Periplasmic chaperone FkpA is essential for imported colicin M toxicity

Summary

Chaperones facilitate correct folding of newly synthesized proteins. We show here that the periplasmic FkpA chaperone is required for killing *Escherichia coli* by colicin M entering cells from the outside. Highly active colicin M preparations were inactive against *fkpA* mutant cells; 10-fold dilutions killed *fkpA*+ cells. Three previously isolated spontaneous mutants tolerant to colicin M carried a stop codon or an IS1 insertion in the peptidyl-prolyl-cis-trans-isomerase (PPIase) domain (C-domain) of FkpA, which resulted in deletion of the domain. A randomly generated mutant carried a G148D mutation in the C-domain. A temperature-sensitive mutant tolerant to colicin M carried a Y25N mutation in the FkpA N-domain. Mutants transformed with wild-type *fkpA* were colicin M-sensitive. Isolated FkpA-His reduced colicin M-His cleavage by proteinase K and renatured denatured colicin M-His in vitro; renaturation was prevented by the PPIase inhibitor FK506. In both assays, periplasmic SurA-His had no effect. No other tested periplasmic chaperone could activate colicin M. Among the tested colicins, only colicin M required FkpA for activity. Colicin M bound to cells via FhuA was inactivated by trypsin; unbound colicin M retained activity. We propose that colicin M unfolds during import across the outer membrane, FkpA specifically assists in folding colicin M into an active toxin in the periplasm and PPIase is essential for colicin M activity. Colicin M is a suitable tool for the isolation of FkpA mutants used to elucidate the functions of the FkpA N- and C-domains.

Introduction

Colicins are toxic proteins produced by *Escherichia coli* that kill sensitive *E. coli* cells (Cascales *et al.*, 2007). Colicin M is synthesized by *E. coli* cells carrying a ColBM plasmid. This colicin lyses sensitive cells by interfering with murein (peptidoglycan) biosynthesis (Braun *et al.*, 1974; Schaller *et al.*, 1982). In the absence of colicin M, the lipid-linked precursor of murein biosynthesis, undecaprenyl- *N*-acetyl-muramyl pentapeptide- *N*-acetyl-glucosamine (lipid II), is transferred across the cytoplasmic membrane and incorporated into murein. Undecaprenyl pyrophosphate is released and converted to the monophosphate, which re-enters the reaction cycle. Colicin M inhibits this undecaprenylphosphate (lipid) carrier regeneration step of murein synthesis (Harkness and Braun, 1989). This finding was recently confirmed and the exact target of colicin M was identified; colicin M is a phosphatase that cleaves the phosphate bond between the lipid moiety and the pyrophosphoryl group of lipid II (El Ghachi *et al.*, 2006), forming 1-pyrophospho-*N*-acetyl-muramyl pentapeptide-*N*-acetyl-glucosamine and undecaprenol instead of undecaprenyl pyrophosphate; undecaprenol does not enter the murein biosynthesis cycle.

Colicin M, like other colicins, is only bactericidal when provided from outside cells (Harkness and Braun, 1990) because it has access to the target only while entering the cells. Cells synthesizing colicin M are protected by an immunity protein that inactivates colicin M in the periplasm before it reaches its target in the cytoplasmic membrane (Ölschläger *et al.*, 1991; Groß and Braun, 1996).

Uptake of colicin M into sensitive cells requires energy provided by the proton motive force across the cytoplasmic membrane (Braun *et al.*, 2002). Uptake requires the FhuA outer membrane receptor and the TonB, ExbB and ExbD proteins, which form an energy-coupling device between the cytoplasmic membrane and the outer membrane (Braun, 1995; Postle and Kadner, 2003). Mutations in any of these genes render cells resistant to colicin M. We previously isolated mutants of *E. coli* tolerant to colicin M (*tolM*). These mutants are insensitive to colicin M, but
the mutations map close to the streptomycin-resistance gene \( rpsL \) and not among the genes required for uptake (Braun et al., 1980). We have also isolated a temperature-sensitive \( E. \ coli \) mutant tolerant to colicin M at 42°C but sensitive at 30°C. This mutation also maps close to \( rpsL \) (Schaller et al., 1981), but we were unable to identify the mutated gene(s).

In a current study aimed at defining the role of periplasmic chaperones in the assembly of the FhuA outer membrane protein, we discovered that mutants in the \( fkpA \) gene, which encodes a periplasmic chaperone, are specifically resistant to high colicin M concentrations. No other tested chaperone mutant conferred colicin M resistance. As \( fkpA \) maps close to \( rpsL \), \( tolM \) might be identical to \( fkpA \).

Periplasmic chaperones assist in the assembly of outer membrane proteins (Mogensen and Otzen, 2005; Betton, 2007). They also prevent aggregation of misfolded periplasmic protein derivatives (Arié et al., 2001; Ramm and Plückthun, 2001; Hu et al., 2006). FkpA consists of a mainly \( \alpha \)-helical N-domain with a predicted chaperone function and an anti-parallel \( \beta \)-pleated sheet C-domain with peptidyl-prolyl-cis-trans-isomerase (PPIase) activity (Fig. 1). FkpA is inhibited by the immunosuppressant FK506 (Saul et al., 2004). FkpA and other chaperones are synthesized in response to extracytoplasmic stress (Sklar et al., 2007). FkpA and other periplasmic chaperones have been to date only implicated in assisting folding and refolding, and preventing misfolding of newly synthesized exported proteins and have not been related to the activation of an imported protein. Here we show that \( tolM \) is identical to \( fkpA \) and that FkpA is essential for the activity of imported colicin M.

