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Displacement of OmpF loop 3 is not required for the membrane translocation of colicins N and A in vivo

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Abstract The pore-forming colicins N and A require the porin, OmpF, in order to translocate across the outer membrane of *Escherichia coli*. We investigated the hypothesis that in vivo, colicins N and A may traverse the outer membrane through the OmpF channel. In order to accommodate a polypeptide in the pore, the mid-channel constriction loop of OmpF, L3, would need to undergo a conformational change. We used five OmpF cystine mutants, which fix L3 in the conformation determined by X-ray crystallography, to investigate L3 movement during colicin activity in vivo. Sensitivity to colicins N and A of *E. coli* cells expressing these OmpF cystine mutants was determined using cell survival and in vivo potassium efflux and fluorescence assays. Results indicate that gross movement of L3 is not required for colicin N or A activity and that neither of these colicins crosses the outer membrane of *E. coli* through the lumen of the OmpF pore.

Key words: Porin; OmpF; Disulfide; Colicin sensitivity; *Escherichia coli*

1. Introduction

The non-selective, outer membrane porins of *Escherichia coli*, OmpC, OmpF and PhoE, allow the passive diffusion of small hydrophilic molecules with a molecular weight of less than 600 Da, into and out of the periplasm. They are currently the only transmembrane channels with known high resolution 3D structures [1–7]. They all form trimers and the non-selective porins, such as *E. coli* OmpF, consist of 16-stranded β-barrels with each monomer forming a pore. Within each pore a long polypeptide loop (L3) runs along one side of the barrel wall and narrows the pore to create the 'eyelet' or 'constriction' region. As well as being a general diffusion pore, OmpF also serves as a cell-surface receptor for phages and colicins A and N [8,9].

Colicins are plasmid-encoded bacteriocins produced by *E. coli* which kill closely related bacteria. The toxic action of colicins occurs in three stages associated with three separate structural domains: (1) receptor recognition and binding (central R-domain); (2) translocation across the cell envelope (N-terminal T-domain); (3) lethal action in the form of either pore formation in the cytoplasmic membrane or nuclease activity in the cytoplasm (C-terminal P-domain) (for reviews see [10–12]). Colicins N and A are pore-forming colicins belonging to the A group of colicins [13] which require the tol QRAB proteins for translocation across the outer membrane [14]. Although they both require OmpF as receptor for full activity in vivo [9,15,16], colicin N has also been shown to bind to the related porins OmpC and PhoE with similar binding affinities in vitro [17]. Nevertheless cells expressing only PhoE/OmpC show greatly reduced sensitivities to colicin N [16,18] and hence OmpF must play a role in later stages of translocation.

Colicin A requires an additional receptor, BtuB, but in the presence of low salt concentrations (10 mM phosphate buffer), this requirement can be by-passed (receptor by-pass conditions) [18,19]. However, under these conditions, neither colicin N or A can by-pass its requirement for OmpF (although colicin A can substitute the closely related OmpC) in what is likely to be the translocation step. Hence the porins are a part of the translocation pathway across the outer membrane. Other colicins which do not require OmpF use related proteins, e.g. TolC (colicin E1) [20,21], Tsx (colicin K) [22,23] and FepA (colicin B) [24,25], which are all β-barrel outer-membrane proteins with channel forming activity. The bacteriocin (pesticin) which is active against the bubonic plague agent *Yersinia pestis* also requires a related iron uptake receptor [26].

It is thought that the interaction with their receptors induces a conformational change or unfolding in the colicins, resulting in the entry of the colicin into the translocation machinery composed of the tolQRAB proteins. [27–29]. Studies on colicin A C-terminal fragment mutants indicate that colicin A unfolds on binding to its receptor [28] and calorimetric measurements on colicin N indicate an exothermic structural rearrangement upon binding to OmpF [17]. It has been proposed that upon receptor binding, colicins N and A unfold and that part of the polypeptide translocates through the pore of OmpF [27,30]. The strongest evidence for this hypothesis has been presented where a single OmpF mutation, G119D, was identified as being colicin N resistant [30]. This mutation is located on L3 deep in the OmpF pore and X-ray structural analysis revealed that D119 was protruding into the channel subdividing it into two sub-compartments [30]. It was also shown that the mutation did not affect the β-barrel structure or the surface exposed loops.

Recently, in vivo ESR spectroscopy studies have shown that the addition of colicin B affected the mobility of a probe on the gating loop of its receptor, FepA. As this loop controls pore conductivity, it is proposed that the observed effect was due to the colicin polypeptide passing through the FepA pore, which is likely to be much larger in diameter than OmpF [31].

