Vectorial transport and folding of an autotransporter virulence protein during outer membrane secretion

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Summary

Autotransporter (AT) proteins are a large and diverse family of extracellular virulence proteins from Gram-negative bacteria, characterized by a central β-helix domain within the mature virulence protein. It is not clear how these proteins cross the outer membrane (OM) quickly and efficiently, without assistance from an external energy source such as ATP or a proton gradient. Conflicting results in the literature have led to several proposed mechanisms for AT OM secretion, including a concerted process, or vectorial secretion with different directionalities. We introduced pairs of cysteine residues into the passenger sequence of pertactin, an AT virulence protein from Bordetella pertussis, and show that OM secretion of the passenger domain stalls due to the formation of a disulphide bond. We further show that the C-terminus of the pertactin passenger domain β-helix crosses the OM first, followed by the N-terminal portions of the virulence protein. In vivo proteolytic digestion shows that the C-terminus is exposed to the extracellular milieu during stalling, and forms stable structure. These AT secretion and folding features can potentially facilitate efficient secretion.

Introduction

Bacterial pathogenesis relies on the secretion of protein virulence factors to the cell surface, where these proteins can interact and interfere with host cell components. Protein transport into and across cellular membranes is a complex and fascinating process, but many of the mechanistic details remain unclear. The transport of proteins across the outer membrane (OM) of Gram-negative bacteria is particularly intriguing, as no less than seven distinct transport mechanisms have been described (Henderson et al., 2004; Bingle et al., 2008). This mechanistic diversity might reflect diverse solutions to the challenge of secreting proteins quickly and efficiently in the absence of an obvious external energy source, such as ATP or a proton gradient (Thanassi et al., 2005).

Autotransporter (AT; also known as Type Va) virulence proteins were originally named because it appeared that all essential components for OM transport were contained within the AT polypeptide sequence itself: an N-terminal signal sequence (for inner membrane transport), a central passenger domain that constitutes the mature virulence factor, and a C-terminal porin domain (Henderson et al., 1998; Dautin and Bernstein, 2007; Dautin et al., 2007). The classical model for AT OM secretion is that the porin domain forms a pore in the OM, through which the central passenger domain passes (Fig. 1A). More recently, however, controversy has emerged regarding the directionality of OM secretion (i.e. transport of the passenger domain through the OM from N→C, versus C→N), the extent of passenger folding that is compatible with OM secretion (Skillman et al., 2005; Jong et al., 2007), and the degree to which other proteins, such as Omp85, are required for OM transport. Indeed, an alternative model has emerged in which the entirety of OM transport occurs as one concerted step, after passenger domain folding in the periplasm (Bernstein, 2007) (Fig. 1A). Importantly, crystal structures of AT porin domains revealed that the central pore is too narrow (1–2 nm) to accommodate a folded passenger domain (Oomen et al., 2004; Barnard et al., 2007), indicating that if the passenger domain does cross the OM through the C-terminal porin, the passenger must maintain or achieve a non-native conformation in the periplasm, prior to OM transport. Yet several studies suggest that the passenger domain can adopt a stable, protease-resistant conformation in the periplasm (Skillman et al., 2005; Ieva et al., 2008), which remains capable of traversing the OM. These results have been interpreted as indirect evidence towards a concerted translocation mechanism, bypassing secretion through the C-terminal β-barrel (Bernstein, 2007). Yet, experiments introducing a calmodulin domain within the passenger domain of haemoglobin protease showed that OM secretion can be stalled by the formation of stable structure within the periplasm (Jong et al., 2007), offering indirect evidence for OM secretion via threading through the...
C-terminal porin domain. This controversy led us to test the AT OM secretion mechanism directly, by stalling the passenger domain during secretion across the OM, and analysing the extracellular exposed segment of the passenger domain.

