

# Oligomerization of type III secretion proteins PopB and PopD precedes pore formation in *Pseudomonas*

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***Pseudomonas aeruginosa* is the agent of opportunistic infections in immunocompromised individuals and chronic respiratory illnesses in cystic fibrosis patients. *Pseudomonas aeruginosa* utilizes a type III secretion system for injection of toxins into the host cell cytoplasm through a channel on the target membrane (the ‘translocon’). Here, we have functionally and structurally characterized PopB and PopD, membrane proteins implicated in the formation of the *P.aeruginosa* translocon. PopB and PopD form soluble complexes with their common chaperone, PcrH, either as stable heterodimers or as metastable heterooligomers. Only oligomeric forms are able to bind to and disrupt cholesterol-rich membranes, which occurs within a pH range of 5–7 in the case of PopB/PcrH, and only at acidic pH for PcrH-free PopD. Electron microscopy reveals that upon membrane association PopB and PopD form 80 Å wide rings which encircle 40 Å wide cavities. Thus, formation of metastable oligomers precedes membrane association and ring generation in the formation of the *Pseudomonas* translocon, a mechanism which may be similar for other pathogens that employ type III secretion systems.**

**Keywords:** pore formation/translocon/type III secretion

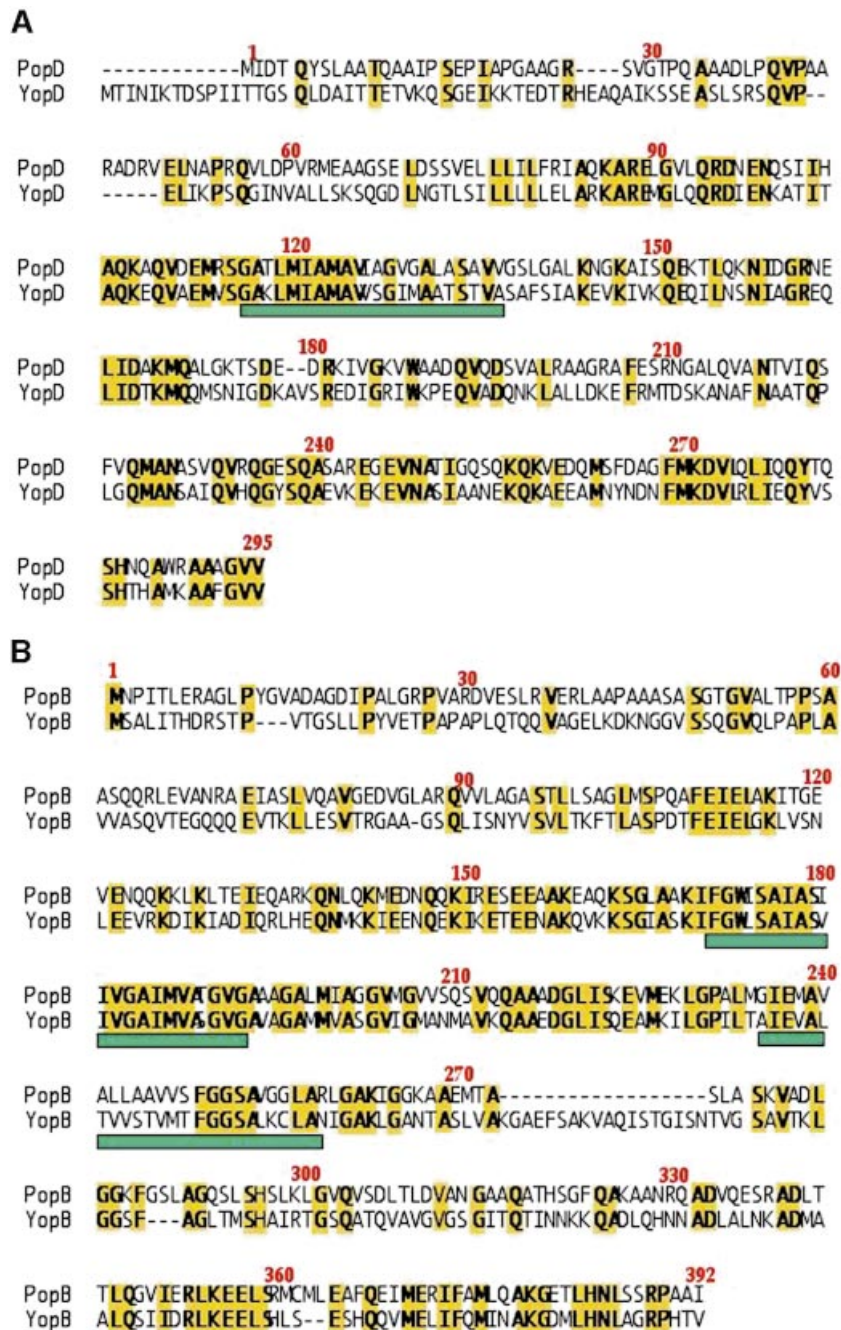
## Introduction

*Pseudomonas aeruginosa* is a Gram-negative, opportunistic pathogen, and is the causative agent of a large number of nosocomial infections as well as acute illness in immunocompromised individuals, such as AIDS, cancer and severe burn patients. In addition, chronic respiratory infections associated with *P.aeruginosa* colonization are a major cause of mortality for cystic fibrosis patients (Lyczak *et al.*, 2000). *Pseudomonas aeruginosa* employs a type III secretion system (TTSS), which is common among pathogenic bacteria, for the injection of at least four effector molecules (ExoS, ExoT, ExoY, ExoU) into the cytoplasmic compartments of target cells once a contact-dependent signal between bacterium and host cell

has been established. Upon entry into the cytoplasm, such effectors disturb the cellular cytoskeleton and initiate inflammatory and apoptotic processes, eventually leading to cell death (Finck-Barbancon, 1997; Frank, 1997; Yahra *et al.*, 1998).

