Lipids assist the membrane insertion of a BAM-independent outer membrane protein

Gerard H. M. Huysmans1,*, Ingrid Guilvout1,2, Mohamed Chami3, Nicholas N. Nickerson4,† & Anthony P. Pugsley2

Like several other large, multimeric bacterial outer membrane proteins (OMPs), the assembly of the Klebsiella oxytoca OMP PulD does not rely on the universally conserved β-barrel assembly machinery (BAM) that catalyses outer membrane insertion. The only other factor known to interact with PulD prior to or during outer membrane targeting and assembly is the cognate chaperone PulS. Here, in vitro translation-transcription coupled PulD folding demonstrated that PulS does not act during the membrane insertion of PulD, and engineered in vivo site-specific cross-linking between PulD and PulS showed that PulS binding does not prevent membrane insertion. In vitro folding kinetics revealed that PulD is atypical compared to BAM-dependent OMPs by inserting more rapidly into membranes containing E. coli phospholipids than into membranes containing lecithin. PulD folding was fast in d1C14:0-phosphatidylethanolamine liposomes but not d1C14:0-phosphatidyglycerol liposomes, and in d1C18:1-phosphatidylcholine liposomes but not in d1C14:1-phosphatidylcholine liposomes. These results suggest that PulD efficiently exploits the membrane composition to complete final steps in insertion and explain how PulD can assemble independently of any protein-assembly machinery. Lipid-assisted assembly in this manner might apply to other large OMPs whose assembly is BAM-independent.

The outer membrane of Gram-negative bacteria contains many proteins with diverse functions that, besides making the membrane semi-permeable to nutrients and solutes, are critical to cellular organisation, fitness and survival1. It is therefore crucial for the bacterium to ensure that the outer membrane contains the right amount of functional proteins. Once passaged through the Sec-translocon, outer membrane proteins (OMPs) rely on one or several periplasmic proteins to chaperone them to the outer membrane2. Many OMPs have a β-sheet transmembrane topology and are passed on from these chaperones to the outer membrane embedded β-barrel assembly machinery (BAM) that catalyses their membrane insertion3. Although the insertion of proteins into the inner membrane is linked to the hydrolysis of ATP and the proton-motive force4, such classical energy sources are not available to catalyse protein insertion into the outer membrane. BAM is proposed to help overcome the energy barrier required for OMP insertion in the presence of lipids with phosphatidylethanolamine (PE) headgroups5–7.

Unlike the pathway followed by most OMPs, the assembly of some, but not all8, large α-helical and β-sheet secretion pores is BAM-independent9,10. Membrane insertion of BAM-dependent OMPs might occur through a process of β-strand augmentation during which BAM forms a pore-chimera with the growing substrate OMPs11,12. How large BAM-independent OMP oligomers might insert into
the membrane remains unclear. However, their assembly might rely on a different membrane insertion mechanism that, regardless of the transmembrane secondary structure, prevents the formation of a large open channel in the membrane. Here we address this question using the secretin PulD as a model system.

Secretins are a large and important class of outer membrane proteins that form multimeric export portals of secretion systems for enzymes, virulence factors, surface pili and filamentous phages11. PulD from the type II secretion system from *Klebsiella oxytoca*14 is a prototype of the secretin family. In *K. oxytoca*, and when expressed in its entirety in *E. coli*, this system secretes the enzyme pullulanase (PulA)14. PulD consists of a modular periplasmic N-domain containing four subdomains (named N0–N3)15,16, a membrane embedded C-domain that is conserved throughout the secretin family15,17, and a C-terminal S-domain that interacts with a PulD-dedicated chaperone, the lipoprotein PulS18,19. PulD targeting to the outer membrane occurs via the Lol-pathway and is strictly PulS-dependent20. In the absence of PulS, PulD inserts into the inner membrane and induces a stress response that includes massive production of the protein PspA21,22. Whereas the nature of the PulD transmembrane topology remains to be determined, its BAM-independence for outer membrane assembly is well-established9.

Many OMPs, including PulD, can fold spontaneously in vivo in the presence of liposomes23,24, providing a method to dissect the roles of chaperones during the folding of these proteins in a controlled in vitro environment5,7,26–28. An equivalent approach has not yet been used for PulS in PulD assembly. To address whether PulS has additional roles besides outer membrane targeting and how PulD overcomes the energetic barrier for efficient assembly, we took advantage of the spontaneous in vitro folding of PulD in a coupled transcription-translation reaction containing liposomes. In vitro, PulD folding achieves optimal efficiency when only a short sequence of the N-terminus and the N3-subdomain precede the C- and S-domains25. Truncation of N0–N2 does not affect PulD efficiency when only a short sequence of the N-terminus and the N3-subdomain precede the C- and S-domains25. Truncation of N0–N2 does not affect in vivo assembly, indicating that all in vivo interactions required for correct assembly are present in this truncated PulD variant25. We previously showed that this truncated secretin, PulD28–42/259–660, folds via a multistep mechanism: membrane adsorbed monomers dodecamerise into a prepore that then inserts into the membrane29. Secretins produced in this manner are indistinguishable from secretins purified from native membranes according to their secondary and quaternary structure and their biochemical properties. Here, we report the effects of adding PulS to the in vitro PulD synthesis reaction and of cross-linking PulS to PulD in vivo on the acquisition of native state determinants. We further examine the effects of changes in the membrane composition to in vitro folding kinetics and propose a folding model in which membrane lipid properties directly influence membrane insertion without the assistance of proteinaceous co-factors. In what follows, we use the terms ‘folding’ and ‘assembly’ to distinguish between the in vitro and the in vivo processes, respectively.

**Results**

**sPulS facilitates rapid PulD28–42/259–660 multimerisation in lecithin liposomes.** We previously observed that PulD28–42/259–660 multimerisation in vitro is inversely dependent on the concentration of lecithin in the coupled synthesis and insertion reaction29. To find conditions under which the effects of adding PulS to the in vitro coupled transcription-translation reaction could be measured, we added a non-lipidated form of PulS (sPulS) to the reaction mixture in the presence of increasing amounts of lecithin before commencing PulD28–42/259–660 synthesis. Although the overall production of PulD28–42/259–660 is lower at lecithin concentrations above 27 mM, we have shown previously that this does not impair the analysis of initial PulD multimerisation29. sPulS was used because the presence of a lipid anchor would (1) require the use of detergent that interferes with liposome integrity and (2) physically restrain PulS on the lipid surface rather than being free in solution to recruit PulD monomers. We established previously that PulS produced in this way interacts efficiently with PulD19,30,31.