**Results**

**fkpA mutants are insensitive to colicin M**

FhuA is a multifunctional protein that serves as receptor for colicin M and the phages T1, T5 and \( \varphi 80 \) and as transporter for ferrichrome and the structurally related antibiotic albomycin (Braun et al., 2004). We have studied the role of periplasmic chaperones in the incorporation of complete FhuA and separately synthesized FhuA cork and FhuA barrel into the outer membrane of \( E. \ coli \) (Braun et al., 2003). Incorporation of active FhuA protein into the outer membrane of \( E. \ coli \) is determined by measuring the receptor and transport activities.

One mutant with a deleted periplasmic chaperone gene, \( E. \ coli \) JW3309 \( \Delta fkpA \), was insensitive to an undiluted colicin M sample; when this sample was diluted up to \( 10^4 \), it inhibited growth of the BW25113 parent strain (Table 1). Complementation of strain JW3309 with wild-type \( fkpA \) cloned in plasmid pYH17 fully restored sensitivity to colicin M (Table 2). Strains mutated in the periplasmic chaperones Skp, SurA, PpiD or PpiA were as

| Strain          | Albomycin | Colicin M | T5 | \( \varphi 80 \) |
|-----------------|-----------|-----------|----|---------------|
| BW25113 \( fkpA \) wild-type | 5         | 4         | 5  | 5             |
| JW3309 \( fkpA \) | 5         | i         | 5  | 5             |
| AB2847 \( fkpA \) wild-type | 5         | 4         | 5  | 5             |
| Mo3 \( fkpA40 \) | 5         | i         | 5  | 4             |
| Mo4 \( fkpA41 \) | 5         | 4         | 5  | 5             |
| Mo6 \( fkpA42 \) | 5         | i         | 5  | 5             |
| K458 \( fkpA43ts^a \) | 5         | 4         | 4  | 5             |
| AB2847\( ^b \) | 5         | 4         | 4  | 2             |
| K458 \( fkpA43ts^b \) | 5         | (3)       | 4  | 2             |
| H8589 \( ompT fkpA40 \) | 5         | i         | 4  | 5             |

a. Cells were grown at 30°C.

b. Cells were grown at 42°C.

The \( E. \ coli \) BW25113 and AB2847 are \( fkpA \) strains. The stock solutions of the ligands were diluted as described in the Experimental procedures, spotted onto nutrient agar plates seeded with the strains listed, and incubated; the zones of growth inhibition were determined. The numbers indicate the highest dilution that caused a clear zone of growth inhibition. i, insensitive; number in parentheses, very turbid inhibition zone.
sensitive to colicin M as the wild-type strain (Table S1; the 

Table 2. Complementation of fkpA mutants.

| Strain          | Temperature (°C) | Colicin M |
|-----------------|------------------|-----------|
| JW3309 FkpA    | 42               | 5         |
| JW3309 FkpA43-His | 42             | 4 (6)     |
| JW3309 FkpA42-His | 37              | 1         |
| Mo3 FkpA      | 42               | 5         |
| Mo3 FkpA-His  | 27               | 3 (5)     |
| K458 FkpA     | 42               | 4 (5)     |
| K458 FkpA-His | 27               | 4         |
| K458 FkpA43-His | 27              | 4         |

a. FkpA indicates that cells were transformed with pYH17 encoding FkpA.
b. Insensitive.
c. FkpA-His indicates that cells were transformed with pYH15 encoding FkpA-His. FkpA44-His indicates that cells were transformed with pH 16 encoding the temperature-sensitive FkpA44-His.

Table 2 shows that the complementation of fkpA mutants revealed that the temperature-sensitive mutant K458 was sensitive at 27°C and 42°C. When mutant K458 was complemented with the temperature-sensitive FkpA (FkpA43-His) encoded on pYH16, cells were sensitive at 27°C and insensitive at 42°C. Strain JW3309 fkpA transformed with wild-type fkpA became sensitive, displayed temperature sensitivity when transformed with fkpA443 and remained insensitive when transformed with fkpA442. These data clearly relate colicin M sensitivity to FkpA.

fkpA restores activity of denatured colicin M

To examine whether FkpA affects the structure of colicin M, the proteins were purified from E. coli BL21(DE3) transformed with pYH15, which carries fkpA with an encoded C-terminal His tag, and from E. coli BL21 fhuA transformed with pMLD237, which carries cma with an encoded N-terminal His tag. The fhuA mutant lacks the colicin M receptor and is therefore not killed by colicin M synthesized in the absence of the colicin M immunity protein (Harkness and Braun, 1990). The fkpA and cma genes were transcribed by the phage T7 RNA polymerase under lacUV5 control. After IPTG induction, three- to fivefold more FkpA was in the culture supernatant than in the cell pellet, owing to cell lysis (90%). In contrast, more than 90% of colicin M was cell-associated. FkpA-His and colicin M-His were purified by affinity chromatography on Ni-NTA agarose. No proteins other than FkpA-His and colicin M-His were observed after SDS-PAGE (Fig. 2).

