⌘ *Author's Choice*

Energy-dependent Immunity Protein Release during *tol*-dependent Nuclease Colicin Translocation*

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Nuclease colicins bind their target receptor in the outer membrane of sensitive cells in the form of a high affinity complex with their cognate immunity proteins. Upon cell entry the immunity protein is lost from the complex by means that are poorly understood. We have developed a sensitive fluorescence assay that has enabled us to study the molecular requirements for immunity protein release. Nuclease colicins use members of the *tol* operon for their translocation across the outer membrane. We have demonstrated that the amino-terminal 80 residues of the colicin E9 molecule, which is the region that interacts with TolB, are essential for immunity protein release. Using *tol* deletion strains we analyzed the cellular components necessary for immunity protein release and found that in addition to a requirement for *tolB*, the *tolA* deletion strain was most affected. Complementation studies showed that the mutation H22A, within the transmembrane segment of TolA, abolishes immunity protein release. Investigation of the energy requirements demonstrated that the proton motive force of the cytoplasmic membrane is critical. Taken together these results demonstrate for the first time a clear energy requirement for the uptake of a nuclease colicin complex and suggest that energy transduced from the cytoplasmic membrane to the outer membrane by TolA could be the driving force for immunity protein release and concomitant translocation of the nuclease domain.

Membrane translocation is a formidable challenge for folded proteins. Eukaryotes have an array of dedicated translocation machineries to accomplish this feat, for example during mitochondrial import of cytosolic precursor proteins for which it has recently become clear that there is a surprising diversity in targeting signals, import routes, and translocation complexes (1, 2). It is now widely accepted that the mitochondrial genome originated from within the (eu)bacterial domain of life, so it should perhaps not come as a surprise that certain features of mitochondrial import have evolved from these ancestors.

Gram-negative bacteria possess two membranes to protect them from the external world, separated by a layer of peptidoglycan and the periplasmic space. Their outer membrane, with its asymmetrical composition of lipopolysaccharide (LPS)[2] and phospholipids, forms an impressive barrier to most substances with the exception of small hydrophilic nutrients that can diffuse through the resident porins (3). Processes that require an energy input at the outer membrane, such as iron siderophore uptake, therefore often rely on energy generated by ion gradients at the cytoplasmic membrane (4). Energy-transducing systems such as the *ton* and *tol* systems in *Escherichia coli* harvest energy generated at the cytoplasmic membrane and transduce it to the outer membrane. These two systems have a number of features in common, and cross-complementation between the two systems has been observed (5).

The energy transducing capacity of the *ton* system is somewhat better defined and is accomplished by three proteins: the cytoplasmic membrane proteins ExbB and ExbD, which form a heteromultimeric complex that interacts with TonB (4). As a result, TonB undergoes a conformational change in response to the PMF of the cytoplasmic membrane, which allows it to traverse the periplasm and make contact with nutrient-loaded outer membrane receptors, thereby facilitating active import (6). The homology between ExbB/D, TolQ/R, and the PMF-responsive flagellar motor proteins MotA and MotB is well established, and the cumulative evidence now suggests that they act as energy-harvesting complexes (7–9). Evidence of an evolutionary relationship between TolA and TonB comes from work demonstrating structural similarities between the *Pseudomonas aeruginosa* TolAIII globular domain and the carboxyl-terminal domain of *E. coli* TonB despite the very low sequence conservation (10). The activities of TonB and TolA are also critically dependent on a conserved SHLS motif in their transmembrane region, the mutation of which affects the interaction with their respective energy-harvesting complexes (11, 12). The cellular function of the *tol* system in *E. coli* is, however, less clear. It is thought that the Tol proteins play a role in maintaining cell envelope integrity through a network of interactions spanning the cytoplasmic membrane, periplasm, and outer membrane (13).

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[2] The abbreviations used are: LPS, lipopolysaccharide; PMF, proton motive force; NDR, natively disordered region; LB, Luria-Bertani; DCCD, *N,N′*-dicyclohexylcarbodiimide; DTT, dithiothreitol; PBS, phosphate-buffered saline; RFU, relative fluorescence unit(s); CCCP, carbonylcyanide *m*-chlorophenylhydrazone; Mes, 4-morpholineethanesulfonic acid; BisTris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane.

Both energy-transducing systems have been parasitized by the colicins, plasmid-encoded antibacterial proteins produced by *E. coli*, and phages for their translocation into the cell, but the energy requirements for these processes are not unequivocal (14). Group A colicins use the *tol* system and group B colicins the *ton* system in a process whereby interactions of their amino-terminal translocation domains with the Tol or Ton proteins in the periplasm ultimately lead to the entry of their carboxyl-terminal cytotoxic domain into the cell. In common with most colicins, the DNase-type colicin E9 consists of three functional domains: the killing activity is contained in its carboxyl-terminal DNase domain; the central section contains the receptor-binding domain, which binds the vitamin B_{12} receptor, BtuB, in the outer membrane; and the amino-terminal translocation domain is needed for the entry of the cytotoxic domain into the target cell. The first 83 residues of this translocation domain, commonly referred to as the NDR, contain the OmpF and TolB binding sites (17, 18). Upon synthesis colicin E9 forms a high affinity interaction with its cognate immunity protein, Im9, also encoded by the colicin operon. This heterodimeric complex formation protects colicin-producing cells against DNA damage and potential suicide prior to release of the complex in the environment. The nature of the complex formation between colicin E9 and Im9 and other colicin-immunity complexes has been well characterized, and in the case of colicin E9-Im9 the interaction is strong, as reflected by its dissociation constant on the order of 10^{-14} M under physiological conditions (19). Despite the high avidity of this interaction, the DNase domain of colicin E9 appears to have only a marginally stabilizing effect on Im9 (20).

Currently much progress is being made to unravel the early events that take place after receptor binding, where it has been shown that the colicin E9 NDR enters the periplasm through the OmpF lumen where it interacts with TolB, possibly displacing it from its interaction with Pal (18, 21–24). It was also recently demonstrated that the receptor binding and translocation domains remain in contact with their binding partners in the outer membrane and the periplasm, respectively, when the DNase domain gains access to the cytoplasm (25). In contrast, the molecular mechanisms that govern the loss of the immunity protein from the colicin complex and the cell entry of the DNase domain are less well documented. Because of the strength of the interaction between the colicin and its cognate immunity protein, it has been proposed that removal of the immunity protein from the complex would require a cellular energy source. One recent report investigating immunity protein loss from the colicin E2-Im2 complex qualitatively concluded that receptor binding alone does not lead to immunity protein release and that a functional *tol* translocation complex is required to establish immunity protein release (26).