At low lecithin concentrations, initial PulD28–42/259–660 multimerisation is too fast to measure a contribution of sPulS (Fig. 1a and b). However, at 53 mM lecithin, initial PulD28–42/259–660 multimerisation was markedly lower in the absence of sPulS (Fig. 1b, open squares) than in its presence (Fig. 1b, filled squares). A PulD variant lacking the S-domain, PulDΔS28–42/259–598, is unable to interact stably with PulS18,32. PulDΔS28–42/259–598 synthesis would therefore be expected to show a large inverse dependence on the lecithin concentration at high concentrations even in the presence of sPulS. Rapid degradation of PulDΔS28–42/259–598 and its poor recognition by the anti-PulD antibody prevented in depth analysis of PulDΔS28–42/259–598 multimerisation. Nonetheless, immunoblots clearly show that initial PulDΔS28–42/259–598 multimerisation was very low at 53 mM lecithin with sPulS present in the reaction mixture before synthesis (Fig. 1c). This result thus demonstrates that the sPulS-mediated increased initial multimerisation of PulD28–42/259–660 was specific to the binding of the S-domain of PulD28–42/259–660 to sPulS.

The observed increase in initial PulD28–42/259–660 multimerisation in the presence of sPulS could stem from a direct kinetic advantage by rapid association of the PulD28–42/259–660/sPulS complex with the lecithin surface, from a PulS-induced conformational advantage for PulD28–42/259–660 oligomerisation, or from a change in the PulD28–42/259–660 folding mechanism. If sPulS binding induces the formation of small oligomers prior to dodecamerisation, then the membrane dependent multimerisation reaction would reduce in order, lowering its inverse dependence on the lecithin concentration: dodecamerisation by monomeric addition results in a dependence of the initial multimerisation of up to twelve, hexamerisation of dimers in a dependence of up to six and so forth. Such oligomers can be observed by creating mixed multimers between full-length PulD (PulD50) and PulD28–42/259–660, which separate as a regular 13-step ladder by SDS-polyacrylamide electrophoresis29. If sPulS helps form oligomers that are obligatory...
intermediates in dodecamerisation, then distinct steps should disappear when PulD fl and PulD 28–42/259–660 synthesised separately in the presence of sPulS are mixed post-synthesis 29. However, PulD 28–42/259–660 formed regular ladders with PulD fl in the presence or absence of sPulS (Fig. 1d), making it unlikely that sPulS induces the formation of small obligate oligomers prior to dodecamerisation.

sPulS does not accelerate other PulD28–42/259–660 folding steps in lecithin liposomes. Since sPulS caused more rapid initial multimerisation of PulD 28–42/259–660 at 53 mM lecithin, it was next investigated whether the effect of PulS extended to subsequent kinetic steps in PulD28–42/259–660 folding. PulD28–42/259–660 folding under these conditions was monitored by SDS-treatment and by subjecting PulD 28–42/259–660 to trypsinolysis at increasing time points after 10 min synthesis (Fig. 2a). Despite the more rapid PulD28–42/259–660 multimerisation in the presence of sPulS immediately after 6 min synthesis, sPulS did not significantly accelerate other phases in PulD28–42/259–660 folding (Fig. 2b). Previously, PulD28–42/259–660 folding was characterised by two rate constants in a multistep sequential process with simultaneous acquisition of SDS- and urea-resistance, followed by trypsin-resistance of the native protein core upon membrane insertion 29. Here, data scattering warranted data fitting to a single exponential equation only. The rate constant of 0.18 ± 0.01 min⁻¹ agreed well with the fast rate constant of 0.14 ± 0.04 min⁻¹ reported in the absence of sPulS 29. As was the case in the absence of sPulS, trypsin-resistance in lecithin liposomes was acquired after a delay of approximately 20 min (Fig. 2b). Together, the data demonstrate that sPulS only improves the efficiency of initial steps in PulD28–42/259–660 folding in lecithin liposomes in vitro.

---

**Figure 1. Effect of sPulS on PulD folding.** (a) Initial multimerisation of PulD 28–42/259–660 in the presence of 0.2 μg/μl sPulS and increasing quantities of lecithin (as indicated) after 6 min of PulD-synthesis. (b) Double logarithmic plot of the initial multimerisation of PulD28–42/259–660 in (a). Errors represent S.D. over 3 independent measurements. Initial PulD multimerisation in the absence of sPulS is also shown (□) (from ref. 29). (c) Initial multimerisation of PulD ΔS 28–42/259–598 in the presence of 0.2 μg/μl sPulS and increasing quantities of lecithin (as indicated). PulD ΔS 28–42/259–598 degradation results in multiple bands below the multimer (MuΔS); the approximate position of the monomer is indicated (MoΔS). (d) Mixed multimer formation between PulD 28–42/259–660 and and PulD fl in equimolar ratios in the presence of 53 mM lecithin and 0.2 μg/μl PulS as indicated. Mu and Mo indicate the migration position of multimeric and monomeric PulD 28–42/259–660 species, respectively. Mu fl indicates the position of full-length PulD.
E. coli lipids do not enhance sPulS dependent PulD28–42/259–660 folding. Lipids with phosphatidylcholine (PC)-headgroups (like lecithin) have proven successful for the in vitro folding of many OMPs from their chemically denatured state, but bacterial membranes rarely contain PC-headgroups. The inner leaflet of bacterial outer membranes consists mostly of lipids with phosphatidylethanolamine (PE)-headgroups (up to 90%) and phosphatidylglycerol (PG)-headgroups. Most chemically denatured bacterial OMPs fold only inefficiently in vitro in membranes derived from native sources like E. coli. However, PulD28–42/259–660 multimerised efficiently after 10 min synthesis in the presence of E. coli polar extract lipids (Fig. 2c). The absence of a delay in the acquisition of trypsin-resistance indicated that PulD28–42/259–660 acquired its final native state efficiently and faster in the presence of E. coli lipids, whereas PulD28–42/259–660 membrane insertion into E. coli membranes, the effect of adding sPulS prior to PulD28–42/259–660 synthesis on the acquisition of trypsin-resistance was measured. As in lecithin liposomes, the addition of sPulS to the synthesis reaction did not increase the rate of PulD28–42/259–660 folding into its trypsin-resistant native state in the presence of E. coli lipids (Fig. 2d,e). Thus, whereas PulD28–42/259–660 membrane insertion is rapid in the presence of liposomes prepared from E. coli lipids, the addition of sPulS did not reveal an additional kinetic advantage in the presence of these lipids. We cannot exclude that the PulS lipid-anchor plays an additional role...
role in the PulD folding process, for example by creating membrane defects or enforcing a particular orientation with respect to the membrane.

