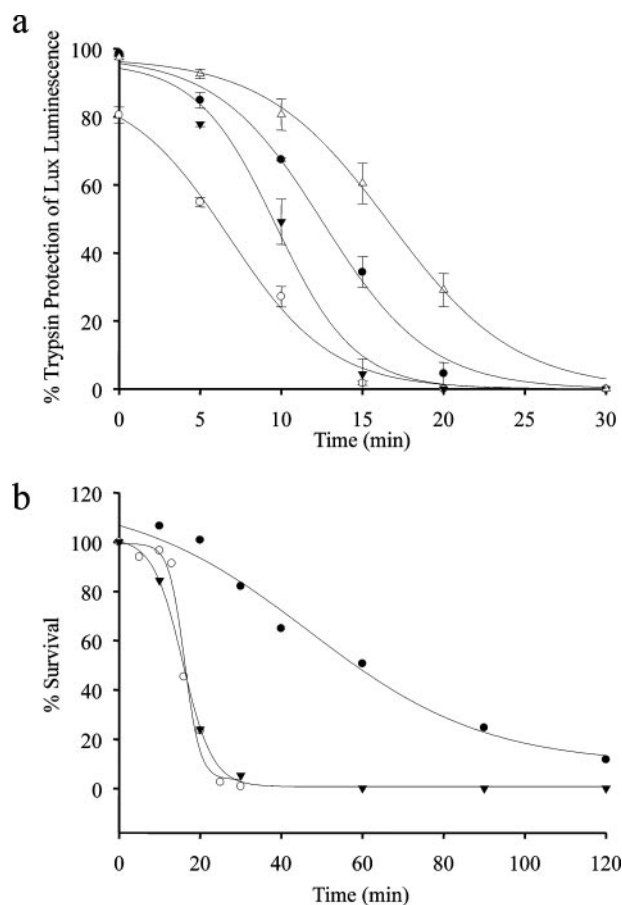


FIGURE 3. Colicin DNase charge controls the cell survival half-life of wild type and anionic phospholipid-depleted *E. coli* cells. *a*, trypsin protection of DNA damage-induced Lux luminescence by DNase type colicins in *E. coli* DPD1718 cells containing a *lux* reporter system. See “Experimental Procedures” for details. The percentage protection data, calculated by comparison with the luminescence induced in the absence of trypsin, are shown for colicins E7 (○), E8 (▼), E9 (●), and E9 K21E/K45E (△). All of the experiments were conducted in duplicate. *b*, protection of colicin E9-induced cell death of HDL11 in the absence (●) and presence (▼) of 100 μ M IPTG and for strain BW25113 (○), which is wild type for phospholipid biosynthetic enzymes, after the addition of trypsin. $\sim 10^8$ colony-forming units were incubated with 80 nM colicin, and intoxication stopped with trypsin treatment at the times indicated. The data are presented as the percentage of survival relative to a no colicin control. All of the half-life data ($\tau_{1/2}$) from trypsin protection assays are shown in Table 1.

cated DNase, the latter reported by a Lux reporter system. Previous work from our laboratories has shown that DNase colicins are strong inducers of the SOS response (42) and that coupling of an SOS-inducible promoter to the *lux* gene cluster provides a convenient measure of DNA damage caused by a translocating DNase colicin that is independent of cell killing (27). Fig. 3*a* shows the time course for trypsin protection of Lux luminescence for colicins with DNase domains of differing overall positive charge added to DPD1718 cells, which are wild type for anionic phospholipid content; +3 (E9 DNase K21E/K45E), +7 (E9 DNase), +9 (E8 DNase), and +13 (E7 DNase). The data show almost a 3-fold difference in the half-life ($\tau_{1/2}$) of trypsin protection, with the most positively charged colicin having a much shorter $\tau_{1/2}$ (~ 7 min) than the least charged variant (> 17 min), consistent with electrostatic attraction gating entry to the cytosol (Table 1).

TABLE 1

Kinetics of cell survival and colicin-induced DNA damage

The data are presented as the half-life ($\tau_{1/2}$ in min) of cell survival or DNA damage in the presence of the colicins indicated. Strains BW25113 and DPD1718 are wild type for phospholipid biosynthetic enzymes. HDL11 in the presence of IPTG has wild type levels of anionic phospholipids, and in the absence of IPTG has low levels of anionic phospholipid. The rates for DPD1718 were calculated from Lux reporter assays (Fig. 3a) and for BW25113 and HDL11 from cell survival assays (Fig. 3b).