In pathogens carrying the TTSS, secretion of effectors into target cells requires assembly of a complex macromolecular structure that spans both bacterial membranes (the ‘secretin’) and includes a 500 Å long ‘needle’ structure traversed by a 20–30 Å wide channel through which toxins may travel (Blocker *et al.*, 2001; Cordes *et al.*, 2003). Access of toxins into the target cytoplasm requires disruption of the cellular membrane at the site of contact by a proteinaceous pore (the ‘translocon’), itself presumably formed by proteins that are delivered through the TTSS needle. In the well-studied *Yersinia* TTSS, the pathogenicity arsenal is encoded by a large virulence plasmid (which codes for >20 proteins), the proposed members of the translocation machinery being encoded by the *lcrGVsycDyopBD* operon. Notably, YopB and YopD are two proteins suggested as forming the translocon itself (Håkansson *et al.*, 1996; Neyt and Cornelis, 1999b), and possess functional counterparts in other pathogenic bacteria, such as enteropathogenic *Escherichia coli* (EspB/EspD; Ide *et al.*, 2001) and *Shigella* spp. (IpaB/IpaC; Ménard *et al.*, 1994).

In *Yersinia* spp., evidence for the formation of a channel on the target membrane by YopB and YopD comes from the detection of YopB/YopD-dependent lytic activity on sheep erythrocytes (Håkansson *et al.*, 1996; Neyt and Cornelis, 1999b), as well as the measurement of an electrophysiologically detectable signal upon incubation of *Yersinia* YopB/YopD-expressing cells with artificial liposomes (Tardy *et al.*, 1999). Interestingly, however, YopD has been detected within the cytosol of infected cells, casting doubt on the role played by this protein in channel formation (Francis and Wolf-Watz, 1998). It is of note that PopB and PopD, expressed by the *Pseudomonas pcrGVHpopBpopD* operon, are capable of functionally complementing *yopB* and *yopD* mutants of *Yersinia pseudotuberculosis*, suggesting that these proteins may play similar roles in the infection process (Frithz-Lindsten *et al.*, 1998). In addition, in the *Pseudomonas* system, the soluble protein PcrV (like LcrV in *Yersinia*) has also been suggested to be a member of such a channel, based on its localization at the exterior of the bacterial cell upon host cell contact, its requirement for effector delivery and cytotoxicity, and its ability to induce conductance increase in lipid bilayer membranes (Pettersson *et al.*, 1999; Sawa *et al.*, 1999; Dacheux *et al.*, 2001; Holmström *et al.*, 2001). However, the role that PcrV could play in channel formation is unclear. To date, no structural information is available regarding the TTSS translocon for any pathogenic system, and the process of host cell membrane