Here we have presented data that for the first time demonstrate a role for the individual Tol proteins and address the issue of energy requirements for immunity protein release. We observed, by using a previously described disulfide-"locked" colicin construct and domain deletion mutants thereof, that entry of the amino-terminal 80 residues of the colicin translocation domain and its interaction with TolB are essential fac-tors for immunity protein release. We have also demonstrated a crucial role for TolA and its transmembrane region in this process, showing that immunity protein release from the colicin complex is an energy-dependent process governed by the cytoplasmic membrane PMF. Finally we have provided a rationale for how an energized Tol system might lead to immunity protein loss and concomitant colicin uptake in sensitive cells.

EXPERIMENTAL PROCEDURES

Plasmids, Bacterial Strains, and Media—*E. coli* DH5α was used as the host strain for cloning and mutagenesis. *E. coli* BL21(DE3) (Novagen) was the host strain for the expression vector pET21a (Novagen) to facilitate the purification of poly-histidine-tagged proteins. *E. coli* DPD1718 has been described earlier (27) and contains a fusion of the *E. coli recA* promoter region to the *Photorhabdus luminescens luxCDABE* reporter genes. *E. coli* strain LMG194 (Invitrogen) containing a *lacZ* deletion (F-Δ*lacZ*74 *gal*E *thi rps*L Δ*pho*A (PvuII) Δ*ara*714 *leu*::Tn10) and transformed with pAG1 was used for the immunity protein release experiments. Plasmid pAG1 is derived from pML261 and contains a 2.4-kb EcoRI-HindIII fragment that encodes the complete *btuB* gene in the vector pUC8 (28). All other strains and constructs are listed in Table 1. All cultures were routinely grown in LB broth, in M9 minimal medium supplemented with 1 mM $MgCl_2$, 0.2% (w/v) glucose, and 0.2% (w/v) casamino acids (for the energy requirements analysis), or on plates of LB agar supplemented where required with ampicillin (100 μg/ml), chloramphenicol (34 μg/ml), or kanamycin (50 μg/ml).

Protein Purification—ColE9-Im9 complexes were purified by metal chelate chromatography with PBS elution buffer containing 0.5 M NaCl and 1 M imidazole, pH 7.4. Where necessary the immunity protein was removed from the colicin complex by diluting the complex 1:2 in 6 M GnHCl, 2 mM DTT followed by preparative Sephacryl S-100 size exclusion chromatography. Fractions containing the free colicin were pooled, dialyzed against 50 mM Mes buffer, pH 6.0, containing 1 mM DTT, and applied to a Mono S 5/50 column (GE Healthcare) equilibrated in the same buffer. Stepwise elution in the same buffer containing 1 M NaCl resulted in an efficient concentration of the free colicin. The free colicin proteins were then refolded by dialysis against PBS containing 1 mM DTT and stored. The MV16 immunity protein (Im9 with C23S and S6C for labeling) was purified in much the same way as for the colicin complex but was concentrated after affinity chromatography via binding to a Mono Q 5/50 column (GE Healthcare) equilibrated in 20 mM BisTris propane, pH 6.5, containing 1 mM DTT followed by stepwise elution in the same buffer containing 1 M NaCl. It was then stored at −20 °C until labeling. The TolAIII constructs YZ48 and YZ74 (Table 1) were purified from *E. coli* BL21(DE3) cells by metal chelate chromatography as described above for the colicin constructs followed by dialysis against 20 mM Tris-HCl, pH 7.6, containing 100 mM NaCl. TolB was purified from *E. coli* BL21(DE3) cells containing pMV37 by metal chelate chromatography as described above followed by Sephacryl S-100 size exclusion chromatography in 20 mM Tris-HCl, pH 8.0, 150 mM NaCl.

Diamide Oxidation and Immunity Protein Labeling—Uncomplexed colicin mutant proteins containing the disulfide lock in their receptor-binding domain were oxidized using *N,N,N′,N′*-tetramethylazodicarbamide as described (29). Oxidized proteins are indicated as ColE9^{S-S}. Immunity protein MV16 was dialyzed against PBS and unfolded in 3 M GnHCl prior to labeling with a 25-fold molar excess of Alexa Fluor 594-C$_5$-maleimide (Invitrogen) as described previously (30). Alexa Fluor 594-labeled MV16 is referred to as Im9^{AF}.

Colicin Activity Assays—The *lux* reporter assay was used to test the activity of wild-type and mutant colicin constructs (31). DNA cleavage assays using linearized pUC18 were performed on free colicin proteins to check for DNase activity essentially as described previously (32). Spot tests with purified ColE9-Im9 protein were performed to check the sensitivity of the *tol* deletion strains before and after transformation with pAG1 essentially as described previously (33).

Immunity Protein Release Assay—Wild-type *E. coli* and *E. coli tol* mutants from overnight cultures were grown to mid-log ($A_{600 \, nm} \sim 0.4–0.6$) in LB broth or M9 minimal medium containing 1 mM MgCl$_2$, 0.2% (w/v) glucose, and casamino acids. Alexa Fluor-labeled colicin complexes were formed by preincubation of oxidized free colE9^{S-S} constructs with Im9^{AF} at a molar ratio of 1:1.5 and were subsequently added to 1 ml of cells in duplicate (10 nM final concentration). Receptor binding was allowed for 5 min at 37 °C after which cells were spun for 2 min at 6,000 rpm and resuspended in prewarmed LB or M9 minimal medium. The cells then underwent an extra wash step before a 100-μl aliquot was taken for analysis of the cell-bound RFU. RFU were measured using a Victor^2 1420 multi-label plate reader (Wallac) controlled by Wallac 1420 software. A reducing agent to resume cell killing (2 mM DTT, final concentration) or PBS (control cells) was added to the remainder of the cells, and they were incubated for a further 30 min at 37 °C. The cells were then spun for 1 min at 10,000 rpm, and the RFU in 100 μl of supernatant of the control and DTT-treated cells were measured in 96-well plates (optical bottom, Nunc) in triplicate. The immunity protein release data presented were obtained by subtracting the value of the RFU released in the supernatant of control cells from that in the DTT-induced cells. In all immunity protein release assays, control cells preincubated with colE9^{S-S}-Im9^{AF} but not treated with DTT were included in order to account for possible nonspecific effects such as cell surface proteolysis or vesicle shedding. Error bars represent the means ± S.E. of at least three independent experiments.