The in vitro PPIase activity of purified FkpA-His (Fig. 2, lane 5) was tested with an established refolding assay using RNase T1 denatured in 5.6 M guanidine hydrochloride (Ramm and Plückthun, 2000). RNase refolding was monitored spectroscopically as the increase in fluorescence over time. FkpA-His (35 nM) increased RNase T1 refolding 27-fold over spontaneous refolding, and 10 nM FkpA-His increased refolding eightfold. The immunosuppressant FK506 (12 μM) completely inhibited RNase T1 refolding by 35 nM FkpA.

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The same FkpA-His sample used for RNase T1 refolding was used to refold colicin M-His denatured in 5 M guanidine hydrochloride. With the denaturing conditions used (10°C overnight), a residual colicin M activity of 2–5% remained. Incubation with FkpA-His for 2 h at 37°C increased colicin M activity 10-fold to 25–50% of the original activity of the native sample (Table 3). Addition of FK506 completely inhibited the increase of colicin M activity by FkpA-His. The purified FkpA313-His protein, which has a G148D mutation in the PPIase domain, did not activate denatured colicin M. Purified SurA-His and lysozyme had no effect. These results indicate a specific activation of colicin M by FkpA and that PPIase activity is essential for activation. Attempts to spectroscopically monitor colicin M denaturation and renaturation yielded no suitable wavelength at which fluorescence changes reflected structural changes.

FkpA reduces inactivation of colicin M by proteinase K

To further test interaction of colicin M with FkpA, we examined whether FkpA-His prevents degradation of colicin M-His by proteinase K. Colicin M-His was inactivated by proteinase K through degradation, and FkpA-His partially prevented the degradation of colicin M-His (Table 4). Protection of colicin M-His by active FkpA-His was specific as the inactive FkpA313-His mutant and SurA-His did not inhibit colicin M-His inactivation.

**Table 3.** Reactivation of denatured colicin M by FkpA.

| Proteins                          | Colicin M activity (%) |
|-----------------------------------|------------------------|
| Native colicin M-His              | 100                    |
| Native colicin M-His + FkpA-His   | 100                    |
| Denatured colicin M-His           | 2–5                    |
| Denatured colicin M-His + FkpA-His| 25–50                  |
| Denatured colicin M-His + ethanol | 25–50                  |
| Denatured colicin M-His + FkpA-His + FK506 | 2–5          |
| Denatured colicin M-His + FkpA313-His | 3–4               |
| Denatured colicin M-His + SurA-His | 3–4                    |
| Denatured colicin M-His + lysozyme | 3–4                    |

Colicin M-His was denatured in 5 M guanidine hydrochloride at 10°C.

**Table 4.** FkpA reduces inactivation of colicin M by proteinase K.

| Sample                           | Diameter of lysis zones (mm) |
|----------------------------------|------------------------------|
|                                  | Colicin M-His dilution       |
|                                  | 10°  10¹  10²  10³            |
| Colicin M-His                    | 0    0    0    0              |
| Colicin M-His and FkpA-His       | 7.5  5    0    0              |
| Colicin M-His and FkpA313-His    | 0    0    0    0              |
| Colicin M-His and SurA-His       | 0    0    0    0              |
| Colicin M-His, no proteinase K   | 10.5 8    5.5   0             |

All samples were treated with proteinase K unless indicated otherwise. Colicin M-His samples were applied to a nutrient agar plate seeded with the sensitive *E. coli* AB2847 strain. After incubation overnight, the diameter of the zones of growth inhibition was measured.
M synthesized by fkpA+ and fkpA40 mutant cells did not result in killing of the fkpA40 mutant. We incubated colicin M purified from fkpA+ and fkpA mutant cells with lipid II and determined the release of undecaprenol by thin-layer chromatography. Colicin M isolated from E. coli HS859 fkpA40 and from E. coli BL21 fhuA fkpA+ (not shown) cleaved approximately 50% of the added lipid II (Fig. 3). These results indicated that FkpA does not activate newly synthesized colicin M.

We also tested whether isolated colicin M could be activated by isolated FkpA. Lipid II was incubated with colicin M isolated from fkpA40 cells and increasing concentrations of FkpA. Colicin M (0.6 μg) cleaved 50–60% of the substrate (Fig. 3) and was only slightly activated by added FkpA. These results support the finding that active colicin M is synthesized in fkpA+ and fkpA cells.

