

Bacterial outer membrane secretin PulD assembles and inserts into the inner membrane in the absence of its pilotin

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Dodecamerization and insertion of the outer membrane secretin PulD is entirely determined by the C-terminal half of the polypeptide (PulD-CS). In the absence of its cognate chaperone PulS, PulD-CS and PulD mislocalize to the inner membrane, from which they are extractable with detergents but not urea. Electron microscopy of PulD-CS purified from the inner membrane revealed apparently normal dodecameric complexes. Electron microscopy of PulD-CS and PulD in inner membrane vesicles revealed inserted secretin complexes. Mislocalization of PulD or PulD-CS to this membrane induces the phage shock response, probably as a result of a decreased membrane electrochemical potential. Production of PulD in the absence of the phage shock response protein PspA and PulS caused a substantial drop in membrane potential and was lethal. Thus, PulD-CS and PulD assemble in the inner membrane if they do not associate with PulS. We propose that PulS prevents premature multimerization of PulD and accompanies it through the periplasm to the outer membrane. PulD is the first bacterial outer membrane protein with demonstrated ability to insert efficiently into the inner membrane.

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Introduction

The secretin PulD is the only integral outer membrane component of the type II secretion system (T2SS) or secretin by which the Gram-negative bacterium *Klebsiella oxytoca* secretes the amylolytic enzyme pullulanase (d'Enfert *et al*, 1989). Thus, PulD probably forms the conduit through which pullulanase crosses the outer membrane. Like other secretins involved in type II (Bitter *et al*, 1998; Brok *et al*, 1999) and

type III (Crago and Koronakis, 1998; Burghout *et al*, 2004) secretion, type IV pilus biogenesis (Bitter *et al*, 1998; Collins *et al*, 2004) and filamentous bacteriophage secretion (Opalka *et al*, 2003), PulD forms multimers, in this case, a dodecamer (Nouwen *et al*, 1999; Chami *et al*, 2005). When examined by electron microscopy, PulD dodecamers resemble a ca 15 nm deep cylinder with a central plug (Chami *et al*, 2005). Secretin multimers do not dissociate in SDS, and some, including PulD, do not even dissociate at 100°C. Unlike classical outer membrane proteins that are extracted by at least some non-ionic detergents, only the ionic, denaturing detergent SDS (Hardie *et al*, 1996a; Bitter *et al*, 1998) and zwitterionic detergents such as ZW3-14 at high salt concentrations solubilize PulD (Nouwen *et al*, 1999).

A dodecameric core domain of PulD, PulD-C, obtained by limited proteolysis with trypsin (Nouwen *et al*, 2000; Chami *et al*, 2005) and comprising amino acids 262–616 of the 660 amino-acid polypeptide, with a single nick after amino acid 297, includes the integral outer membrane- and plug-forming regions (Chami *et al*, 2005). The region N-terminal to this domain, the N domain, constitutes part of the outer membrane-distal wall of the cylinder that plunges deep into the periplasm (Nouwen *et al*, 1999, 2000; Chami *et al*, 2005). These features are consistent with a model in which secretins form a closed outer membrane channel that opens to allow the selective release of specific exoproteins such as pullulanase from the periplasmic compartment. The driving force for secretion could be the piston-like motion of an elongating and retracting pilus-like structure, the pseudopilus, of which PulG is the major component in *K. oxytoca* (Köhler *et al*, 2004; Chami *et al*, 2005).

The region C-terminal to the C domain of PulD, the S domain, is the binding site for a lipoprotein chaperone, the pilotin PulS (Daefler *et al*, 1997), which is specifically required to protect PulD from proteolysis and to direct it to the outer membrane (Hardie *et al*, 1996a,b). Twelve copies of PulS copurify with the PulD dodecamer during purification (Nouwen *et al*, 1999, 2000). In the absence of PulS, PulD associates with the inner membrane and induces the phage shock response, resulting in the appearance of large amounts of PspA protein (Hardie *et al*, 1996a,b). Other *Klebsiella*-derived proteins are not required for PulD multimerization and outer membrane localization in *Escherichia coli*, but secretin assembly in the T2SSs of some other bacteria requires ancillary factors, notably the ExeA and ExeB proteins in *Aeromonas hydrophila* (Ast *et al*, 2002) and the ExeB-like protein OutB in *Erwinia chrysanthemi* (Condemine and Shevchik, 2000), the lipoproteins Tgl and PilW in *Myxococcus xanthus* and *Neisseria meningitidis*, respectively (Carbonnelle *et al*, 2005; Nudleman *et al*, 2006), and the general outer membrane insertion factor Omp85 in *N. meningitidis* (Voulhoux *et al*, 2003; reviewed in Bayan *et al*, 2006).

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Peptide insertions in the C domain of PulD disrupt its ability to form multimers (Guilvout *et al*, 1999). Knowledge of the precise junction between the N and C domains (Chami *et al*, 2005) allowed us to amplify, clone and express DNA encoding only the C and S domains fused to the PulD signal peptide and the first 14 amino acids of PulD that are required for its export. We report studies of the multimerization, targeting and membrane insertion of this PulD fragment (hereafter referred to as PulD-CS) and of the complete PulD multimer.