Although a colicin unfolding step has been implicated in its translocation across the outer membrane, work with disul-

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phide bonded colicin A P-domain helices [32] shows that the translocation of this domain to the inner membrane is not inhibited by the presence of the disulphide bonds. This provides strong evidence that the extensive unfolding of P-domain to allow it through the pore does not occur. On the other hand, recent evidence has shown that colicin N T-domain, which interacts with Tol, is unstructured and this may allow the T-domain to penetrate the OmpF pore [33,34]

However, the space available in the OmpF pore when L3 is in the conformation determined by X-ray crystallography is 11×7 Å compared to 11×19 Å at the extracellular mouth of the pore and 22×15 Å at the periplasmic side [1]. Clearly a polypeptide, which may contain secondary structural elements, will be unable to fit through the pore with L3 in place (Fig. 1A,B). It therefore seems feasible that, to enable colicin polypeptide translocation, L3 must adopt a different conformation to create enough space in the pore. In view of this possible involvement of L3 movement in colicin translocation, we have used several OmpF cystine mutants (which form disulphide bonds between two introduced cysteine residues [35]), which restrict L3 to the conformation determined by X-ray crystallography, to investigate L3 movement during colicin activity in vivo using cell survival, in vivo K^+ efflux and fluorescence assays.

2. Materials and methods

2.1. Bacterial strains, plasmids and media

Production of plasmids which encode the OmpF cystine mutants have previously been described [35]. Cystine mutants were chosen from the lowest-energy pairs predicted by the SSBOND program [36]. The porin deficient *E. coli* strain BZB1107 [30] was used to express OmpF cystine mutants. Cells were grown in Luria-Bertani (LB) broth with the addition of 10 mM DTT (Clelands reagent; DL-dithiothreitol, Sigma) at 37°C with shaking. Ampicillin was added as required.

2.2. Protein purification

Colicin N was purified from *E. coli* BZB1019 (hsdR) carrying pChap4 [15] as previously described [37]. Colicin A protein was supplied by F. Pattus. Protein concentrations were determined by UV absorption at 280 nm (using the extinction coefficient of 49 500 M^{-1} cm^{-1} for colicin N).

2.3. Cell survival assays

Cell survival assays have been previously described [18,38–41]. BZB1107 cells expressing OmpF cystine mutants were grown in LB broth with DTT to an OD_{600nm} of 0.5. Cells were washed with 10 mM oxidised DTT (*trans*-4,5-dihydroxy-1,2-dithiane, Sigma) in 100 mM sodium phosphate buffer, pH 7.4 (buffer A) then resuspended in buffer A. 1 ml of cells (5×10^8 cells/ml) were incubated with colicin at 100 molecules/cell for 15 min at 37°C. 18 ml of fresh LB was added and the cells were grown at 37°C for 2 h. Percentage survival was determined by the ratio of OD_{600nm} of colicin treated to untreated cells. Cells expressing OmpF single cysteine mutants were grown to an OD_{600nm} of 0.5 in LB. For normal cell survival conditions, cells were washed in buffer A. For by-pass conditions [30,41,42], cells were washed in 10 mM phosphate buffer pH 7.4 (buffer B). Assays proceeded as for cystine mutants.

2.4. In vivo K^+ efflux assays

Assays measuring K^+ efflux resulting from colicin pore insertion into the cytoplasmic membrane of sensitive *E. coli* have previously been described [18,48]. Cells expressing OmpF cystine mutants were grown to an OD_{600} of 0.75 in 10 mM DTT. Cells were resuspended in 0.2% glucose, 1 mM KCl and 10 mM oxidised DTT in Buffer A and incubated at 37°C for 15 min. Cells were washed in 10 mM oxidised DTT in buffer A and resuspended in buffer A with 0.3% glucose, to a concentration of 2×10^9 cells/ml. An aliquot of 10 ml of this suspension was used per assay with a colicin multiplicity of 1000, 400 or 100 molecules/cell added after 150 s. K^+ efflux was measured using a K^+-selective valinomycin electrode (BPS NICO). Total internal cellular K^+ concentrations were determined by heating a second aliquot of cells to 75°C for 30 min to release all internal K^+. Total internal K^+ values were used to calculate proportion of internal K^+ remaining over a time course of 1400 s.

2.5. In vivo fluorescent depolarisation assay

Cell sensitivity assays using membrane-potential sensitive fluorescent probes have previously been described [40,43,44]. As with the cell survival assay, BZB1107 cells expressing OmpF mutants were grown in LB broth with DTT at 10 mM to an OD_{600nm} of 0.5. Cells were washed with oxidised DTT at 10 mM in buffer A then resuspended in buffer A to a concentration of 5×10^9 cells/ml. Measurements were made on an Aminco SLM 8100 spectrofluorometer at 25°C with a 1 cm pathlength cuvette (Hellma). For each assay, 0.3 ml of cells were mixed with 2.7 ml of buffer A. In the spectrofluorometer, 300 μl of a 1 μM stock of ANS (8-anilino-1-naphthalene sulphonic acid, Sigma) was added to the cells after 50 s. After 200 s colicin was added at a multiplicity of either 100 or 400 molecules/cell. The fluorescence increase was monitored for at least 20 min.