ATP Measurements—Intracellular ATP levels were measured using the ATP bioluminescence assay kit CLSII (Roche Applied Science) according to the manufacturer's recommendations. Briefly, 1 ml of *E. coli* cells was concentrated into 50 μl followed by cell lysis through the addition of 150 μl of dimethyl sulfoxide. Ice-cold milliQ water was added (1 ml), and ATP levels in 100 μl of the mixture were measured using a 10-s integration time on a Lucy 1 luminometer (Anthos Labtech) at 22 °C.

Surface Plasmon Resonance Analysis—The TolB construct MV37 (50 nM, 600 response units) was immobilized onto a CM5 sensor chip, pre-equilibrated in HBS-EP running buffer (BIAcore AB) via amine coupling. Two-minute injections of the TolAIII constructs YZ48 and YZ74 over a concentration range of 5 to 80 μM were performed at a flow rate of 30 μl/min. After each analyte injection, the sensor chip surface was regenerated using a 2-min pulse of 10 mM glycine, pH 1.8. Global analysis using BIAevaluation software 3.1 was used to fit corrected surface plasmon resonance responses to the theoretical 1:1 Langmuir binding model.

RESULTS

Validation of the Alexa Fluor-labeled Immunity Protein and Its Release—The native cysteine residue at position 23 in Im9 was replaced by a serine residue, and we introduced a serine to cysteine mutation at position 6. This location was chosen for labeling with Alexa Fluor 594-C$_5$-maleimide because it is not involved in binding the DNase domain of colicin E9 (34). The biological activity of the resulting Im9^{AF} was ascertained using a pUC18 cleavage assay and the DNase activity of colicin E9. Fig. 1*A* shows the cleavage of $\sim$ 125 ng of linearized pUC18 by free colicin E9 (*lane 2*), which is inhibited to a similar extent by either Im9 (MV16) or Im9^{AF} at a molar ratio of 1:1.5 (*lanes 3* and *4*, respectively). To synchronize cell killing we used a previously described colicin construct (29), colE9^{S-S}, which upon oxidation contains a disulfide bond in its receptor-binding domain (Table 1). This construct binds to sensitive *E. coli* cells but remains inactive until the disulfide bond is reduced, at which point the colicin resumes its biological activity. Complex formation between Im9^{AF} and colE9^{S-S} was confirmed using size exclusion chromatography (Superdex 75 10/300 GL, GE Healthcare) (data not shown). The activity of the ColE9^{S-S}-Im9^{AF} complex *in vivo* was examined using our *lux* reporter assay for DNA damage (Fig. 1*B*) (31). Treatment of the reporter strain *E. coli* DPD1718 with reduced ColE9^{S-S}-Im9 or ColE9^{S-S}-Im9^{AF} generated similar amounts of luminescence, indicating that the fluorescence label on the immunity protein was not impeding the biological activity of the colicin complex. We routinely observed a small amount of background luminescence generated by the disulfide-locked colicin complexes. This is likely to have been caused by trace amounts of reduced protein remaining after the diamide oxidation process.

The amount of Im9^{AF} released from *E. coli* LMG194 (pAG1) cells after a 30-min incubation period in the presence and absence (control) of DTT is shown in Fig. 1*C*. The low levels of background activity of the oxidized construct (Fig. 1*B*) gave rise to a small amount of RFU released by the control cells. In the presence of DTT, the released Im9^{AF} increased $\sim$3-fold. The RFU resulting from Im9^{AF} released by the control cells were subtracted in all further experiments. The use of pAG1-containing *E. coli* LMG194 cells, in which BtuB comprises about 20–40% of the outer membrane proteins, enabled us to increase the detection sensitivity of our assay. We did however repeat all of the experiments using cells with native levels of BtuB in order to ascertain that the transformation with pAG1 had not unduly compromised the cell physiology.