All three AT passenger domain structures solved to date adopt a right-handed β-helix topology at the C-terminus of the passenger domain (Fig. 2) (Emsley et al., 1996; Otto et al., 2005; Gangwer et al., 2007), despite diverse lengths, sequences and functions (Table 1). Indeed, >97% of all ATs are predicted to contain β-helical structure (Junker et al., 2006). In addition, we have recently shown that the C-terminal rungs of β-helical structure in the passenger domains of Bordetella pertussis pertactin and Escherichia coli Pet are markedly more stable than the N-terminal portions of these passenger domains (Junker et al., 2006; Renn and Clark, 2008). These results led us to propose a model for AT OM secretion in which the free
energy released upon the progressive folding of the β-helical C-terminus of the passenger, after its transport across the OM, either actively drives forward OM secretion, or at the very least prevents backsliding of the passenger back into the periplasm (Junker et al., 2006) (Fig. 1A). Our model made two crucial predictions: (i) the direction of passenger secretion across the OM is C→N, and (ii) the stable C-terminal core observed during AT passenger folding in vitro is also populated in vivo, during OM secretion. Here we use the pertactin AT from B. pertussis to test these predictions, and discuss these results in light of current models for OM secretion.

Results

The Luirink laboratory has previously shown that introduction of a pair of widely spaced Cys residues into an AT passenger domain produces a disulphide-bonded loop within the periplasm, and these loops can block OM secretion (Jong et al., 2007), possibly because the loop is too large to pass through the AT porin. To test the extent of passenger domain transport and folding during OM secretion, we employed a similar strategy, engineering three Cys-pairs into three distinct locations within the passenger domain of the pertactin AT (wild-type pertactin contains no Cys residues) (Fig. 1B and C). The amino acid residues selected for cysteine mutations are all >40 aa apart from one another, and surface-exposed according to the pertactin passenger domain crystal structure (Emsley et al., 1996). In vitro folding experiments confirmed that these mutations do not affect the structure or folding properties of the pertactin passenger domain (not shown). Low-level expression of full-length pertactin from a constitutive tac promoter (see Experimental procedures for details) resulted in the accumulation of processed passenger domain for only wild-type pertactin, and a mutant in which a long loop (35 aa; residues 260–294) in the passenger domain is replaced with three alanines (Fig. 3). Conversely, low-level expression did not produce significant quantities of processed passenger, or unprocessed pre-protein, for any of the Cys-pair constructs. However, upon overexpression, unprocessed pre-protein was detectable for all constructs, although again, processed passenger accumulated only for wild-type pertactin and the loop-deleted construct (Fig. 3). Presumably, during low-level expression, the clearance of the stalled pre-protein via the protein degradation machinery occurs at a rate at least as fast as pertactin pre-protein production. In contrast, during overexpression, pertactin synthesis overwhelms both pro-

Table 1. β-Helical autotransporters have diverse sequences, lengths and functions.

<table>
<thead>
<tr>
<th>Property</th>
<th>Haemoglobin protease</th>
<th>Pertactin</th>
<th>VacA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organism</td>
<td>E. coli</td>
<td>B. pertussis</td>
<td>H. pylori</td>
</tr>
<tr>
<td>Function</td>
<td>Protease</td>
<td>Adhesion</td>
<td>Pore formation</td>
</tr>
<tr>
<td>Destination</td>
<td>Extracellular release</td>
<td>OM surface</td>
<td>Extracellular release</td>
</tr>
<tr>
<td>Length*</td>
<td>1377 aa</td>
<td>910 aa</td>
<td>1288 aa</td>
</tr>
</tbody>
</table>

*a. Lengths reported here are for the full-length protein (including signal sequence, passenger and porin domains). For pertactin and haemoglobin protease, structures of the full-length passenger have been solved (see Fig. 2). For VacA, the structure of only the C-terminal 55 kDa domain was solved; the full-length passenger is 88 kDa.
cessing and clearance, leading to a steady-state accumulation of unprocessed pre-protein.

The cysteine mutations could lead to misfolding, aggregation, and/or mistargeting of pertactin. To address this, we tested whether the Cys-pair constructs were processed correctly to produce a mature passenger domain under reducing conditions. As shown in Fig. 4A, during low-level expression overnight in the presence of reducing agent, all three Cys-pair constructs are cleaved at the OM, indicating that in the absence of disulphide bond formation these constructs are correctly processed. Furthermore, similar intensities were detected for the bands corresponding to the mature passenger domain for wild-type pertactin and the Cys-pair constructs, indicating that the cysteine mutations themselves (independent of disulphide bond formation) had no negative effects on AT biogenesis. Correct processing was also observed when these constructs were overexpressed in the presence of reducing agent (Fig. 4A).