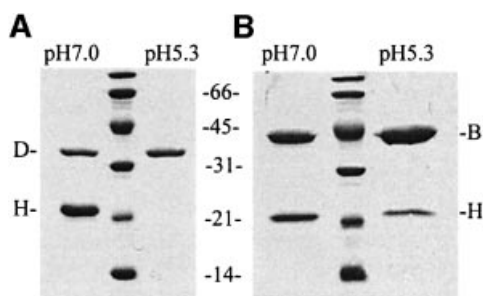


**Fig. 1.** Sequence alignments between (A) PopD and YopD, and (B) PopB and YopB from *P.aeruginosa* and *Yersinia pseudotuberculosis*, as performed by BLAST. PopD and YopD display 40% identity, while PopB and YopB display 43%. Identical residues are shown in bold and in yellow highlights, while transmembrane regions are indicated with green bars.

destabilization in bacterial infections remains largely uncharacterized.

In this work, we functionally and structurally characterize the *Pseudomonas aeruginosa* PopB/PopD translocation channel by expressing the Pop membrane proteins complexed to their common chaperone (PcrH). The PopB/PcrH complex is a mixture of oligomeric, metastable species, while PopD is only oligomeric at acidic pH and in the absence of PcrH. PopB/PcrH complexes bind to and lyse artificial membranes below pH 7.0, while PopD can only be effectively introduced into and disrupt membranes at acidic pH, once the

chaperone has been released. Both processes are independent of PcrV function. Negative-staining electron microscopy shows that the PopB/PcrH oligomer is a Y-shaped, 110 Å long and 90 Å wide molecule, while upon membrane association, PcrH-free PopB and PopD form ring-like structures of 80 Å in outer diameter and with a 40 Å wide centralized hole. The extensive biochemical characterization of the components of a type III secretion-translocation pore, as well as structural insight provided by electron microscopy data for the Pop system, allows us to propose a model for translocon pore formation and insertion.



**Fig. 2.** Complex formation properties of PopB and PopD with PcrH at different pH values. (A) Lane 1, peak fraction of PopD/PcrH purified at pH 7.0; lane 2, PopD purified at pH 5.3 has lost its chaperone PcrH. (B) Lane 1, peak fraction of PopB/PcrH purified at pH 7.0; lane 2, PopB/PcrH at pH 5.3 has released most PcrH, as compared with the neutral pH complex. Samples were separated on 12% SDS-PAGE and molecular weight markers are indicated.

## Results

### *PcrH* allows soluble expression of *PopB* and *PopD* in *E. coli*

PopB (392 amino acids) and PopD (295 amino acids) contain two and one putative transmembrane binding region, respectively (Figure 1) and, largely due to functional complementarity and sequence identity with the YopB and YopD of *Yersinia* (43 and 40%, respectively), have been postulated to require an intrabacterial chaperone for stability, possibly PcrH (LcrH/SycD in *Yersinia*; Neyt and Cornelis, 1999a). Bicistronic vectors capable of co-expressing PopB and PcrH, as well as PopD and PcrH, in the *E. coli* cytoplasm were constructed and soluble complexes were purified in both cases, indicating that PcrH is highly efficient at complexation of the Pop molecules. Notably, expression of all three proteins concomitantly from a *pcrHpopBpopD* tricistronic system permitted the purification of PopB/PcrH and PopD/PcrH binary complexes with biochemical characteristics that were indistinguishable from those described for the ones produced with the bicistronic systems.

### *PopB/PcrH* and *PopD/PcrH* binary complexes exist in multimeric forms

Gel filtration of PopD/PcrH between pH values of 7.0 and 9.0 revealed a unique, symmetric peak eluting at 13.5 ml on a Superdex 200 column, indicating a 1:1 complex between PopD (31 kDa) and PcrH (21 kDa) (Figure 2A). This stoichiometry was confirmed by native mass spectrometry of PopD/PcrH performed at physiological pH resulting in a mol. wt 52 674 Da (theoretical expected mol. wt 52 640 Da).

Interestingly, the PopD/PcrH sample gel filtered at pH 5.3 eluted as a broad peak running much earlier than the 1:1 complex at an elution volume (11 ml), which corresponds approximately to that of the 158 kDa marker protein aldolase. SDS-PAGE analysis revealed that at acidic pH the chaperone dissociates from the membrane protein, which then oligomerizes, as detected by gel filtration (Figure 2A). Native mass spectrometry analyses indicated that PcrH-free PopD consists of a heterogeneous mixture of oligomers. Hence, *in vitro*, a decrease in pH can act as a 'switch' that induces the release of PcrH from

PopD and promotes oligomerization of the latter. Notably, even in the absence of the chaperone, PopD does not aggregate at acidic pH.