To ensure that the increase in fluorescence in the cell supernatants was due to the presence of Im9^{AF} and not caused by the loss of the fluorescently labeled colicin complex from the cell membrane, we routinely analyzed the cell supernatants (derived from assays with active colicin constructs) for activity in
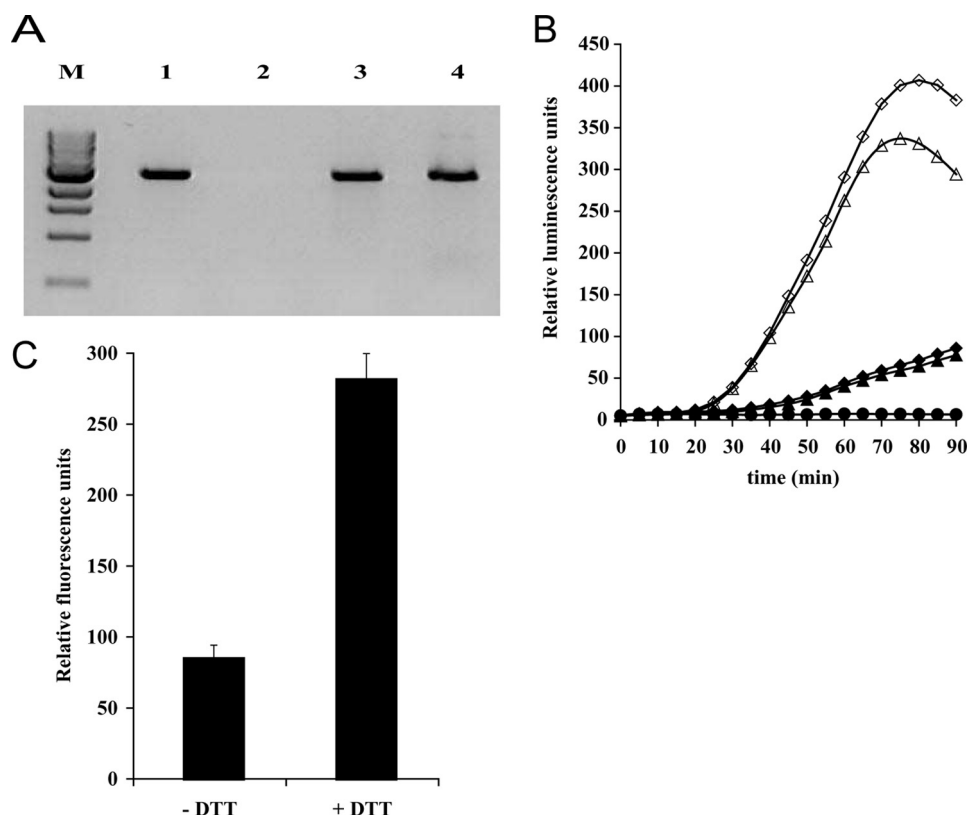
FIGURE 1. **Validation and characterization of the immunity protein release assay.** *A*, 125 ng of NdeI-linearized pUC18 (*lane 1*) and its cleavage by the DNase activity of free colicin E9 (700 ng, *lane 2*), free colicin E9 preincubated with a 1:1.5 molar excess of Im9 (MV16) (*lane 3*), or Im9^{AF} (*lane 4*). *Lane M*, DNA ladder. *B*, DNA damage-induced luminescence (in relative luminescence units) in *E. coli* DPD1718 cells by incubation with 4 nM oxidized ColE9^{S-S}-Im9 (▲), 4 nM oxidized ColE9^{S-S}-Im9 in the presence of 1 mM DTT (△), 4 nM oxidized ColE9^{S-S}-Im9^{AF} (◆), 4 nM oxidized ColE9^{S-S}-Im9^{AF} in the presence of 1 mM DTT (◇), and LB medium (●). *C*, immunity protein released (Im9^{AF}, expressed as relative fluorescence units) from *E. coli* LMG194 (pAG1) cells preincubated with 10 nM ColE9^{S-S}-Im9^{AF} followed by a 30-min incubation period in the absence and presence of 2 mM DTT at 37 °C.

TABLE 1

E. coli **strains and plasmids used**

Strain or plasmid	Genetic description	Source
Strains		
DPD1718	*lac::recA-luxCDABE*	Ref. 27
LMG194	F-Δ*lac*X74 *gal*E *thi rps*L Δ*pho*A (PvuII) Δ*ara*714 *leu*::Tn10	Invitrogen
JC7782	1292 *tolA* (stop after codon 40)	Ref. 70
K-12Δ*tolB*	BW25113 *tolB*::K$_m$	Ref. 22
TPS13^a	GM1 *tolQ13* (stop after codon 36)	Ref. 70
TPS300	GM1 *tolR*::Cm	Ref. 70
JC9776	8056 Δ(*orf1 tolQ tolR tolA*)::Cm	Ref. 11
Plasmids		
ptolA	pAR3, N-His$_6$-tolA	Ref. 70
ptolA$_{H22A}$	pAR3, N-His$_6$-tolA H22A	This work
ptolA$_{Y352H,V407E}$	pAR3, N-His$_6$-tolA Y352H,V407E	This work
pYZ48	pET21a, N-His$_6$-tolAIII	This work
pYZ74	pET21a, N-His$_6$-tolAIII Y352H,V407E	This work
pMV37	pET21a, TolB Δ1–22	This work
pBH29 (colE9^{S-S}-Im9)	pET21a, ColE9 Y324C, L447C/Im9	Ref. 29

^{a} The TolQ mutation has a polar effect on the expression of TolR (69).

the *lux* reporter assay (under reducing conditions), which we have previously shown to be sensitive down to picomolar concentrations of colicin (31).

The NDR of the Colicin Translocation Domain Is Important for Immunity Protein Release—We subsequently used our assay to study regions of the colicin molecule that could be involved in immunity protein release. Amino-terminally deleted colicin