Strain	Colicin			
	E7	E8	E9	E9 (K21E/K45E)
HDL11 – IPTG	<5	37	60	75
HDL11 + IPTG	<3	15	18	25
Wild type (BW25113)	1	10	16	12
Lux (DPD1718)	7	10	13	17

We also conducted trypsin protection experiments using *E. coli* HDL11 where cell death kinetics was monitored by the number of colony-forming units as a function of time. Fig. 3b shows the time course for survival of HDL11 cells treated with colicin E9 (+7) in the presence and absence of IPTG. Cell survival $\tau_{1/2}$ in the presence of colicin E9 for IPTG-induced HDL11 cells (~18 min) is essentially the same as that for the standard lab strain *E. coli* BW25113 ($\tau_{1/2}$ = ~16 min), consistent with IPTG restoring the anionic phospholipids in the cell to wild type levels. Strikingly, the time taken to kill *E. coli* HDL11 cells that have not been IPTG-induced increases by more than 3-fold ($\tau_{1/2}$ = ~60 min) for the same colicin, consistent with the suggestion that IM charge gates entry of the nuclease to the cytoplasm.

Measurements of cell survival half-life were made for the full complement of DNase colicins in this study and compared with the trypsin protection of Lux luminescence data (Table 1). The data reiterate the relationship between nuclease charge and the rate of cell killing and show how this becomes attenuated when the charge on the IM of the bacterium or enzyme is decreased. The most extreme examples of this are colicin E9 K21E/K45E (the least charged of the variants with +3), where $\tau_{1/2}$ for cell killing of HDL11 IPTG is ~75 min but that reduces to ~25 min in the presence of IPTG, and colicin E7 (the most charged variant), where $\tau_{1/2}$ for cell entry is ~5 min and is largely insensitive to IM charge. Because trypsin can only access regions of the colicin that are displayed on the surface of the OM, our data are consistent with recent evidence suggesting that DNase colicins remain attached to their OM receptor during passage to the periplasm (37). Moreover, we show that the IM translocation step is gated by the degree of positive charge on the DNase domain, with highly charged enzymes having significantly faster rates of cell entry.

Translocation of Colicin Nuclease Domains across the Cytoplasmic Membrane Requires the AAA⁺ ATPase FtsH—The dependence of DNase colicin cell entry and cytotoxicity on its net positive charge suggests that colicin nuclease domains partition into the IM. This raises the question of how they then move to the cytoplasm? In the case of bacterial toxins such as ricin that act on eukaryotic cells, it has been proposed that retrotranslocation from the endoplasmic reticulum (ER) via the Sec61 channel that normally functions in protein export is responsible (43, 44). We therefore investigated the possibility that colicin nuclease domains could be retrotranslocated by the known protein conducting channels of the *E. coli* cytoplasmic membrane: the Sec and Tat pathways.

Strains with deletions in *tatAE*, *tatB*, or *tatC* are completely defective in the export of Tat-dependent substrates and display pleiotropic cell envelope defects (16). However, the sensitivity of these strains to colicin E9 was unchanged relative to the wild type strain, indicating that the Tat pathway is not involved in colicin retrotranslocation (data not shown). The key genes of the Sec transport system cannot be deleted, but cold-sensitive mutations in SecD, SecE, SecF, and SecY that disrupt this pathway are known (45). These mutants display an export defect at all temperatures, which becomes more pronounced at lower temperatures. However, such mutants (e.g. *secY205*, *secY39*, *secY125*, *secD1*, and *secE501* described by Matsumoto *et al.* (17)) showed no colicin insensitivity at 37 °C or at temperatures close to the nonpermissive temperature (data not shown), suggesting that the Sec complex is also not required for colicin import, although we cannot formally rule this out as a possible protein conducting channel because other regions of the channel may be involved.

