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Insertion of an outer membrane protein in *Escherichia coli* requires a chaperone-like protein

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Only one of the characterized components of the main terminal branch of the general secretory pathway (GSP) in Gram-negative bacteria, GspD, is an integral outer membrane protein that could conceivably form a channel to permit protein transport across this membrane. PulD, a member of the GspD protein family required for pullulanase secretion by *Klebsiella oxytoca*, is shown here to form outer membrane-associated complexes which are not readily dissociated by SDS treatment. The outer membrane association of PulD is absolutely dependent on another component of the GSP, the outer membrane-anchored lipoprotein PulS. Furthermore, the absence of PulS resulted in limited proteolysis of PulD and caused induction of the so-called phage shock response, as measured by increased expression of the *pspA* gene. We propose that PulS may be the first member of a new family of periplasmic chaperones that are specifically required for the insertion of a group of outer membrane proteins into this membrane. PulS is only the second component of the main terminal branch of the GSP for which a precise function can be proposed.

Keywords: chaperone/general secretory pathway/outer membrane protein/phage shock response/PulD

Introduction

The main terminal branch of the general secretory pathway (GSP) is required for the secretion of a wide variety of extracellular proteins by Gram-negative bacteria. At least 14 different proteins are components of, or are implicated in, the assembly of the secretion machinery by which proteins are transported across the outer membrane. One of the most intriguing features of this branch of the GSP is that proteins are translocated across the outer membrane in a fully or almost fully folded conformation (Hirst and Holmgren, 1987; Pugsley, 1992; Bortoli-German *et al.*, 1994; Hardie *et al.*, 1995). This implies that their transport across this membrane differs, for example, from the translocation of locally unfolded secretory precursor proteins across the bacterial cytoplasmic membrane by the Sec pathway which forms the first part of the GSP (for review, see Pugsley, 1993).

GspD is the only integral outer membrane protein component of this branch of the GSP and thus the only component likely to form a channel that might permit protein transport across this membrane. Intriguingly, outer membrane proteins with a high degree of sequence identity to GspD are required for protein secretion via GSP-independent pathways in several Gram-negative bacteria (Michiels *et al.*, 1991; Allaoui *et al.*, 1993; Genin and Boucher, 1994), for transformation of *Haemophilus influenzae* (Tomb *et al.*, 1991) and for the assembly and secretion of filamentous bacteriophages such as f1 (Russel, 1994a), pili (Martin *et al.*, 1993) and S-layer (Thomas and Trust, 1995). All of these GspD homologues show particularly high levels of sequence conservation in their C-terminal halves, whereas their N-terminal halves are conserved only within members of the same family (Genin and Boucher, 1994).

Studies on the bacteriophage f1-encoded member of this family of proteins, gpIV, show that its C-terminal half is required for insertion into the outer membrane and that discrete multimers containing up to 12 monomers can be stabilized by chemical cross-linking reagents (Kazmierczak *et al.*, 1994). Russel has proposed that gpIV multimers in the outer membrane form the walls of a large gated channel through which the bacteriophage is extruded (Russel, 1994b). This implies that gpIV differs dramatically from the best characterized outer membrane proteins, the porins, which form stable homotrimers (Cowan *et al.*, 1992). Each porin monomer in the trimer forms a distinct channel which excludes molecules over ~650 Da (Cowan *et al.*, 1992), far smaller than a folded polypeptide or a filamentous bacteriophage. One would therefore expect gpIV and its homologues to assemble into the outer membrane in a completely different way to the porins. It may be relevant that gpIV and its homologues lack the C-terminal phenylalanine residue which is essential for the efficient outer membrane insertion and assembly of porins (Struyvé *et al.*, 1991). This again suggests that outer membrane insertion of GspD and related proteins occurs by a different mechanism from that proposed for porins (Eisele and Rosenbusch, 1990; Sen and Nikaido, 1990; de Cock *et al.*, 1990).

PulD and some of its homologues have been shown to induce the phage shock response, manifested by the appearance of large amounts of the protein PspA (Brisette *et al.*, 1990; Possot *et al.*, 1992). The phage shock response was originally reported to result from filamentous phage infection and was later shown to be due to the production of phage-encoded gpIV (Brisette *et al.*, 1990; Russel, 1994b). In addition, the phage shock response is induced by heat shock, osmotic shock, ethanol treatment (Brisette *et al.*, 1990) and lipid depletion (Bergler *et al.*, 1994). The precise induction signal is unknown, but it has been suggested that it may result from membrane bilayer

Table I. *Escherichia coli* K-12 strains carrying *pul* genes from *K.oxytoca* integrated into the chromosome at the 5′ end of the *malPQ* operon

Strain	Status of *pul* genes														
	S	*A*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*	*L*	*M*	*N*	*O*
PAP7232	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PAP7447	+	+	+	Δ	+	+	+	+	+	+	+	+	+	+	+
PAP7446	Tn*5*	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PAP7228	+	+	+	+	+	+	Δ	+	+	+	+	+	+	+	+
PAP7245	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Δ
PAP3175	+	+	+	+	Δ	+	+	+	+	+	+	+	+	+	+

These strains are all derived from the laboratory collection strain MC4100 [Δ(*lac-argF*)*U169 araD139 relA1 rps150*]. The *pul* genes were introduced into this strain by P1 transduction from strains described in d'Enfert *et al.* (1987); see also d'Enfert and Pugsley (1989) (*pulS*::Tn*5*), Pugsley and Dupuy (1992) (Δ*pulG3*, Δ*pulO*) and Possot *et al.* (1992) (Δ*pulE3*). The construction of the Δ*pulD* mutation is described in the text. All strains carry F′ lacI^{q1} pro^{+} Tn*10*. Δ represents a deletion.

Table II. Plasmids carrying *pul* genes under endogenous or *lacZ* promoter control

Plasmids	Vector	*pul* genes														
		S	*A*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*	*L*	*M*	*N*	*O*
pCHAP362[a]	pHSG575	–	–	–	*	–	–	–	–	–	–	–	–	–	–	–
pCHAP378[b]	pBR322	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
pCHAP710[c]	pACYC184	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+
pCHAP40[d]	pACYC184	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+
pCHAP231[d]	pBR322	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pCHAP230[d]	pEMBL9	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–

[a]d'Enfert *et al.* (1989); [b]d'Enfert and Pugsley (1989);[c]Kornaker and Pugsley (1989); [d]d'Enfert *et al.* (1987); +, gene present; *, gene present under *lacZp* control; –, gene absent.

perturbation, changes to the osmotic balance in the periplasm or increased proximity of the cytoplasmic and outer membranes (Brisette *et al.*, 1990; Bergler *et al.*, 1994; Russel, 1994b).

As a representative of the main terminal branch of the GSP, we have studied the secretion of the surface-anchored lipoprotein pullulanase produced by *Klebsiella oxytoca*. The genes encoding the pullulanase-specific components of the GSP have been cloned and shown to be functional in *Escherichia coli* (d'Enfert *et al.*, 1987). One of the cloned genes, *pulD*, codes for a member of the GspD family of proteins (d'Enfert *et al.*, 1989), and here we describe experiments showing that PulD forms SDS-resistant multimers. In addition, we demonstrate that the stability and association of PulD with the outer membrane depends on the presence of another Gsp protein, PulS (GspS), itself peripherally associated with the outer membrane (d'Enfert and Pugsley, 1989). We propose that the 12 kDa lipoprotein, PulS, is a member of a new family of membrane proteins with a periplasmic domain which has chaperone-like activity.