An equally relevant concern is whether the disulphide-mediated stalling at the OM causes the pertactin AT to reach an equilibrium conformation that is incompatible with maturation to the mature virulence protein. To test this, we examined the reversibility of stalling. Wild-type pertactin and Cys-pair constructs were first overexpressed under oxidizing conditions, followed by 3 h growth under reducing conditions. To prevent interference from newly synthesized protein during growth under reducing conditions, cells were gently pelleted and resuspended in medium lacking IPTG prior to the addition of reducing agent. After treatment with reducing agent, the band corresponding to pre-protein disappears, replaced by a band corresponding to processed passenger domain for all three formerly stalled Cys-pair constructs (Fig. 4B), indicating that the conformations adopted by the stalled constructs remain compatible with pertactin biogenesis.

Expression using the constitutive promoter alone does not lead to the accumulation of detectable quantities of the stalled constructs in the OM (Fig. 3), indicating the processed passenger domains detected after IPTG removal are derived from pertactin constructs synthesized under oxidizing conditions.

To determine what portion of the passenger domain is exposed outside the cell during stalling, we used a non-specific protease to digest the exposed portions of the overexpressed pertactin constructs, and analysed the resulting digestion fragments that were released from the cell surface into the digestion solution; this approach resulted in the detection of a major fragment of 28 kDa (Fig. 5A). An important question is whether this fragment arises from an extracellularly exposed portion of the pertactin passenger domain, or whether proteinase K digestion compromises the integrity of the OM, perhaps...
The C-terminal rungs of the AT passenger β-helix are exposed first during OM secretion, and fold during secretion. A. The C-terminus of the passenger domain is outside the cell when OM secretion is stalled via disulphide bond formation. Proteinase K digestion of intact cells overexpressing pertactin constructs released a stable 28 kDa core for wild-type, loop-deleted, and the two pertactin constructs with N-terminal Cys-pairs (1 and 2), but not for a construct with a C-terminal Cys-pair (3), nor for a construct expressing only the passenger domain in the cytoplasm (‘passenger’). After centrifugation to remove intact cells and cell debris, the protease digestion buffers were separated by SDS-PAGE and analysed by Western blotting, probed with an anti-pertactin polyclonal antibody. B. The pertactin passenger domain fragment released from the cell surface upon proteinase K digestion is resistant to digestion over time. Aliquots from the supernatant of the cell digestion solution were collected over time, separated by SDS-PAGE, and resolved by silver staining. Shown are results for Cys-pair construct 1 (S151C/V202C); equivalent results were obtained for wild-type pertactin (not shown). C. The protease-resistant stable structure formed on the surface of the cell matches the in vitro stable core (Junker et al., 2006). MALDI-TOF mass spectrum of the purified 28 kDa fragment from pertactin S151C/V202C (Cys-pair 1) contains five peaks identifiable as pertactin fragments, all located in the C-terminus of the passenger domain; amino acid residue ranges are labelled. Fragments also identified for the in vitro stable core (Junker et al., 2006) are marked with a star (★). The mass-to-charge ratios for the five fragments are: 1341, 1448, 1748, 3390 and 3675.

Fig. 5. The C-terminal rungs of the AT passenger β-helix are exposed first during OM secretion, and fold during secretion. A. The C-terminus of the passenger domain is outside the cell when OM secretion is stalled via disulphide bond formation. Proteinase K digestion of intact cells overexpressing pertactin constructs released a stable 28 kDa core for wild-type, loop-deleted, and the two pertactin constructs with N-terminal Cys-pairs (1 and 2), but not for a construct with a C-terminal Cys-pair (3), nor for a construct expressing only the passenger domain in the cytoplasm (‘passenger’). After centrifugation to remove intact cells and cell debris, the protease digestion buffers were separated by SDS-PAGE and analysed by Western blotting, probed with an anti-pertactin polyclonal antibody. B. The pertactin passenger domain fragment released from the cell surface upon proteinase K digestion is resistant to digestion over time. Aliquots from the supernatant of the cell digestion solution were collected over time, separated by SDS-PAGE, and resolved by silver staining. Shown are results for Cys-pair construct 1 (S151C/V202C); equivalent results were obtained for wild-type pertactin (not shown). C. The protease-resistant stable structure formed on the surface of the cell matches the in vitro stable core (Junker et al., 2006). MALDI-TOF mass spectrum of the purified 28 kDa fragment from pertactin S151C/V202C (Cys-pair 1) contains five peaks identifiable as pertactin fragments, all located in the C-terminus of the passenger domain; amino acid residue ranges are labelled. Fragments also identified for the in vitro stable core (Junker et al., 2006) are marked with a star (★). The mass-to-charge ratios for the five fragments are: 1341, 1448, 1748, 3390 and 3675.