constructs (disulfide-locked and in complex with Im9^{AF}) were added to sensitive *E. coli* cells transformed with pAG1, and the amount of immunity protein released was measured 30 min after the addition of 2 mM DTT. Fig. 2 shows the background-corrected results for the wild-type and amino-terminally deleted constructs. All colicin constructs used showed similar levels of cell bound fluorescence prior to DTT treatment (data not shown). The immunity protein release data correlate well with the *in vivo* biological activity of the constructs (18). Colicin constructs that are biologically inactive (Δ1–38, Δ1–60, and Δ60–80) showed very little immunity protein release, whereas constructs that showed partial activity (Δ1–7 and Δ1–30) had intermediate levels of immunity protein release. These results point to the colicin translocation domain and more precisely the NDR (residues 1–83) as an important region within the colicin molecule for immunity protein release from the complex. This part of the translocation domain contains interaction sites for two proteins, OmpF and TolB (18, 22, 35, 36). Although essential for colicin uptake, we and others have shown that OmpF recruitment in isolation is not sufficient to cause immunity protein release (18, 26, 30). It was proposed that the NDR of colicin E9 competitively recruits TolB from its interaction with Pal in the presence of Ca^{2+} ions (21). Our data suggested that the interaction of the colicin translocation domain with TolB was important for immunity protein release. We therefore analyzed two colicin mutants with single alanine mutations in their TolB-interacting region that result in the loss of the TolB interaction and biological activity (37, 38). Although severely affected in their biological activity, both W39A and W46A ColE9^{S-S}-Im9^{AF} complexes showed only a 60% reduction in immunity protein release following binding to LMG194 (pAG1) cells. We reasoned that this may have been caused by the increased levels of BtuB in the outer membrane due to the use of pAG1. We therefore analyzed the biological activity of the two colicin mutants using our *lux* reporter strain transformed with pAG1 to directly compare biological activity with immunity protein release in *btuB*-up-regulated cells. Under these conditions, the two colicin mutants exhibited ~20% of the wild-type activity (data not shown), which may explain the lack of complete inhibition of the immunity protein release. For inactive constructs we cannot rule out that some of the background-corrected RFU detected could have been derived from released fluorescently labeled colicin
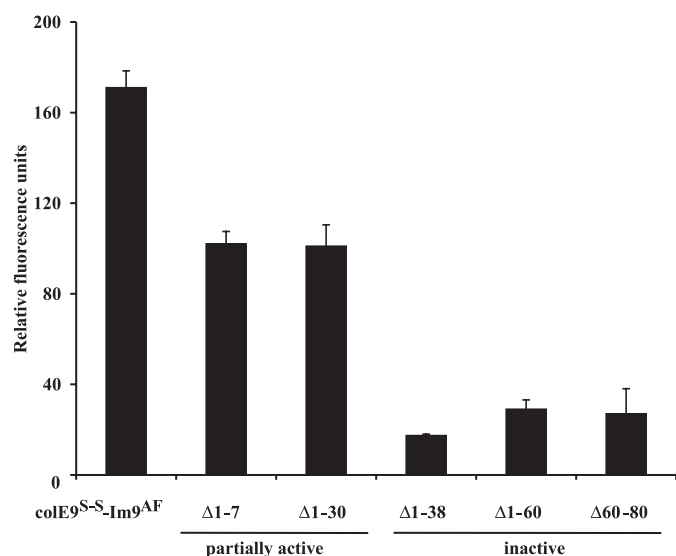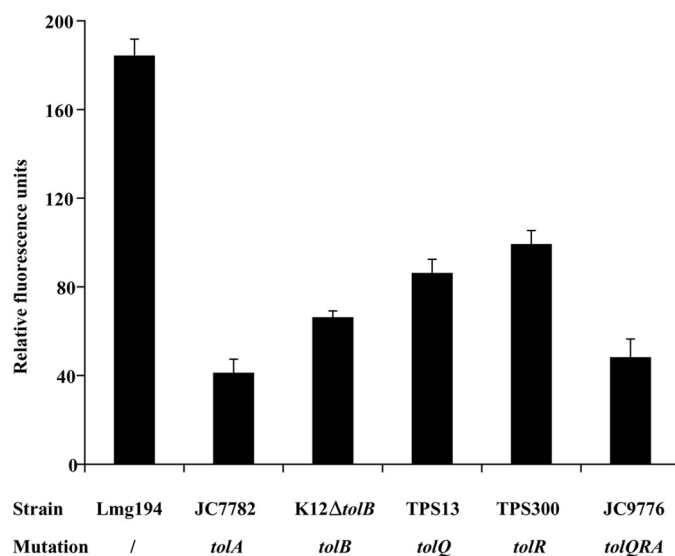
FIGURE 2. **Immunity protein release from amino-terminally deleted colicinE9 constructs.** The background-subtracted release (relative fluorescence units) as described under "Experimental Procedures" is shown for *E. coli* LMG194 (pAG1) cells preincubated with each ColE9^{S-S}-Im9^{AF} construct (10 nM) in the presence and absence of 2 mM DTT after a 30-min incubation period at 37 °C. The amino-terminally deleted colicin E9 constructs have been described by Housden *et al.* (18).

FIGURE 3. **Immunity protein release from wild-type and *tol* mutant *E. coli* strains.** Background-subtracted release (relative fluorescence units) as described under "Experimental Procedures" is shown for *E. coli* LMG194 (pAG1) and *tol* mutant cells (see Table 1) transformed with pAG1 and preincubated with 10 nM ColE9^{S-S}-Im9^{AF} construct after a 30-min incubation period at 37 °C in the presence and absence of 2 mM DTT.

complex. We consider this unlikely, however, in view of the normal levels of cell-bound fluorescence obtained with all constructs prior to reduction of the disulfide lock (data not shown) and the fact that a similar amount released by the control cells would have been subtracted.

Overall the data show that cell entry of the translocation domain of a nuclease colicin via OmpF and its subsequent interaction with TolB are essential steps for immunity protein release during colicin uptake. It is conceivable that the cellular entry of the NDR leads to a conformational rearrangement of the cell-bound colicin, which ultimately promotes dissociation of the immunity protein and subsequent DNase uptake across the outer membrane via an as yet unknown mechanism.

Analysis of tol Mutant Strains Suggests That in Addition to TolB, TolA Plays a Key Role in Immunity Protein Release—Nuclease colicins have a requirement for members of the *tol* operon to cross the periplasm and reach their target in the cytoplasm (14). We therefore set out to analyze immunity protein release upon ColE9^{S-S}-Im9^{AF} binding to a series of *tol* deletion strains (Table 1). The results show that the *tolA* and the *tolQRA* deletion strains are the most severely compromised in their immunity protein release (Fig. 3) in line with their tolerant phenotype (data not shown). Perhaps surprisingly, the immunity protein release was not completely abolished in these two deletion strains. When the experiment was repeated in M9 minimal medium as opposed to LB medium the release was further reduced but still not abolished. At this point the explanation for this residual release is unclear, but it may reflect the heterogeneous nature of the BtuB receptors in the outer membrane (71). Although it seems difficult to reconcile the tolerant phenotype with the residual immunity protein release, there may be other processes subsequent to immunity protein release that also rely on a functional *tolQRA* complex because immunity protein release is a relatively early event in the colicin

uptake followed by the nuclease domain having to cross two membranes (and the periplasm) before cell death occurs.

The single *tolQ* and *tolR* deletion strains were only intermediately affected, in line with the hazy zones of cell killing observed at 100 nM and 1 μM of colicin in the spot tests (data not shown). The *tolB* deletion strain released amounts of immunity protein higher than anticipated in view of its tolerant phenotype in the spot tests (data not shown). This could indicate that the higher amount of BtuB, due to the use of pAG1 in combination with the absence of TolB, may have destabilized the outer membrane. We therefore analyzed the wild-type and *tol* mutant strains without pAG1. The results (data not shown) showed a trend similar to those described above, but the immunity protein release from the *tolB* deletion strain was much more reduced. Transformation of the *tolB* deletion strain with wild-type *tolB* in the low copy number vector pACYC184 restored immunity protein release to ~70% of the wild-type level (data not shown).

Overall, the results extend the findings of Duché *et al.* (26), who showed qualitatively that a functional translocation complex is necessary for immunity protein release. Our quantitative analysis has now enabled us to investigate the relative importance of the individual Tol proteins and suggests that in addition to TolB, TolA also plays a significant role in the immunity protein release. Interestingly, no direct interaction between a nuclease colicin and TolA has yet been demonstrated, which suggests that the role of TolA in the cellular uptake of colicin E9 may be indirect.