Results

PulD-CS is targeted to the outer membrane together with PulS

Two forms of PulD-CS were generated in this study, one without additional amino acids and one with a His tag inserted in the S-domain (PulD-CS_{His}), as in the previously-characterized full-length PulD_{His} (Chami *et al*, 2005) (see Materials and methods). PulD-CS and PulD-CS_{His} exhibited identical properties and were used interchangeably. Total cell extracts of *E. coli* producing PulD-CS together with PulS were treated with phenol to dissociate any PulD-CS multimers (Hardie *et al*, 1996a) and examined by SDS-PAGE (on a 10% acrylamide gel) and immunoblotting. A single immunoreactive band that was absent from control cells without PulD-CS was detected at ca 46 kDa (not shown). Analysis of membrane fragments separated by floatation through sucrose gradients revealed PulD-CS predominantly in the dense, outer membrane fractions (Supplementary Figure S1A), as is full-length PulD (Hardie *et al*, 1996a; Supplementary Figure S1B). Thus, PulD-CS localizes to the outer membrane in cells producing PulS_{His}. However, PulD-CS was unable to substitute for PulD to promote pullulanase secretion (not shown).

To determine whether the properties of PulD were affected by removal of the N domain, membranes containing PulD-CS were treated with a range of detergents under different conditions. Four conditions lead to quantitative extraction of dodecameric PulD from *E. coli* envelope preparations: the ionic detergent SDS, the zwitterionic detergent *N*-tetradecyl *N,N*-dimethyl-3-ammonio-1-propanesulfonate (ZW3-14) with 0.25 M NaCl at pH 7.5 or 8 or without salt at pH 10, and simultaneous action of the nonionic detergent octylpolyoxyethylene (octylPOE) and lysozyme (25 µg/ml) at pH 10. Lysozyme cannot replace NaCl for extraction by ZW3-14 at pH 7.5 or 8, and high salt does not permit PulD extraction by octylPOE with lysozyme at pH 7.5–8 (Supplementary Figure S2 and data not shown).

PulD-CS in cell envelopes with PulS was extracted exclusively under conditions that also permitted PulD extraction; that is, ZW3-14 with NaCl at pH 7.5 and octylPOE with lysozyme at pH 10, but not ZW3-14 without NaCl at pH 7.5, or octylPOE without lysozyme at pH 10 or at pH 7.5 (Table I). Thus, the properties of PulD that contribute to its unusual requirements for extraction from the outer membrane are entirely determined by the C (and S) domains.

PulD-CS forms PulD-like multimers in the outer membrane

The amount of PulD-CS monomer detected by immunoblotting was considerably lower when the cell extracts were not treated with phenol, suggesting the presence of PulD-CS multimers with properties similar to those of PulD (Hardie *et al*, 1996a). PulD-CS multimers were not detected by immunoblotting, however (data not shown). Therefore, PulD-CS_{His} was produced in *E. coli* together with PulS, solubilized in ZW3-14 with NaCl, purified by cobalt affinity chromatography and examined by SDS-PAGE and Coomassie blue staining. Substantial amounts of a slow-migrating protein were detected (indicated by an arrow in Figure 1, lane 3).

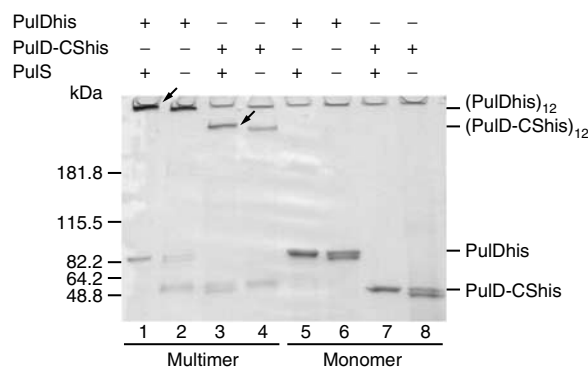


Figure 1 SDS-PAGE analysis of PulD_{His} and PulD-CS_{His} purified by cobalt affinity chromatography from envelopes of *E. coli* cells with and without PulS or PulS_{His}. The strains used were PAP105(pCHAP585) (with PulS) or PAP5198 (without PulS). PulD_{His} and PulD-CS_{His} multimers ((PulD_{His})₁₂ and (PulD-CS_{His})₁₂, respectively) were dissociated into monomers (PulD_{His} and PulD-CS_{His}) with phenol. Proteins were separated on a 4–15% acrylamide gradient gel and stained with Coomassie blue. The positions of molecular size markers (kDa) are shown on the left. Arrows indicate the multimeric forms of PulD_{His} and PulD-CS_{His}. Note that PulD_{His} and PulD-CS_{His} migrate more slowly than expected from their mass (68.7 and 45.6 kDa) in this electrophoresis system.

Table I Extraction of PulD and PulD-CS by different detergents from strains with and without PulS

	ZW3-14 NaCl pH 7.5	ZW3-14 pH 7.5	octylPOE pH 10	octylPOE lysozyme pH 10	octylPOE lysozyme pH 7.5
PulD/PulS _{His}	+	–	–	+	–
PulD-CS _{His} /PulS	+	–	–	+	–
PulD-CS _{His}	+	–	–	+	–
OmpA	+	(+)	(+)	+	(+)
SecG	+	+	+	+	+

Detergents were used at 3%, NaCl at 250 mM and lysozyme at 50 µg/ml. Buffers were 50 mM Tris-HCl (pH 7.5) or CHES-NaOH (pH 10.0). Total membranes were incubated in the different conditions during 1 h. The soluble fraction was separated from the debris by ultracentrifugation. Equal volumes of both fractions (pellet and supernatant) were treated with phenol. The amount of PulD or PulD-CS_{His} and SecG solubilized was estimated after immunoblotting. The distribution of OmpA in the two fractions was deduced after Ponceau red staining of the nitrocellulose sheet. +, >70% in soluble fraction; (+), 25–70% in soluble fraction; –, <25% in soluble fraction (see Supplementary Figure S1).