releasing a protease-resistant structure that has formed within the periplasm. To investigate the integrity of the cell membranes during the digestion experiments, the following controls were performed: (i) digestion of cells overexpressing only the pertactin passenger domain, lacking a signal sequence and the β-porin domain (and therefore expressed in the cytoplasm) did not produce any detectable fragments (Fig. 5A), confirming the integrity of the inner membrane during the digestion procedure. (ii) The viability of cells expressing Cys-pair or wild-type pertactin was indistinguishable, as was the viability of cells treated with proteinase K versus undigested cells (not shown). (iii) Proteinase digestion did not release stable fragments from any protein other than pertactin (Fig. 5B, and mass spectrometry results below).

We also directly tested the integrity of the OM, to determine whether the pertactin stable core could form in the periplasm, by analysing the concentration of the periplasmic marker protein maltose-binding protein (MBP). In order to minimize cell stress, in vivo proteolytic digestion was performed directly in the culture medium during pertactin expression. Strikingly, the concentration of MBP inside the periplasm did not change significantly after proteinase K digestion (Fig. 6A), indicating MBP is neither leaking out of the cell nor digested by proteinase K entering the periplasm. Yet, because the stress on the cells under these conditions is significantly lower than in digestion experiments with higher cell concentrations, protease concentration, and additional centrifugation steps (Fig. 5A and B), we also determined whether digestion under these gentler conditions produces the same pertactin 28 kDa proteolytic fragment as seen in Fig. 5. Indeed, proteinase K digestion following IPTG induction of wild-type pertactin produced the same 28 kDa fragment shown in Fig. 5A (Fig. 6B).

More importantly, the fragment is also detected for the N-terminal and central Cys-pair constructs, it is not detected for the empty vector or the C-terminal Cys-pair construct, also consistent with the results shown in Fig. 5.

The lack of MBP digestion in the periplasm might alternatively be due to a remarkably high resistance of MBP to digestion by proteinase K. Therefore, we tested the resistance of MBP to proteinase K digestion by determining the MBP concentration in the medium before and after digestion. As a result of normal cell turnover, a small amount of MBP is detectable in concentrated culture medium under all growth conditions (Fig. 6C, left). After proteinase K digestion, this small amount of MBP rapidly disappeared (Fig. 6C, right), demonstrating that MBP is not resistant to proteolytic digestion under these conditions, and strengthening our conclusion that proteinase K and the pertactin fragments described here are located extracellularly.

Finally, as an additional control to demonstrate surface exposure of pertactin, we performed immunofluorescence microscopy on live, intact cells expressing wild-type pert-
actin or stalled Cys-pair constructs (Fig. 7). Using a polyclonal antibody raised against the pertactin passenger domain, we detected pertactin at the outer cell periphery for all constructs except the C-terminal Cys-pair (A479C/T524C), presumably because very little of the passenger domain is exposed extracellularly in this construct (see below).

Having established the extracellular location of proteinase K and the pertactin digestion fragments, we proceeded to analyse the digestion products in detail. In the absence of protease, the digestion buffer contains low concentrations of correctly processed pertactin for the wild-type and loop-deleted constructs, showing a fraction of the mature virulence protein is released from the OM due to the weak non-covalent interaction with an unknown partner at the OM. Conversely, proteinase digestion of cells expressing wild-type pertactin released one major fragment with a size of 28 kDa into solution after removal of cells, and a less abundant 35 kDa fragment. Bands of the same size were also observed in digestion solution for cells expressing the