Results

PulD forms large, SDS-resistant, multimeric complexes

PulD was shown to exist in two forms by SDS–PAGE of whole cell extracts from wild-type *K.oxytoca* strain UNF5023 or *E.coli* bearing the complete *pul* gene complex on the chromosome (PAP7232; Table I) or cloned on a plasmid (pCHAP231; Table II). Whilst a monomeric form of PulD was clearly visible at the expected position (~69 kDa; d'Enfert *et al.*, 1989), >90% of the protein migrated very slowly and was retained within the stacking gel (see representative results in Figure 1). These complexes were not detected in earlier studies; thus PulD is far more abundant than previously assumed. Immunoblot analyisis of similar extracts with antisera raised against the major outer membrane proteins OmpF and OmpA, or with antisera raised against the Gsp proteins PulE, PulF and PulG, indicated the total absence of these proteins in

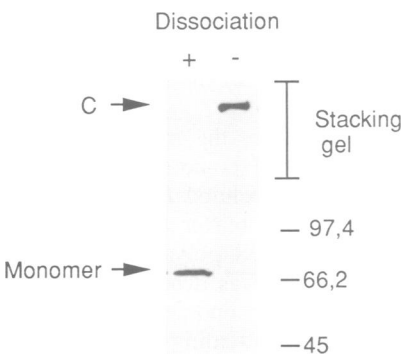

Fig. 1. Two forms of PulD protein. PAP7232 was grown to late exponential phase with maltose and the cells were collected by centrifugation for 2 min. One aliquot was resuspended directly in SDS–PAGE sample buffer (lane –) and the other was extracted with phenol before resuspension in SDS–PAGE sample buffer (lane +). Equivalent amounts of each sample were subjected to SDS–PAGE on a 9% acrylamide gel and separated proteins were electrotransferred onto nitrocellulose for immunoblot analysis with anti-PulD–PhoA85. Molecular size markers (kDa) are shown along with the position of the 4.5% stacking gel. Arrows mark the position of the multimeric complex (C) and monomeric forms of PulD.

the stacking gel. Proteins could be detected at the position of PulD in the stacking gel by staining with silver, irrespective of whether or not the cell extracts contained PulD. Trace amounts of lipid-containing material were also found at this position in extracts of cells metabolically labelled with [^{3}H]palmitate, irrespective of the presence or absence of PulD (data not shown).

The large PulD complex may represent its functional form in the outer membrane or might be formed upon contact with SDS. In an attempt to distinguish between these possibilities, whole or lysed cells were treated with various agents [10% Tween 20, 10% Triton X-100, 5 M urea, 2% octylpolyoxyethylene (OP), 2% *n*-octyl-β-D-glucopyranoside (OG), 2% Brij 35 or 2% sodium deoxycholate (DOC)] prior to solubilization in SDS. Although OP, OG, Brij and DOC solubilized up to 50% of PulD, none of these agents increased the monomer:multimer ratio. Solubilization in SDS at temperatures between 30 and 80°C instead of 100°C, or substitution of lithium dodecylsulfate for the SDS, were also without effect. Prior treatment of cell extracts with 30% formic acid, which dissociates certain membrane protein complexes (J.T.Buckley and S.Normark, personal communications), decreased the yields of the large complex while only partially increasing the yield of monomeric PulD. Prior extraction with chloroform–methanol to reduce the lipid content of the membrane extracts, or with lysozyme to degrade peptidoglycan, also failed to prevent the appearance of the large complexes (data not shown). However, phenol treatment, which extracts lipopolysaccharide (LPS) and denatures proteins, completely prevented its appearance and resulted in a corresponding increase in the recovery of the monomeric form of PulD (Figure 1).

To determine whether the monomeric form of PulD separated by SDS–PAGE could associate to form the large complex, it was electroeluted from the gel, redissolved in SDS–PAGE loading buffer and subjected to electrophoresis on a second SDS–polyacrylamide gel. This time, only monomeric PulD was detected by immunoblotting, indicating failure of the monomer to form a complex upon re-exposure to SDS, providing additional evidence that complex formation is not induced by SDS. Further confirmation was obtained from the inability of a variant of PulD lacking its N-terminal signal sequence (thereby remaining cytoplasmic) to form multimers. Electroelution and re-electrophoresis of the multimeric form separated by SDS–PAGE resulted in the recovery of ~10% of the PulD protein in the monomeric form. Thus, the complex is not totally resistant to dissociation by SDS, but prolonged heating at 100°C in the presence of this detergent did not appear to increase the yields of monomeric form (data not shown).

The presence of fatty acids at the same position as PulD in the stacking gel (see above) and the fact that the multimers were dissociated by phenol treatment suggested that the PulD complex might contain LPS. To test the possible involvement of LPS carbohydrates in complex formation, pCHAP231 was introduced into *E.coli* K-12 derivatives carrying Δ*rfaGPSB1*, Δ*rfa1*, Δ*rfaC*, Δ*rfaK2*, Δ*rfaQ*, Δ*rfaS* or *rfaZ2* mutations affecting core carbohydrate modification (Klena *et al.*, 1992; Parker *et al.*, 1992). In every case, the yields and ratio of the monomeric and multimeric forms of PulD were the same as in the corresponding wild-type strains (data not shown).

PulD forms heterologous cross-linkable complexes

Complexes of the PulD homologue gpIV were observed previously following treatment with chemical cross-linking agents (Kazmierczak *et al.*, 1994). To see whether similar complexes could be observed with PulD, cells were treated with formaldehyde, *bis*(sulfosuccinimidyl)-suberate (BS^3) and dithio*bis*(succinimidylpropionate) (DSP), and the cross-linked proteins were partially dissociated by treatment with phenol and/or hot SDS (100°C). Treatment with any of the three cross-linking agents caused the disappearance of monomeric PulD and the appearance of a smear near the top of an immunoblotted SDS–PAGE. When formaldehyde or BS^3-treated samples were treated with phenol, intermediate-sized complexes were obtained. Successive treatment of formaldehyde cross-linked material with phenol and then hot SDS resulted in extensive dissociation of PulD in slowly migrating complexes, with a corresponding increase of monomeric PulD and abundant intermediate sized complexes (Figure 2). Interestingly, the ~95 kDa band (Figure 2) was only observed in cross-linked, phenol-treated samples. This and the other complexes observed do not migrate as a set of homomultimers (PulD dimer, trimer, etc.), which is in contrast to the previous observations with gpIV (Kazmierczak *et al.*, 1994). This may be due to non-linear size resolution in the Tris–glycine gels used, or to the association of PulD with other proteins. Nevertheless, the data indicate that the monomeric form of PulD observed upon SDS–PAGE is probably part of a multi-meric complex in which it can be stabilized by cross-linking. We think it likely that this complex is the same as that which is detected in the absence of cross-linking and which is dissociated inefficiently by SDS.