**Colicin M changes its structure when bound to cells**

It is likely that colicin M is unfolded during import across the outer membrane into the periplasm. To examine whether colicin M changes its conformation when it binds to the FhuA outer membrane receptor, we tested its trypsin sensitivity. This approach was guided by our earlier finding that unpurified colicin M is resistant to degradation by trypsin but is trypsin-sensitive when bound to susceptible E. coli cells (Schaller et al., 1981). Purified colicin M (0.1 μg ml⁻¹) was added to exponentially growing cultures of the wild-type fhuA strain AB2847 and the ΔfhuA strain MB97 to exclude unspecific cell adsorption of colicin M as the cause of trypsin sensitivity. Cultures were treated with trypsin, and the remaining colicin M activity in the culture supernatant was determined in a plate assay with a colicin M-sensitive strain. A surplus of trypsin inhibitor was added prior to spotting the spent medium onto plates seeded with the colicin M-sensitive strain to avoid trypsin action while colicin M entered the indicator bacteria. The control medium contained colicin M but no cells. No colicin M activity was found in the supernatant of the fhuA+ strain, only a slight reduction in colicin M activity was observed in the supernatant of the ΔfhuA strain, and no reduction in colicin M activity was found in the control (Table 5), i.e. colicin M was degraded by trypsin only in cultures of fhuA− cells.

We monitored cell growth in the presence of colicin M and colicin M with trypsin in another experiment. A surplus of colicin M (1 μg ml⁻¹) was used to monitor the action of colicin M after addition of trypsin inhibitor. Trypsin prevented cell lysis by colicin M (Fig. 4). Addition of trypsin inhibitor resulted in the lysis of cells by colicin M that had not been degraded by trypsin (Fig. 4). When colicin M was completely inactivated by trypsin (initial colicin M concentration of 0.1 μg ml⁻¹; Table 5), addition of trypsin inhibitor did not result in cell lysis (not shown), which supports the conclusion that colicin M and not FhuA was degraded by trypsin. In the presence of trypsin inhibitor, synthesis of FhuA would render cells susceptible to colicin M killing. We and others have previously shown that FhuA in cells and isolated outer membranes is not degraded by trypsin (Hoffmann et al., 1986; Moeck et al., 1996; Bonhivers et al., 2001).

**Temperature-sensitive FkpA shows an increased sensitivity to proteinase K**

To examine whether temperature-sensitive FkpA43 assumes a structure that differs from wild-type FkpA, isolated FkpA43 was incubated with proteinase K at 4°C. The low temperature was used to delay cleavage so that the larger fragments formed could be identified by SDS-PAGE. FkpA43 was cleaved by proteinase K to smaller fragments than those formed by cleavage of wild-type FkpA (Fig. 5), which indicates that FkpA43 and wild-type FkpA have different conformations. Although FkpA-43

### Table 5. Degradation of cell-bound colicin M by trypsin.

| Strain       | Col M-His | Col M-His + trypsin + inhibitor |
|--------------|-----------|---------------------------------|
| AB2847 fhuA- | 9         | 0                               |
| MB97 fhuA    | 9         | 8                               |
| Uninoculated medium | 9   | 9                               |

The numbers indicate the diameter of lysis zones in mm obtained by spotting 10 μl of spent medium onto a nutrient agar plate seeded with the colicin M (Col M)-sensitive strain E. coli AB2847.
was active at 27°C, its altered structure was clearly discernible at 4°C.

**Discussion**

In this study, we show for the first time that the activity of an imported protein, colicin M, depends specifically on a single periplasmic chaperone, FkpA. Four *fkpA* mutants were completely insensitive to colicin M, and a temperature-sensitive *fkpA* mutant was nearly insensitive at the non-permissive temperature.

The Y25N replacement in FkpA43 causing the temperature sensitivity is located in helix 1, which is part of the interface between the two FkpA subunits that form a dimer (Fig. 1) (Saul *et al*., 2004). It is likely that the amino acid replacement weakens the interaction between the two subunits so that inactive monomers are formed at 42°C. The altered structure of FkpA43 was evident even at 4°C, at which it was degraded by proteinase K to a greater extent than wild-type FkpA. The phenotype of the colicin-resistant *fkpA* mutants and also the restoration of colicin M sensitivity of the mutants by wild-type FkpA and the acquisition of the temperature-sensitive phenotype of *fkpA* mutants when transformed with *fkpA*43ts clearly relate colicin M sensitivity to FkpA. Conformational changes in colicin M elicited specifically by active FkpA was demonstrated by the partial restoration of activity of denatured colicin M by added FkpA and lack of restoration by inactive FkpA313, SurA and lysozyme. Moreover, only active FkpA reduces cleavage of colicin M by proteinase K. In this case, only active FkpA binds to colicin M, or the FkpA PPIase activity constantly shifts the equilibrium...
between a sensitive and resistant form of colicin M towards the resistant form.