PulS attachment does not prevent PulD assembly in vivo. The lack of any effect on late steps in PulD\textsuperscript{fl} folding kinetics might reflect that the PulD\textsuperscript{fl}-PulS complex dissociates once it adsorbs onto the lipid surface or forms a dodecamer. Complex dissociation could be a prerequisite for the formation of a native, secretion-competent PulD complex in the outer membrane. To examine further whether this is the case, a series of cysteine variants in PulD\textsuperscript{fl} and PulS was created that would cross-link spontaneously upon interaction in vivo. The choice of residues for substitution by cysteines was based upon the crystal structures of PulS and that of the PulS homologue OutS containing the binding peptide of the S-domain of the PulD homologue OutD (Fig. 3a). OutS has the

Figure 3. Cross-linking efficiency of cysteine bridges between PulD\textsuperscript{fl} and PulS in vivo. (a) Cartoon representation of K. oxytoca PulS (green) containing the binding peptide of PulD (red) based on the OutD-OutS complex (PDB 4K0U). Residues lining the bottom of the binding cleft in PulS and interacting residues on PulD\textsuperscript{fl} are shown as sticks in yellow and magenta, respectively, and those substituted into cysteines are labelled. Two side-chain orientations are shown for Q95, as in the deposited PulS structure (PDB 4A56). (b) Cross-linking efficiency between PulD\textsuperscript{fl} and PulS variants, as indicated. (c) Specificity of the PulD\textsuperscript{fl}-Q95C cross-link. The cysteine cross-link was reduced upon DTT treatment and multimers dissociated by phenol extraction, as indicated. OmpF was used as a loading control before and after phenol extraction. In (b,c) immunoblots after SDS-PAGE of total cell extracts were stained with anti PulD, anti PulS and anti OmpF antibodies as indicated. Numbers on the left side of blots in (b) and (c) indicate migration positions of the molecular weight markers in kDa. Mu\textsuperscript{fl} and Mo\textsuperscript{fl} indicate the migration position of multimeric and monomeric PulD\textsuperscript{fl} species, respectively. * Indicates likely cross-linked dimers between two PulD\textsuperscript{fl} monomers with cysteines; ** Indicates trimmed PulD\textsuperscript{fl} monomers that escape outer membrane targeting.
same structural fold as PulS and functionally interacts in a similar way with OutD as PulS does with PulD$^{36,37}$. In fact, OutS can substitute for PulS$^{30}$. Of the residues lining the PulS binding cleft, Q38 on $\alpha$-helix 1 and Q95 on $\alpha$-helix 3 appeared to be good candidates (in distance and orientation relative to the PulD peptide) for substitution by cysteines: Q38C might cross-link with two residues of the PulD S-domain, A649C and F654C, while Q95C might cross-link with A643C of the PulD S-domain (Fig. 3a). All of the pairs tested produced PulD$^{\alpha}$ multimers, albeit less efficiently than observed with wild-type PulD$^{\alpha}$ and wild-type PulS (Fig. 3b). An upper shift in the electrophoretic migration of PulD$^{\alpha}$A643C monomers recognized by anti-PulD antibodies indicated that it cross-linked with high efficiency to PulS$^{\alpha}$Q95C, while PulD$^{\alpha}$A649C and PulD$^{\alpha}$F654C showed no or limited cross-linking with PulS$^{\alpha}$Q38C, respectively (Fig. 3b). PulD$^{\alpha}$A643C monomers and multimers were also recognized efficiently by anti-PulS antibodies (Fig. 3b). Cross-linking was specific between the engineered cysteines A643C and Q95C, as efficient cross-linking was not achieved when either of the variants was produced in the presence of the wild-type binding partner (Fig. 3c). The slower migrating cross-linked PulD$^{\alpha}$A643C-PulSQ95C hetero-dimer disappeared upon treatment with dithiothreitol (DTT), which resulted in a concomitant increase in the amount of non-cross-linked PulD$^{\alpha}$A643C$^{\alpha}$ monomers. In addition, broadening of the multimer band upon DTT treatment also suggested that PulD$^{\alpha}$A643C$^{\alpha}$ multimers were cross-linked to PulSQ95C (Fig. 3c). Phenol treatment dissociated the multimers and predominantly resulted mainly in an increase in the amount of cross-linked PulD$^{\alpha}$A643C-PulSQ95C hetero-dimers (Fig. 3c). Neither phenomenon occurred with any of the other combinations or between wild-type proteins and the variants (Fig. 3c).

Having established that at least the majority of the PulD$^{\alpha}$A643C$^{\alpha}$ multimers formed were cross-linked to PulSQ95C, we next investigated whether these multimers behaved like the wild-type upon production in E. coli; i.e., do they induce the Psp response and permit PulA secretion. A Psp response is induced when PulD multimers associate with or insert into the inner membrane. For example, PulS$^{\alpha}$A643C-PulSQ95C hetero-dimer disappeared upon treatment with dithiothreitol (DTT), which resulted in an increase in the amount of non-cross-linked PulD$^{\alpha}$A643C$^{\alpha}$ monomers. In addition, broadening of the multimer band upon DTT treatment also suggested that PulD$^{\alpha}$A643C$^{\alpha}$ multimers were cross-linked to PulSQ95C (Fig. 3c). Phenol treatment dissociated the multimers and predominantly resulted mainly in an increase in the amount of cross-linked PulD$^{\alpha}$A643C-PulSQ95C hetero-dimers (Fig. 3c). Neither phenomenon occurred with any of the other combinations or between wild-type proteins and the variants (Fig. 3c).

Having established that at least the majority of the PulD$^{\alpha}$A643C$^{\alpha}$ multimers formed were cross-linked to PulSQ95C, we next investigated whether these multimers behaved like the wild-type upon production in E. coli; i.e., do they induce the Psp response and permit PulA secretion. A Psp response is induced when PulD multimers associate with or insert into the inner membrane. For example, PulS$^{\alpha}$A643C-PulSQ95C hetero-dimer disappeared upon treatment with dithiothreitol (DTT), which resulted in a concomitant increase in the amount of non-cross-linked PulD$^{\alpha}$A643C$^{\alpha}$ monomers. In addition, broadening of the multimer band upon DTT treatment also suggested that PulD$^{\alpha}$A643C$^{\alpha}$ multimers were cross-linked to PulSQ95C (Fig. 3c). Phenol treatment dissociated the multimers and predominantly resulted mainly in an increase in the amount of cross-linked PulD$^{\alpha}$A643C-PulSQ95C hetero-dimers (Fig. 3c). Neither phenomenon occurred with any of the other combinations or between wild-type proteins and the variants (Fig. 3c).
background, a small proportion of the PulD<sub>0A643C</sub> multimers apparently inserted into the inner membrane and formed small pores, as is the case with wild-type PulD and PulS<sup>21,22</sup>.

PulS production and lipidation are critical to achieve PulA secretion through PulD<sup>30,38</sup>. Regardless of the presence of the substituted cysteines and regardless of the formation of a disulfide bridge, all multimeric secretins secreted PulA efficiently (Fig. 4b). Hence, it appears that PulD<sub>0A643C-PulS<sub>095</sub></sub> cross-linked secretins are fully functional and that PulS dissociation is not required to allow PulD assembly and function.

**Final PulD<sub>28–42</sub>/PulS<sub>259–660</sub> folding steps are efficient in phosphatidylethanolamine, but not in phosphatidylglycerol.** The fast acquisition of the trypsin-resistant state in the presence of *E. coli*-lipids, compared to that in the presence of lecithin, demonstrated the importance of the lipid composition for efficient PulD<sub>28–42</sub>/PulS<sub>259–660</sub> folding. To explore this observation further, PulD<sub>28–42</sub>/PulS<sub>259–660</sub> was produced in the presence of a series of liposomes with different headgroup compositions and hydrophobic thicknesses. Whether PulD<sub>28–42</sub>/PulS<sub>259–660</sub> could form native multimers in these liposomes was tested by electron microscopy (EM) of PulD<sub>28–42</sub>/PulS<sub>259–660</sub> solubilised from the liposomes by dodecylmaltoside. These procedures provide well-established, standard criteria for determining the extent of PulD<sub>28–42</sub>/PulS<sub>259–660</sub> folding and assembly<sup>15,25,29</sup>. Data from a series of comparisons are shown in Fig. 5. PulD<sub>28–42</sub>/PulS<sub>259–660</sub> appears as two stacked rings when examined by EM<sup>15</sup>. As previously, particle averaging revealed two orientations: a stack when viewed in the plane of the membrane (side view) and a disc perpendicular to the axis of symmetry (top view) (Fig. 5).