PulD is protected from degradation by PulS

To assess the possibility that the formation of the large PulD complex required other Pul components, existing *E.coli* mutants which no longer secrete pullulanase due to mutations in different *pul* genes were analysed by immunobloting with anti-PulD. No change in the profile of PulD was revealed in strains carrying Δ*pulG* (PAP7228), Δ*pulO* (PAP7245), Δ*pulA* [MC4100(pCHAP710)], Δ*pulE* (PAP3175) or Δ*pulF-O* [MC4100(pCHAP230); Table I]. However, the monomeric form of PulD was apparently degraded to two products of 66 and 45 kDa in the absence of *pulS* [PAP7446, or MC4100(pCHAP40)] (Figure 3). The absence of PulS did not markedly affect the ratio of monomeric to complexed forms of PulD. However, phenol dissociation of the complexed forms of PulD produced in strains lacking PulS showed that it contained only the 66 and 45 kDa breakdown products. Thus, PulS prevents the appearance of PulD breakdown products in both the complexed and monomeric forms of the protein

To investigate whether the stoichiometry of PulD and PulS is important in this protective interaction, the ratio of the two proteins was altered by manipulating the copy number and expression levels of the two genes. Only full-length monomeric PulD was detected in a strain bearing

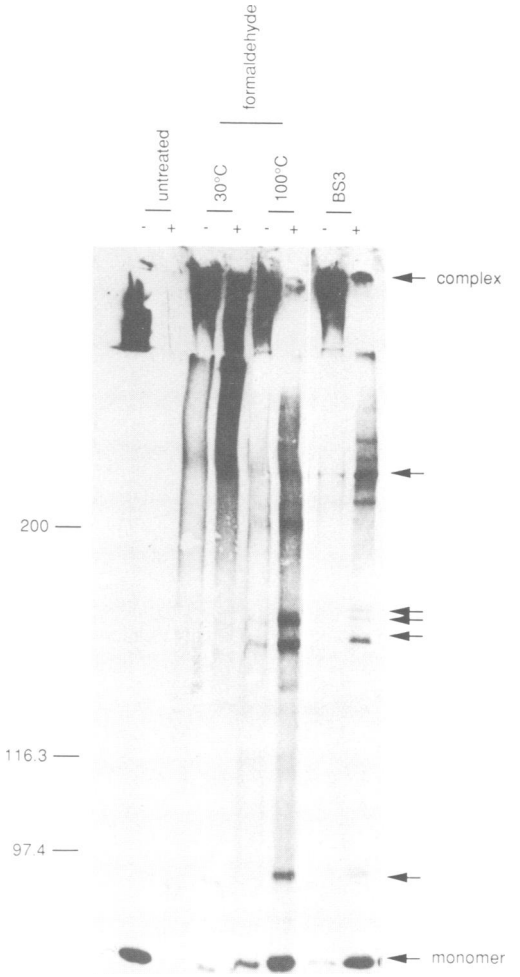

Fig. 2. Cross-linking of PulD complexes. Exponentially grown MC4100(pCHAP231) was subjected to cross-linking by formaldehyde or BS^3 as described in Materials and methods. Cell extracts were either resuspended directly in SDS–PAGE sample buffer (–) or extracted with phenol (+). In the case of the formaldehyde-treated cells, duplicate sets of samples were prepared, one was heated to 30°C, the other to 100°C, before application to the 7% acrylamide SDS–PAGE. The first two lanes show a sample of the same culture not exposed to cross-linking agents, but incubated in parallel in the same buffer. The gel was immunoblotted with anti-PulD, and the molecular size markers are shown in kDa. Arrows mark the position of monomeric and complexed forms of PulD.

a single chromosomal copy of all of the genes in the *pul* cluster (PAP7232). When the level of PulD produced by this strain was increased by isopropyl-β-D-thiogalactoside (IPTG) induction of a plasmid-borne *lacZp–pulD* operon fusion (pCHAP362), the majority of PulD appeared in the degraded form. Production of full-length PulD was restored if the strain also carried *pulS* on a multiple copy number plasmid (pCHAP378) (Figure 4). Thus, protection of PulD is only achieved when sufficient PulS is present, although, due to unavailability of anti-PulS serum, we could not determine the exact stoichiometry of the two proteins. The dependence of PulD assembly and stabilization on the amount of PulS also suggests that these proteins interact directly, and not through another component which would be limiting under these conditions.

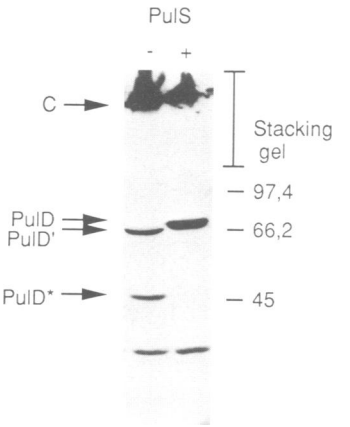

Fig. 3. PulS protects PulD from proteolytic degradation. Maltose-induced, exponentially grown cells of strains MC4100(pCHAP40)($PulS^-$) and MC4100(pCHAP710)($PulS^+$) were harvested and examined by SDS–PAGE and immunoblotting without phenol treatment as described for Figure 1. The positions of molecular weight markers are shown in kDa and the different forms of PulD are indicated by arrows. The positions of the PulD complex (C), full length monomeric PulD (PulD), degradation products of 60 kDa (PulD′) and 45 kDa (PulD*) are indicated. The faster migrating product recognized by the anti-PulD–PhoA is also present in extracts of cells lacking PulD.

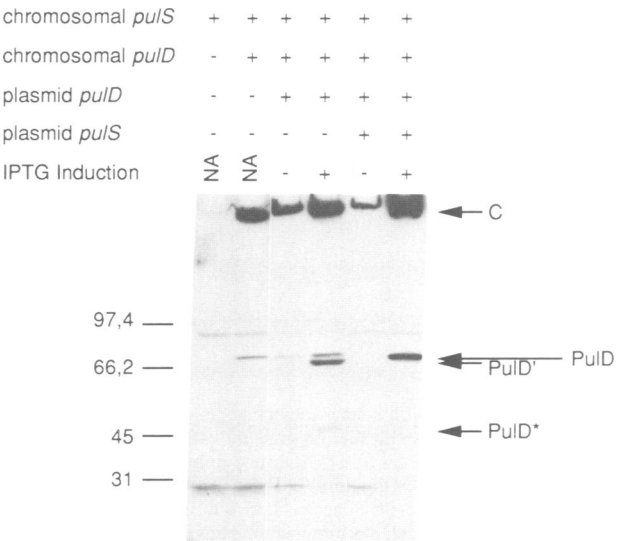

Fig. 4. Titration of PulS by increased production of PulD. The strains PAP7447, PAP7232, PAP7232(pCHAP362) and PAP7232(pCHAP362/PCHAP378) were grown in the presence of maltose to late logarithmic phase. The two strains containing pCHAP362 were grown in parallel with and without IPTG for induction of *pulD*. A sample of each strain was centrifuged for 2 min to harvest the cells which were resuspended in SDS–PAGE sample buffer, sonicated and analysed by immunoblot using anti-PulD. Other details are as for Figures 1 and 2.