2.6. Western blotting

To determine that the disulphide bonds had formed in vivo, cells used in the K^+ efflux assays were prepared for SDS-PAGE using non-reducing and reducing conditions and run on 12% (w/v) acrylamide gels. Samples were electroblotted onto nitrocellulose and after blocking with milk powder solution they were incubated with a rabbit polyclonal antiserum raised against the OmpF sequence 236-NATPITNKFTNTSGFANK-253. Blots were revealed using whole anti-rabbit IgG linked to horseradish peroxidase (Sigma).

3. Results

3.1. Formation of disulphide bonds in vivo

Sites of the disulphide bonds used in this study are shown together superimposed on the known structure of OmpF (Fig. 1C). Three of the disulphides are between L3 and barrel wall (D107C-S177C, E117C-D312C and E117C-A333C) and two are within L3 itself (V105C-F129C and Y124C-D127C). Purified mutant porins have been introduced into artificial lipid bilayers and their single channel conductances measured [35]. The single channel conductance of a wild-type OmpF channel was measured at 827 pS. The mutant porins did show variations in single channel conductance, with channel sizes being measured at between 694–875 pS. Hence the channel diameter may be affected by the introduced mutations.

Disulphide bond formation in vivo was analysed using Western blotting. A previous study has shown that single cysteine residues within the OmpC pore could not be labelled without prior treatment with DTT [45]. This indicated that some unknown component of growing cells can react with these cysteine residues. In order to maximise disulphide bond formation in OmpF double cysteine mutants, cells expressing these mutants were grown in DTT then washed with oxidised DTT. Disulphide bond formation in vivo is evident from the increased mobility of mutant OmpF polypeptide when run under non-reducing conditions (Fig. 2). This may be due to the restricted dimensions of the denatured molecule [46]. However, for V105C-F129C, the disulphide bonded mutant runs as native OmpF and the reduced form runs slower than the native form. Interestingly, the L3 tip to barrel wall mutants, E117C-D312C and E117C-A333C, appear not to form disulphide bonds at the desired level in vivo under these conditions. For the E117C-D312C mutant this is evident from the lack of a faster migrating polypeptide and for E117C-

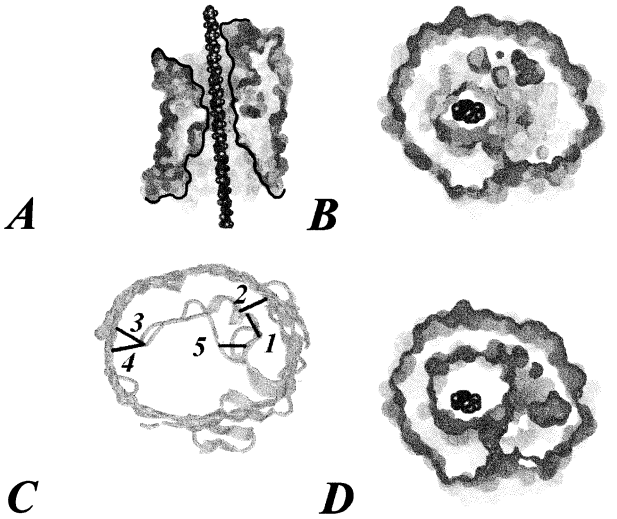

Fig. 1. OmpF molecular modelling. A: Molecular surface model showing space available for translocation of poly-Ala through the OmpF pore. Section through OmpF from the side showing a fully extended poly-Ala in van der Waals representation. Model shows limited space available at the 'eyelet' region for translocation of this simple polypeptide model when L3 is in the conformation determined by X-ray crystallography. B: Same model as A but now looking down through the pore from the cell surface but with OmpF cell surface exposed loops removed. Models A and B show that a colicin polypeptide would be unable to fit through the pore unless L3 undergoes a large conformational shift. C: Cystine mutants were chosen from the lowest-energy pairs predicted by the SSBOND program [36]. Sites of the disulphide bonds are shown superimposed on the known structure of OmpF and are: 1, V105C-F129C; 2, D107C-S177C; 3, E117C-D312C; 4, E117C-A333C and 5, Y124C-D127C. D: OmpF model, as B but with L3 truncated to residue 107. The model shows that more space is now available for poly-Ala translocation but there is unlikely to be enough space available for a colicin polypeptide which may contain secondary structural elements.

A333C the presence of two polypeptides on the Western blot; one running as wild-type and the other polypeptide migrating faster than wild-type indicates only partial disulphide bond formation. This was also observed in the purified protein, which formed the desired disulphide bond at 80% efficiency [35]. The treatment of the cells with DTT/oxidised DTT thus ensures the maximum disulphide bond formation in 3 of the 5 mutants. However we do not discount the possibility that the E117C-D312C forms a disulphide bond as will be discussed in the next section. The Western blots also show that the level of expression of wild-type and mutant OmpF are similar.