A first important difference between lecithin- and *E. coli*-liposomes is the composition of the lipid headgroups. To investigate whether the headgroup-composition of the membrane influences PulD<sub>28–42</sub>/PulS<sub>259–660</sub> folding into its native state, PulD<sub>28–42</sub>/PulS<sub>259–660</sub> folding was initiated in synthetic liposomes prepared from phospholipids with acyl chains containing 14 carbons. Bilayers containing C<sub>14</sub>-lipids are thicker than C<sub>12</sub>-bilayers. However, C<sub>14</sub>-lipids might be considered rather large, the mole-fraction of the PG-fraction used in the PE-fraction used is the highest that allows one to probe the importance of the PE-headgroup properties whilst maintaining membrane fluidity at the PulD<sub>28–42</sub>/PulS<sub>259–660</sub> synthesis temperature (30 °C).

PulD<sub>28–42</sub>/PulS<sub>259–660</sub> multimerised with high efficiency after 90 min into dC<sub>14</sub>:0PC- and dC<sub>14</sub>:0PC/PE-liposomes (90 ± 10 % and 94 ± 2 %, respectively; Fig. 5a,b). Multimerisation was much less efficient in dC<sub>14</sub>:0PE-liposomes (up to 55 ± 13 % on average; Fig. 5c), presumably due to the proximity of the phase transition temperature in the presence of dC<sub>14</sub>:0PE<sup>42,43</sup>. However, whereas at most approximately a third of PulD<sub>28–42</sub>/PulS<sub>259–660</sub> multimers acquired trypsin-resistance within 90 min in the presence of dC<sub>14</sub>:0PC- and dC<sub>14</sub>:0PC/PE-liposomes (16 ± 5 % and 35 ± 4 % of total PulD, respectively, Fig. 5a,b) more than half of the multimerised PulD<sub>28–42</sub>/PulS<sub>259–660</sub> was trypsin-resistant after 20 min in dC<sub>14</sub>:0PC/PE-liposomes (35 ± 10 % of total PulD, Fig. 5c). Although the amount of trypsin-resistant PulD<sub>28–42</sub>/PulS<sub>259–660</sub> multimers in dC<sub>14</sub>:0PC- and dC<sub>14</sub>:0PC/PE-liposomes was modest after 90 min, a significantly higher amount acquired trypsin-resistance after overnight incubation, indicating that the high trypsin-sensitivity measured at shorter times was not due to the physicochemical properties of the lipid composition used or to saturation of the liposomes with inserted PulD<sub>28–42</sub>/PulS<sub>259–660</sub> multimers (Fig. 5a,b). EM confirmed that PulD<sub>28–42</sub>/PulS<sub>259–660</sub> multimers attained their native structure in all of the membranes used (Fig. 5a–c). Thus, although the yield of PulD<sub>28–42</sub>/PulS<sub>259–660</sub> multimers was lower in the presence of dC<sub>14</sub>:0PC/PE-liposomes, the data suggest that late stages in PulD<sub>28–42</sub>/PulS<sub>259–660</sub> assembly occur more rapidly into dC<sub>14</sub>:0PC/PE-liposomes than into dC<sub>14</sub>:0PC- and dC<sub>14</sub>:0PC/PG-liposomes.

Because of the low yield of the PulD<sub>28–42</sub>/PulS<sub>259–660</sub> multimers in dC<sub>14</sub>:0PC/PE-liposomes, PulD<sub>28–42</sub>/PulS<sub>259–660</sub> assembly into liposomes containing 60 % dC<sub>12</sub>:0PC and 40 % dC<sub>12</sub>:0PG was compared with that in liposomes containing 90 % dC<sub>14</sub>:0PC and 10 % dC<sub>12</sub>:0PE. The latter liposomes remain more fluid than dC<sub>14</sub>:0PC/PE-liposomes with the same headgroup ratio and should produce a higher yield of PulD<sub>28–42</sub>/PulS<sub>259–660</sub> multimers. After 90 min PulD<sub>28–42</sub>/PulS<sub>259–660</sub> multimerised with high yields in the presence of both types of liposomes (87 ± 3 % and 89 ± 1 % in dC<sub>12</sub>:0PC/PG-liposomes and dC<sub>12</sub>:0PC/PE-liposomes, respectively (Fig. 6)). However, multimerisation was markedly slower in dC<sub>12</sub>:0PC/PG-liposomes at 0.049 ± 0.009 min<sup>−1</sup> compared to 0.115 ± 0.008 min<sup>−1</sup> in dC<sub>14</sub>:0PC/PE-liposomes (Fig. 6). Trypsin resistance was consistently higher after 20–60 min in dC<sub>12</sub>:0PC/PE-liposomes, while it remained low throughout the first 90 min in dC<sub>12</sub>:0PC/PG-liposomes (Fig. 6). Overnight incubation allowed the acquisition of higher trypsin resistance in dC<sub>12</sub>:0PC/PG and dC<sub>12</sub>:0PC/PE-liposomes (Fig. 6).

Together, the data indicate that PulD<sub>28–42</sub>/PulS<sub>259–660</sub> folding was accelerated in dC<sub>12</sub>:0PC and dC<sub>14</sub>:0PC-liposomes by including lipids with PE-headgroups.

**Thick bilayers accelerate final PulD<sub>28–42</sub>/PulS<sub>259–660</sub> folding steps.** Besides the differences in lipid headgroup composition between the *E. coli* and lecithin-liposomes, the lecithin bilayer has a higher hydrophobic thickness than *E. coli*-bilayers. *E. coli* lipids predominantly contain acyl chains that are 16 carbons in length<sup>44</sup>, but soy bean lecithin primarily contains lipids with 18 carbon long acyl-chains<sup>45</sup>. Considering that the hydrophobic thickness of the outer membrane is slightly less than that of bilayers...
of *E. coli* phospholipids (the acyl chains on LPS molecules usually have 14 carbons¹⁴⁰) the increased bilayer thickness of lecithin might delay the acquisition of PulD²⁸–⁴²/²⁵⁹–⁶⁶⁰ trypsin-resistance. The folding of PulD²⁸–⁴²/²⁵⁹–⁶⁶⁰ was therefore measured in synthetic liposomes containing only diC¹⁴:¹₅PC or diC¹₆:¹₅PC lipids, as well as in diC¹₄:¹₅PC-liposomes, all of which remain fluid under the experimental conditions used. PulD²⁸–⁴²/²⁵⁹–⁶⁶⁰ SDS-resistance was acquired quickly in the presence of diC¹₄:¹₅PC-, diC¹₆:¹₅PC- and