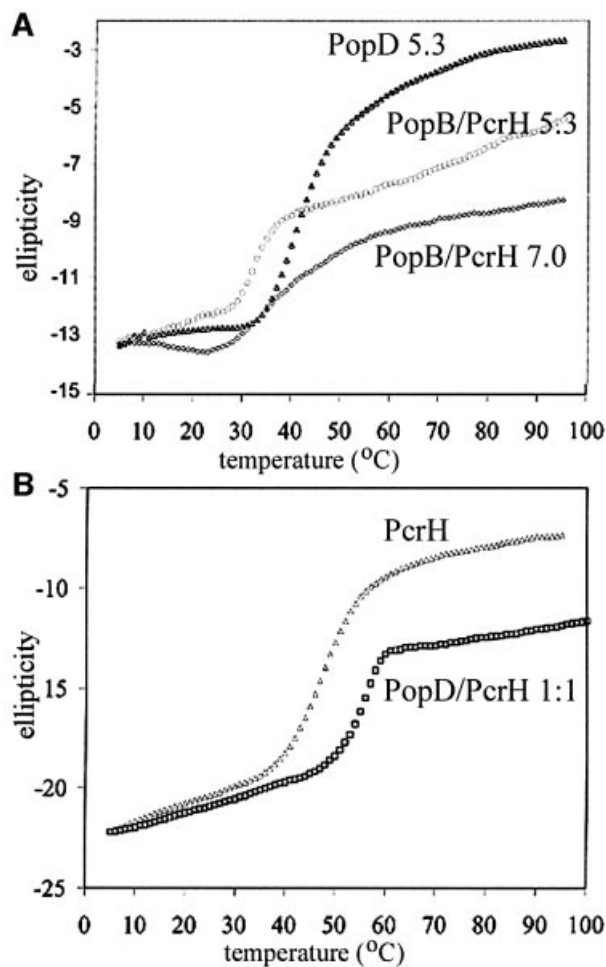
Purification of the binary complex between PopB (45 kDa) and PcrH (21 kDa) showed that it eluted as a single, broad peak at ~10 ml of a Superdex 200 column using several pH values between 5.0 and 9.0. This elution volume is consistent with an oligomeric species the molecular weights of which range between 300 and 500 kDa, based on marker proteins. Notably, a decrease in pH also induces release of PcrH from PopB, as in the case for PopD/PcrH, albeit not to completion (Figure 2B). Native mass spectrometry performed on the PopB/PcrH oligomer at both acidic and physiological pH values identified only the presence of a species with a mol. wt ~45 kDa, most probably the PopB monomer; no oligomers could be detected under the experimental conditions used. This observation suggests that the conditions required for the experiment may destroy the interaction between PopB and PcrH, in addition to the oligomers themselves. This result would be expected if the interaction between PopB and PcrH, as well within the oligomeric molecule, were mostly of hydrophobic nature. In addition, in contrast to PopD/PcrH, no heterodimeric complex between PcrH and PopB could be isolated.

### *PopB/PcrH* and *PopD* oligomers are metastable molecules

Circular dichroism studies were undertaken in order to verify the stability of the distinct complexes (1:1 versus oligomeric) at different pH values. All complexes tested produced CD spectra characteristic of  $\alpha$ -helical proteins (data not shown). The PopB/PcrH complex displayed transition temperatures ( $T_m$ ) of ~32°C at pH 5.3 and 40°C at pH 7.0, both of which are indicative of low thermostability (Figure 3A). Similarly, the acidic, oligomeric form of PopD melted at 42°C (Figure 3A). PcrH alone showed a unique  $T_m$  of 48°C (Figure 3B), and the 1:1 complex of PopD/PcrH analyzed at neutral pH displayed a  $T_m$  of ~55°C (Figure 3B), indicating increased thermostability of the PopD/PcrH heterodimer compared with the chaperone on its own. These results support the idea that although PopB/PcrH may exist in metastable form in a range of pHs, the low pH form (with much less PcrH) is less stable than the one purified at physiological pH. PopD, on the other hand, exists in two distinct conformations; one, complexed in 1:1 stoichiometry with PcrH, is relatively stable; a second one, oligomeric and dissociated from PcrH, melts at a temperature that is 13°C lower than the one measured for the 1:1 complex, and resembles the proposed metastable conformation of low pH PopB.