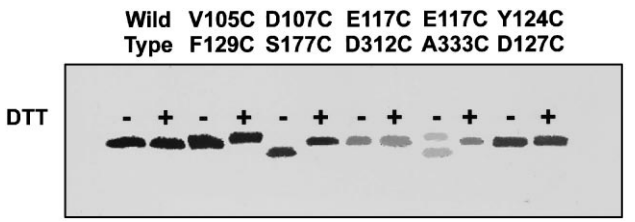

Fig. 2. Electrophoretic migration of OmpF disulphide mutants in vivo revealed using Western blotting. Protein in cells expressing OmpF disulphide mutants were separated on a 12% polyacrylamide gel under non-reducing (−DTT) and reducing (+DTT) conditions and electroblotted onto nitrocellulose. OmpF mutants were revealed using an antibody raised against the OmpF sequence 236-NATPITNKFTNTSGFANK-253. Disulphide bond formation is evident from the increased mobility of mutant OmpF polypeptide when run under non-reducing conditions.

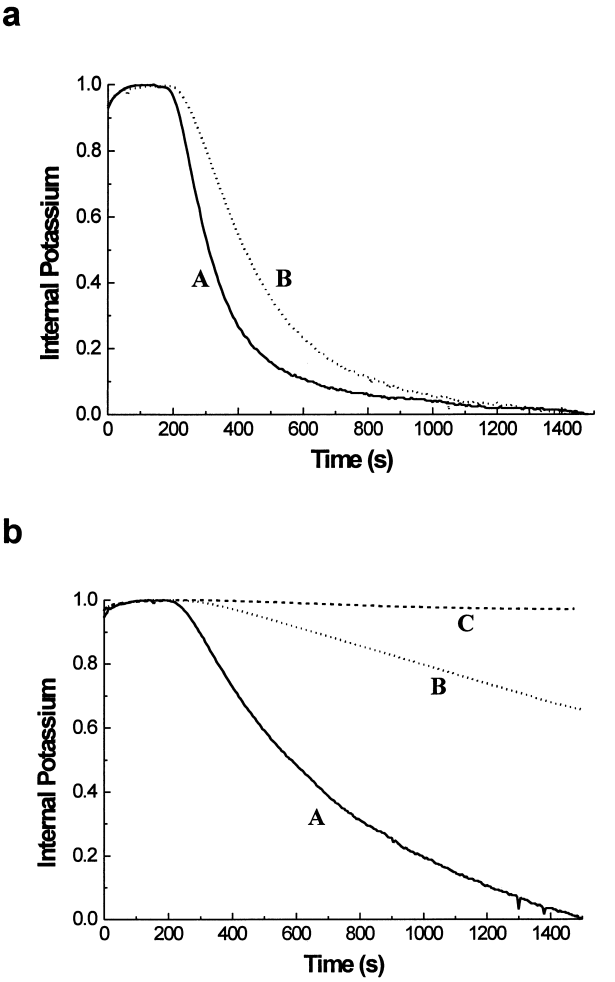

Fig. 3. Remaining internal K^+ in cells expressing wild-type and mutant OmpF after the addition of colicin. a: After the addition of colicin N at 150 s to cells expressing wild-type OmpF at 100 molecules per cell, K^+ efflux was 1.5 nmol K^+/mg dry cell weight/s (A). The only cystine mutant which caused resistance to colicin N at this multiplicity was E117C-D312C which reduced K^+ efflux to 0.85 nmol K^+/mg dry cell weight/s (B). b: K^+ efflux from wild-type cells was 0.49 nmol K^+/mg dry cell weight/s after the addition of colicin A at 100 molecules per cell (A). None of the cells expressing OmpF cystine mutants had any resistance to colicin A. The resistance shown by cells expressing D107C-S177C, with a K^+ efflux of 0.094 nmol K^+/mg dry cell weight/s, was due to the single mutation D107C (B). Cells expressing the control colicin resistant mutant R168C had a K^+ efflux rate calculated at 0.01 nmol K^+/mg dry cell weight/s (C).

3.2. Cell survival

Cell survival assays showed that only one OmpF cystine mutant conferred resistance to colicins at 100 molecules/cell. The mutant, D107C-S177C, caused a 52% cell survival rate with colicin A at this multiplicity but afforded no protection against colicin N. Cell survival assays using cells expressing D107C and S177C single OmpF mutants revealed that resistance to colicin A was due to the single mutation D107C. Cell sensitivity returned to normal levels under by-pass conditions. Surprisingly, we did not detect any survival of cells expressing