PulS is required for outer membrane association of PulD

Both PulD and PulS are located in the outer membrane (see Introduction). Thus, PulS may interact with (and thereby protect) PulD either before or after its insertion in the outer membrane. In the latter case, PulD should be located in the outer membrane irrespective of the presence of PulS, while in the former, PulD might be located in the outer membrane only in strains expressing

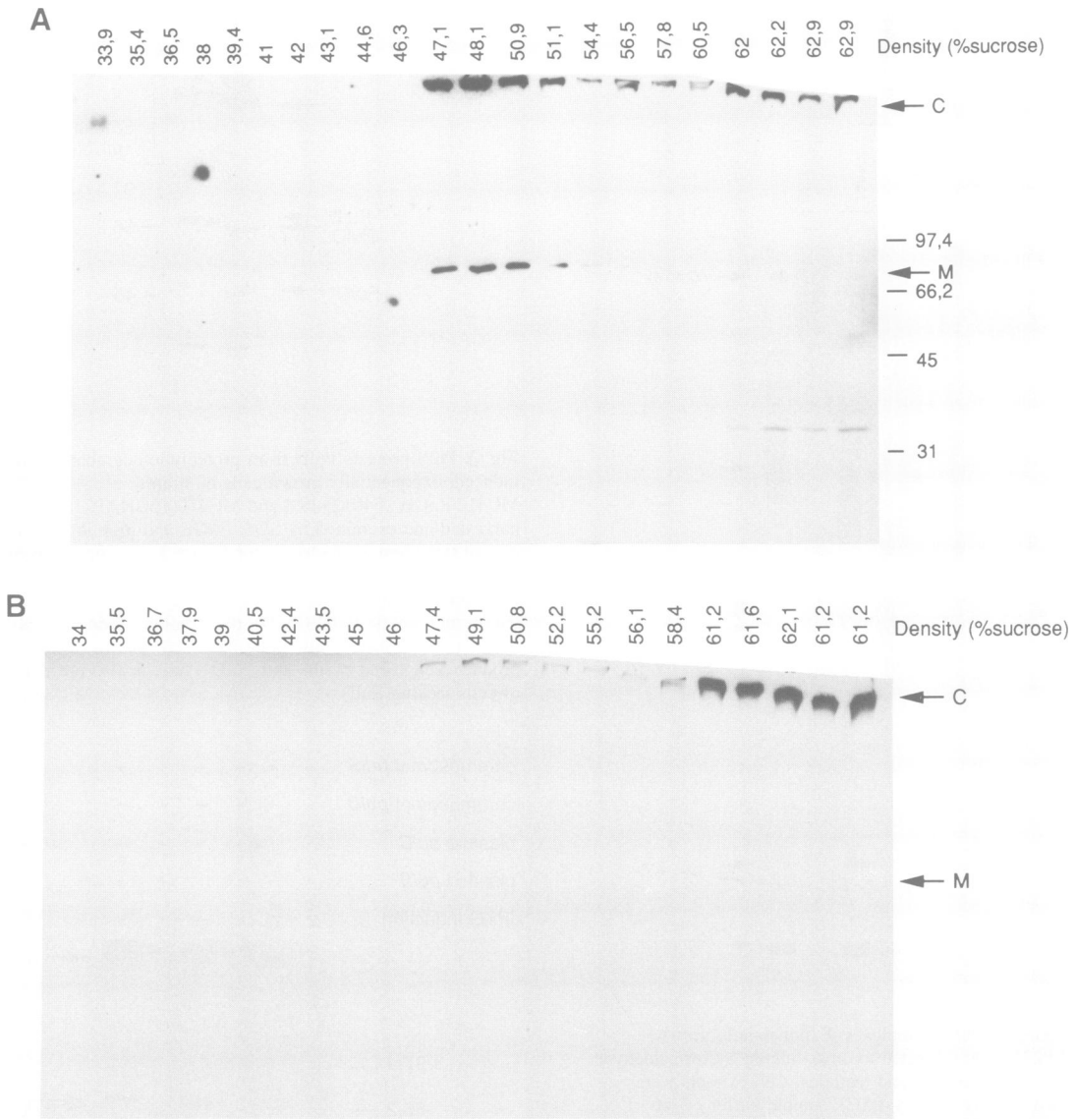

Fig. 5. PulS is required for outer membrane association of PulD. Cell lysates of MC4100(pCHAP710) (**A**) and MC4100(pCHAP40) (**B**) were applied to the bottom of a 32–60% sucrose gradient which was centrifuged as described in Materials and methods. Aliquots from each fraction from the gradient were analysed by SDS–PAGE and immunoblotting using anti-PulD–PhoA85. The positions of the multimeric (C) and monomeric (M) forms of PulD are indicated by the arrows. The sucrose concentration (% w/w) in each fraction is also marked. Examination of the nitrocellulose membrane by staining with Ponceau S prior to immunoblotting revealed that outer membrane porins and OmpF were located in fractions containing 46–52% sucrose, that cytoplasmic membrane was enriched in the top five fractions and that cytoplasmic and other proteins were located in the bottom 6–7 fractions. Molecular size markers (kDa) are shown.

pulS. To examine this, total cell extracts from strains expressing *pulD* in the presence or absence of *pulS* [MC4100(pCHAP710) and MC4100(pCHAP40), respectively] were subjected to flotation sucrose gradient analysis. This method was chosen because it provides clear separation of membrane-associated proteins (which float up the gradient) and soluble proteins or large protein aggregates (which remain at the bottom). In the presence of PulS, most of the complexed and monomeric forms of PulD were detected in the same fractions as the outer membrane porins and OmpA, indicating their almost exclusive association with this membrane (Figure 5). In the absence of PulS, however, the complexed form of PulD and the monomeric 66 kDa breakdown product remained almost entirely at the bottom of the gradient, together with soluble cytoplasmic and periplasmic proteins, indicating that neither form was associated with either the cytoplasmic or outer membranes (Figure 5). When the analysis was repeated by sedimentation sucrose gradient centrifugation, the two forms of PulD produced by the strain expressing *pulS* sedimented rapidly to reach equilibrium in fractions containing outer membrane porins and OmpA, while the two forms detected in cells lacking PulS sedimented more slowly and did not reach equilibrium within 19 h (data not shown).