### *PopB* and *PopD* oligomers bind to and disrupt artificial membranes

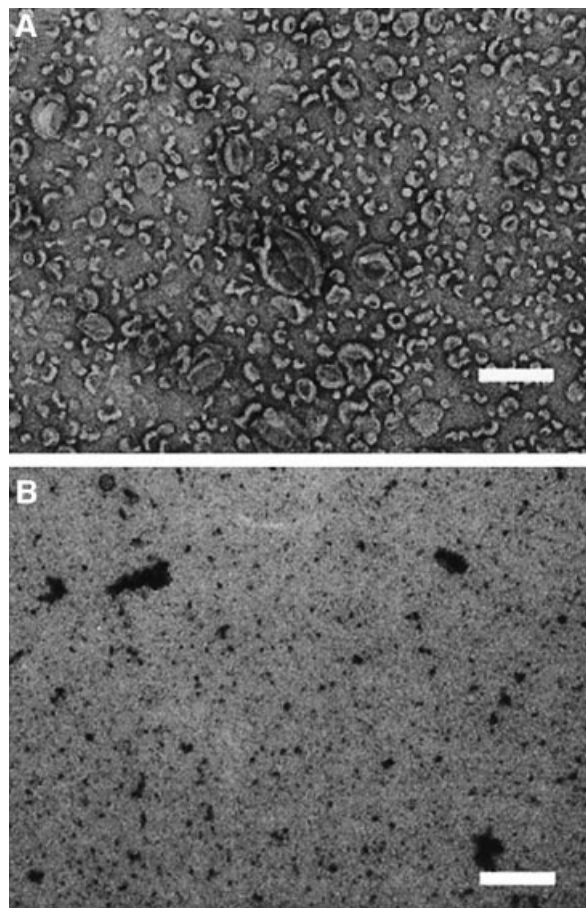
PopB and PopD have been suggested to be the putative components of the proteinaceous pore that is formed on host cell membranes upon bacterial attack. In addition, *P.aeruginosa* cytotoxicity has been reported to be linked to the presence on host cell membranes of lipid rafts, regions that are enriched in sphingolipids and cholesterol (Grassmé *et al.*, 2003). In order to verify whether membrane association of PopB and PopD required charged lipids and/or cholesterol and sphingomyelin, we



**Fig. 3.** Stability measurements of PcrH alone and in complex with PopB and PopD. **(A)** Oligomeric PopD, PopB/PcrH in low pH buffer (pH 5.3) and oligomeric PopB/PcrH in physiological buffer conditions (pH 7.0) were unfolded by increase of temperature and the circular dichroism signal was recorded at 222 nm. Although all three complexes measured at 0.1 mg/ml showed variable minima at 222 nm the measured ellipticity was adjusted to the same starting value for better visualization of the overlaid curves. **(B)** Thermostability measurements of PcrH and PopD/PcrH, both recorded at pH 7.0 at a concentration of 0.1 mg/ml and 222 nm. The starting value of the measured ellipticity of PopD/PcrH was adjusted to that of PcrH for better visualization of the overlaid curves.

incubated PopB/PcrH (pH values between 5.3 and 9.0), PopD/PcrH (pH 7.0–8.5) and PopD (pH 5.3) with artificial liposomes and separated soluble from membrane-bound proteins on sucrose gradients. Control liposomes, which were not incubated with protein, repeatedly migrated on fractions 1 and 2 of the gradients, while visibly lysed liposomes (in fact, lipid/protein aggregates and micelles) migrated on lower fractions (fractions 3–5). Liposomal lysis was evidenced by the appearance of white precipitate, which was detected by a clear spectral modification in the range of 220–260 nm (data not shown). Such lysis was protein concentration- and pH-dependent, with faster lytic activity occurring with increasing Pop concentration and decreasing pH. In addition, lysed liposomes were clearly distinct from unlysed, control liposomes as shown by negative-staining electron microscopy (Figure 4A and B).