FkpA is not required for the activity of newly synthesized colicin M as colicin M isolated from fkpA mutant cells was active in vivo and in vitro. FkpA is only required for cell lysis when added from the outside. Then how is colicin M inactivated during uptake and how does FkpA activate colicin M in the periplasm? It is unlikely that colicin M is synthesized in both a prolyl-cis and a prolyl-trans form and that only the cis form is taken up and activated by FkpA in the periplasm. In this case, addition of FkpA together with colicin M to cells would inactivate colicin M, which was not observed. The target of colicin M resides at the outer surface of the cytoplasmic membrane. The lipid carrier translocates the hydrophilic murein precursor to the periplasmic side of the membrane, where it is incorporated into murein. To reach its target, colicin M binds to FhuA in the outer membrane, which presumably transfers the colicin into the periplasm as energy coupling of FhuA to TonB is required for colicin M sensitivity. Mutants in the TonB box of FhuA are colicin M-resistant. However, we cannot exclude that binding of colicin M to FhuA requires TonB, as binding of phages T1 and φ80 to FhuA requires TonB (Schöffler and Braun, 1989). It is unknown whether the entire colicin M is taken up into the periplasm. The C-proximal activity domain entering the periplasm would suffice. Colicin M, like all studied colicins, is composed of three structural and functional domains: an N-terminal translocation domain, a central receptor binding domain and a C-terminal activity domain. A 24 kDa colicin M fragment that lacks 5 kDa from the N-terminus binds to the FhuA receptor but does not kill cells (Dreher et al., 1985). Point mutations that inactivate colicin M activity reside in the C-terminal domain (PlisI et al., 1993). Regardless whether the entire colicin or only the activity domain is translocated across the outer membrane, it most likely unfolds partially and rearranges its functional domains.

The finding that colicin M becomes trypsin-sensitive upon binding to cells via FhuA suggests a conformational change of colicin M close to the cell surface, when it is still accessible to trypsin. For other colicins, there is evidence that colicin domains physically separate from each other during import but most of them remain chemically linked. For example, colicin E2 remains bound to the outer membrane receptor when its nuclease domain enters the cytoplasm (Sharma et al., 2007). In the crystal structures of the pore-forming colicin Ia (Wiener et al., 1997) and the nuclease E3 (Soelaiman et al., 2001), the receptor binding domains are separated over a large distance from the translocation and activity domains. The three domains must further separate during import so that the activity domains can insert into the cytoplasmic membrane and enter the cytoplasm. When colicin Ia is bound to its Cir receptor, the translocation and activity domains of colicin Ia extend ~80 Å away from Cir and ~150 Å from the lipid bilayer (Buchanan et al., 2007). From the structure of the colicin E2 binding domain bound to its BtuB receptor, it was inferred that the projection of the binding domain in the plane of the outer membrane is ~70 Å and that of the entire colicin ~100 Å (Sharma et al., 2007). The structural data imply that for translocation of the activity domain across the outer membrane and subsequent insertion into the cytoplasmic membrane or uptake into the cytoplasm, large movements of the translocation and activity domains are required, and these movements are most likely accompanied by substantial structural changes.

Experimental evidence for structural changes of the activity domain has been obtained with those colicins that are exported with immunity proteins, which are tightly bound to the activity domains. These colicins undergo structural changes in the activity domains to dissociate from the immunity proteins (Zakharov and Cramer, 2004; Cascales et al., 2007). Colicin A shows an increased activity after urea treatment (Benedetti et al., 1992) and disulphide bonds engineered into the pore-forming domain delay translocation (Duché et al., 1994). Similar structural changes are expected for colicin M during uptake into sensitive cells. Trypsin sensitivity of cell-bound colicin M and the requirement for FkpA for colicin M activity support a change in conformation. It is difficult to determine such a structural change of the imported colicin M in the periplasm as a few molecules (approximately 10) suffice to kill a cell (Schaller et al., 1981). Therefore, we denatured purified colicin M in vitro and determined restoration of colicin M activity by FkpA. Colicin M denatured in 5 M guanidine hydrochloride was activated by FkpA. Although the denatured colicin M most likely does not reflect the form of colicin M after translocation across the outer membrane, restoration of activity demonstrates interaction of FkpA with colicin M such that folding into a native conformation is enhanced. FkpA may cause refolding by catalysing proline cis-trans isomerization, and may accelerate spontaneous folding by binding to the correctly folded protein, which is withdrawn from the equilibrium with incorrectly folded protein forms. As FK506 inhibits refolding, it is likely that the PPlase activity of FkpA is required. Colicin M contains 15 proline residues (5.5%), which is higher than the average proline content (4.8%) of all protein sequences in the Swiss-Prot database (http://expasy.org/sprot/relnotes/relstat.html). One or several of the proline residues could serve as cis-trans isomerization substrates. However, refolding of colicin M does not necessarily involve proline cis-trans isomerization as FkpA increased solubility of an antibody fragment devoid of cis-prolines in the periplasm (Bothmann and Plückthun, 2000).

Our results indicate that colicin M can be used to isolate fkpA mutants for which there is no other selection procedure. Mutations in chaperones do not lead to a