Complementation Analysis Points to the Importance of the Transmembrane Region of TolA in Immunity Protein Release—TolA is a 44-kDa protein necessary for the import of all group A colicins (39). It is anchored in the cytoplasmic membrane via a single transmembrane region important for maintaining interactions with TolQ and TolR. Its periplasmic domain consists of an elongated central region rich in α-helical structure (TolA II)
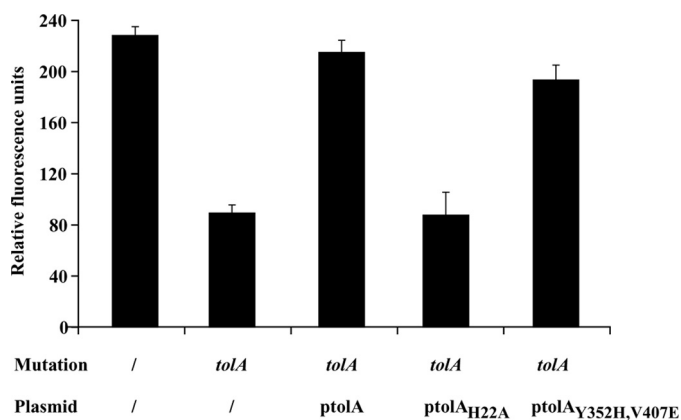
FIGURE 4. **Immunity protein release from the *tolA* mutant *E. coli* strain (JC7782) and its complementation by wild-type and mutant *tolA*.** Background-subtracted release (relative fluorescence units) as described under "Experimental Procedures" is shown for *E. coli* JC7782 (pAG1) and *E. coli* JC7782 (pAG1) complemented by plasmids encoding wild-type *tolA* (ptolA), *tolA* H22A (ptolA$_{H22A}$), and *tolA* Y352H,V407E (ptolA$_{Y352H,V407E}$) preincubated with 10 nM ColE9^{S-S}-Im9^{AF} construct after a 30-min incubation period at 37 °C in the presence and absence of 2 mM DTT.

and a globular carboxyl-terminal domain (TolA III) (40). Deletion of *tolA* leads to a *tol* phenotype that is characterized by: outer membrane blebbing, release of the periplasmic content, increased sensitivity to cholic acid and SDS, and defective O-antigen polymerization (39, 41). Because of this pleiotropic phenotype, we decided to investigate the specificity of the observed inhibition of the *tolA* deletion strain on immunity protein release via complementation with wild-type *tolA*. Fig. 4 shows that immunity protein release is restored to near wild-type levels in JC7782 (pAG1) transformed with a plasmid encoding the wild-type *tolA* gene (ptolA, Table 1). Full sensitivity to colicin E9 was also re-established (data not shown).

TolA undergoes a conformational change in response to the PMF, which is regulated by interactions of its transmembrane region with those of TolQ and TolR (8, 42). TolA also interacts with Pal in an energy-dependent manner, an interaction that is important for outer membrane integrity (43). These properties of TolA have been shown to be critically dependent on a conserved SHLS motif within its transmembrane region (11). One residue in particular, His-22, has been singled out as being essential for the energy-dependent conformational change of TolA in accordance with TonB (44). We created an alanine mutation of this residue in full-length TolA and analyzed the ability of a plasmid (ptolA$_{H22A}$, Table 1) expressing this mutant TolA protein to complement the *tolA* phenotype. The data in Fig. 4 show that this construct is unable to restore immunity protein release to the wild-type level, and the mutant TolA H22A also did not restore colicin sensitivity (data not shown). It is interesting that a single point mutation in the transmembrane region of TolA has such a dramatic effect. This indicates that an energy-dependent conformational change of TolA is essential for nuclease colicin uptake and concomitant immunity protein release.

TolA has also been shown to interact with the amino terminus of TolB in the periplasm via its globular carboxyl-terminal domain. Using a yeast two-hybrid screen, Walburger *et al.* (45) identified a TolA mutant containing two point mutations in its

TolAIII domain (Y352H and V407E), which results in the loss of this interaction. This double mutant was also reported to have a decreased sensitivity to colicin E3 and a loss of outer membrane stability. The residue Tyr-352 was also identified as an important residue for the TolA interaction with TolB via a genetic screen (46). To investigate the functional importance of the TolA-TolB interaction in immunity protein release, we decided to analyze a plasmid (ptolA$_{Y352H,V407E}$, Table 1) expressing the double mutant TolA Y352H,V407E in our immunity protein release assay for complementation of the *tolA* phenotype. Surprisingly, this mutant rendered *E. coli* JC7782 cells sensitive to colicin E9 to a similar degree as wild-type TolA (data not shown) and restored immunity protein release to ~85% of the wild-type level (Fig. 4). We therefore decided to verify the absence of an interaction between TolA Y352H,V407E and TolB *in vitro* by purifying the soluble TolAIII mutant protein (pYZ74, Table 1) and comparing its interaction with TolB (pMV37, Table 1) to that of the wild-type TolAIII (pYZ48, Table 1) by using surface plasmon resonance. The K_d values obtained for the interaction of both constructs with TolB (4.1 μM for wild-type TolAIII and 23.6 μM for the mutant TolAIII) showed that although the interaction of the mutant TolAIII with TolB was weaker compared with the wild-type TolAIII, it was not abolished, which could explain our *in vivo* complementation results. This suggests that we cannot disregard the TolA-TolB interaction as being important in the immunity protein release process. Moreover, we have recently identified interactions between TolA and TolB that are critical for immunity protein release and colicin translocation, but this finding will be reported elsewhere.[3]

The results suggest that the energy-dependent conformational change of TolA is an important determinant for immunity protein release and point to a role for TolA in transducing cellular energy in a manner similar to that described for TonB (4). It is possible that the interaction of a colicin-bound TolB with TolA allows for energy "transfer" to the colicin, or it could enable direct access of the colicin translocation domain to the energized cytoplasmic membrane.