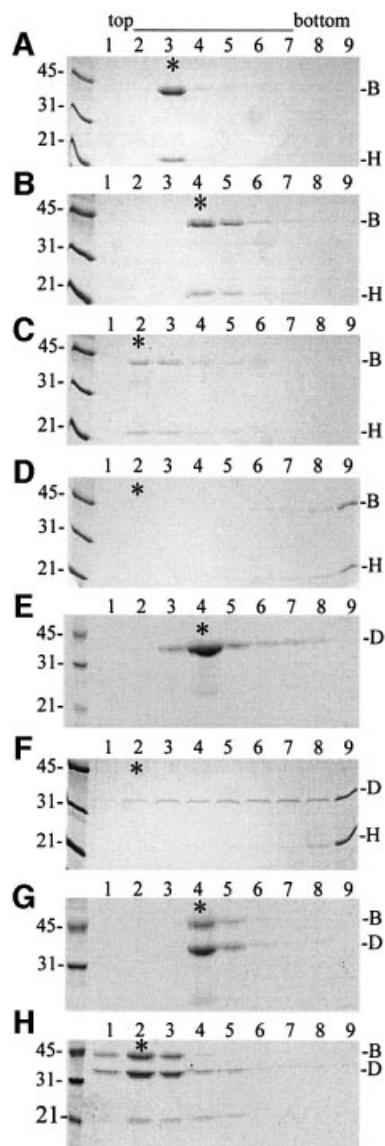
Our results show that PopB binds and lyses liposomes containing phosphatidylserine (PS) and cholesterol



**Fig. 4.** Negative-staining electron microscopy studies of intact and lysed liposomes. **(A)** Intact L1 liposomes. **(B)** The same liposomes as in **(A)**, after incubation with equimolar amounts of PopB and PopD. Scale bar, 200 nm.

(liposome composition L1), indicated by the presence of PopB in fraction 3 on a sucrose gradient (Figure 5A). Similarly, liposomes containing PS, cholesterol and sphingomyelin (L2) were lysed efficiently between pH 5 and 7.0, with the lipoprotein molecules migrating up to fraction 4 and 5 in a sucrose gradient (Figure 5B). Notably, when PS was exchanged against positively charged phospholipids such as phosphatidyl-ethanolamine (PE), no binding or lysis was observed in the presence (L3) or absence (L4) of cholesterol, independent of the pH (data not shown), demonstrating that negatively charged phospholipids play an important role in Pop binding. In addition, low pH PopB complexes only attached, but were unable to lyse, cholesterol-free liposomes (L5; Figure 5C). At pH 8.5 no significant binding or lysis of any of the five liposome types was achieved by PopB, as most of the protein was found at the bottom of the gradient (Figure 5D). The differences in binding efficiency at different pH values suggest that the protonation state of key residues, which could be related to a release of PcrH, may play an important role in membrane recognition by PopB.

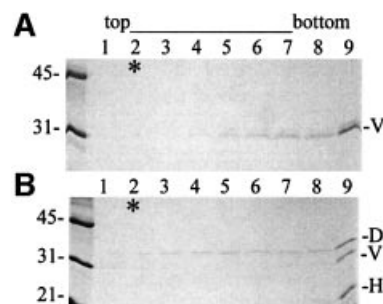
Notably, small amounts of PcrH could be detected in the liposome-containing fractions in experiments performed at acidic pH. This behavior seems to reflect non-specific association of the chaperone with the membranous fraction, since in control experiments where free chaperone



**Fig. 5.** PopB and PopD complexes interact with and disrupt liposomes *in vitro*. (A) Flotation of PopB/PcrH with L1 liposomes, pH 5.3. (B) Flotation of PopB/PcrH with L2 liposomes, pH 5.3. (C) Flotation of PopB/PcrH with L5 liposomes, pH 5.3. (D) Flotation of PopB/PcrH with L2 liposomes, pH 8.5. (E) Flotation of PopD with L1 liposomes, pH 5.3. (F) Flotation of PopD/PcrH with L2 liposomes, pH 8.5. (G) Flotation of equimolar amounts of PopD and PopB with L1 liposomes, pH 5.3. (H) Flotation of equimolar amounts of PopD and PopB with L5 liposomes, pH 5.3. All samples from the sucrose gradients were separated on a 12% SDS-PAGE. The position of a clear lipid band within the gradient is marked by an asterisk.

was added to PopB/PcrH proteoliposomes and the mixture was sucrose gradient purified, there were increased amounts of PcrH in the floating liposome fractions (not shown). In addition, other preliminary results indicate that PopB elutes as a single peak that is not associated with PcrH when PopB/PcrH purified lysed proteoliposomes (Figure 5B) are solubilized with 1%  $\beta$ -octyl-glucopyranoside and subsequently gel filtered (data not shown). Hence, the presence of PcrH in liposome-containing fractions in the experiments described above seems to be artifactual.