**Fig. 6.** Proteinase K digestion does not disrupt OM integrity.
A. Western blot of whole cell lysates probed with a polyclonal antibody against the periplasmic marker protein MBP. The MBP concentration in the periplasm does not vary significantly upon expression of pertactin constructs, or proteinase K digestion.
B. Proteinase K digestion in culture medium produces the 28 kDa pertactin passenger fragment detected using harsher digestion conditions (Fig. 5). Western blot of concentrated culture medium before pertactin expression, after expression, and after expression followed by 10 or 30 min of proteinase K digestion. The 28 kDa digestion fragment is detectable for all pertactin constructs except the C-terminal Cys-pair. The Western blot was probed with a polyclonal antibody against pertactin.
C. A Western blot of concentrated culture medium shows the rapid disappearance of MBP in the culture medium after addition of proteinase K. The small amount of MBP present in the culture medium due to normal cell turnover (left) is completely digested after 10 min of proteinase K in the medium (right).
loop-deleted construct (Fig. 5A). The loop deletion did not significantly affect the sizes of the fragment bands, indicating that the loop is not a component of these fragments. Moreover, the portion of the passenger that is N-terminal to the loop (residues 35–259) is too small (22.9 kDa) to produce fragments of these sizes, suggesting that both bands represent fragments from the C-terminus of the passenger domain. Digestion of the two N-terminal Cys-pair constructs also produced one major fragment of 28 kDa; a 35 kDa fragment was not detected. The 28 kDa fragment is stable, as shown by the resistance of this fragment to proteinase K digestion over time when produced by cells expressing pertactin with the N-terminal Cys-pair (Fig. 5B). The uniform size of the 28 kDa fragment produced upon digestion of cells expressing wild-type, loop-deleted, and N-terminal Cys-pair pertactin constructs suggests that this fragment is derived from the same portion of the pertactin passenger domain. Digestion of cells expressing the N-terminal Cys-pair construct did not produce the larger 35 kDa fragment, indicating either that OM stalling affects the stability of this part of the extracellular exposed passenger segment, or that portions of the passenger domain that constitute the 35 kDa fragment remain buried within the OM or the periplasm. Digestion of cells grown under reducing conditions resulted in the detection of a 28 kDa fragment for all three Cys-pair constructs (data not shown), indicating the cysteine mutations themselves do not affect the stability of the stable core.

In contrast, no fragments were detected upon proteinase K treatment of cells expressing the C-terminal Cys-pair construct (Fig. 5A). The lack of fragments suggests this stalled construct does not expose a large stable extracellular fragment of the passenger domain. Consistent with this explanation, we were unable to detect extracellular exposure of this C-terminally stalled passenger domain by immunofluorescence microscopy. Taken together, the large size of the 28 kDa fragment, its lack of sensitivity to loop-deletion, and its appearance for only the N-terminal and central Cys-pair constructs, are consistent with extracellular exposure of the C-terminus of the pertactin passenger, presumably while the stalled N-terminus is lodged in the periplasm (Fig. 1C, left). Hence we conclude that OM secretion of pertactin occurs vectorially, and from C-terminus to N-terminus.

For unequivocal identification of the proteinase K-resistant fragment formed during OM secretion, we performed in-gel trypsin digestion of the 28 kDa fragment, followed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. The mass spectrum contains six major peaks, five of which were identified as pertactin tryptic peptides (Fig. 5C). All five peptides are located in the C-terminus of the pertactin passenger. Four peptides correspond to tryptic peptides previously detected during the identification of the C-terminal stable core produced during equilibrium unfolding of the isolated pertactin passenger domain in vitro (Junker et al., 2006). The fifth peptide corresponds to the C-terminal boundary of the passenger domain, which was not present in the construct used in vitro. The existence of a protease-resistant stable core indicates that the C-terminus of the passenger domain can adopt a compact structure prior to the completion of OM secretion.

**Discussion**

The introduction of paired cysteine residues at various locations throughout the passenger domain of pertactin, and subsequent disulphide bond formation in the oxidizing environment of the periplasm, was sufficient to reversibly stall the pertactin OM secretion process at three distinct positions. We have used these stalled Cys-pair constructs to show that OM secretion of the pertactin AT passenger domain is a vectorial process, and that the stable C-terminal core is the first portion of the passenger domain exposed outside the OM. This C-terminus can adopt stable, protease-resistant structure prior to the completion of secretion, perhaps contributing to efficient OM secretion. These results are incompatible with an OM secretion mechanism in which a folded or partially folded passenger domain is transported across the OM in a concerted fashion. Moreover, the capacity of a Cys-pair to completely block OM secretion when placed at three dis-
tinct locations along the passenger sequence suggests that, for pertactin, significant folding or compaction of any portion of the passenger domain within the periplasm does not occur during OM secretion. These results are at odds with those of the Goldberg lab, who identified a protease-resistant portion of the IcsA passenger domain in periplasmic fractions (Brandon and Goldberg, 2001).