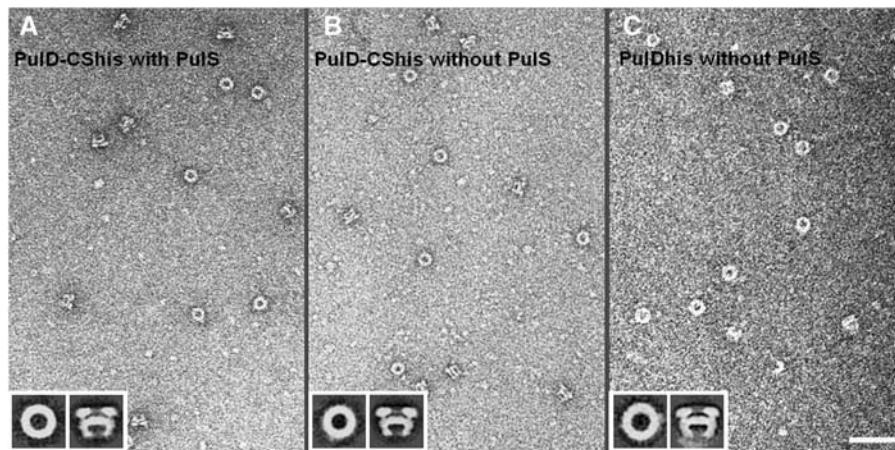


Figure 2 Electron microscopy of purified PulD-CSHis and PulDHis particles negatively stained with uranyl acetate. (A) PulD-CSHis purified from membranes of *E. coli* PAP105 with PulS; inset, averaged images of two major classes: top views ($n = 171$) and side views ($n = 233$). (B) PulD-CSHis purified from membranes of *E. coli* PAP5198 without PulS; inset, averaged images of two major classes: top views ($n = 177$) and side views ($n = 300$). (C) PulDHis purified from *E. coli* PAP5198 without PulS; inset, averaged images of two major classes: top views ($n = 272$) and side views ($n = 253$). The scale bar corresponds to 50 nm and the inset baseline corresponds to 25 nm.

This band migrated faster than PulDHis (compare lanes 1 and 3 in Figure 1) but did not react with anti-PulD (not shown).

Phenol treatment of the purified protein prior to SDS-PAGE caused these multimers to disappear and generated a band (Figure 1, lane 7) that migrated faster than monomeric PulDHis (compare lanes 5 and 7 in Figure 1) and reacted with PulD antibodies (not shown). We conclude that PulD-CSHis forms large, defined multimers and that PulD antibodies (Hardie *et al*, 1996a) react with PulD-CSHis only when it is dissociated.

To determine whether the PulD-CSHis multimers are structurally similar to C domain dodecamers obtained by proteolysis, negatively stained samples of purified PulD-CSHis complexes were examined by transmission electron microscopy. Abundant ring-like structures almost indistinguishable from those of trypsin-resistant PulD-C dodecamers (Nouwen *et al*, 1999; Chami *et al*, 2005) were observed (Figure 2A). These particles were 14 nm in diameter and had a stain-filled 7 nm diameter cavity, indicating that, like PulD (Chami *et al*, 2005), they are dodecameric. Many cup-and-saucer-like side-views of individual PulD-CS particles were observed (Figure 2A), in contrast to the low amounts of almost exclusively 'cup-to-cup' double complexes previously found in PulD-C (Chami *et al*, 2005). This change in behaviour suggests that amino acids present in PulD-CS but absent from PulD-C obtained by proteolysis (i.e., the S domain and additional amino acids at the N-terminus) prevent double dodecamer formation. These data indicate that the N domain influences neither the outer membrane targeting nor the multimerization of PulD.

PulS is not required to form PulD or PulD-CS multimers

Previous studies indicated the formation of a PulD complex in the absence of PulS (Hardie *et al*, 1996b), but it was not characterized and could correspond to heterogeneous aggregates. PulD-CSHis was partially degraded in the absence of PulS (not shown), as reported previously for PulD (Hardie *et al*, 1996b). A protease-deficient strain (PAP5198) was used to reduce this problem. PulD-CSHis produced by this strain without PulS was extracted from the cell envelope with ZW3-14 with NaCl, purified by cobalt affinity chroma-

tography and examined by SDS-PAGE. Data in Figure 1 revealed the presence of a PulD-CSHis multimer that co-migrated with PulD-CSHis produced by cells with PulS (lanes 3 and 4). Thus, PulD-CSHis forms discrete multimers in the absence of PulS. Likewise, PulDHis migrated as a discrete multimeric band in the absence of PulS (Figure 1, lane 2). When dissociated by phenol (lane 8), PulD-CSHis without PulS migrated as two bands. N-terminal sequencing of each band revealed a unique sequence (EEF) corresponding to the N-terminus of the short N-terminal region of PulD that is retained in this construct, indicating that the faster-migrating band results from proteolysis near the C-terminus.

Electron microscopy of negatively stained samples of PulD-CSHis and PulDHis complexes from envelopes of the strain without PulS revealed multimers that were virtually indistinguishable from those of the same protein produced in a strain with PulS (Figure 2B and C). The diameters of the PulD-CSHis particles were identical (14 nm) to those of the same particles purified from the outer membrane. The external diameter of the PulDHis particles was larger (16 nm), as expected from previous studies (Nouwen *et al*, 1999; Chami *et al*, 2005).