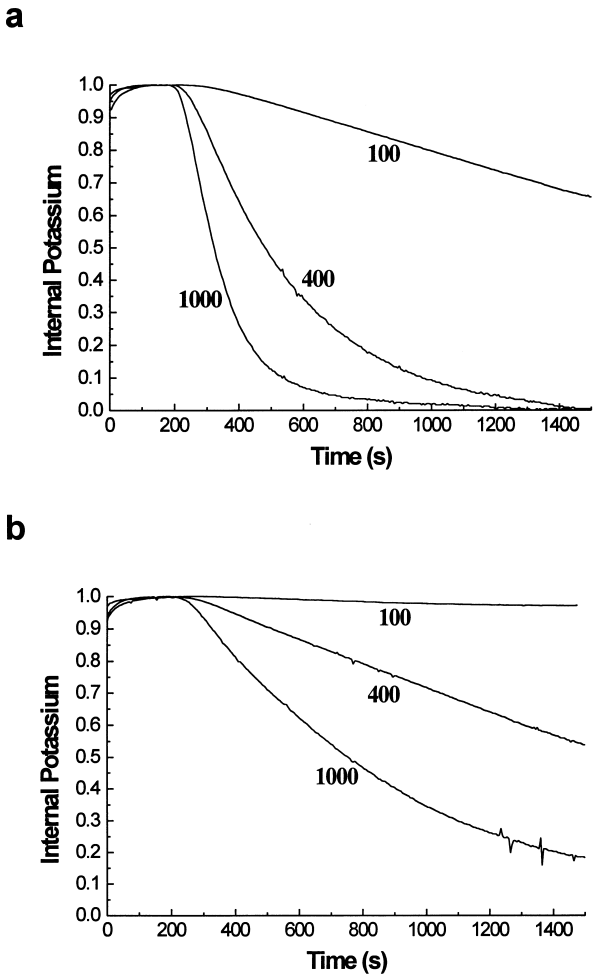

Fig. 4. Remaining internal K^+ in cells expressing D107C (a) and R168C (b) at colicin A multiplicities of 100, 400 and 1000 per cell. Increasing the multiplicity of colicin A from 100 to 1000 per cell increased K^+. K^+ efflux rates for cells expressing D107C were 0.09, 0.66 and 1.54 nmol K^+/mg dry cell weight/s and 0.01, 0.13 and 0.38 nmol K^+/mg dry cell weight/s for cells expressing R168C at colicin A multiplicities of 100, 400 and 1000 per cell respectively. Rates for cells expressing wild-type OmpF were 0.49, 1.54 and 2.99 nmol K^+/mg dry cell weight/s at the same multiplicities.

OmpF R168C, our control colicin N and A resistant mutant [47], when colicin N was added at a multiplicity of 100 molecules/cell. When colicin A was added at the same multiplicity, 92% of the cells survived. Cell sensitivity, as with D107C, returned under by-pass conditions.

3.3. K^+ efflux measurements

Addition of colicin N to cells expressing the OmpF cystine mutants always resulted in an efflux of cytoplasmic K^+. After the addition of colicin N at 100 molecules/cell, K^+ efflux from cells expressing wild-type OmpF was rapid, with a rate of K^+ efflux of 1.5 nmol K^+/mg dry cell weight/s (calculated from the linear part of the graph and assuming that 2×10^9 cells correspond to 1 mg cell dry weight/ml, [48]). Cells expressing the OmpF mutants V105C-F129C, D107C-S177C, E117C-A333C and Y124C-D127C exhibited a K^+ efflux at the same rate as cells expressing wild-type OmpF. Cells expressing the remaining mutant, E117C-D312, showed a reduction in K^+ efflux (Fig. 3a). The reduced rate was 0.85 nmol K^+/mg dry cell weight/s and was not caused by the single OmpF mutations E117C or D312C, which allowed wild-type K^+ efflux from cells. This slight resistance was overcome when the colicin N multiplicity was increased to 400 molecules per cell.

Evidence for the formation of a disulphide bond for this mutant, which was not obvious from Western blot analysis, comes from two observations. The reduced K^+ efflux was not seen in (i) cells expressing E117C-D312C which were not treated with DTT/oxidised DTT and (ii) cells expressing the single mutations E117C or D312C. Therefore we propose that the disulphide bond for this mutant does form under treatment conditions and that this does not cause a large increase in the SDS-PAGE mobility of the denatured polypeptide. The similar PhoE L3 'tip to barrel-wall' cystine mutant, K18C-E110C [49] which forms a disulphide bond at high efficiency also shows no difference in mobility of the reduced and oxidised form on normal SDS-PAGE gels.