PopD was only able to recognize artificial membranes at acidic pH, that is, in its oligomeric, PcrH-free form. Under



**Fig. 6.** PcrV does not interact with membranes *in vitro* and does not affect PopD/PcrH membrane interaction. (A) Flotation of PcrV with L2 liposomes, pH 7.0; experiments performed at pH values between 5.3 and 8.0 displayed the same results. (B) Flotation of PcrV and PopD/PcrH with liposomes containing the same lipid composition as in (A), incubated at pH 8.5. Asterisks represent fractions where liposomes floated. Samples were separated on 12% SDS-PAGE.

these conditions, PopD bound to and lysed liposomes containing PS and cholesterol (L1), as indicated by its reduced mobility on a sucrose gradient (fraction 4; Figure 5E). Notably, the 1:1 PopD/PcrH complex did not bind any of the five different types of liposomes efficiently, although small amounts of PopD devoid of PcrH migrated up in sucrose gradients, while most of the protein was still present at the bottom (Figure 5F; results with L2 shown). These observations suggest that the 1:1 form of PopD/PcrH, much like the PopB/PcrH complex at basic pH, does not recognize membranes efficiently.

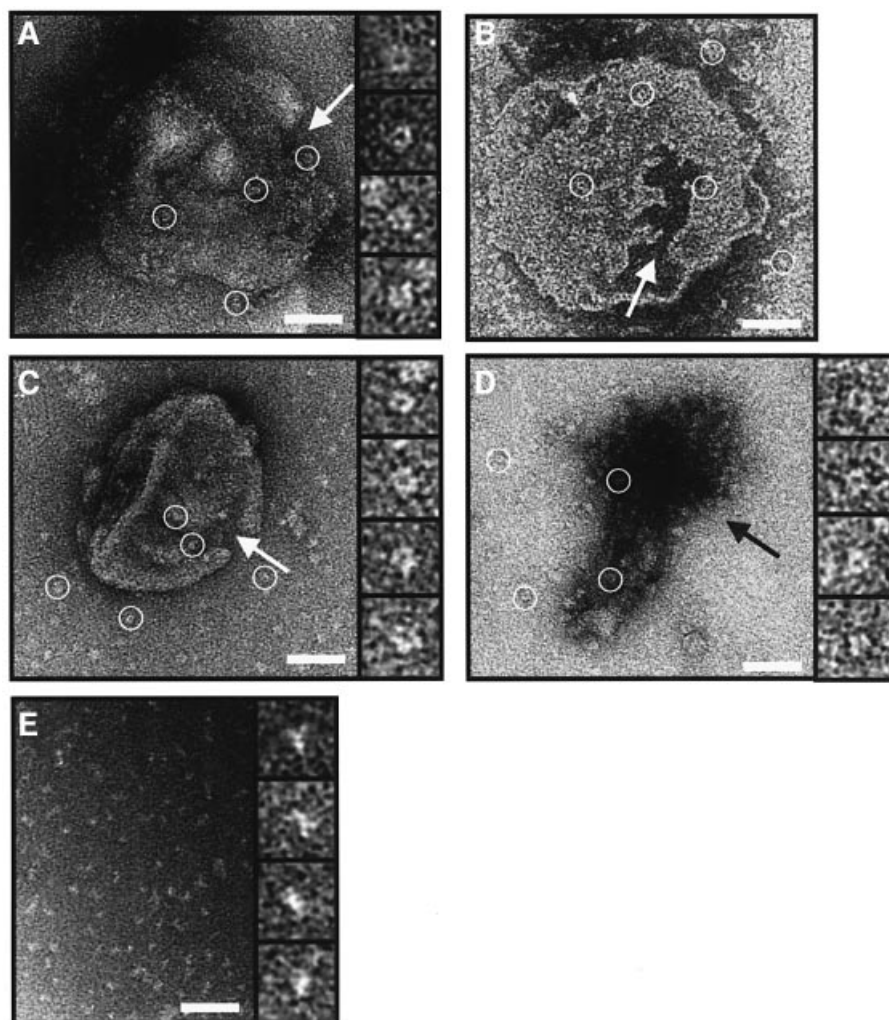
An equimolar mixture of PopB and PopD at acidic pH efficiently lysed liposomes (L1) containing PS and cholesterol. The lysed lipid-protein aggregates migrated mostly up to fraction 4 on a sucrose gradient (Figure 5G). As seen with low pH PopB/PcrH alone (see Figure 5C), a mixture of low pH PopD and PopB/PcrH floated and thus interacted with liposomes containing PS and no cholesterol (L5), but was not able to lyse them, as indicated by the fact that the proteins were detected on the top fractions of a sucrose gradient (Figure 5H), as well as by direct analysis of fraction 2 by electron microscopy (see below). Thus, membrane binding seems to be a cholesterol-independent process, while lysis requires the presence of cholesterol for both Pops.