PulS prevents mislocalization of PulD-CS and PulD to the inner membrane

Immunoblotting of phenol-treated fractions obtained after floatation sucrose gradient centrifugation of envelopes from the protease-deficient strain revealed PulD-CS and full-length PulD monomers predominantly in the inner membrane fraction in the absence of PulS (Supplementary Figure S1C and D). To determine whether these monomers were created by phenol dissociation of PulD-CS and PulD multimers, respectively, proteins were examined by SDS-PAGE without phenol extraction and stained with Coomassie blue. In both cases, a distinct, slow-migrating band was observed (Figure 3) that was absent from control cells without PulD or PulD-CS. Furthermore, these multimers were found exclusively in the same fractions as the authentic inner membrane proteins SecG (detected by immunoblotting) and PspA (detected by protein staining), while outer membrane porins and OmpA were found exclusively in the outer membrane fraction by

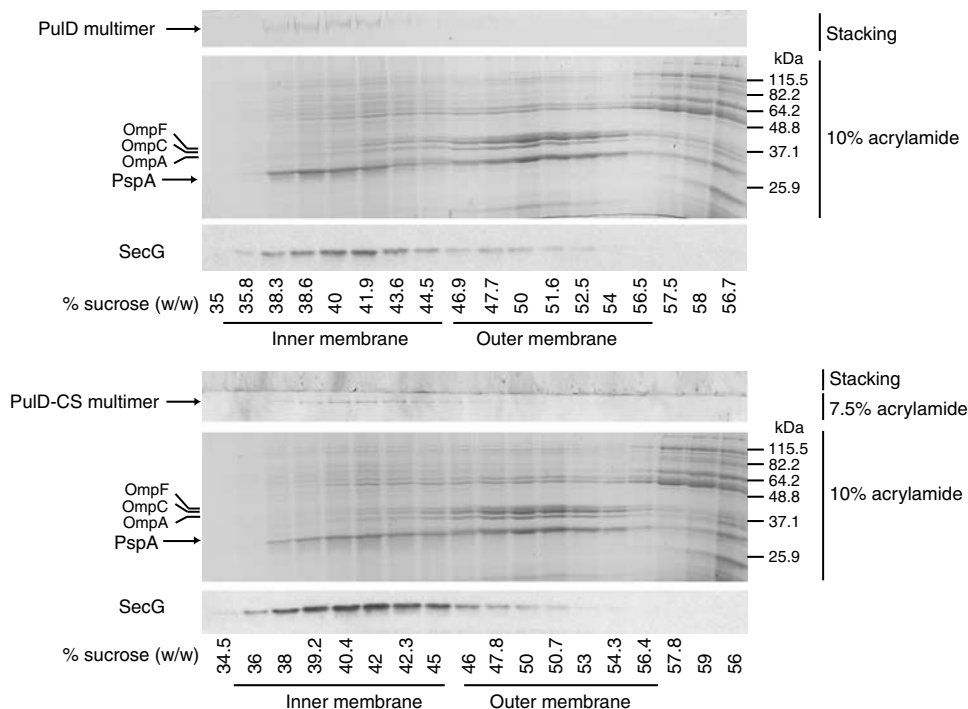


Figure 3 Inner membrane localization of PulD (upper panel) and PulD-CS (lower panel) multimers produced in the absence of PulS and DegP detected by separating membranes in sucrose floatation gradients. Fractions from the gradients were loaded onto 7.5 or 10% acrylamide-SDS gels and stained with Coomassie blue (note that PulD-CS multimers were detected using the 7.5% acrylamide gel whereas PulD multimers were detected in the stacking gel). SecG was detected by immunoblotting with specific antibodies. The outer membrane fractions contain PspA and SecG. The band migrating close to the position of PspA but in the outer membrane fraction is not PspA.

Coomassie blue staining (Figure 3). These data rule out the possibility that PulD-CS or PulD aggregate when PulS is absent, since aggregated proteins remain at the bottom of the centrifuge tube, or that the inner membrane fraction is contaminated with outer membranes. Furthermore, nonaggregated secretin-like particles identical to those purified from total membranes were found exclusively in the inner membrane fractions from these gradients (not shown). Thus, PulD-CS and PulD multimers both localize to the inner membrane in the absence of PulS.

To determine whether the PulD and PulD-CS multimers were intimately associated with inner membrane vesicles, they were analysed directly by electron microscopy of negatively stained, large inner membrane vesicles obtained in a cell disrupter. Approximately 5% of the vesicles displayed a few secretin-like rings (Figure 4) that were clearly separated and completely absent from inner membrane vesicles without secretin. Some smaller inner membrane vesicles obtained by disrupting cells in a French pressure cell contained one or two secretin particles (Supplementary Figure S3), confirming that the particles seen in the larger vesicles (Figure 4) were not physically linked. The diameter of the secretin particles was close to that of the purified particles. The stain-filled centre of the barrel has a diameter of 7 nm, as in the purified particles. The vesicles containing secretin rings were clearly different from outer membrane sheets (Supplementary Figure S4).

Localization of PulD-CS to the inner membrane does not change its solubilization properties

Data in Figure 4 indicate the PulD-CS multimers are inserted into the inner membrane in the absence of PulS. To validate

this interpretation, cells of strain PAP5198 producing PulD-CSHis without PulS were resuspended in different concentrations of urea, which should release peripheral membrane proteins but not integral inner membrane proteins, and then disrupted in a French pressure cell. Addition of urea prior to disruption should enable it to access both inner and outer surfaces of the inverted inner membrane vesicles that form under these conditions. Total cell proteins and proteins in the soluble (supernatant) and sedimented (pellet) fractions separated by ultracentrifugation were extracted with phenol and examined by SDS-PAGE and immunoblotting. The peripheral inner membrane protein PspA (see below) was extracted efficiently by 4 M urea and partially by 2 M urea, whereas the integral inner membrane protein SecG was not (Figure 5). Only small amounts of PulD-CSHis were extracted by urea, indicating that it behaves like an integral inner membrane protein. Urea (4 M) was also unable to extract PulDHis from the inner membranes of cells without PulS (data not shown). Inspection of membranes after extraction with 4 M urea did not reveal any change in the numbers or distribution of secretin particles (not shown). Only detergents that extracted PulD or PulD-CSHis from the outer membrane extracted PulD-CSHis from the inner membrane of cells without PulS (Table 1).