Compared to colicin N, colicin A at 100 molecules per cell caused a slightly lower K^+ efflux with a rate of 0.49 nmol K^+/

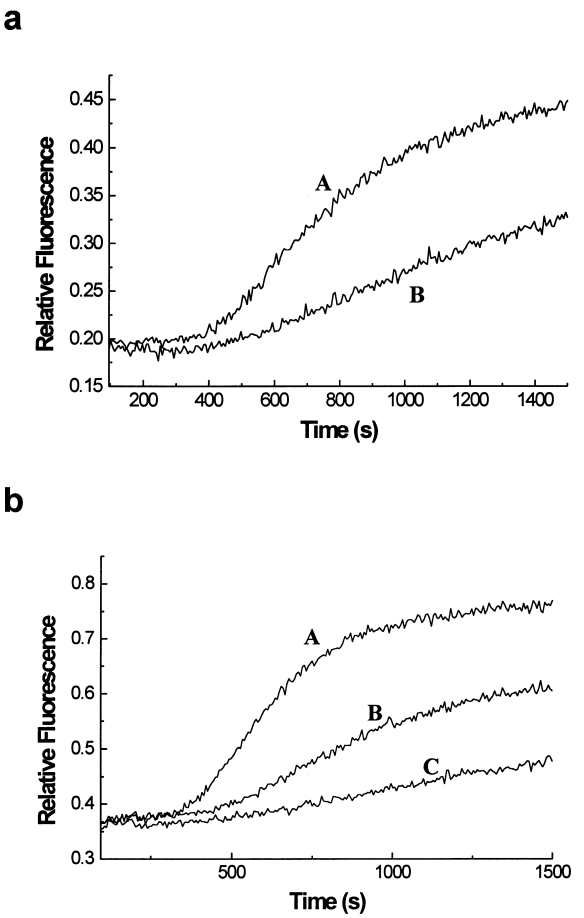

Fig. 5. Fluorescent depolarisation assay. Spectra show the change in the fluorescence of the potential sensitive probe, ANS, caused by membrane depolarisation after the addition of colicin after 200 s. a: Fluorescence change in cells expressing wild-type OmpF after the addition of colicin N at 100 molecules per cell (A). The only cystine mutant which caused a deviation from wild-type fluorescence change was E117C-D312C at 100 colicin N molecules per cell (B). b: None of the cystine mutants caused a deviation from wild-type fluorescence change (A) at a colicin A multiplicity of 400 per cell. The decrease in fluorescence seen for D107C-S177C expressing cells was due to the single mutation D107C (B). Our control mutant R168C shows a large decrease in depolarisation (C).

mg dry cell weight/s for all cells expressing OmpF wild-type or cystine mutants, except D107C-S177C. The reduced rate in K^+ efflux from cells expressing D107C-S177C, 0.094 nmol K^+/mg dry cell weight/s, was, as with the cell sensitivity assay, due to the single mutation D107C (Fig. 3b). This resistance was reduced, but not overcome, when colicin A multiplicity was increased to 400 and 1000 per cell (Fig. 4a).

3.4. Fluorescent depolarisation assay

The in vivo ANS fluorescence assay results were similar as those from the K^+ efflux assays. All cells expressing OmpF cystine mutants were sensitive to colicin N to the same extent as cells expressing wild-type OmpF at a multiplicity of 400 colicin molecules per cell. When the multiplicity was reduced to 100 colicin N molecules per cell, cells expressing E117C-D312C showed, as in K^+ efflux assays, a slight resistance (Fig. 5a). When colicin A was used, again, resistance was seen for the D107C expressing cells (Fig. 5b) but no resistance was established for OmpF cystine mutant expressing cells.

We could not detect any colicin N resistance in cells expressing OmpF R168C at multiplicities of 100, 400 and 1000 molecules per cell using both the K^+ efflux and fluorescence assays. However a resistance to colicin A was established using both assay systems (Fig. 3bFig. 5b). K^+ efflux rates at 100, 400 and 1000 colicin A molecules per cell are shown (Fig. 4b). Since we have been able to use our K^+ efflux assay to detect very small changes in sensitivity, we must conclude that in spite of published data to the contrary [47] the OmpF R168C mutant does not cause resistance to colicin N and must be referred to as a colicin A resistant mutant only.

4. Discussion

Using cystine scanning mutagenesis of OmpF L3, we have attempted to answer the question whether colicins N and A translocate across the outer membrane of *E. coli* through the pore of OmpF. It is clear that for a polypeptide to translocate through the pore, the internal L3 must undergo a conformational change to create enough space within the pore. We have demonstrated that for four of our OmpF double cysteine mutants, V105C-F129C, D107C-S177C, E117C-D312C and Y124C-D127C, we can maximise the formation of the disulphide bond in vivo by treatment of the cells with DTT/oxidised DTT, thereby fixing L3 in the conformation determined by X-ray crystallography [1]. The fifth mutant, E117C-A333C, formed a disulphide bond at much reduced levels in vivo. A mixed population of bonded and unbonded porin was present in our experiments and it was to be expected that cells expressing this mutant had a K^+ efflux the same as cells expressing wild-type OmpF.

L3 movement has previously been implicated in porin voltage gating. It has now been shown that this is not the case [35,50,51]. Single channel conductance measurements on wild-type OmpF presumably relate to a channel size with L3 in the conformation determined by X-ray crystallography. By comparing single channel conductances of our OmpF cystine mutants to the value for wild-type OmpF, we conclude that although channel sizes vary, no major changes were seen in the size of the mutant channels [35].