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severe impairment of the cell physiology as chaperones can mutually replace each other functionally (Rizzitello et al., 2001). For example, the periplasmic chaperone Skp binds to the OmpA protein. skp mutants have fewer outer membrane proteins (Chen and Henning, 1996). skp mutants show no physiological phenotype, but skp degP double mutants do not grow at 37°C and they accumulate proteins in the periplasm (Schäfer et al., 1999). Single mutations in the periplasmic chaperone gene surA lead to lower levels of porins and unfolded monomeric LamB maltoporin in the periplasm and cause hypersensitivity to bile salts, detergents and hydrophobic antibiotics (Rouviere and Gross, 1996; Rizzitello et al., 2001; Betton, 2007). surA degP double mutations are lethal. Also mutations in fkpA, which encodes the periplasmic chaperone studied here, lead to no observable phenotype. FkpA suppresses the formation of inclusion bodies in the periplasm by the folding-defective maltose binding protein MalE31 (Arié et al., 2001) and assists in the formation of a functional antibody fragment linked to a filamentous MalE31 (Arié et al., 2001) and assists in the formation of a functional antibody fragment linked to a filamentous (Hais et al., 2000). A degP fkpA double mutant stops growing 3 h after shifting from 37°C to 40°C (Arié et al., 2001). FkpA mutants and colicin M as the natural substrate would be instrumental for correlating FkpA structure to FkpA function. The results of the present study indicate that the PPIase activity is essential for colicin M activation. The PPIase inhibitor FK506 inhibits colicin M renaturation, the G148D mutant in the PPIase domain is inactive and the N-proximal chaperone fragments of 127, 140 and 163 residues in the fkpA mutants are not sufficient to confer colicin M sensitivity.

The other colicins tested in our study, unlike colicin M, do not require FkpA and the other periplasmic PPlases/chaperones. Pesticin, which acts as a muramidase in the periplasm (Vollmer et al., 1997), is also active in an fkpA mutant. Localization of the target site does not play a role for colicin M activation by FkpA. The pore-forming colicins assume their active conformation while they insert into the cytoplasmic membrane and folding of the cytoplasmic RNase and DNase colicins may be assisted by cytoplasmic chaperones.

Our data are consistent with the proposal that colicin M unfolds during import and FkpA assists refolding of colicin M in the periplasm to its active form. Active colicin M can then access its substrate in the outer leaflet of the cytoplasmic membrane and cleave the phosphate bond between undecaprenol and PP-N-acetyl-muramyl pentapeptide-N-acetyl-glucosamine.

**Experimental procedures**

**Bacterial strains and plasmids**

The bacterial strains and plasmids used and their sources are listed in Table 6. The previously isolated tolM mutants were obtained by screening for colonies that grew on nutrient agar plates containing colicin M (Braun et al., 1980). Those colonies that were specifically insensitive to colicin M but fully sensitive to the FhuA ligands albomycin and phages T1 and T5 were collected. Sensitivity to these ligands excluded mutations in fhuA, tonB, exbB and exbD. Sensitivity to colicins B and Ib supported the conclusion of functional tonB, exbB and exbD genes. Unaltered sensitivity to antibiotics for which the outer membrane forms a partial permeability barrier suggested that the outer membrane structure was not altered.

Temperature-sensitive tolM mutants were screened on nutrient agar plates containing colicin M and ferrichrome as the sole iron source. Ferrichrome can be used as the iron source only when the fhuA, tonB, exbB and exbD genes are functional; use of ferrichrome therefore excludes mutations in these genes as the cause of colicin M insensitivity. Colonies that were colicin M-insensitive at 42°C but sensitive at 30°C were collected.

The plasmid encoding colicin M modified at the N-terminus by a (His)6 tag was obtained from D. Mengin-Lecreulx (Université Paris-Sud, Orsay, France). Cells freshly transformed with the plasmids were used in the assays. fkpA40 and rpsL were co-transduced with phage P1 (selection for streptomycin resistance) from E. coli Mo3 into E. coli BL21(DE3) to yield strain HS859. Primers FkpAxba (GCTCTAGAGTATGTAGATTTGTTCG ACAACGC) and FkpAxho (GATCTCGAGTTTTTTAGCAGAATCTGCGG) were both used to amplify the fkpA gene from E. coli AB2847. Plasmid pYH15 was generated by cloning the Xbal/Xhol-digested PCR product into plasmid pET25b; the recombinant FkpA contained a C-terminal His tag. Plasmid pYH16 was generated in the same way by cloning the PCR product of E. coli K458 into pET25b. Plasmid pYH16 was generated by cloning the PCR fragment of the fkpA region of AB2847 into plasmid pT7-6 using the primers FkpAprim GCTTCTAGAGTATGTAGATTTGTTCG ACAACGC and FkpAend GACCAATTGCACTCCTTTTCAGGAGCCTGTCG (MunI).

fkpA313 was obtained by mutagenesis of E. coli AB2847 with N-methyl-N′-nitro-N-nitrosoguanidine (Miller, 1972) and selection for colicin M insensitivity and albomycin sensitivity (Braun et al., 1980). As albomycin requires the same genes for translocation across the outer membrane as colicin M, the procedure selects against colicin M uptake mutants. From the resulting strain SIP1275, fkpA chromosomal DNA was amplified by PCR with primers FkpA1 AGTATGTAGATTTGTTCG ACAACGC and FkpA2 GTTTTTTACAGAATCTCGGG CTTTTCAGGAGCCTGTCG (MunI).

fkpA313 was isolated from cells of E. coli BL21(DE3) fkpA transformed with pYH15 encoding FkpA-His. Cells were grown with shaking at 37°C in Luria–Bertani (LB) medium (tryptone 10 g l⁻¹, yeast extract 5 g l⁻¹, NaCl 5 g l⁻¹) with 50 μg ml⁻¹ ampicillin to an OD of 0.5 at 600 nm; 1 mM IPTG

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Table 6. E. coli K-12 strains and plasmids used in this study.