PulD-CS induces the phage shock response

The phage shock response, characterized by the massive production of the 26 kDa peripheral membrane protein PspA, is induced following permeabilization of the *E. coli* inner membrane (Darwin, 2005). Production of PulD-CS in the absence of PulS caused the appearance of a prominent

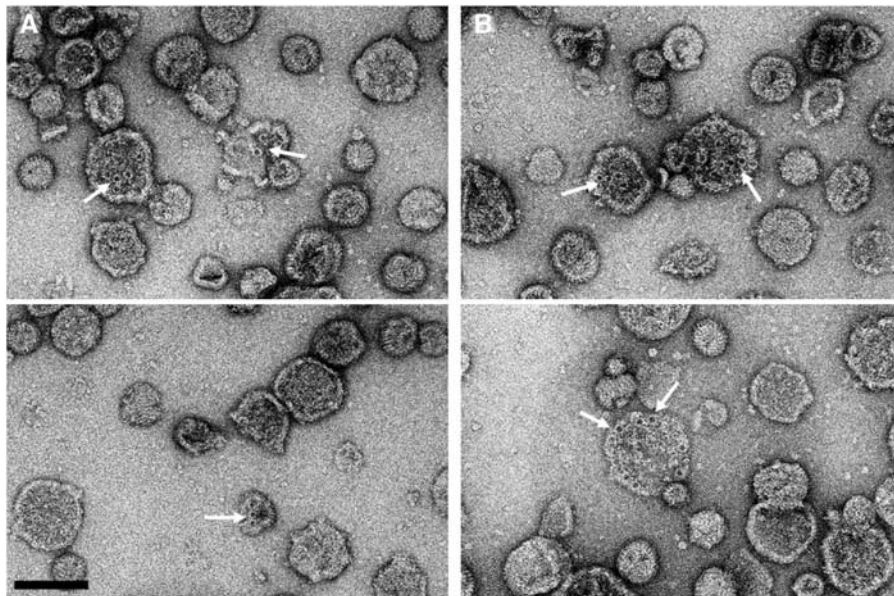


Figure 4 Electron microscopy of inner membrane vesicles purified from protease-deficient *E. coli* producing PulD (A) or PulD-CSHis (B) without PulS. Arrows indicate ring-like particles corresponding to secretin particles. The scale bar corresponds to 100 nm.

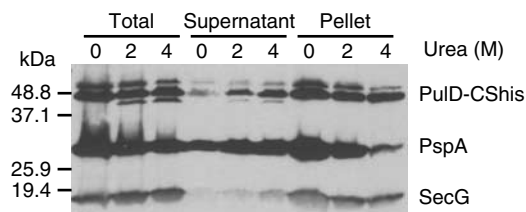


Figure 5 Extraction of PulD-CSHis, PspA and SecG with urea. Samples were treated with phenol and then subjected to SDS-PAGE and immunoblotting with specific antibodies. Samples were derived from the same amount of starting material. Size markers (kDa) are indicated on the left.

26 kDa inner membrane protein (Figure 3) that we identified as PspA (Brissette *et al*, 1990) by immunoblotting (Figure 6). Thus, insertion of PulD-CS into the inner membrane induces the phage shock response. Variants of PulD with substantially reduced ability to form multimers due to the insertion of a 24 amino-acid peptide in the C domain (Guilvout *et al*, 1999) did not induce PspA production when produced without PulS (two examples are shown in Figure 7), whereas variant D640::TEV, which formed multimers efficiently (Guilvout *et al*, 1999), induced PspA production (Figure 7).

The induction of the phage shock response would be consistent with PulD-induced partial dissipation of the transmembrane electrochemical potential ($\Delta\psi$) (Kleerebezem *et al*, 1996). To test this idea, the $\Delta\psi$ of *E. coli* cells producing PulD or PulD-CS without PulS was measured by their ability to accumulate ^3H -triphenyl phosphonium (TPP^+) (Possot *et al*, 1997). Production of PulD or PulD-CS (encoded by the high copy number pUC18-derived plasmids pCHAP3671 and pCHAP3711 under *lacZp* control) in the absence of PulS consistently caused a modest (ca 10%) drop in the $\Delta\psi$ (not shown). These plasmids could not be introduced into strain MC4100 ΔdegP *pspA::km F'lacI^{q1}* (lacking DegP and PspA), despite the presence of the *lacI^{q1}* allele to repress *lacZp*

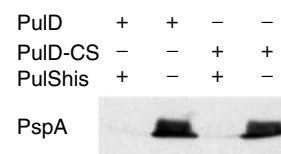


Figure 6 Induction of PspA caused by production of PulD or PulD-CS in the absence of PulS. Proteins from pooled inner membrane fractions (Supplementary Figure S1) were separated by SDS-PAGE and immunoblotted with PspA-specific antibodies.