While our results suggest that the passenger domain crosses the OM prior to the formation of stable structure, the reversibility of the disulphide-mediated stalling indicates that, even during prolonged dwell times in the periplasm, the pertactin passenger domain is protected from proteolytic digestion. Interactions with periplasmic chaperones such as SurA and/or SkpB might protect the passenger domain during transit through the periplasm; such interactions have recently been demonstrated by the Goldberg lab for IcsA (Wagner et al., 2009). Alternatively, the mechanism for folding from N→C-terminus after transport across the inner membrane into the periplasm might expose the AT polypeptide to itself in a way that produces a fundamentally different folding mechanism, with dramatically different folding kinetics, from the C→N-terminal vectorial folding mechanism used after secretion across the OM. Supporting this idea, in vitro, when the full length of the passenger domain can interact with all of itself all at once, the isolated passenger domain folds extremely slowly (Junker et al., 2006), perhaps mimicking kinetically trapped but collapsed states that might form during extremely slow N→C-terminal vectorial folding in the periplasm.

Our results are compatible with previous proposals that the β-helical structure found in AT passenger domains is relevant for AT biogenesis, rather than for the function of the mature virulence protein (Junker et al., 2006; Kajava and Steven, 2006). In the absence of an external energy source, the free energy released upon folding outside the OM could provide a driving force for efficient OM secretion, for example, by providing a template structure of β-strands that promote fast, efficient folding of subsequently secreted N-terminal portions of the passenger domain. Indeed, folding after secretion must be orders of magnitude faster than the extremely slow folding reactions observed in vitro (Junker et al., 2006) in order to approach a rate comparable to that of the B. pertussis doubling time. More passively, the formation of folded β-helical structure might merely prevent backsliding of the passenger domain into the periplasm during secretion (Junker et al., 2006; Renn and Clark, 2008). Another possible rationale for the passenger domain stable C-terminal core comes from the work of Rachel Fernandez on the pertactin homologue BrkA, showing that deletion of 92 residues at the extreme C-terminus of the passenger domain dramatically decreases passenger domain stability and folding (Oliver et al., 2003). Hence the C-terminal β-strands might be involved in a capping mechanism to stabilize the β-helix, protecting it from aggregation or proteolytic degradation (Richardson and Richardson, 2002).

**Experimental procedures**

**Molecular biology**

The pertactin gene and promoter region was amplified from plasmid p41869D, and subcloned into pET21b (Novagen), resulting in pP.93WT. Pertactin is expressed from pP.93WT under the control of a constitutive tac promoter, and an IPTG-inducible promoter. Cys-pair constructs were first produced by site-directed mutagenesis of plasmid pPERPLC01, which encodes just the pertactin passenger domain (Junker et al., 2006), followed by subcloning of the mutated regions into pP.93WT. A fourth Cys-pair construct (S434C/A479C) was also constructed, but preliminary experiments revealed incorrect processing under reducing conditions (data not shown). To delete the loop spanning residues 260–294, the regions 5′- and 3′- to the loop were PCR amplified from pPERPLC02 and ligated together into pET21b, resulting in pPETPLC01 Δloop. The loop deletion construct was then subcloned into pP.93WT as for the cysteine mutants.

**Outer membrane purification**

HB101 cells expressing pertactin under the control of the endogenous promoter were prepared by inoculation of 100 ml Luria–Bertani (LB) plus 100 µg ml⁻¹ ampicillin with a single colony from a freshly streaked plate, and grown overnight at 37°C. Cultures were pelleted at 5000 g for 10 min and resuspended in 50 mM Tris pH 8.8. Cell lysis was induced by sonication for 90 s, with intervals of 30 s on/30 s off, followed by centrifugation for 15 min at 15 000 g to pellet cell debris. The cleared supernatant was transferred to an ultracentrifuge tube and membranes were pelleted for 45 min at 140 000 g using a Beckman 70.1 Ti rotor. To selectively resuspend the inner membrane, membrane pellets were vortexed in 100 mM phosphate buffer pH 8 containing 1% Sarkosyl. OMs were pelleted by an additional centrifugation for 90 min at 140 000 g.