---

**Figure 5. Kinetics of PulD²⁸–⁴²/²⁵⁹–⁶⁶⁰ folding in C¹₄:₀-liposomes containing heterogeneity in the lipid headgroups.** (a) Multimerisation kinetics by acquisition of SDS- (■) and trypsin- (☐) resistance in the presence of 53 mM diC¹₄:₀-phosphatidylcholine (PC) liposomes. (b) Multimerisation kinetics by acquisition of SDS- (■) and trypsin- (☐) resistance in the presence of 53 mM diC¹₄:₀-phosphatidylcholine/glycerol (PC/PG) liposomes (60:40 mol/mol). (c) Multimerisation kinetics by acquisition of SDS- (■) and trypsin- (☐) resistance in the presence of 53 mM diC¹₄:₀-phosphatidylcholine/ethanolamine (PC/PE) liposomes (90/10 mol/mol). Errors represent S.D. over 3 independent measurements. (a–c) also show trypsin-resistance after overnight (O/N) incubation in the respective liposomes. Mu³ and Mo³ indicate the migration position of multimeric and monomeric PulD²⁸–⁴²/²⁵⁹–⁶⁶⁰ species, respectively. (a–c) include field images obtained by transmission electron microscopy of negatively stained PulD²⁸–⁴²/²⁵⁹–⁶⁶⁰ multimers solubilised from the respective liposomes in dodecylmaltoside (scale bar is 100 nm). The arrow and arrowhead show the top and side view, respectively. The insets show the average images of top (left) and side (right) view.
diC18:1PC-liposomes with rates of 0.125 ± 0.049, 0.103 ± 0.051 and 0.189 ± 0.110 min⁻¹, respectively and with efficiencies of 97 ± 7%, 101 ± 5% and 98 ± 5% after 90 min (Fig. 7a–c). However, whereas only 21 ± 5% and 23 ± 6% was trypsin-resistant after 90 min in the presence of diC14:1PC- and diC16:1PC-liposomes, respectively (Fig. 7a,b), the majority of the multimerised PulD28–42/259–660 (72 ± 18 %) adopted the trypsin

---

**Figure 6. Kinetics of PulD28–42/259–660 folding in C12:0-liposomes containing heterogeneity in the lipid headgroups.** (a) Multimerisation kinetics by acquisition of SDS- (■) and trypsin- (☐) resistance in the presence of 53 mM diC12:0-phosphatidylcholine (PC/PG) liposomes (60/40 mol/mol). (b) Multimerisation kinetics by acquisition of SDS- (■) and trypsin- (☐) resistance in the presence of 53 mM diC12:0-phosphatidylcholine/ethanolamine (PC/PE) liposomes (90/10 mol/mol). Errors represent S.D. over 3 independent measurements. (a,b) also show trypsin-resistance after overnight (O/N) incubation in the respective liposomes. Mu′ and Mo′ indicate the migration position of multimeric and monomeric PulD28–42/259–660 species, respectively.
Figure 7. Kinetics of PulD<sup>28–42/259–660</sup> folding in phosphatidylcholine (PC) liposomes with increasing hydrophobic thickness. (a) Multimerisation kinetics by acquisition of SDS- (■) and trypsin- (☐) resistance in the presence of 53 mM diC<sub>14:1</sub>PC-liposomes. (b) Multimerisation kinetics by acquisition of SDS- (■) and trypsin- (☐) resistance in the presence of 53 mM diC<sub>16:1</sub>PC-liposomes. (c) Multimerisation kinetics by acquisition of SDS- (■) and trypsin- (☐) resistance in the presence of 53 mM diC<sub>18:1</sub>PC-liposomes. Errors represent S.D. over 3 independent measurements. (a–c) also show trypsin-resistance after overnight (O/N) incubation in the respective liposomes. Mu<sup>tr</sup> and Mo<sup>tr</sup> indicate the migration position of multimeric and monomeric PulD<sup>28–42/259–660</sup> species, respectively.
resistant state gradually with increasing multimerisation in \( \text{d}C_{18:1}\text{PC} \)-liposomes (Fig. 7c). Higher levels of trypsin-resistance were also achieved in the presence of \( \text{d}C_{16:1}\text{PC} \)- and \( \text{d}C_{18:1}\text{PC} \)-liposomes after overnight incubation (Fig. 7a,b). Thus, although the final stages of PulD\( ^{28-42}/\text{259-660} \) folding were slow in thick lecithin-liposomes, PulD\( ^{28-42}/\text{259-660} \) folding occurred rapidly in the presence of pure \( \text{d}C_{18:1}\text{PC} \)-liposomes. In contrast, while PulD\( ^{28-42}/\text{259-660} \) folding was favoured in thinner \( E. \text{coli} \)-membranes, folding was slow in pure \( \text{d}C_{16:1}\text{PC} \)-liposomes.

The results suggest that the hydrophobic thickness of the membrane affects the rate of PulD\( ^{28-42}/\text{259-660} \) folding; however, hydrophobic thickness alone is not a critical determinant in PulD\( ^{28-42}/\text{259-660} \) folding.

**Discussion**

This report examines the factors required for the folding and assembly of the OMP PulD, whose biogenesis is independent of the general OMP-specific assembly machinery (BAM)\(^{23} \). Previous *in vivo* and *in vitro* studies\(^{20,21,46} \) demonstrated that the lipoprotein PulS plays an essential role in delivering PulD to the outer membrane. Here we show that PulS can improve the efficiency of early PulD multimerisation steps *in vitro*, but we failed to observe any major influence on later steps in PulD folding corresponding to the transition of the prepore into the native structure\(^{25} \). Nonetheless, PulS does not have to dissociate from its substrate for PulD to complete this transition. These observations clearly show that the PulD assembly pathway is quite different from the general pathway used by most OMPs, in which the broad specificity chaperones SurA and Skp must release their substrates near the membrane surface, passing them on to the membrane, for example by sensing lateral pressure. Formation of the PulD prepore\(^{39} \) to organise the membrane insertion, it would not need a system such as BAM that lowers the energy. Insertion in this manner likely requires a high level of organisation to measure the amount of energy stored in the membrane. Membrane insertion could depend on an as yet-unidentified assembly machinery, although this seems unlikely in view of its ability to insert both into artificial, protein-free liposomes *in vitro* and into the *E. coli* inner membrane when PulS is absent *in vivo*\(^{21,22} \). However, we observe that the lipid composition of the membrane influences PulD folding *in vitro* in an unusual fashion. Neither the lipid headgroup composition nor the hydrophobic thickness themselves appeared to be critical for folding. If the headgroup composition were critical, then PulD\( ^{28-42}/\text{259-660} \) folding would have been slow in \( \text{d}C_{18:1}\text{PC} \)-liposomes, whereas the contrary was observed. If membrane thickness were the critical factor, then PulD\( ^{28-42}/\text{259-660} \) folding would have been more efficient in lecithin compared to *E. coli* lipid, whereas the contrary was observed. Instead, we propose that general physical membrane properties, like membrane curvature and membrane-stored energy, drive efficient PulD insertion, both *in vitro* and *in vivo*. Stored energy is high in PE-containing bilayers (as in *E. coli* extract liposomes) because of the non-bilayer packing conformations of the PE-lipids that lead to an increase in curvature stress\(^{51,52} \), and also in thick membranes composed of lipids with long saturated or mono-unsaturated acyl chains (as in \( \text{d}C_{18:1}\text{PC} \))\(^{53} \). In contrast, thick lecithin liposomes, which contain a high number of poly-unsaturated acyl chains, and thin PC-/PG-liposomes form highly elastic membranes with little stored energy.