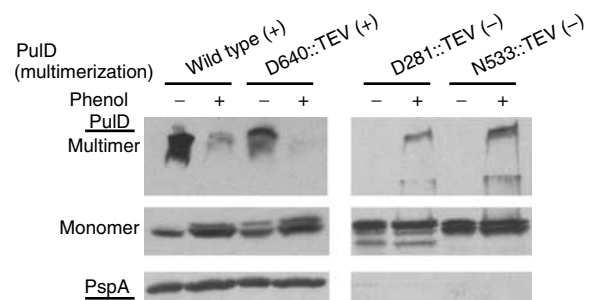


Figure 7 PspA induction by PulD derivatives with linker insertions in the C or S domains. Total cell extracts were analysed by SDS-PAGE with or without phenol treatment. Immunoblots were developed with antibodies against PulD and PspA. All extracts are derived from the same amount of starting material.

promoter activity. However, this strain could be transformed with the low copy number plasmid pCHAP362 in which *pulD* is also under *lacZp* control (d'Enfert *et al*, 1989). When grown in liquid cultures, this strain showed a substantial drop in $\Delta\psi$ 2 h after IPTG-induced PulD production (Table II). This drop in $\Delta\psi$ is probably insufficient to kill the cells, implying that the $\Delta\psi$ drops further when PulD production is continued or that other events, such as futile ATP hydrolysis to restore the $\Delta\psi$, caused cell death.

Table II Measurement of $\Delta\psi$ in cells of strain MC4100 *F'lacI^{q1} ΔdegP* with or without *pspA::Km* producing pCHAP362-encoded PulD

DegP	PspA	PulD	³ H-TPP ⁺ in/out	$\Delta\psi$ (mV)
–	+	–	703	171
–	+	+	654	169
–	–	–	553	165
–	–	+	133	127

Cells were grown exponentially for 2 h in the presence of IPTG before being washed and resuspended in 0.1 M phosphate buffer with 0.4% glycerol and 1 mM KCl (pH 7.6). Accumulation of ³H-TPP⁺ was measured as the ratio of radioactivity inside the cells and outside and used to calculate the $\Delta\psi$. Cells without PulD carried the vector DNA.

Discussion

The C-terminal half of the outer membrane secretin PulD (PulD-CS) is an autonomous multimerization and membrane insertion module. The N-terminal half of the polypeptide does not influence either of these properties. The pilotin protein PulS, which binds to the S domain, is not required for correct multimerization but targets secretin to the outer membrane since, in its absence, apparently normal PulD multimers assemble in the inner membrane.

Four independent lines of evidence indicate that PulD and/or PulD-CS inserts into, rather than associates with the inner membrane in the absence of PulS. First of all, very little PulD-CS or PulD is stripped from inner membrane vesicles by urea at concentrations that remove most of the peripheral membrane protein PspA. Second, only detergent extraction conditions that release PulD-CS from the outer membrane (of cells with PulS) can release it from the inner membrane (of cells without PulS). Indeed, the conditions required to extract PulD and PulD-CS are much more drastic than those needed to solubilize other outer membrane proteins, suggesting that factors other than merely dissolving the lipids around the secretin complex and protecting the hydrophobic regions thereby exposed are involved.

Third, electron micrographs revealed PulD-CS or PulD complexes in around 5% inner membrane vesicles. Large vesicles often contained several secretins that were not in direct contact and were in the same orientation, indicating that they are not micro-aggregates and are inserted into the membrane, rather than lying on the surface. Small vesicles (obtained in a French pressure cell) contained only single secretin particles (data not shown), indicating that the limited clustering of secretin particles in large vesicles does not reflect their physical association.

Finally, the induction of the phage shock response by cells producing PulD (or PulD-CS) without PulS is consistent with the formation by the former of a channel in the inner membrane that decreases the $\Delta\psi$ across this membrane (Kleerebezem *et al*, 1996). Although a high level production of export-defective outer membrane proteins or defects in the Sec protein export pathway can also induce high level PspA production, (Kleerebezem and Tommassen, 1993), the underlying mechanisms remain unclear. Induction of the phage shock response by mislocalized PulD cannot be explained by a defect in PulD export across the inner membrane by the Sec system, since PulS probably affects only PulD transport to the outer membrane. PulD has a plug in the centre of the

oligomer (Chami *et al*, 2005) but electrophysiological data indicate that a small conductance can be detected in lipid bilayers containing PulD when a voltage is applied across the membrane (Nouwen *et al*, 1999). The electrical potential across the inner membrane might cause these channels to open, allowing protons to leak into the cell. The massive amounts of PspA protein produced might prevent complete dissipation of the membrane potential (Hankamer *et al*, 2004), allowing the cells to remain viable despite the presence of secretin channels in the inner membrane (Model *et al*, 1997). This would be entirely consistent with the observed lethality of PulD in cells lacking PspA and PulS.

The fact that unusually harsh conditions are required to solubilize PulD from both the outer and inner membrane (see above) suggests that it might have an atypical membrane anchor or an unusually strong interaction with membrane lipids. Like other outer membrane proteins, PulD could be anchored in the membrane by antiparallel amphipathic β -strands. PulD has rather less β -strand structure than other outer membrane proteins, consistent with the fact that a large proportion of the protein is exposed on the periplasmic side of the membrane (Chami *et al*, 2005), as in the trimeric TolC protein (Koronakis *et al*, 1997). In this scenario, multimerization and membrane insertion and organization could be similar to that of the heptameric *Staphylococcus aureus* haemolysin (Montoya and Gouaux, 2003). Alternatively, like the *E. coli* Wza protein involved in capsular polysaccharide excretion, which forms rings that are superficially similar to PulD dodecamers (Beis *et al*, 2004; Dong *et al*, 2006), the transmembrane segments of PulD could be amphipathic α -helices. Since the structures of PulD-CS and PulD in the inner and outer membranes are apparently identical, it seems reasonable to assume that the same β -strands or α -helices span the lipid bilayer irrespective of the membrane in which PulD is inserted.