The PMF Provides the Main Driving Force for Immunity Protein Release—Because of the striking effect of the *tolA* deletion strain on the immunity protein release and the lack of complementation by the TolA H22A mutant, we decided to investigate the energy requirements for immunity protein release. The interaction between nuclease colicins and their cognate immunity protein is strong; the K_d is on the order of 10^{-14} M, which also raises the question as to what is the energy source for immunity protein release upon colicin cell entry. To ascertain whether the PMF is involved, sensitive *E. coli* cells were grown to mid-log phase, at which point ionophores were added and remained present during colicin binding (10 min) and during the DTT-induced immunity protein release. The effect of the chemicals on the cellular ATP content was also analyzed. None of the treatments altered colicin binding as judged by measuring cell-bound fluorescence (data not shown). The protonophore CCCP was added at three concentrations (10, 20, and 50

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[3] M. Vankemmelbeke, D. A. Bonsor, and C. Kleanthous, unpublished observations.

TABLE 2

Percent inhibition of immunity protein release by ionophores and ATPase inhibitors and their effect on intracellular ATP levels

The percentage of inhibition was calculated by comparing the background-corrected immunity protein release from *E. coli* LMG194 (pAG1) cells in the presence and absence of the compounds. Cells were pretreated for 10 min (a minimum of 30 min in the case of arsenate) before analyzing the immunity protein release. DCCD treatment was performed on cells transferred into 100 mM Tris-HCl, pH 7.6, 1 mM EDTA. ATP levels were determined using a bioluminescence assay kit (CLSII) and are presented as a percentage of the ATP levels of untreated cells. Values are the mean ± S.E. of at least three independent experiments.

Treatment (concentration)	% Inhibition	% ATP
CCCP (10 μM)	41 ± 5	47 ± 2
CCCP (20 μM)	69 ± 10	50 ± 4
CCCP (50 μM)	89 ± 9	47 ± 4
Nigericin (2 μM)	10 ± 3	96 ± 7
Valinomycin (5 μM)	51 ± 0.3	95 ± 11
Nigericin (2 μM) + valinomycin (5 μM)	75 ± 1	89 ± 1
Arsenate (50 mM)	39 ± 8	34 ± 2
DCCD (100 μM)	64 ± 4	20 ± 6

μM), and the analysis of the amounts of Im9^{AF} released compared with untreated control cells showed a dose-dependent increase in inhibition of immunity protein release over this concentration range (Table 2). This finding suggests that the PMF contributes to immunity protein release. These concentrations of CCCP reduced the cellular ATP levels by about 50% (Table 2). Nigericin (at 2 μM) has been shown to selectively collapse the proton gradient (ΔpH) component of the PMF (47). When the cells were permeabilized by 1 mM EDTA, nigericin reduced the immunity protein release by only 10% and had a negligible effect on the intracellular ATP levels (Table 2). Valinomycin (at 5 μM), which in the presence of 1 mM EDTA and 100 mM KCl decreases the transmembrane electrical potential (Δψ) (47), caused a 50% reduction in immunity protein release (Table 2). No significant effect on the cellular ATP content was observed with this treatment (Table 2). The combination of valinomycin and nigericin will also collapse the PMF, and Table 2 shows that when combined (at the concentrations used for the individual treatments) these two ionophores resulted in a 75% inhibition of the immunity protein release with only a marginal effect on ATP levels. Taken together these data argue for an involvement of the cytoplasmic membrane PMF in immunity protein release, suggesting that the transmembrane electrical potential is the important component.

We also investigated whether energy generated by ATP hydrolysis was involved. Mid-log *E. coli* cells were transferred into minimal medium in the absence of glucose and casamino acids and grown for a further 1 h, at which point their ATP levels had dropped to 30% of the original value. We analyzed the immunity protein release under these conditions and observed no inhibition (data not shown). Preincubation of cells with 50 mM arsenate for a minimum of 30 min was required in order to reduce the cellular ATP content to 30% of the original level; this led to a 40% inhibition in immunity protein release (Table 2). We believe that the inhibition observed by the addition of arsenate could have been an indirect effect due to the length of preincubation and the high concentration used, in view of the lack of inhibition, despite a similar ATP level under starvation conditions. The F_0F_1-type proton-translocating ATPase inhibitor DCCD at 100 μM reduced the cellular ATP content by ~80%, which led to a roughly 60% inhibition of the immunity protein release (Table 2). Although this seems to suggest the

involvement of ATP, we cannot rule out the possibility that the inhibition could have been the consequence of the general reactivity of DCCD with glutamic acid residues and not the direct result of the reduction in cellular ATP content. Sodium orthovanadate, an inhibitor of P-type Na$^+$-translocating and other ATPases, did not inhibit the immunity protein release at 100 μM (data not shown). We consider the reduction in ATP levels in the absence of any chemical treatment (under conditions of starvation), and therefore devoid of secondary effects, to be the most informative. We therefore propose that the direct participation of ATP hydrolysis is not involved in the immunity protein release.

It has been suggested previously for colicins E2, E3, and K that colicin action can be divided into two stages, a first stage during which cells can be rescued by trypsin treatment and a second irreversible stage during which cell damage occurs. The transition from stage I to stage II could be inhibited by compounds that disrupt the energized state of the cytoplasmic membrane (48). Our results suggest that in the case of nuclease colicins this energy-dependent transition coincides with the immunity protein release and concomitant translocation of the nuclease domain.

DISCUSSION

We have provided evidence for the first time of the energy dependence of immunity protein release from a nuclease colicin complex upon cell entry and assigned an essential role to TolA and TolB in this process. Our investigation of the energy requirements suggests an involvement of the cytoplasmic membrane PMF and indicates that the process may be driven predominantly by the transmembrane electrical potential. We propose that the observed inhibition of immunity protein release by collapsing the PMF is caused by an effect on the *tol* system, as ionophores have been shown to inhibit the energy-dependent TolA-Pal interaction and also to affect the TolQ-TolR channel activity (8, 43, 49). Although there is experimental evidence for the energy dependence of *ton*-dependent colicin and phage uptake, the reports on the energy dependence of *tol*-dependent colicin uptake have not been consistent (14). From these data and our results it seems likely that different colicins have different energy requirements; it will therefore be interesting to determine whether this coincides with a different use of the *tol* system to gain entry into the cell or whether additional processes are involved.