| Strain/Plasmid | Genotype | Reference |
|----------------|----------|-----------|
| **Strain**     |          |           |
| AB2847         | aroB thi tsx malT | Schaller et al. (1982) |
| BL21(DE3)      | F'ompTgal dcm lon hsdS (r0- m0) λ(DE3) lacI lacUV5-T7 gene 1 | Studier and Moffatt (1986) |
| BL21 fhuA      | BL21(DE3) fhuA | This institute |
| BW25113       | lacI 39083 ΔlacZ4787 hsdS514 Δ araBAD) 567 Δ (rhaBAD) 568 rph-1 | Keio Collection |
| JW0013         | BW25113 ΔntrK | Keio Collection |
| JW0052         | BW25113 ΔsurA | Keio Collection |
| JW0157         | BW25113 ΔdegP | Keio Collection |
| JW0427         | BW25113 ΔclpP | Keio Collection |
| JW0431         | BW25113 ΔppID | Keio Collection |
| JW3309         | BW25113 ΔfkuA | Keio Collection |
| JW3326         | BW25113 ΔppIA | Keio Collection |
| JW3584         | BW25113 ΔsecB | Keio Collection |
| JW4103         | BW25113 ΔgroL | Keio Collection |
| KA48           | AB2847 fpkA43 (tolM-ts) | Schaller et al. (1981) |
| MB97           | AB2847 ΔfkuA | Braun et al. (2003) |
| MC4100         | F' ΔlacU169 araD139 rpsL150 relA1 ptsF ribfB5301 | Casadaban (1976) |
| MC4100Skp      | F' ΔlacU169 araD139 rpsL150 relA1 ptsF ribfB5301 proAB::Tn10 Δskp | Schäfer et al. (1999) |
| Mo3            | AB2847 fpkA40 (tolM40) | Braun et al. (1980) |
| Mo4            | AB2847 fpkA41 (tolM41) | Braun et al. (1980) |
| Mo6            | AB2847 fpkA42 (tolM42) | Braun et al. (1980) |
| SIP127         | AB2847 fpkA131 | This study |
| **Plasmid**    |          |           |
| pASKSurA       | pASK75 encoding SurA-His | Behrens et al. (2001) |
| pMLD237        | pET2430::cma encoding Cma-His | El Ghachi et al. (2006) |
| pSP127/56      | pET25b encoding FkpA13-Hisb | This study |
| pYH15          | pET25b encoding FkpA-Hisb | This study |
| pYH16          | pET25b encoding FkpA43-Hisb | This study |
| pYH17          | pT7-6 encoding FkpA | This study |

a. The strains of the Keio Collection, Japan, were described by Baba et al. (2006).
b. FkpA-His denotes the FkpA protein with a C-terminal His tag.

was then added. After 3 h incubation, the culture was centrifuged, 50 ml of the clear supernatant was diluted 1:2 with buffer A (50 mM Na-phosphate buffer, 300 mM NaCl, 10 mM imidazole, pH 8.0), and the sample was applied to a Ni-NTA agarose column (1.5 ml bed volume) (Qiagen, Hilden, Germany) equilibrated with buffer A and washed with 10 ml of 50 mM Na-phosphate buffer, 300 mM NaCl, 20 mM imidazole, pH 8.0. The His-tagged proteins were eluted with 50 mM Na-phosphate buffer, 300 mM NaCl, 20 mM imidazole, pH 8.0. The fractions obtained were analysed by SDS-PAGE for their content of the desired proteins and impurities. FkpA313-His was isolated accordingly from the FkpA313-His encoding plasmid pSP127/56 to an electrophoretically homogeneous form.

SurA-His was isolated from MC4100 pASKSurA as described (Behrens et al., 2001).

(His)b-colicin M was synthesized in E. coli BL21 fhuA and purified on a Ni-NTA-agarose column as described (El Ghachi et al., 2006).

**Sensitivity tests**

Sensitivity to colicin M and albomycin and to the phages T5 and φ80 was tested. Aliquots (7 μl) of 10-fold dilutions of colicin M (0.25 mg ml⁻¹ stock solution), phage T5 and phage φ80 stock solutions, and a threefold dilution of an albomycin stock solution were spotted onto LB (10 g bacto tryptone, 5 g yeast extract, 5 g NaCl per l) agar plates seeded with the strains to be tested, as described previously (Braun et al., 1974). The results are given as the last 10-fold dilution that resulted in a clear zone of growth inhibition. For example, a colicin M titer of 4 means that a 10⁴-diluted stock solution inhibited cell growth. By plotting colicin M concentrations against the diameters of growth inhibition, a linear standard curve was obtained, which was used to estimate the degree of colicin M refolding by FkpA.