The flotation gradient analysis was repeated using total cell extracts from strains PAP7232 and PAP7446 (*pulS::*Tn*5*) to determine whether these observations were also valid for strains carrying the *pul* genes in the chromosome instead of on high copy number plasmids. Similar results were obtained (data not shown). Furthermore, other Pul proteins were shown not be involved in

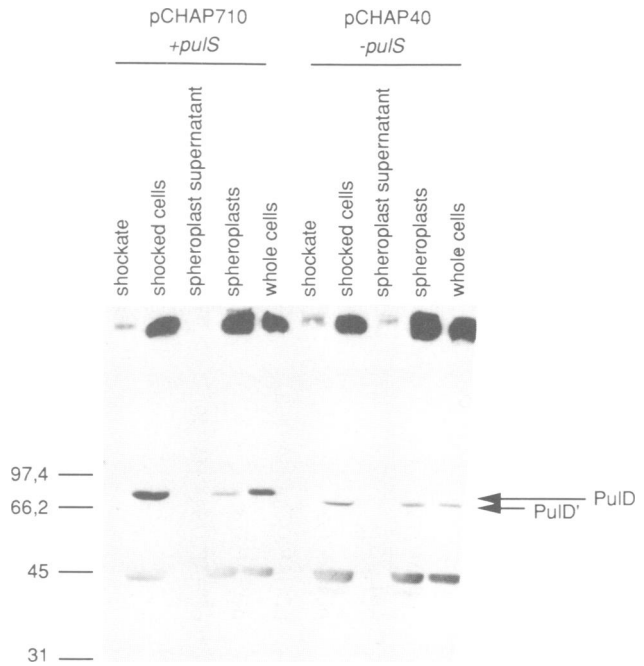

Fig. 6. PulD is not released with periplasmic proteins. MC4100(pCHAP710) and MC4100(pCHAP40) were grown to early exponential phase in the presence of maltose, and subjected to osmotic shocks or spheroplasting. Aliquots of the fractions obtained were analysed by immunoblotting with anti-PulD. Samples loaded onto the gel were equivalent to the same amount of starting material. The positions of molecular weight markers are shown in kDa, and the positions of unprocessed PulD, and degraded PulD, PulD', are shown by arrows. Periplasmic fractions released by spheroplasting and osmotic shock are denoted spheroplast supernatant, and shockate respectively. Prior staining of the nitrocellulose membrane with Ponceau S revealed the expected presence of periplasmic proteins in these fractions.

the association of PulD with the outer membrane by a similar analysis of extracts of strain MC4100(pCHAP362) which expresses *pulD* alone, and strain MC4100 (pCHAP362/pCHAP378) which expresses both *pulS* and *pulD*. Outer membrane association of PulD was only observed in the latter case (data not shown).

To determine whether PulD produced in the absence of PulS was located in the periplasm, cells of the strains MC4100(pCHAP710) and MC4100(pCHAP40) were subjected to osmotic shock or were converted into spheroplasts to release periplasmic proteins. In both cases, the two forms of PulD (monomeric and complexed) remained cell associated instead of being released with periplasmic proteins (Figure 6). However, PulD is almost certainly translocated across the cytoplasmic membrane in the absence of PulS because PulD–PhoA hybrids are periplasmic (d'Enfert *et al.*, 1989), and a variant of PulD lacking the N-terminal signal peptide was detected at ~70 kDa and did not form complexes nor was it degraded to the 66 and 45 kDa forms (data not shown). Thus, PulD produced in the absence of PulS probably forms periplasmic aggregates or complexes that contain both 66 and 45 kDa breakdown products. These complexes are probably too large to be released by osmotic shock or spheroplast preparation. Interestingly, monomeric forms of the two breakdown products were not released by either of these treatments, again suggesting that all of PulD exists in the cell only in its complexed/aggregated form from which monomers are released inefficiently by SDS.

Regions of PulD protected from degradation by PulS

Both the 66 and 45 kDa PulD degradation products that appeared in the absence of PulS reacted with antiseum raised against PulD–PhoA81, which contains only the first 99 amino acids of mature PulD (d'Enfert *et al.*, 1989). Thus, the 45 kDa product, and probably also the 66 kDa product, retain most or all of this N-terminal region of PulD, suggesting that PulS protects the C-terminal region of PulD from degradation. To extend these observations, we determined the effects of changes to the extreme C-terminus of PulD. The pCHAP3504-encoded PulD variant, which has six histidine residues fused to its C-terminus, was not only protected by PulS (as judged by immunoblot analysis), but could restore pullulanase secretion in strain PAP7447(Δ*pulD*) (data not shown), indicating that it is functional. As expected, this complementing ability was dependent on the level of expression of *pulS*. Another variant of PulD (encoded by pCHAP3511) with a single additional glutamate at its C-terminus also behaved like wild-type PulD (data not shown). Therefore, small changes at the C-terminus of PulD do not interfere with PulS protection, although the physical interaction implied by this protection has not been demonstrated.

In a previous study, we constructed two PulD–PhoA hybrid proteins containing, respectively, the N-terminal 395 (hybrid 121) and 99 (hybrid 85) residues of PulD (d'Enfert *et al.*, 1989). Hybrid 85 was shown previously to be mainly periplasmic (d'Enfert *et al.*, 1989). Re-examination of these hybrids revealed the presence of a small but readily detectable amount of complexed or aggregated material that was not dissociated by SDS and which remained in the stacking gel upon SDS–PAGE (Table III). When flotation gradient analysis was performed with lysates of strains producing these hybrids, all of the multimers co-fractionated with the outer membrane porins, indicating peripheral or integral association with the outer membrane (Table III). Furthermore, trace amounts (hybrid 81) or 50% (hybrid 121) of the monomeric forms of these hybrids were also found associated with the outer membrane (Table III). These studies suggest that the N-terminal region of PulD (fused to PhoA) can spontaneously associate with or insert into the outer membrane. Recovery of the two forms of the protein in outer membrane fractions was not increased by the presence of PulS protein (data not shown).

PulS reduces PulD-induced expression of the phage shock response

The phage shock response, manifested by the appearance of large amounts of the protein PspA, is induced by high level production of gpIV (Brisette *et al.*, 1990) or PulD (Possot *et al.*, 1992), as well as by heat shock, osmotic shock, ethanol (Brisette *et al.*, 1990) and lipid depletion (Bergler *et al.*, 1994). It has been suggested that this induction may result from membrane bilayer perturbation, changes to the osmotic balance in the periplasm or increased proximity of the cytoplasmic and outer membranes (Brisette *et al.*, 1990; Bergler *et al.*, 1994; Russel,

Table III. Multimerization and distribution of PulD–PhoA hybrid polypeptides in the envelope fraction of *E.coli* cells

Hybrid	Amino acids of PulD	Multimers (%)	Monomer (%)		Multimer (%)	
			OM	sol.	OM	sol.
PulD–PhoA81	99	20	5	95	100	0
PulD–PhoA121	395	5–10	50	50	100	0

Total cell extracts from cells producing the hybrids were subjected to flotation gradient analysis. Proteins in fractions collected from the gradient were separated by SDS–PAGE, and proteins containing PulD were detected by immunoblotting, and the percentage of protein present in the multimeric (complexed) form and the percentage of each form of the protein in the outer membrane (OM) and soluble (sol.) fractions were determined.

1994b). The induction of PspA by PulD and the role of PulS was re-investigated by immunodetection of PspA in cells carrying different complements of *pul* genes, and by measuring β-galactosidase activity in a strain carrying a *pspA–lacZ* operon fusion in the chromosome (strain MC3 transformed with plasmids carrying various *pul* genes) or on pAD3 (in strains carrying *pul* genes in the chromosome) (Bergler *et al.*, 1994).