For overexpression, 100 ml LB medium supplemented with 100 µg ml⁻¹ ampicillin was inoculated with 2 ml overnight culture and grown at 37°C to a cell density of OD₆₀₀ = 0.4–0.6. Overexpression was induced by the addition of IPTG (500 µM). After 2 h, cells were pelleted and the OM was purified as described above. In some preparations, 0.01 M β-mercaptoethanol was added to reduce disulphide bonds formed in the periplasm (Jose et al., 1996; Jose and Zangen, 2005).

**In vivo protease digestion**

In order to reduce cell envelope stress and potential release of periplasmic proteins into the proteinase K digestion solution, in these experiments pertactin constructs were overexpressed for only 45 min prior to protease digestion. Cells were grown, induced for 45 min, and pelleted as for OM
preparations. The pertactin passenger domain alone was expressed from plasmid pPERPLC02 in E. coli strain BL21(DE3)pLysS. Cell pellets were resuspended in 1 ml fresh LB medium supplemented with 7.5 mM CaCl₂. Digestion was induced by the addition of freshly prepared proteinase K to a final concentration of 0.1 mg ml⁻¹. After 10 min incubation at room temperature, cells were centrifuged at 21 000 g for 5 min, and the supernatant was removed. To inactivate proteinase K, supernatants were boiled for 10 min. Digestion fragments were resolved by SDS-PAGE and detected either by Western blotting (using an anti-pertactin passenger polyclonal antibody) or silver staining. To test OM integrity during proteinase K digestion, cells were induced for 21 000 g for 5 min, and the supernatant was removed. For each dilution, 50 μl was spread on an LB agar plate supplemented with 100 μg ml⁻¹ ampicillin. After 18 h growth at 37°C, the number of colonies was counted and with the dilution factor used to compute the number of colony-forming units in the original culture. This assay was repeated at least three times for each construct.

Cell viability assay

Log-phase cell cultures expressing wild-type pertactin, the S151C/V202C Cys-pair double mutant, or an empty vector, each with or without proteinase K digestion, were serially diluted. For each dilution, 50 μl was spread on an LB agar plate supplemented with 100 μg ml⁻¹ ampicillin. After 18 h growth at 37°C, the number of colonies was counted and with the dilution factor used to compute the number of colony-forming units in the original culture. This assay was repeated at least three times for each construct.

In-gel tryptic digests

The N-terminal Cys-pair construct S151C/V202C was over-expressed in strain HB101 grown in 1 l of culture medium as described above. After in vivo digestion and inactivation of proteinase K, protein fragments were precipitated by addition of 100% TCA (final ratio of 1:4 TCA : protein solution). After 10 min on ice, the precipitant was pelleted, washed twice with acetone, dried and resuspended in SDS loading buffer, and resolved using SDS-PAGE. The 28 kDa fragment was the major component of these samples (see, for example, the lack of other bands in Fig. 5B), so no additional purification was performed. This band was cut from the gel, diced into small pieces, and vortexed in 300 μl 25 mM ammonium carbonate with 50% acetonitrile. After 10 min, the liquid phase was removed. This washing procedure was repeated until the washing solution was visibly free of Coomassie dye. Gel pieces were dried with a Speed-Vac at 30°C, rehydrated with trypsin (100 ng μl⁻¹) in 25 mM ammonium carbonate, and incubated overnight at 37°C. The gel pieces were then removed from the supernatant and the peptides were extracted by repeatedly vortexing and sonicating in 50% acetonitrile containing 5% formic acid. MALDI-TOF mass spectrometry was performed as previously described (Junker et al., 2006).

Fluorescence microscopy

Induced, log-phase cultures of HB101 cells expressing wild-type pertactin, Cys-pair constructs, or nothing (none) were collected by centrifugation at 1000 g for 5 min, resuspended and washed twice in phosphate-buffered saline (PBS). Live cells were applied to poly-L-lysine-coated coverslips for 30 min, washed three times with PBS, incubated with the anti-pertactin polyclonal antibody for 10 min, washed three additional times with PBS, followed by a 10 min incubation with the Cy3-conjugated secondary antibody. Images were collected using an Applied Precision DeltaVision Core fluorescence microscope (1.42 numerical aperture, 100× objective), and deconvolved using constrained iterative deconvolution. Exposure time was 40 ms.

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