E. coli lacks a multicomponent degradation pathway in the IM akin to the ER-associated protein degradation pathway of the ER. However, the IM-bound AAA⁺ protease FtsH has been shown to be responsible for the dislocation and proteolysis of misfolded or misincorporated membrane proteins (46). FtsH possesses N-terminal membrane spanning sections with cytoplasmically located AAA⁺ ATPase and zinc metalloprotease domains and forms a hexameric ring located in the *E. coli* cytoplasmic membrane (46). The involvement of FtsH in colicin translocation has been largely ignored because of early reports showing the apparent lack of specificity by FtsH point mutants tolerant of both nuclease and pore-forming colicins (47, 48). However, it is likely that the genetic background in which the original tests were conducted compounded these mutant phenotypes. In the work of Matsuzawa *et al.* (47), the strain UM21, which was isolated as a spontaneous mutant tolerant to colicin E3, showed additional tolerance to colicins E2, D, Ia, and Ib. Tolerance in this strain was shown to be due to a single amino acid change in FtsH, H421Y, which inactivates FtsH function (48). However, there are genetic differences between UM21 and AR3291, a strain where FtsH has been deleted (see below), because UM21 displays the additional phenotype of temperature sensitivity. This phenotype is thought to be due to a combination of a lack of FtsH and an unidentified mutation in the parent strain of UM21, an assertion that is supported by the observation that the FtsH H418Y mutant does not show temperature sensitivity in a AR3291 background (49).

Given the uncertainties with the original reports that *E. coli* FtsH mutants were tolerant of both pore-forming and nuclease colicins, we revisited this issue using well characterized deletion strains. FtsH is an essential gene in *E. coli*, with lethality stemming from the overproduction of LPS and a lethal shift in the balance of the LPS:phospholipid ratio. This is a consequence of the loss of FtsH-mediated cleavage of LpxC, a key enzyme in LPS biosynthesis. The lethality can be suppressed in a *sfhC21* mutant background, where the fatty acid biosynthesis enzyme FabZ (R-3-hydroxy-acyl-ACP-dehydrase) is up-regulated and restores balance to the LPS:phospholipid ratio (49). We tested the FtsH deletion strain AR3291 (Δ *ftsH sfhC21*) and the parent strain AR3289 (*sfhC21*) for sensitivity to the nuclease