**Degradation of colicin M by trypsin**

Susceptibility of purified colicin M to degradation by trypsin was tested with a plate assay and in liquid culture. In the plate assay, colicin M-His (0.1 μg ml⁻¹) dissolved in the elution buffer used for Ni-NTA chromatography, supplemented with 0.1% dodecylmaltoside, was incubated in LB medium for 20 min with 0.3 mg ml⁻¹ trypsin at 30°C; 1.5 mg ml⁻¹ trypsin inhibitor was then added. Samples contained no cells. E. coli AB2847 or its fhuA deletion derivative MB97. The cultures were centrifuged, and colicin M activities were determined in the supernatant by spotting 10 μl onto plates seeded with E. coli AB2847. The diameter of the lysis zones was...
measured. In the liquid assay, colicin M-His (1 µg ml⁻¹) was added after 60 min incubation to exponentially growing cells of *E. coli* AB2847 in LB medium. A second culture received 0.3 mg ml⁻¹ trypsin together with colicin M-His. A third culture received colicin M and trypsin and then 1.5 mg ml⁻¹ trypsin inhibitor after 160 min incubation. Growth of the cultures was monitored at 578 nm for 5 h.

**Reactivation of colicin M by FkpA**

Colicin M (0.4 mg ml⁻¹) was incubated overnight in 0.1 M Tris-HCl, pH 8, 5 M guanidine hydrochloride at 10°C. The solution was then diluted 100-fold by adding 50 mM Tris-HCl, pH 8, 50 mM NaCl, 5 mM dithiothreitol and incubated for 2 h at 37°C without addition or after addition of 17 µg ml⁻¹ isolated FkpA-His; FkpA-His and 100 µM FK506 dissolved in ethanol (1% final concentration in the assay); 13 µg ml⁻¹ isolated FkpA313-His; 18 µg ml⁻¹ isolated SurA-His; 20 µg ml⁻¹ hen egg white lysozyme; or 1% ethanol. Colicin M activity was determined by spotting 3 µl on a nutrient agar plate seeded with the indicator strain *E. coli* AB2847. The diameter of the zones of growth inhibition was measured and compared with a standard curve.

**Reduction in proteinase K cleavage of colicin M by FkpA**

Colicin M-His (37 µg ml⁻¹) was incubated in 0.1 M Tris-HCl, 50 mM NaCl, 5 mM CaCl₂, 5 mM dithiothreitol with 0.25 mg ml⁻¹ proteinase K for 13 min on ice. The reaction was stopped by adding 5 mM phenylmethanesulfonylfluoride. Additional assays contained 22 µg ml⁻¹ FkpA-His, 13 µg ml⁻¹ FkpA313-His or 18 µg ml⁻¹ SurA-His and were pre-incubated for 30 min at 37°C. Colicin M activity was determined by spotting undiluted and 10-fold-diluted samples onto nutrient agar plates seeded with *E. coli* AB2847.

**Determination of PPlase activity in vitro**

The *in vitro* PPlase activity of FkpA was determined using an RNase T1 refolding assay (Ramm and Plückthun, 2001). RNase T1 (Sigma) was unfolded by overnight incubation in 5.6 M guanidine hydrochloride in 0.1 M Tris-HCl, pH 8 at 10°C. Refolding was initiated by 80-fold dilution to a final concentration of 0.2 µM in 50 mM Tris-HCl, pH 8, 50 mM NaCl and 10 mM FkpA-His (dimer) purified on a Ni-NTA agarose column (Fig. 2, lane 5). The folding reaction was followed at 10°C with a Jasco FP-6500 spectrofluorometer at 323 nm after excitation at 295 nm. Inhibition of the PPlase activity by FK506 was determined with 10 µM FK506 (final concentration) dissolved in ethanol. Controls contained denatured RNase T1 with FkpA, with and without ethanol, or only denatured RNase T1.

**Cleavage of lipid II by colicin M**

Cleavage of radiolabelled lipid II by colicin M was determined essentially as described in El Ghachi *et al.* (2006). Purified colicin M (0.6 µg in 16 µl 0.1 M Tris-HCl, 20 mM MgCl₂, 150 mM NaCl, pH 7.5) isolated from *E. coli* BL21(DE3) carrying wild-type *fpkA* or mutant *fpkA40* was incubated with 0.8 nmol of lipid II dissolved in 4 µl of 0.5% Triton X-100. After incubation for 30 min at 37°C, the reaction was stopped by raising the temperature for 1 min to 100°C. The reaction products were separated on silica gel thin-layer plates (Sili-cagel 60 Merck) with the solvent chloroform/methanol/water/32% ammonia (88:48:10:1, by vol.). The products were visualized by exposure to iodine vapour. The uncleaved substrate and the reaction product 1-pyrophosphoryl-muramyl pentapeptide-N-acetyl-glucosamine showed Rᵱ values of 0.5 and 0.1 respectively. The areas of silica gel containing the uncleaved substrate and the product and the area in between were scraped off the plate and counted in a liquid scintillation counter. Lipid II was kindly provided by Eefjan Breukink, University of Utrecht, through Waldemar Vollmer.

**Transport of ferrichrome**

Transport of [⁵⁷Fe⁺⁻⁻⁻]ferrichrome was determined essentially as previously described (Braun *et al.*, 2003). Exponentially growing cells were harvested and incubated with 1 µM [⁵⁷Fe⁺] (specific activity 81.5 kBq in 0.048 µg) and 5 µM deferri-ferrichrome. Samples were withdrawn after 5, 10, 15 and 25 min and filtered; the filters were dried, and the radioactivity was determined in a liquid scintillation counter.

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Supplementary material

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