The quantitative analysis of the *tol* mutant strains has allowed us to confirm the importance of the *tol* translocation machinery and to assign essential roles to both TolA and TolB in the immunity protein release process. We also present data showing that TolA with a single point mutation (H22A) in the conserved transmembrane helical SHLS motif loses its functional role in colicin uptake and immunity protein release. This strengthens the hypothesis that an energy-dependent conformational change of TolA is required for its role in nuclease colicin uptake. More recently, mutational analysis of the transmembrane helices of TolQ and TolR and the TolR periplasmic domain have demonstrated that the TolQ-TolR complex forms a putative ion channel, the func-

tion of which may be regulated by the TolR periplasmic domain (9, 49). Taken together these data suggest that, by analogy with the *ton* system, the *tol* cytoplasmic membrane system is a dynamic complex responsible for harvesting energy from the cytoplasmic membrane and transducing it to the outer membrane via TolA. Through the analysis of domain deletions and point mutations within the NDR of the colicin translocation domain, we have shown that although the interaction of this region with TolB in the periplasm is a clear requirement for immunity protein release, it is not sufficient in the absence of an "energized" TolA. We hypothesize that energy transferred to a TolB-bound colicin, possibly through a conformational change via the TolA-TolB interaction, results in immunity protein release and subsequent translocation of the DNase domain across the outer membrane. This would explain the need for an energized TolA in the absence of any evidence for a direct interaction of a nuclease colicin with TolA. Free colicin E9 remained inactive on the *tolA* mutant strain (data not shown), indicating that the involvement of TolA in nuclease colicin uptake is not limited to providing energy for the immunity protein removal but also involves assisting the concurrent entry of the DNase domain across the outer membrane. The energy-dependent interaction of TolA with Pal may also hold the key, although this is somewhat harder to conceive given that a *pal* mutant shows very little tolerance to nuclease colicins (52). The interaction of colicin with TolB may thus serve predominantly to hold the colicin molecule sufficiently long and in the correct orientation at the cell surface for the translocation domain to access the energy source necessary for immunity protein release and DNase translocation. Alternatively it may constitute a signaling event to allow uptake of the DNase domain. It is currently not entirely clear how this latter event occurs, but at least one study suggests that a destabilized nuclease domain devoid of immunity protein could use OmpF to cross the outer membrane (50). More recently the *tol-* dependent pore-former colicin N was shown to insert in the outer membrane at the periphery of OmpF, thereby displacing LPS from its interaction with OmpF (51). This could indicate that its translocation might happen at the protein/lipid interface, but it remains to be established whether this is a general feature of all colicins.

Alternatively, it is possible that in the context of colicin biology, an "energized *tol* system" serves to provide "competent" colicin uptake sites in accordance with their localization at sites of membrane adhesion, their involvement in maintaining the outer membrane integrity, and LPS biogenesis. It has been shown that treatment of cells with colicin A results in the increased presence of the Tol proteins at contact sites between the inner and outer membrane (53). These adhesion sites between the inner and outer membrane of *E. coli* cells (originally named Bayer's junctions) have been shown to play an important role in the macromolecular export of capsular polysaccharides and LPS biosynthesis and are also known to be porin insertion and phage entry sites (54–56). They have also been implicated in the cell division process via the formation of periseptal annuli (57). Interestingly, the Tol proteins have recently been shown to be recruited to the cell division site at a late stage in the division process, and division defects of a *tolA* mutant fit with this observation (58, 59). More recently it was demonstrated that phages preferentially bind to positions at mid-cell and at the cell poles, locations that are also favored by FtsH (60). It is therefore tempting to speculate that colicins have a similar preference for these "specialized" sites and that recruitment of the Tol proteins to these sites enables colicin uptake. It is currently not clear whether these regions comprise membrane and/or protein bridges and whether translocation across both membranes occurs simultaneously or via a soluble intermediate. If a colicin nuclease domain traverses the periplasm as a soluble intermediate, then it would be hard to imagine that this could be accomplished without the aid of a periplasmic chaperone.

There are some striking similarities between certain aspects of eukaryotic mitochondrial import of preproteins from the cytosol and colicin import into bacterial cells. The two processes involve unidirectional transport across two membranes in order to reach the site of action, which in both cases occurs at sites of membrane adhesion (53, 61). The mitochondrial outer and cytoplasmic membranes also possess dedicated translocation machineries that work in a cooperative manner to ensure efficient translocation into the mitochondrial matrix (62). Unfolding has been shown to enable more efficient translocation into the mitochondria and similarly to speed up colicin import into sensitive cells (63, 64). For mitochondrial import, the different energy sources required are dictated by the type of translocase used and the final destination (mitochondrial cytoplasmic membrane or matrix), which could also explain the different energy requirements among the colicins (2, 14). The majority of mitochondrial proteins are targeted to the mitochondrial matrix via their amino-terminal positively charged presequences, the initial translocation of which is critically dependent on the membrane potential (2, 65). Moreover the difference in net positive charge of these presequences was found to form the basis of the differential sensitivity to CCCP, suggesting an electrophoretic effect of the membrane potential on the positively charged presequences (65). Interestingly, the difference in net positive charge of the DNase domains of colicins E2, E7, E8, and E9 has recently been shown to affect their rate of uptake, so it is possible that the different energy requirements for colicin uptake are also dictated by the net positive charge of their effector domains (33). A final step in mitochondrial import is the proteolytic cleavage of the amino-terminal targeting sequence in the mitochondrial matrix. Proteolytic processing of imported nuclease colicins has thus far been shown for colicin D (*ton*-dependent) and colicin E7 (*tol*-dependent) and also inferred for colicin E2 (23, 66–68). The cleavages were critically dependent on conserved basic residues, the mutations of which resulted in the loss of import of the nuclease domains into the cytoplasm. The nature of the enzymes involved, however, was different, a metalloprotease in the case of colicin E7 and a serine protease for colicin D, which may be a reflection of their different translocation pathways. Recently, a cytoplasmic membrane AAA$^+$ ATPase and metalloprotease, FtsH,

has been implicated in the charge-dependent nuclease domain translocation into the cytoplasm (33).

We have shown here for the first time a direct energy requirement for immunity protein release from a nuclease colicin complex upon cell entry. We propose that this energy requirement may not be a general feature for all colicins but could be related to the nature of the colicin, its requirements for crossing the outer membrane, and its final destination in the cell.

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