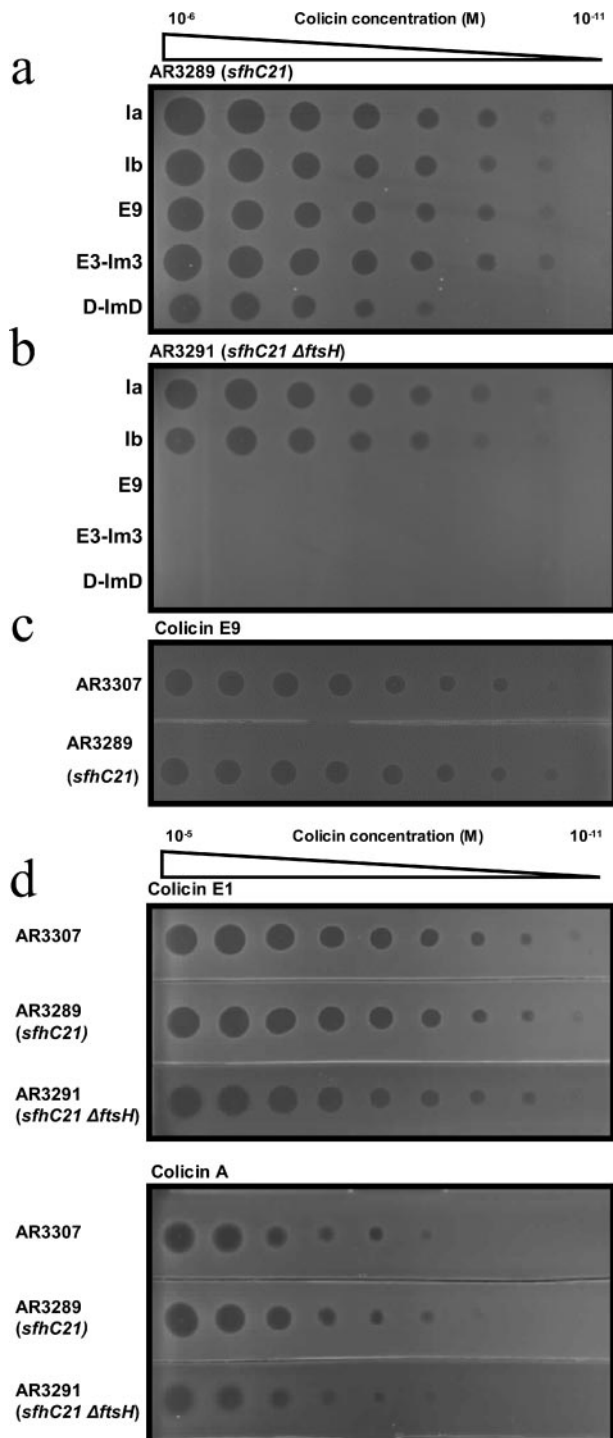


FIGURE 4. FtsH is required for nuclease but not pore-forming colicin-mediated *E. coli* cell death. *a*, cytotoxicity plate assay showing *E. coli* AR3289 (*sfhC21*) is sensitive toward both pore-forming and nuclease colicins. A lawn of AR3289 (*sfhC21*) was spotted with a 5-fold serial dilution of colicins Ia and Ib (pore-formers) and nuclease colicins. *b*, cytotoxicity plate assay, as described for *a*, showing *E. coli* AR3291 (Δ *ftsH sfhC21*) is sensitive toward pore-forming but completely resistant toward nuclease colicins. *c*, *E. coli* strain AR3307, the parent strain, and AR3289 (*sfhC21*) are both sensitive toward colicin E9. *d*, cytotoxicity plate assay showing that all of the strains used in this study, AR3307, AR3289 (*sfhC21*), and AR3291 (*sfhC21 ΔftsH*), are sensitive toward the pore-forming colicins E1 and A.

type colicins E3, D, and E9 and the pore-forming colicins Ia and Ib in spot tests using purified colicins (Fig. 4, *a* and *b*). Nuclease type colicins were unable to kill strain AR3291 (Δ *ftsH sfhC21*) at

any concentration tested, whereas we observed no difference in the sensitivity of AR3291 and AR3289 to colicins Ia and Ib. The possibility that the presence of the *sfhC21* mutation could affect nuclease colicin translocation in some way can be ruled out because AR3289 (*sfhC21*) and the parent strain AR3307 show identical sensitivity to colicin E9 (Fig. 4*c*). Colicins Ia and Ib are Ton-dependent pore-forming toxins; hence we also tested Tol-dependent pore-forming colicins, E1 and A, and found no difference in their ability to kill AR3291 (Δ *ftsH sfhC21*), AR3289 (*sfhC21*), or AR3307 (Fig. 4*d*).

We tested AR3289 and AR3291 for sensitivity to a number of additional pore-forming and nuclease type colicins in stab tests. The pore-forming colicins N and B were active against both AR3289 (*sfhC21*) and AR3291 (Δ *ftsH sfhC21*), whereas the nuclease type colicins E5 (tRNase), E2, E7, and E8 (DNases) were active only against AR3289 (*sfhC21*) (data not shown). Similar to the pore-forming colicins, colicin M, which kills cells through the inhibition of murein synthesis, was able to kill both AR3289 (*sfhC21*) and AR3291 (Δ *ftsH sfhC21*) (data not shown). It has been shown recently that the lethal effect of colicin M is due to its enzymatic degradation of membrane-bound undecaprenyl phosphate-linked peptidoglycan precursors (50), which suggests that like the pore-forming colicins, this toxin does not have to pass into the cytoplasm. We conclude that deletion of FtsH renders cells insensitive to all known families of nuclease colicins (DNase, rRNase, and tRNases) but does not affect sensitivity toward pore-forming colicins or colicin M. Importantly, resistance to nuclease colicins is independent of the translocation route across the OM because AR3291 (Δ *ftsH sfhC21*) is resistant to both Ton-dependent (colicin D) and Tol-dependent (colicins E2–E9) colicins. Conversely, all Ton- and Tol-dependent pore-forming colicins are able to kill this strain. We speculate that FtsH is the route by which colicin nuclease domains make their way to the cytoplasm following their electrostatically driven association with the IM. We cannot discount the possibility that the involvement of FtsH is indirect, with the deletion phenotype caused either by the retention of a protein or proteins within the IM that inhibit nuclease colicin translocation or by a more general membrane stress response (46). This seems unlikely for two reasons: (i) Pore-forming colicins are still active, which argues that the IM has not been grossly affected. (ii) To our knowledge, resistance to colicins has never been attributed to any general, outer envelope stress response but is invariably associated with mutations in specific proteins that play a role in their import. Nevertheless, initial attempts at identifying cross-linked adducts *in vivo* between nuclease colicins and FtsH by Western blotting have thus far been unsuccessful (data not shown), a result that is unsurprising given that colicins kill cells at the single molecule level.