The unique resistance to colicin N by cells expressing the mutant E117C-D312C at a colicin multiplicity of 100 per cell after treatment of the cells with DTT/oxidised DTT may indicate that small conformational changes are needed at the tip of L3 for full activity of colicin N. Since cell sensitivity returned when the multiplicity of toxin increased, this resistance is probably due to impeded binding of colicin N to OmpF. The colicin N resistant OmpF mutation, G119D [30], which is two residues from the tip of L3, caused a distortion of L3 and reduced receptor binding. This implies that, for colicin N activity, L3 needs to be either in a defined conformation which is distorted by mutation or that it needs flexibility. However, we cannot be confident that the mutant E117C-D312C forms a disulphide bond at 100% efficiency and therefore must explore the possibility that the tip of L3, from residue 107, where we have produced a disulphide bond at 100%, may undergo a conformational shift. Examination of a truncated OmpF L3 model (Fig. 1D), with the tip of the loop being residue 107 rather than 117, reveals more space available for poly-Ala translocation but it still remains improbable that a partially unfolded colicin polypeptide, which may contain some secondary structural elements as well as bulky sidechains, is able to translocate through this model pore. Therefore a possibility exists that an unstructured T-domain may penetrate the pore in order to bind to tol A [29,33] but since the tol A binding site is distant from the N-terminus this would require the translocation of 65 residues including two tryptophans [34].

The formation of OmpF disulphide bonds had no effect on the activity of colicin A. Although the single mutation, D107C and our control mutant, R168C, conferred resistance to colicin A, cell survival assays carried out under by-pass conditions restored cell sensitivity for both mutants. This suggests that the mutations D107C and R168C are required for colicin A receptor binding only.

The results here demonstrate that the formation of disulphide bonds between L3 and the barrel wall and within L3 itself, at various positions along L3, had little effect on colicin N and A activities on cells expressing these mutants in vivo. With L3 fixed in the conformation determined by X-ray crystallography not enough space is available for the translocation of a *folded* polypeptide. Duché et al. [32] showed that disulphide bonds which fixed the *folded* conformation of colicin A also had no effect upon translocation. Thus from two different uses of disulphide bond technology we must conclude that the *whole* colicin molecule does not translocate through the pore of OmpF. Our results further imply that, since the single mutations D107C and R168C have no effect on colicin N, colicins N and A may interact with OmpF at different receptor sites. However, the reason for the receptor binding resistance shown by D107C to colicin A remains intriguing since this mutation is located deep in the OmpF pore on the periplasmic side of L3.

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References

[1] Cowan, S.W., Schirmer, T., Rummel, G., Steiert, M., Ghosh, R., Paupit, R.A., Jansonius, J.N. and Rosenbusch, J.P. (1992) Nature 358, 727–733.
[2] Hirsch, A., Breed, J., Saxena, K., Richter, O., Ludwig, B., Diederichs, K. and Welte, W. (1997) FEBS Lett. 404, 208–210.