PspA clearly was produced in greater amounts when *pulD* was expressed in the absence of *pulS*, irrespective of the presence of other Pul proteins (Figure 7). The two PulD variants with extended C-termini also induced PspA production when PulS was absent (data not shown). Production of the PulD–PhoA hybrid proteins 85 and 121 did not induce PspA (data not shown). Examination of chromosomal *pspA–lacZ* expression levels (Table IV) confirmed that PulS reduced PulD induction of the phage shock response. Similar results were observed when *pspA–lacZ* expression from pAD3 was measured in strains carrying the *pul* gene cluster in the chromosome with mutation in *pulS* (PAP7446). This time, however, only a 2-fold increase in *pspA–lacZ* expression was observed when the *pulS* gene was mutated. This is probably because the phage shock response is only strongly induced when relatively high levels of PulD are produced; this would not be achieved with a single chromosomal copy of this gene.

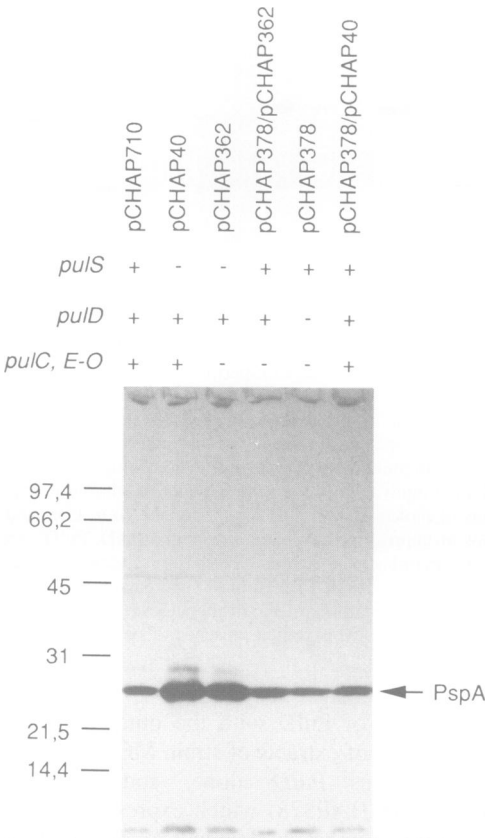

Fig. 7. PulD induces the phage shock response when produced in large amounts in the absence of PulS. Strain MC4100 carrying the indicated plasmids was grown to late logarithmic growth phase and the cells harvested by centrifugation and resuspended in SDS–PAGE sample buffer for SDS–PAGE and immunoblot analysis with anti-PspA. The positions of PspA and molecular size markers (kDa) are shown. After anaylsis, the nitrocellulose membrane was stripped and re-probed with anti-PulD to confirm the presence of PulD and its breakdown products.

Discussion

The sole integral outer membrane component of the main terminal branch of the GSP (GspD) has homologues in a number of unrelated membrane translocation systems including GSP-independent secretion (Michiels *et al.*, 1991; Allaoui *et al.*, 1993; Genin and Boucher, 1994), type IV pilus assembly (Martin *et al.*, 1993), DNA uptake (Tomb *et al.*, 1991), filamentous phage assembly and secretion (Russel, 1994a) and assembly of S-layers (Thomas and Trust, 1995). Therefore, GspD almost certainly plays a pivotal role in secretion, and may form the core of a gated channel in the outer membrane through which the secretory proteins are transported. In order to perform such a function, GspD would be expected to form an outer membrane complex quite distinct from that formed by the porins. Evidence for the existence of such a complex has already been reported in the case of gpIV, the bacteriophage f1-encoded GspD homologue involved in phage morphogenesis and extrusion (Kazmierczak *et al.*, 1994). Here we show that the GspD protein required for pullulanase secretion in *K.oxytoca*, PulD, also forms multimeric complexes. These PulD multimers were detected both by chemical cross-linking, as reported previously for gpIV and, more importantly, as large complexes which were not readily dissociated by SDS but which were dissociated by phenol treatment. The observed partial solubilization of this PulD complex by non-ionic detergent might facilitate its purification for biochemical and biophysical analysis.

Outer membrane localization of the PulD complexes was abolished in the absence of another GSP component, the 12 kDa peripheral outer membrane lipoprotein PulS (GspS). Although the PulD complexes formed in the

Table IV. β-Galactosidase activities of *pspA–lacZ* fusion induced by PulD

Strain	*pul* genes present	β-Galactosidase activity (Miller units)
MC3(pACYC184)	–	20
MC3(pCHAP710)	*S C D E F G H I J K L M N O*	120
MC3(pCHAP40)	*C D E F G H I J K L M N O*	630
MC3(pCHAP362)	*D (lacZp* control)	973
MC3(pCHAP362/pCHAP378)	*S D (lacZp* control)	114

Strains were grown in LB media with IPTG (for pCHAP362) or maltose (for *pulC* promoter present in pCHAP710 and pCHAP40) and appropriate antibiotics. β-Galactosidase activities were measured in exponentially growing cells and are expressed in Miller units as the average of 3–5 independent experimental values. Where β-galactosidase levels were low, the presence of a PspA–lacZ fusion was verified by treating the cells with 10% ethanol, identification of blue colonies on plates containing X-gal, and subjecting them to heat shock.

absence of PulS were not examined in detail, they are similar to the outer membrane-associated complexes formed in the presence of PulS in that they are resistant to dissociation by SDS but are dissociated by phenol. However, the complexes were found to contain only the 66 and 45 kDa breakdown products of PulS, and are probably located in the periplasm. Thus, we propose that PulS is a membrane-anchored periplasmic protein that facilitates the insertion of PulD into the outer membrane. We have not determined whether degradation of PulD in the absence of PulS is the cause or a consequence of its failure to insert into the outer membrane, although we note that truncated forms of PulD comprising the N-terminal half of the protein are at least partially associated with the outer membrane even in the absence of PulS. The possibility that PulS forms an integral part of the outer membrane PulD complex has also not been ruled out, and may be an explanation for its requirement at stoichiometric levels and for the presence of PulD-containing, cross-linkable products in the range of 90 kDa. It is also possible, however, that high level production of PulD results in titration of the chaperone function of PulS.

PulS is only the second component of the main terminal branch of the GSP for which a defined role has been clearly demonstrated (for review, see Pugsley, 1993) and is the first periplasmic chaperone shown to be absolutely required for the correct insertion of an outer membrane protein. Interestingly, *pulS* is the only gene in the *pul* gene cluster which is constitutively expressed; expression of all other *pul* genes is under the control of the maltotriose-sensing, positive activator protein MalT (d'Enfert *et al.*, 1987). Perhaps this ensures PulS is always available to receive PulD and aid its correct membrane insertion.

What makes PulD and its homologues different from the 'classical' outer membrane proteins such as the porins? Besides its ability to form large, detergent-resistant multimers (see above), PulD homologues differ from porins and many other outer membrane proteins in that the C-terminal residue is not an aromatic amino acid (usually phenylalanine). Furthermore, studies presented here indicate that the C-terminus of PulD can be extended without affecting its functionality, whereas Struyvé *et al.* (1991) have shown that the integrity of the C-terminus of PhoE porin is essential for efficient outer membrane insertion. Thus, the mode of assembly and membrane insertion of GspD and related proteins would be expected to differ from that of porins, which explains the requirement for a specific periplasmic chaperone-like activity which could be provided by PulS by virtue of its peripheral attachment to the periplasmic face of the outer membrane.