Different secretins require different proteins for multimerization and outer membrane insertion (reviewed in Bayan *et al*, 2006). Formation of stable PilQ multimers in *Neisseria* requires the lipoprotein PilW (Carbonnelle *et al*, 2005). PilW is not needed for PilQ outer membrane association, however (Carbonnelle *et al*, 2005). Likewise, Tgl, which is related to PilW, is required for multimerization of the PilQ secretin in *Myxococcus xanthus* (Carbonnelle *et al*, 2005; Nudleman *et al*, 2006). In *A. hydrophila*, multimerization of the T2SS secretin ExeD requires the inner membrane proteins ExeA (an ATPase) and ExeB (Ast *et al*, 2002). The pullulanase T2SS does not have Tgl/PilW homologues, and the ExeB homologue, PulB, is not required for pullulanase secretion (Possot *et al*, 2000). Thus, PulD differs from PilQ and ExeD in that dodecamer formation occurs in the absence of all other components of the system of which it is part. Furthermore, PulS differs from PilW and Tgl in that it is required to target its cognate secretin to the outer membrane, and not for multimerization. It seems likely, therefore, that secretins such as PilQ require additional, as yet unidentified factors to modulate multimerization and to target them to the outer membrane.

Omp85/YaeT, an essential outer membrane protein, facilitates insertion of integral outer membrane proteins and porin trimerization (Voulhoux *et al*, 2003; Doerrler and Raetz, 2005; Ruiz *et al*, 2005; Werner and Misra, 2005; Wu *et al*, 2005). *N. meningitidis* PilQ fails to multimerize in the absence of

Omp85 (Voulhoux *et al*, 2003), suggesting that Omp85 and PilW have overlapping functions (Bayan *et al*, 2006). However, since PulD forms dodecamers in the inner membrane in the absence of PulS, and, therefore, without contact with outer membrane protein YaeT (Omp85), it seems reasonable to assume that PulD multimerization and membrane insertion can be independent of YaeT and, indeed, of any other protein. This raises the question of why PulD and PulD-CS insert exclusively into the inner membrane, rather than into both membranes, when PulS is absent. A likely explanation is that PulS binds rapidly to PulD monomers emerging from the inner membrane to prevent their multimerization, and that the lipoprotein sorting pathway (Tokuda and Matsuyama, 2004) leads them immediately to the outer membrane. PulS binding to the outer membrane receptor LolB could trigger a conformational change in the PulD–PulS complex that allows PulD multimerization and insertion. The naturally constitutive expression of the *pulS* gene (d'Enfert and Pugsley, 1989) ensures that PulS is present before PulD synthesis is switched on by maltodextrins in the culture medium (d'Enfert *et al*, 1987). In the absence of PulS, PulD multimers would assemble in the inner membrane by default because they would be too large to diffuse through the peptidoglycan (Demchick and Koch, 1996). Production of plasmid-encoded PulD causes low level induction of the phage shock response even in the presence of PulS (Hardie *et al*, 1996a,b), suggesting that a small amount of PulD is incorrectly localized despite the presence of its pilotin.

These observations raise the question of whether other outer membrane proteins can or do insert into the inner membrane and the role that Omp85/YaeT plays in outer membrane targeting. Misrouting of many outer membrane proteins to the inner membrane could be more detrimental than it is for secretins. For example, the creation of a porin channel in the inner membrane would almost certainly be lethal. At least three interlocking factors appear to prevent such events. First, the structures of intermediates in their folding pathway might enable porins to interact at an early stage with components exclusively located in the outer membrane, such as lipopolysaccharide (de Cock *et al*, 1990, 1996). Second, chaperones such as Skp (Chen and Henning, 1996; Schäfer *et al*, 1999; Harms *et al*, 2001) might prevent their premature folding (Bulieris *et al*, 2003) or illicit association with the inner membrane (Mogensen *et al*, 2005). In their absence, porins would aggregate or be degraded. Third, Omp85/YaeT and associated outer membrane proteins might be absolutely required for the final stages of their membrane insertion and folding (Voulhoux *et al*, 2003; Doerrler and Raetz, 2005; Wu *et al*, 2005); hence, ensuring that even if porins do associate with the inner membrane, they cannot insert in a functional, pore-forming configuration. Misrouting of porins to the inner membrane cannot be studied *in vivo* because of the associated lethality, but their insertion into proteoliposomes might provide a way to test the role of membrane proteins, lipopolysaccharide and chaperones. We note with interest that bacterial outer membrane porins can be reconstituted into lipid bilayers without outer membrane lipopolysaccharide (Kleinschmidt *et al*, 1999) and that routing of a mitochondrial outer membrane porin to the endoplasmic reticulum leads to the assembly of a functional form of this β -barrel protein in the Golgi membranes of eukaryotic cells (Buettner *et al*, 2000).