[3] Kreusch, A. and Schulz, G.E. (1994) J. Mol. Biol. 243, 891–905.
[4] Meyer, J.E.W., Hofnung, M. and Schulz, G.E. (1997) J. Mol. Biol. 266, 761–775.
[5] Przybylski, M. et al. (1996) Protein Sci. 5, 1477–1489.
[6] Schirmer, T., Keller, T.A., Wang, Y.F. and Rosenbusch, J.P. (1995) Science 267, 512–514.
[7] Weiss, M.S. and Schultz, G.E. (1992) J. Mol. Biol. 227, 493–509.
[8] Nikaido, H. and Vaara, M. (1985) Microbiol. Rev. 49, 1–32.
[9] Pugsley, A.P. (1984) J. Bacteriol. 158, 523–529.
[10] James, R., Kleanthous, C. and Moore, G.R. (1996) Microbiology UK 142, 1569–1580.
[11] Lakey, J.H., van der Goot, F.G. and Pattus, F. (1994) Toxicology 87, 85–108.
[12] Lazdunski, C.J. et al. (1988) Biochim. Biophys. Acta 947, 445–464.
[13] Davies, J.K. and Reeves, P. (1975) J. Bacteriol. 123, 102–117.
[14] Webster, R.E. (1991) Mol. Microbiol. 5, 1005–1011.
[15] Pugsley, A.P. (1987) Mol. Microbiol. 1, 317–325.
[16] Tommassen, J., Pugsley, A.P., Korteland, J., Verbakel, J. and Lugtenberg, B. (1984) Mol. Gen. Genet. 197, 503–508.
[17] Evans, L.J.A., Cooper, A. and Lakey, J.H. (1996) J. Mol. Biol. 255, 559–563.
[18] Bourdineaud, J.P., Fierobe, H.P., Lazdunski, C. and Pages, J.M. (1990) Mol. Microbiol. 4, 1737–1743.
[19] Benedetti, H., Frenette, M., Baty, D., Lloubes, R., Geli, V. and Lazdunski, C. (1989) J. Gen. Microbiol. 135, 3413–3420.
[20] Benz, R., Maier, E. and Gentschev, I. (1993) Z.blatt Bakteriol. Int. J. Med. Microbiol. Virol. Parasitol. Infect. Dis. 278, 187–196.
[21] Koronakis, V., Li, J., Koronakis, E. and Stauffer, K. (1997) Mol. Microbiol. 23, 617–626.
[22] Bremer, E., Middendorf, A., Martinussen, J. and Valentin, H.P. (1990) Gene 96, 59–65.
[23] Manning, P.A. and Reeves, P. (1976) Biochem. Biophys. Res. Commun. 71, 466–471.
[24] Liu, J., Rutz, J.M., Feix, J.B. and Klebba, P.E. (1993) Proc. Natl. Acad. Sci. USA 90, 10653–10657.
[25] Rutz, J.R., Lui, J., Goranson, J., Armstrong, S.K., McIntosh, M.A., Feix, J.B. and Klebba, P.E. (1992) Science 258, 471–475.
[26] Rakin, A., Saken, E., Harmsen, D. and Heesemann, J. (1994) Mol. Microbiol. 13, 253–263.
[27] Lazdunski, C.J. (1995) Mol. Microbiol. 16, 1059–1066.
[28] Benedetti, H., Lloubes, R., Lazdunski, C. and Letellier, L. (1992) EMBO J. 11, 441–447.
[29] Benedetti, H., Lazdunski, C. and Lloubes, R. (1991) EMBO J. 10, 1989–1995.
[30] Jeanteur, D. et al. (1994) Proc. Natl. Acad. Sci. USA 91, 10675–10679.
[31] Jiang, X.Q., Payne, M.A., Cao, Z.H., Foster, S.B., Feix, J.B., Newton, S.M.C. and Klebba, P.E. (1997) Science 276, 1261–1264.
[32] Duche, D., Baty, D., Chartier, M. and Letellier, L. (1994) J. Biol. Chem. 269, 24820–24825.
[33] Vetter, I.R., Parker, M.W., Tucker, A.D., Lakey, J.H., Pattus, F. and Tsernoglou, D. (1998) Structure (in press).
[34] Raggett, E.M., Bainbridge, G., Evans, L.J.E., Cooper, A. and Lakey, J.H. (1998) Mol. Microbiol. 28, 1335–1344.
[35] Bainbridge, G., Mobasheri, H., Armstrong, G.A., Lea, E.J.A. and Lakey, J.H. (1998) J. Mol. Biol. 275, 171–176.
[36] Hazes, B. and Dijkstra, B.W. (1988) Protein Eng. 2, 119–125.
[37] El-Kouhen, R., Fierobe, H.P., Scianimanico, S., Steiert, M., Pattus, F. and Pages, J.M. (1993) Eur. J. Biochem. 214, 635–639.
[38] Cavard, D. (1994) FEMS Microbiol. Lett. 116, 37–42.
[39] El-Kouhen, R., Hoenger, A., Engel, A. and Pages, J.M. (1994) Eur. J. Biochem. 224, 723–728.
[40] Evans, L.J.A., Labeit, S., Cooper, A., Bond, L.H. and Lakey, J.H. (1996) Biochemistry 35, 15143–15148.
[41] Fourel, D., Hikita, C., Bolla, J.M., Mizushima, S. and Pages, J.M. (1990) J. Bacteriol. 172, 3675–3680.
[42] Cavard, D. and Lazdunski, C. (1981) FEMS Microbiol. Lett. 12, 311–316.
[43] Brunden, K.R., Cramer, W.A. and Cohen, F.S. (1984) J. Biol. Chem. 259, 190–196.
[44] Cramer, W.A. and Phillips, S.K. (1970) J. Bacteriol. 104, 819–825.
[45] Gokce, I., Bainbridge, G. and Lakey, J.H. (1997) FEBS Lett. 411, 201–205.
[46] Derman, A.I. and Beckwith, J. (1995) J. Bacteriol. 177, 3764–3770.
[47] Fourel, D., Mizushima, S., Bernadac, A. and Pages, J.M. (1993) J. Bacteriol. 175, 2754–2757.
[48] Boulanger, P. and Letellier, L. (1988) J. Biol. Chem. 263, 9767–9775.
[49] Eppens, E.F., Nouwen, N. and Tommassen, J. (1997) EMBO J. 15, 4295–4301.
[50] Eppens, E.F., Saint, N., VanGelder, P., vanBoxtel, R. and Tommassen, J. (1997) FEBS Lett. 415, 317–320.
[51] Phale, P.S., Schirmer, T., Prilipov, A., Lou, K.L., Hardmeyer, A. and Rosenbusch, J.P. (1997) Proc. Natl. Acad. Sci. USA 94, 6741–6745.