The aberrant accumulation of PulD complexes in the periplasm or loosely associated with a membrane but yet not integrated into the outer membrane is shown here to cause high level induction of the phage shock response. The PulS requirement for PulD assembly in the outer membrane thus explains the previously observed induction of the so-called phage shock response by high levels of PulD produced in its absence. Production of gpIV also induces the phage shock response both in bacteriophage f1-infected cells and in cells in which gpIV is expressed in the absence of other phage genes (Brisette *et al.*, 1990). Does this indicate that *E.coli* K-12 has a PulS homologue which can facilitate gpIV insertion into the outer membrane but which cannot cope with high level gpIV production resulting from bacteriophage infection? Even when it is produced at levels which induce the phage shock response, gpIV is apparently at least partially associated with the outer membrane (Brisette and Russel, 1990). However, the membrane fractionation techniques used to examine the location of gpIV (Brisette and Russel, 1990; Russel and Kazmierczak, 1993; Kazmierczak *et al.*, 1994), and of a close relative of PulD which is apparently outer membrane-associated in the absence of a PulS homologue (Hu *et al.*, 1995), may not have been adequate to distinguish between membrane-associated and aggregated periplasmic forms of these proteins. Furthermore, the presence of large SDS-resistant protein complexes or aggregates was not examined in either of these studies. The observed reduction of PulD-induced *pspA* expression by PulS does not help to understand the nature of the signal which triggers the phage shock response or the physiological role of the Psp proteins, which are not required for pullulanase secretion (Possot *et al.*, 1992) or f1 phage production (Brisette *et al.*, 1990).

According to our data, *pulS* homologues should exist in other, related and unrelated secretion systems involving GspD homologues. A *pulS* homologue is present in the GSP gene cluster of *Erwinia chrysanthemi* (Condemine *et al.*, 1992), but the published sequence of this gene indicated that its predicted product is a cytoplasmic protein rather than an outer membrane lipoprotein. However, an overlapping reading frame at the 5′ end of this gene (*outS*) could code for a peptide similar to a lipoprotein signal peptide. Thus, we propose that the published *outS* nucleotide sequence might include an error near its 5′ end. Nucleotide sequence analysis of *gsp* genes from other

bacteria failed to identify a *pulS* homologue, but such a gene might be located at a different chromosomal position to the other *gsp* genes.

The two PulD–PhoA multimers examined also formed multimers and were at least partially associated with the outer membrane (Table III). Thus, the signals for multimerization and outer membrane association are at least partially located within the first 395 or even the first 99 amino acids of PulD. The data suggest that the multimers formed by these two hybrids might be less resistant to denaturation by SDS than full-length PulD, since considerably more of both of these hybrids was present in monomeric form after SDS–PAGE (Table III). These data call for a re-evaluation of results from an earlier analysis of hybrids in which PhoA was fused to gpIV, which indicated that the C-terminal region of this PulD homologue determined its association with the outer membrane (Russel, 1994a). These studies did not recognize the possible existence of large gpIV complexes and their association with the outer membrane. The results of our analysis of PulD–PhoA hybrids are also at variance with our data suggesting that PulS protects the C-terminus of PulD from degradation and, therefore, that the integrity of the C-terminal region of PulD is required for outer membrane association. One explanation for these conflicting observations might be that the C-terminal region of PulD prevents insertion of this protein into the membrane (possibly by causing misfolding or premature aggregation) unless it is chaperoned by PulS.

Materials and methods

Plasmids and strains

Strains of *E.coli* K-12 carrying the complete *pul* gene cluster (with or without mutations in specific *pul* genes) in the chromosome, and plasmids carrying various *pul* genes under the control of the native (*pulC* or *pulS*) or *lacZ* promoters are listed in Tables I and II, respectively. The Δ*pulD* mutation was created by deleting the DNA between the *Bcl*I site close to the 5′ end of the gene and the *Bgl*II site near the 3′ end of the gene in a pUC18 derivative. DNA flanking the deletion was subcloned into M13mp10 and then recombined into the chromosome of strain PAP7232 (Table I) by the method of Blum *et al.* (1989) to produce strain PAP7447.

In order to create a His-tagged PulD for affinity purification, an *Xho*I site was created at the 3′ end of *pulD* by PCR amplification using the oligonucleotides 5′-CCCCCTCGAGTAGATTGCCTCCCAGATTGAA-3′ (containing an *Xho*I site) and 5′-CCCCCAGCTGTTTTCCG-CCACTTCAAGGGC-3′ (containing a *Pvu*II site). This fragment was cloned into the vector pCR1000 (Invitrogen). Using the introduced *Xho*I site and an *Eco*RI site in the vector, the 3′ end of *pulD* was transferred into similar sites of pBluescriptSK+ (Stratagen). Using the vector *Eco*RI and *Kpn*I sites of this construct, the 3′ end of *pulD* was then moved into pUC18Cm, a specially constructed pUC18 derivative in which the *blaM* gene has been replaced by the *cat* gene coding for resistance to chloramphenicol. Following this, the 3′ region of *pulD* was ligated to its 5′ region and the upstream *pulC* and MalT-dependent, maltose-inducible promoter (extending to the *Bam*HI site upstream of *pulC*) by subcloning a *Bgl*II–*Hin*dIII fragment from this construct into the vector pDN18 containing *pulC–pulD* (Nunn *et al.*, 1990; see d'Enfert *et al.*, 1989 for sequence and position of restriction endonuclease sites). The *Bgl*II site is internal to *pulD* and to the *Hin*dIII site derived from pUC18Cm. The His tag was then introduced via the *Xho*I site. An *Xho*I–*Pst*II fragment was subcloned from pET22 which bears the His-encoding region (Invitrogen) into the same sites of the newest construct. Thus, a construct was created bearing the maltose-regulated *pul* promoter, *pulC*, *pulD–his* with pET22 sequences extending to the *Pst*II site in the vector pDN18. pCHAP3504 is the 2.9 kb *Kpn*I–*Pst*I subclone of this construct subcloned into the same sites of pTZ18R (Pharmacia). pCHAP3511 was created by the introduction of the linker 5′-TCGAATGAATTCAT-3′ into the *Xho*I site of pCHAP3504 to introduce an *Eco*RI site and an in-frame stop codon preceded by an additional glutamate at the C-terminus of PulD.

A cytoplasmically located, His-tagged PulD was created by subcloning *pul′CDE′* carried on the 2.5 kb *Sma*I–*Sal*I fragment into pUC18Cm. The DNA carrying *pul′C* plus the signal sequence and the first 29 residues of mature *pulD* was removed by digestion of this construct with *Cla*I–*Eco*RI, filling in and religation, which reforms the *Eco*RI site. The *Eco*RI–*Hin*dIII fragment of this construct was transfered to the vector pTrcHisB (Invitrogen) resulting in the fusion of six histidine residues to the N-terminus of PulD, and expression under the control of the P*trc* promotor.