Materials and methods

Plasmid constructions

To construct plasmids encoding PulD-CS, pCHAP3671 (pUC18 carrying the complete *pulD* gene) (Guilvout *et al*, 1999) was cleaved with *EcoRI* and *BglII*. The fragment corresponding to *pulD* was then cleaved with *ApoI* and *PasI*. The fragments encoding amino acids 1–42 and 259–634 were purified and ligated in the presence of oligonucleotides 5'-AATTTCTCGACCGCCAGCAGCGG ACC-3' and 5'-CTGGGTCCGCTGCTGGCGGTCCGAGA-3'. The resulting fragment was ligated back into pCHAP3671 cleaved with *EcoRI* and *BglII* to give pCHAP3711. To create pCHAP3713, carrying a 6 His codon insert, pCHAP3711 was cleaved with *EcoRI* and *HpaI* and the liberated fragment coding for the N-terminal region of PulD-CS was ligated into pCHAP3678 (pSU18 encoding PulD-his) (Chami *et al*, 2005) cleaved with the same enzymes. The *EcoRI*–*HindIII* fragment from pCHAP3678 and pCHAP3713 were subcloned into pUC18 to give pCHAP3715 (encoding PulD-his) and pCHAP3714 (PulD-CShis), respectively.

Strains, other plasmids and growth conditions

E. coli K-12 strain PAP105 (Guilvout *et al*, 1999) was used for plasmid construction and verification. Strain PAP105 carrying pCHAP3516 (*pulD*) (Daefler *et al*, 1997) and pCHAP5506 (encoding PulS-his) (Chami *et al*, 2005) was used for experiments on the extraction of PulD from the outer membrane. In other cases, plasmid pCHAP585 (Guilvout *et al*, 1999) was used as a source of *pulS*. Strain PAP105(pCHAP1226) carrying all *pul* genes except *pulD* in the chromosome (Possot *et al*, 2000) was used for complementation assays. *E. coli* K-12 strain SF120 lacking DegP, OmpT and Ptr protease (Meerman and Georgiou, 1994) and carrying F' *lacI^{q1}* Tn10 (strain PAP5198) was used to examine proteins in the inner membrane. Derivatives of *E. coli* K-12 strain MC4100 (*araD139 lacU169 relA1 rpsL150 thi mot fliB-5301 deoC ptsF25 rbsR*) carrying *pspA::km* (M Russel) and/or *Δ degP* (N Sassoon and J-M Betton) together with F' *lacI^{q1}* Tn10 and pCHAP362 (*lacZp-pulD*; Cm^R, (d'Enfert *et al*, 1989)) were used to measure the $\Delta\psi$. Plasmids carrying TEV insertions in *pulD* were described elsewhere (Guilvout *et al*, 1999). Bacteria were grown in LB medium (Miller, 1992) containing appropriate antibiotics (100 μ g/ml ampicillin, 25 μ g/ml chloramphenicol) at 30°C with vigorous aeration. Isopropyl-thio- β -galactoside (IPTG) (0.5 mM) was used to induce expression of genes under *lacZp* control. Maltose (0.4%) was used to induce expression of *pul* genes other than *pulS* (which is constitutively expressed).

SDS-PAGE and immunoblotting

Proteins dissolved in buffer containing 2.5% SDS, heated to 100°C for 5 min and separated by SDS-PAGE in 7.5, 10 or 12% acrylamide or on 4–15% acrylamide gradient gels (BioRad) using Tris–HCl–glycine buffers. After electrophoresis, proteins were either fixed and stained with Coomassie blue or were electrotransferred onto nitrocellulose membranes and reacted with PspA, SecG or PulD-specific antibodies and then with horseradish peroxidase-coupled secondary antibodies (Amersham). Bound antibodies were detected by enhanced chemiluminescence (Amersham). Phenol extraction was performed as previously (Hardie *et al*, 1996a). For N-terminal sequence analysis, proteins were transferred onto PVDF membranes (Millipore).

Membrane fractionation and extraction

Unless otherwise indicated, bacteria were broken in a French pressure cell (Aminco) at 1600 bar. A cell disrupter (Constant Systems) was sometimes used at 1200 bar to obtain larger vesicles. In both cases, membranes separated from the soluble fraction by centrifugation at 180 000 g for 30 min were fractionated by floatation through centrifuged sucrose gradients (Robichon *et al*, 2005). Membranes were extracted with detergents for 60 min at room temperature with constant mixing. Soluble material was separated from insoluble material by centrifugation for 30 min at 180 000 g. For extraction of inner membrane proteins with urea, bacteria were resuspended in 50 mM Tris–HCl buffer (pH 7.5) containing 1 mM EDTA or in the same buffer containing 2 M or 4 M urea for 15 min before being disrupted in the French press. Membranes were then separated from soluble proteins as above.

Purification

PulDhis-PulS and PulD-CShis-PulS complexes were purified by cobalt affinity chromatography from enriched outer membrane fractions as previously (Chami *et al*, 2005). PulD-CShis and PulDhis were similarly purified from total envelope fractions or from inner membrane vesicles purified by sucrose gradient fractionation, as above.

Electron microscopy and image processing

The inner and outer membrane vesicles purified by sucrose gradient fractionation were pelleted at 250 000g and the pellets were resuspended in 20mM Tris-HCl buffer (pH 7.5) containing 200mM NaCl to remove the sucrose. Vesicles were sometimes incubated in 4M urea for 30 min to remove surface-bound material and then centrifuged and resuspended as above. Aliquots (5 μ l) of samples were adsorbed onto glow-discharged 200-mesh carbon coated grid and stained with 2% (W/V) uranyl acetate. The micrographs were recorded at an accelerating voltage of 100 kV and a magnification of \times 50 000, using a Hitachi 7000 electron microscope. Purified secretin particles were examined by negative staining as previously (Nouwen *et al*, 1999, 2000). All micrographs were recorded on Kodak SO-163 film.

Reference-free alignment was performed on manually selected particles from digitized electron micrographs using the EMAN image processing package (Ludtke *et al*, 1999). After using a

reference-free alignment procedure, particle projections were classified by multivariate statistical analysis. The class averages with the best signal-to-noise ratio were selected.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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