Complementation of AR3291 (Δ *ftsH sfhC21*) with the plasmid pIFH108 encoding wild type FtsH restored sensitivity to colicin E9 at levels comparable with AR3289 (*sfhC21*). However, restoration of cell killing was not observed on complementation of AR3291 with pIFH108 derivatives expressing FtsH mutants K201N, F228R, E255Q, D307A, E418Q, and H421Y but was restored with FtsH D304A (Fig. 5). These FtsH variants have been characterized extensively by Ogura and co-workers (22–24), who showed that all, with the exception of

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FIGURE 5. Colicin E9-induced killing is not restored in FtsH mutants that cannot support cleavage of the physiological substrate σ^{32} . Sensitivity of *E. coli* AR3291 ($\Delta ftsH$ *sfhC21*) transformed with plasmid pIFH108 encoding wild type (WT) FtsH or derived plasmids encoding the mutant FtsH proteins K201N, F228R, E255Q, D304A, D307A, E418Q, and H421Y and tested for colicin E9 sensitivity. Plasmids and strains have been described previously (22–24).

D304A, are proteolytically inactive against the cytoplasmic FtsH substrate, σ^{32} . Thus, proteolytic activity against σ^{32} mirrors the ability of FtsH to support nuclease type colicin translocation. The positions of these mutations cover most of the functionally important regions of FtsH with Lys²⁰¹ and Glu²⁵⁵ located in the ATPase domain; Phe²²⁸ is thought to form the entrance to the central substrate translocating pore, Asp³⁰⁷ is located in a conserved region of the protein of unknown function, and His⁴²¹ and Glu⁴¹⁸ are active site residues of the protease domain. We tested these mutants against DNase, rRNase, and tRNase colicins and found that all required the ATPase and protease activities of FtsH (data not shown). Currently, *E. coli* LepB is the only membrane-associated protease that has been implicated in nuclease colicin processing, but this is specific to a single, Ton-dependent toxin, colicin D, with an *E. coli* *lepB* mutant not displaying more general resistance against other nuclease colicins such as E2 (13). Our observation that the proteolytic machinery of FtsH is required for both Ton- and Tol-dependent nuclease colicin cytotoxicity provides another candidate for this final processing step that, in contrast to LepB, is independent of the structure of the nuclease or the mode of entry to the periplasm.

DISCUSSION

The present work reveals for the first time the importance of electrostatic charge in colicin nuclease cell entry, suggestive of a direct, electrostatically mediated association between the nuclease and the bacterial IM. Translocation across the IM to the cytoplasm likely involves the hijacking of an endogenous system, in much the same way that OM and periplasmic pro-

teins are commandeered by colicin to expedite translocation across the OM. We show that this latter step does not involve retrograde transport through the Sec or Tat pathways but is instead dependent on FtsH, an IM dislocating protease that degrades misfolded membrane proteins. This raises the intriguing possibility that nuclease domains of colicins are mistaken for misfolded membrane proteins destined for destruction in the cytoplasm.

Our data do not at this stage rule out indirect involvement of FtsH in colicin entry; for example, through the overexpression of an inhibitory IM protein in the FtsH deletion strain that blocks translocation to the cytoplasm. The most parsimonious explanation, however, is that FtsH itself is the IM translocator, which if true would have interesting parallels with toxin import into eukaryotic cells. It is generally acknowledged that bacterial toxins that target mammalian cells parasitize endogenous trafficking pathways to reach their target substrate in the cytoplasm. Cholera, for example, uses a glycolipid specific pathway to reach the Golgi and ER (51), from where retrograde transport via the ER-associated protein degradation pathway, responsible for the dislocation and degradation of unfolded proteins, translocates the toxin to the cytoplasm (52). Importantly, toxins such as cholera are somehow able to avoid the later stages of ubiquitination and degradation by the proteasome. We speculate that by analogy with eukaryotic-specific toxins, colicins avoid being degraded by active FtsH while still requiring active protease for toxicity; for example, proteolytic processing by FtsH might release the colicin nuclease to the cytoplasm. Another possibility is that nuclease colicins are continually degraded by the processive activity of FtsH, but occasionally a complete domain is proteolytically released to the cytoplasm.

Finally, we note that if FtsH is involved directly in colicin import, this would be consistent with its poor unfoldase activity (53), providing a rationale for the destabilization that accompanies colicin nuclease association with anionic phospholipids (29, 30). Similarly, ricin interacts directly with negatively charged membranes, with this association also serving to destabilize the protein prior to translocation into mammalian cells (54).

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