Growth conditions

Cultures were incubated at 30°C in L broth (Miller, 1972) with vigorous shaking. Maltose (0.4%) was added to induce expression of the *pulC-O* operon in L broth buffered to pH 7.2 with 10 mM phosphate. Induction of *lacZp*-controlled expression was achieved by the addition of 1 mM IPTG. Antibiotics were added to the following concentrations: kanamycin 50 μg/ml, chloramphenicol 25 μg/ml, ampicillin 100 μg/ml, tetracycline 16 μg/ml.

Enzyme assays

Pullulanase (d'Enfert *et al.*, 1989) and β-galactosidase (Miller, 1972) were assayed as previously described.

Polyacrylamide gel electrophoresis

Proteins were separated by SDS–PAGE and stained with Coomassie Blue according to Pugsley and Oudega (1987). LDS–PAGE was performed similarly, but with resuspension of proteins in 2% lithium dodecylsulfate, 10% glycerol, 2.5% β-mercaptoethanol, 0.02% bromophenol blue, 60 mM Tris–HCl pH 6.8. The samples were not boiled before application to the polyacrylamide gel, and the running buffer contained LDS in place of SDS. To stain proteins with silver, the gel was fixed for 30 min in 50% methanol/10% acetic acid, and washed in 10% ethanol/5% acetic acid for 30 min. Following a 5 min wash in 0.1% potassium dichromate in 0.2 ml/l nitric acid, the gel was washed for 20 min in 0.2% silver nitrate. Following two brief washes in deionized water, the stain was developed in 3% sodium carbonate/0.25% formaldehyde and the reaction stopped by transferring the gel to 5% acetic acid.

Electroelution was performed in Bio-Rad electro-eluter in 50 mM ammonium bicarbonate/0.1% SDS as recommended by the manufacturer. Trichloroacetic acid (TCA) precipitation of proteins was performed by the addition of TCA to samples to a final concentration of 10%, incubation on ice for 30 min and then 10 min centrifugation to harvest the precipitated proteins, which were washed with cold acetone and resuspended in SDS–PAGE sample buffer.

Immunoblotting

Proteins were transfered to nitrocellulose by semi-dry electrotransfer in 50 mM Tris/40 mM glycine/20% methanol, stained with Ponceau red, and blocked in 5% fat-free milk for 60 min at room temperature. All immunoblotting incubations were performed in TBST (10 mM Tris–HCl pH 8, 150 mM NaCl, 0.05% Tween 20), with copious washing between antibodies and prior to developing by enhanced chemiluminescence (ECL; Amersham). Incubation with the primary and secondary antibody (1:3000 horseradish peroxidase-linked anti-rabbit IgG; Amersham) was for 60 min at room temperature with gentle shaking. The primary antibodies used were a 1:4000 dilution of anti-PulD–PhoA (d'Enfert *et al.*, 1989) and a 1:200 dilution of a freshly prepared antiserum raised against PulD–His lacking its N-terminal signal peptide and purified on a nickel column following solubilization with Gu-HCl as recommended by Qiagen. The resulting serum was purified by affinity chromatography on a CNBr-activated Sepharose column (Pharmacia) loaded with PulD–His purified in the same way according to Harlow and Lane (1988). The other primary antibodies used were a 1:2000 dilution of anti-OmpF (a gift from Jean-Marie Pagès), a 1:2000 dilution of anti-OmpA (a gift from Ulf Henning), a 1:4000 dilution of anti-PhoA (this laboratory), a 1:4000 dilution of anti-PspA (a gift from Marjorie Russel) and 1:2000 dilutions of anti-PulG, anti-PulE and anti-PulF (all from this laboratory). To re-probe nitrocellulose membranes, they were stripped in 100 ml of 62.5 mM Tris–HCl pH 6.7, 2% SDS, 0.715% β-mercaptoethanol at 50°C for 30 min with vigorous shaking. Following extensive washing in TBST, immunoblotting was performed as described above.

Extraction and dissociation of PulD

Phenol extraction was performed essentially as described in Hancock and Nikaido (1978). For chloroform–methanol extraction, cell pellets were resuspended in 0.2 ml of distilled water and sonicated briefly. Chloroform (0.25 ml) was added followed by 0.5 ml of methanol. Samples were vortexed vigorously and frequently for 60 min and then 0.2 ml of distilled water followed by 0.25 ml of chloroform were added. Following further vortexing over 30 min, the samples were centrifuged for 15 min and both phases and the pellicle at the interface were placed in separate tubes. Following TCA precipitation, samples were resuspended in SDS–PAGE sample buffer. Treatment with formic acid involved incubation of cell pellets in formic acid at a final concentration of 30% on ice for 30 min, followed by evaporation in a Speedvac for ~4 h at room temperature to evaporate all the formic acid. The resultant pellet was then resuspended in SDS–PAGE sample buffer.

Cross-linking

Cells were washed and resuspended in phosphate-buffered saline (PBS) to an absorbance at 600 nm of 1.0. An incubation of 30 min at room temperature was carried out with a final concentration of either 0.37% formaldehyde, 0.2 mg/ml DSP or 0.5 mg/ml BS^3. Following addition of 0.1 M Tris–HCl pH 7.4 to quench the reaction, the cells were harvested by centrifugation.

Protein labelling

Cells were labelled with 10 μCi/ml [^{3}H]palmitate for 4 h (Pugsley *et al.*, 1986) in minimal medium followed by resuspension of cells in SDS–PAGE sample buffer. Gels were stained with Coomassie Blue, shaken for 30 min in Amplify (Amersham), dried and exposed to X-ray film.

Subcellular fractionation

Osmotic shock was performed as described by Brockman and Heppel (1968). Spheroplasts were prepared essentially as described in Pugsley *et al.* (1991). The sucrose gradient fractionation of membranes obtained from cells lysed in a French pressure cell was essentially as described previously (Poquet *et al.*, 1993). The sucrose gradients spanning 32–60% sucrose were prepared in 25 mM HEPES (pH 7.5). For sedementation gradients, the lysates were applied to the top of the gradient, and for flotation gradients the whole cell lysates were made 60% sucrose and loaded at the bottom of the gradient. The gradients were centrifuged for 19 h at 49 000 r.p.m. in a Beckman SW55 rotor at 10°C. Fractions of 250 μl were collected from the top of the centrifuged tube and subjected to TCA precipitation before SDS–PAGE and analysis by immunoblotting.

For the preparation of mixed membrane fractions, *E.coli* MC4100(pCHAP231) was grown in LB with maltose induction to late exponential phase. Cells were harvested by centrifugation, washed in 25 mM HEPES (pH 7.4) and resuspended in 1:20 volume of the same buffer. Following disruption in a French pressure cell at 500 p.s.i., cell debris was removed by brief low speed centrifugation. The clear cell lysate was then centrifuged at 40 000 r.p.m. for 60 min at 4°C in a Beckman 75Ti rotor. The membrane-containing pellet was resuspended in 1/50th of the original culture volume of the same HEPES buffer. Aliquots (50 μl) were extracted with detergents or other agents for 30 min on ice and then centrifuged in a microfuge to separate insoluble from soluble proteins for 30 min, and these were then examined by SDS–PAGE and immunoblotting as above.

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