As PulD insertion appears to be tuned to suit the *in vivo* membrane composition, the observations reported here might rationalise why PulD assembly is Bam-independent. BAM comprises five proteins: four peripheral lipoproteins (BamB-E) and one membrane embedded protein, BamA, which forms the central component that catalyses OMP insertion\(^{4} \). High-resolution structures and simulations reveal how the 16-stranded \( \beta \)-barrel of BamA distorts the membrane around strands 1 and 16, providing an access route for OMP insertion into the membrane\(^{6,10,7,48} \) by lowering the energy barrier for OMP membrane insertion. If PulD exploits membrane-stored energy for its membrane insertion, it would not need a system such as BAM that lowers the energy. Insertion in this manner likely requires a high level of organisation to measure the amount of energy stored in the membrane, for example by sensing lateral pressure. Formation of the Prepore prepore\(^{39} \) to organise the C-domains could provide a means to achieve this. Therefore, the stability of the prepore structure could be a critical parameter in determining the fate of assembling PulD secretins.

How general is this phenomenon of BAM-independent OMP assembly? Although BAM-independent assembly was initially reported for PulD\(^{23} \) and then for other secretins in the same family\(^{15} \), the OMPs CsgG, GfC and Wza were also shown recently to exhibit Bam-independent assembly. Like PulD, they also appear to form prepore structures\(^{9,34,55} \). Like PulD\(^{29,39} \), all of these complexes require the coalescence of multiple subunits to form a single transmembrane pore or channel. We hypothesise that a common
assembly mechanism based on achieving a critical stability in the prepore and membrane-assisted inser-
tion represents a new paradigm for complex OMP assembly. The characteristics that determine whether
OMP assembly is BAM-dependent or not might be encoded in the three-dimensional structure of the
OMP, which remains to be determined at high-resolution for secretins. In vitro analysis of the folding of
OMPs with diverse structural features in the presence and the absence of BAM would greatly advance
our understanding of the mechanisms involved.

Methods
Strains, plasmids, cloning and site-directed mutagenesis. Cloning and PulDΔ functional assays
were performed in E. coli K-12 Pap105 (Δ(lac-pro) F’ (lacF’ ΔlacZM15 proAB’ Tn10)). Cells were grown at
30°C in Luria Bertani medium supplemented with ampicillin (100 μg/ml) and chloramphenicol (25 μg/ml)
as appropriate.

Plasmids encoding for PulD variants were obtained by site-directed mutagenesis on the plasmids
pCHAP3635 and pCHAP3629. The first is a pSU18 derivative that allows high levels of PulD produc-
tion for cross-linking and PspA response assays, whilst the second is a pHSG575 derivative for a low
production level used in secretion assays. PulS variants were generated from a pUC19 derived vector
containing the pulS gene (pCHAPS85)Δ2. Primers used for mutagenesis are listed in Table 1.

To produce PulSQ95C in the presence of all other Pul proteins, the pulS gene was mutagenised through
a cloning sequence rather than by site-directed mutagenesis. This was required because of the large size
of the plasmids carrying the entire pul operon. First, the DNA fragment encoding for all the Pul proteins
(except PulDΔ) was amplified from pCHAP585Δ2 and ligated into pCHAP231Δ2 using restriction sites
Psil and HindIII. This created the plasmid pCHAP3402 that encodes for all the Pul proteins except PulD and
has unique Ascl and AsiSI restriction sites flanking a 2929 bp fragment carrying the pulS gene. Two separate,
partially overlapping amplicons were generated to cover the entire 2929 bp: one fragment from
the Ascl-site up to the codon for Q102 on the pulS gene and a second from the codon for S89 on the pulS
gene to the Ascl-site. Primers annealing to the pulS gene carried the required codon change to substitute
amino acid Q95 into C in PulS (Table 1, primers ING339 and 340). Primers annealing near the AsiSI
site were boiled and 0.05 D600nm/ml of each was loaded. Proteins were separated on 10% or 15% polyacryla-
dime (37.5:1 acrylamide/bisacrylamide) gels or gels composed of stacked layers of 10% and 15% and

Analysis of PulDΔ-PulS cross-linking efficiency. Cells were transformed with the appropriate com-
bination of two plasmids, one encoding for wild-type PulDΔ or a single cysteine variant of PulDΔ (A643C,
A649C or F654C) and one for wild-type PulS or a single cysteine variant of PulS (Q38C or Q95C). The
cells of 1 ml of the overnight culture were collected and resuspended in SDS sample buffer (4% SDS,
62.5 mM Tris (pH 6.8), 20% glycerol) supplemented with 10 mM dithiotreitol (DTT), as indicated, to
a density of 10 D600nm/ml. Where indicated, PulD multimers were dissociated by phenol extraction and
dissolved at the same concentration in SDS sample buffer with or without DTT, as indicated. All samples
were boiled and 0.05 D600nm/ml of each was loaded. Proteins were separated on 10% or 15% polyacryla-
dime (37.5:1 acrylamide/bisacrylamide) gels or gels composed of stacked layers of 10% and 15% and
analysed by immunoblotting with antibodies against PspA, PulS, PulD and OmpF, as indicated. Bands were analysed by densitometry.

**PspA induction and PulA secretion assay.** PspA induction was measured from the same cells used in the cross-linking assays. An empty vector and cells producing wild-type PulD in the absence of PulS were used as negative and positive controls, respectively. PulA activity following its secretion to the outer surface was measured upon induction of the entire *pul* operon with 0.4% maltose in cells transformed with one of two plasmids encoding for the entire set of Pul proteins except PulD, pCHAP3402 (for wild-type PulS) or pCHAP3405 (for PulS<sub>Δ</sub>575C), and either pHSG575 (empty vector), pCHAP362 (wild-type PulD<sup>fl</sup>) or pCHAP3406 (PulD<sup>fl</sup><sub>Δ</sub>643C). Pullulanase secretion was measured as a fraction of the pullulanase enzymatic activity on the bacterial surface in whole cells compared to that of octyl-polyoxyethylene lysed bacteria and relative to the activity upon PulA secretion in the presence of wild-type PulS and PulD<sup>Δ</sup>575C.

**Liposome preparation.** Appropriate amounts of lecithin (Sigma), *E. coli* polar extract, diC<sub>12:0</sub>PC, diC<sub>12:0</sub>PE, diC<sub>14:0</sub>PC, diC<sub>14:0</sub>PE, diC<sub>14:1</sub>PC, diC<sub>16:1</sub>PC or diC<sub>18:1</sub>PC (Avanti Polar Lipids) in solvent (as supplied) were dried under a gentle stream of nitrogen followed by evaporation of residual chloroform under vacuum. Dried lipids were hydrated to 20–200 mg/ml (as appropriate), vortexed and sonicated for 15 min in a water bath.

**sPulS production and purification.** Production and purification of sPulS is described elsewhere<sup>19</sup>. Briefly, cells containing the plasmid for the expression of MalE-PulS with an N-terminal hexahistidine-tag were grown to a D<sub>600</sub> = 0.5 and induced with 0.5 mM IPTG for 4 h. Cells were harvested, lysed and debris was removed by centrifugation. The supernatant was applied to a nickel charged HiTrap column for affinity purification. After elution, MalE-PulS containing fractions were dialysed and digested overnight with Factor Xa. sPulS was further purified by cation exchange (HiTrap SP-Sepharose column) and gel filtration (HiLoad 16/60 Superdex 200 column).

**PulD synthesis.** PulD was synthesised by *in vitro* translation using an RTS100 *E coli* kit (5 Prime) as described<sup>25,29</sup> in the presence of 10 ng DNA (pCHAP3731 (PulD<sup>fl</sup>), pCHAP3716 (PulD<sup>fl</sup><sub>28–42/259–660</sub>)), pCHAP3803 (PulD<sup>fl</sup><sub>Δ</sub>575C, PulD<sup>fl</sup><sub>Δ</sub>575C<sub>Δ</sub>28–42/259–660, PulD<sup>fl</sup><sub>Δ</sub>575C<sub>Δ</sub>28–42/259–660, PulD<sup>fl</sup><sub>Δ</sub>575C<sub>Δ</sub>28–42/259–660, PulD<sup>fl</sup><sub>Δ</sub>575C<sub>Δ</sub>28–42/259–660, PulD<sup>fl</sup><sub>Δ</sub>575C<sub>Δ</sub>28–42/259–660), 10 to 60 μg liposomes and 0.2 μg sPulS (as indicated) per μl RTS100 at 30 °C. Although the RTS100 kits were centrifuged at 100 000 g for 30 min before use to remove most of the *E. coli* membranes, the trace amounts that remain are sufficient to allow limited PulD assembly. Synthesis was arrested with 3 ng streptomycin per μl of reaction after 6 min for initial multimerisation experiments, after 10 min in all other kinetic experiments and for at least 6 h for structural characterisation. Synthesis reactions were further incubated for at least 6 h at 30 °C for complete folding to occur. Mixed multimers were produced by priming the reaction with the relevant DNAs in a 1:1 ratio. PulD<sup>fl</sup><sub>Δ</sub>575C<sub>Δ</sub>28–42/259–660 was used as a control for the effects of the addition of sPulS to the reaction mixture. PulD<sup>fl</sup><sub>Δ</sub>575C<sub>Δ</sub>28–42/259–660 no longer has the S-domain that binds PulS and behaves in all experiments performed as PulD<sup>fl</sup><sub>Δ</sub>575C<sub>Δ</sub>28–42/259–660 in the absence of sPulS. Monomeric and multimeric PulD were separated in SDS on a 10 % polyacrylamide gel (37:5:1 acrylamide/bis-acrylamide) gel without heating to 100 °C, transferred to nitrocellulose and analysed by immunoblotting with an antibody raised against native PulD-multimers. Bands corresponding to multimeric and monomeric PulD were analysed by densitometry. Resulting transients were fitted to a single exponential equation using Kaleidagraph 4.0. Fitting parameters are reported in the text.

**Folding kinetics followed by SDS treatment.** Folding transients of PulD<sup>fl</sup><sub>Δ</sub>575C<sub>Δ</sub>28–42/259–660 were obtained by mixing aliquots of the synthesis reaction at the time points indicated with SDS sample buffer in a 1:1 ratio to arrest folding and incubated on ice for 1 h before analysis by SDS-PAGE.

**Folding kinetics followed by limited proteolysis by trypsin digestion.** Trypsin was added to PulD<sup>fl</sup><sub>Δ</sub>575C<sub>Δ</sub>28–42/259–660 aliquots at the times indicated to a final concentration of 4 μg/μl and incubated on ice for 5 min. Reactions were blocked using 150 ng/ml Pefabloc (Interchim) before mixing with SDS sample buffer for analysis. The fraction of trypsin resistant multimers was determined relative to the amount of SDS-resistant multimer at the endpoint of the reaction.

**Protein solubilisation and transmission electron microscopy (TEM).** Liposomes, purified as above, were resuspended in 100 mM Tris, pH 7.5, and 500 mM NaCl and diluted twice in 2% DDM. The lipid to detergent ratio was typically 1:5 (w/w) for solubilisation. For negative staining, 4 μl of sample was adsorbed onto carbon film-coated copper EM grids, washed with three droplets of pure water and subsequently negative stained with 2% (w/v) uranyl-acetate. The prepared grids were imaged using a Philips CM10 TEM (FEI, Eindhoven, The Netherlands) operating at 80 keV. Images were recorded on a side-mounted Veleta 2 K × 2 K CCD camera (Olympus, Germany) at a magnification of 130000. The pixel size at the sample level is 3.7 Å. Image processing was performed in the EMAN2 software package<sup>40</sup>. The images were contrast transfer function corrected and the particles were semi-automatically
selected. e2refine2d was used to classify the particles. This program produces reference-free class averages from a population of mixed, unaligned particle images. The representative class average with the best signal-to-noise ratio were selected and gathered in a gallery.

**References**

1. Nikaiko, H. Molecular basis of bacterial outer membrane permeability revisited. *Microbiol Mol Biol Rev 67*, 593–656 (2003).
2. Solov'eva, T. F., Novikova, O. D. & Portynagina, O. Y. Biogenesis of beta-barrel integral proteins of bacterial outer membrane. *Biochemistry (Moscow) 77*, 1221–36 (2012).
3. Hagan, C. L., Silhavy, T. J. & Kahne, D. Beta-Barrel membrane protein assembly by the Bam complex. *Annu Rev Biochem 80*, 189–210 (2011).
4. Driessen, A. J. & Nouwen, N. Protein translocation across the bacterial cytoplasmic membrane. *Annu Rev Biochem 77*, 643–67 (2008).
5. Gewinski, D. & others Outer membrane beta-barrel protein folding is physically controlled by periplasmic lipid head groups and BamA. *Proc Natl Acad Sci USA 111*, 5878–83 (2014).
6. Nojaim, N. et al. Structural insight into the biogenesis of beta-barrel membrane proteins. *Nature 501*, 385–90 (2013).
7. Patel, G. J. & Kleinschmidt, J. H. The lipid bilayer-inserted membrane protein BamA of Escherichia coli facilitates insertion and folding of outer membrane protein A from its complex with Skp. *Biochemistry 52*, 3974–86 (2013).
8. Vouhoux, R., Bos, M. P., Geurtsen, J., Mols, M. & Tommassen, J. Role of a highly conserved bacterial protein in outer membrane protein assembly. *Science 299*, 262–5 (2003).

9. Dunstan, R. A. et al. Assembly of the secretion pores GspD, Wza and CsgG into bacterial outer membranes does not require the Omp85 proteins BamA or TamA. *Mol Microbiol 97*, 616–29 (2015).
10. Hoang, H. H. et al. Outer membrane targeting of Pseudomonas aeruginosa proteins shows variable dependence on the components of Bam and Lol machineries. *MBio 2*, e00246–11 (2011).
11. Estrada Mallarino, L. et al. TiOmp85, a beta-barrel assembly protein, functions by barrel augmentation. *Biochemistry 54*, 844–52 (2015).
12. Nojaim, N., Rollauer, S. E. & Buchanan, S. K. The beta-barrel membrane protein insertase machinery from Gram-negative bacteria. *Curr Opin Struct Biol 21*, 35–42 (2011).
13. Korotkov, K. V., Gonen, T. & Hol, W. G. Secretins: dynamic channels for protein transport across membranes. *Trends Biochem Sci 36*, 433–43 (2011).
14. Russel, M. Macromolecular assembly and secretion across the bacterial cell envelope: type II protein secretion systems. *J Mol Biol 279*, 485–99 (1998).
15. Chami, M. et al. Structural insights into the secretin PulD and its trypsin-resistant core. *J Biol Chem 280*, 37732–41 (2005).
16. Korotkov, K. V., Pardon, E., Steyaert, J. & Hol, W. G. Crystal structure of the N-terminal domain of the secretin GspD from ETEC determined with the assistance of a nanobody. *Structure 17*, 255–65 (2009).
17. Genin, S. & Boucher, C. A. A superfamily of proteins involved in different secretion pathways in gram-negative bacteria: modular structure and specificity of the N-terminal domain. *Mol Gen Genet 243*, 112–8 (1994).
18. Daelder, S., Guivout, I., Hardie, K. R., Pugsley, A. P. & Russel, M. The OmpA domain of the secretin PulD contains the binding site for its cognate chaperone, PulS, and confers PulS dependence on pilV1 function. *Mol Microbiol 24*, 465–75 (1997).
19. Nickerson, N. N. et al. Outer membrane targeting of secretin PulD protein relies on disordered domain recognition by a dedicated chaperone. *J Biol Chem 286*, 38833–43 (2011).
20. Collin, S., Guivout, I., Nickerson, N. N. & Pugsley, A. P. Sorting of an integral outer membrane protein via the lipoprotein-specific Lol pathway and a dedicated lipoprotein pilotin. *Mol Microbiol 80*, 655–65 (2011).
21. Guivout, I., Chami, M., Engel, A., Pugsley, A. P. & Bayan, N. Bacterial outer membrane secretin PulD assemblies and inserts into the inner membrane in the absence of its pilotin. *Embo J 25*, 5241–9 (2006).
22. Hardie, K. R., Lory, S. & Pugsley, A. P. Insertion of an outer membrane protein in Escherichia coli requires a chaperone-like protein. *Embo J 15*, 978–88 (1996).
23. Collin, S., Guivout, I., Chami, M. & Pugsley, A. P. YaeT-independent multimerization and outer membrane association of secretin PulD. *Mol Microbiol 64*, 1350–7 (2007).
24. Burgess, N. K., Dao, T. P., Stanley, A. M. & Fleming, K. G. Beta-barrel proteins that reside in the Escherichia coli outer membrane in vivo demonstrate varied folding behavior in vitro. *J Biol Chem 283*, 26748–58 (2008).
25. Guivout, I. et al. In vitro multimerization and membrane insertion of bacterial outer membrane secretin PulD. *J Mol Biol 382*, 13–23 (2008).
26. Entzminger, K. C., Chang, C., Myhr, R. O., McCallum, K. C. & Maynard, J. A. The Skp chaperone helps fold soluble proteins in vitro by inhibiting aggregation. *Biochemistry 51*, 4822–34 (2012).
27. McMorrana, L. M., Bartlett, A. I., Huysmans, G. H., Radford, S. E. & Brockwell, D. J. Dissecting the effects of periplasmic chaperones on the in vitro folding of the outer membrane protein PagP *J Mol Biol 425*, 3178–91 (2013).
28. Patel, G. J., Behrens-Kneip, S., Holst, O. & Kleinschmidt, J. H. The periplasmic chaperone Skp facilitates targeting, insertion, and folding of OmpA into lipid membranes with a negative membrane surface potential. *Biochemistry 48*, 10235–45 (2009).
29. Huysmans, G. H., Guivout, I. & Pugsley, A. P. Sequential steps in the assembly of the multicellular outer membrane secretin PulD. *J Biol Chem 288*, 30700–7 (2013).
30. Hardie, K. R., Seydel, A., Guivout, I. & Pugsley, A. P. The secretin-specific, chaperone-like protein of the general secretory pathway: separation of proteolytic protection and piloting functions. *Mol Microbiol 22*, 967–76 (1996).
31. Tosi, T. et al. Pilotin-secretin recognition in the type II secretion system of Klebsiella oxytoca. *Mol Microbiol 82*, 1422–32 (2011).
32. Guivout, I., Nickerson, N. N., Chami, M. & Pugsley, A. P. Multimerization-defective variants of dodecameric secretin PulD. *Res Microbiol 162*, 180–90 (2011).
33. Goldfine, H. Bacterial membranes and lipid packing theory. *J Lipid Res 25*, 1501–7 (1984).
34. Padley, F. B., Gunstone, F. D. & Harwood, J. L. *Bacterial Lipids in The Lipid Handbook* (eds Gunstone, F.D., Harwood, J.L. & Padley, F.B.) pp. 1273 (Chapman & Hall, London, 1994).
35. Rehman, S., Gu, S., Shevchik, V. E. & Pickersgill, R. W. Anatomy of secretin binding to the Dickeya dadiantti type II secretion system pilotin. *Acta Crystallogr D Biol Crystallogr 69*, 1381–8 (2013).
36. Gu, S., Rehman, S., Wang, X., Shevchik, V. E. & Pickersgill, R. W. Structural and functional insights into the pilotin-secretin complex of the type II secretion system. *PLoS Pathog 8*, e1002531 (2012).
37. Shevchik, V. E. & Condemine, G. Functional characterization of the Erwinia chrysanthemi OutS protein, an element of a type II secretion system. *Microbiology 144* (Pt 11), 3219–28 (1998).
38. d'Enfert, C., Ryter, A. & Pugsley, A. P. Cloning and expression in Escherichia coli of the Klebsiella pneumoniae genes for production, surface localization and secretion of the lipoprotein pullulanase. *EMBO J 6*, 3531–8 (1987).
Lipids assist the membrane insertion of a BAM-...Huysmans, G. H. M.

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Huysmans, G. H. M. et al. Lipids assist the membrane insertion of a BAM-independent outer membrane protein. Sci. Rep. 5, 15068; doi: 10.1038/srep15068 (2015).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/