#### **The role of PcrV in PopB, PopD and membrane association**

PcrV is a 30 kDa, soluble protein, which has been identified in the bacterial cytosol as well as outside the bacterium; in addition, it is essential for translocation of effectors into the host cytosol (Sawa *et al.*, 1999) and has been suggested as being a membrane-targeting component of the *Pseudomonas* translocon complex (Holmström *et al.*, 2001). In order to determine a possible role for PcrV in pore formation, liposome flotation experiments with liposomes prepared with five different lipid compositions were performed with purified PcrV both at physiological and acidic pHs; in all cases, PcrV remained at the bottom of the gradient, and did not associate with the liposomal fractions (Figure 6A).

*In vitro*, PopD requires a decrease in pH, and the release of PcrH, in order to bind to liposomes (described above); *in vivo*, this 'switch' could possibly be catalyzed by





**Fig. 7.** Negative-staining electron microscopy analysis shows that PopB and PopD lyse liposomes and form ring-like structures only upon membrane interaction. (A) Electron micrograph of L1 liposome incubated with PopB at pH 5.3, stained with uranyl acetate. (B) Electron micrograph of PopB associated to L1 liposome at pH 5.3, stained with SST. (C) Electron micrograph of PopD associated to L1 liposome at pH 5.3, stained with uranyl acetate. (D) Electron micrograph of PopB and PopD associated to L1 liposomes at pH 5.3, stained with uranyl acetate. (E) Electron micrograph of PopB/PcrH at pH 5.3 prior to membrane association and stained with SST. Scale bars, 500 Å (50 nm). White circles show ring-shaped molecules, which are either attached to a liposome or released. White arrows point to initial sites of membrane destruction in (A), (B) and (C). The black arrow points to ‘ghost’ liposomes, which are no longer visible (due to destruction) but still concentrate ring-shaped Pops in (D). Selected views of distinct molecular entities are shown on the galleries to the right of (A), (C), (D) and (E). For (A), (C) and (D) the three top molecules clearly show ring-like structures (top view of the pore; diameter ~80 Å, wall thickness ~20 Å), whereas the bottom one displays a more rectangular shape, with a channel going through (possibly a side view of the pore). In the (E) gallery, the PopB/PcrH complex is a ~110 Å long, ~90 Å wide Y shaped molecule. Micrographs (A), (C) and (D) correspond to samples purified by sucrose gradient centrifugation.

interaction with a different protein(s). In order to verify if PcrV could play the role of ‘switch molecule’ in the membrane-activation of PopD, flotation experiments with the five distinct types of liposomes were performed in the presence of both PcrV and PopD/PcrH at pH 8.5 (hence, in its membrane-inactive form). These studies showed that PcrV had no effect on the recognition of artificial membranes of any composition by the PopD/PcrH complex (Figure 6B), and the majority of PopD, PcrH and PcrV remained at the bottom of the gradient. Similarly, the same experiment was performed with the PopB/PcrH complex at pH 8.5 (in its membrane-inactive form), and the presence of PcrV also did not alter the lack of membrane recognition by the PopB/PcrH complex at this pH value (not shown). Subsequently, we attempted to detect a potential weak association between PcrV and

PopB/PcrH and PopD/PcrH molecules by co-elution of triple complexes from a Ni<sup>2+</sup> affinity column. However, this did not result in any detectable complexes of PcrV and either PopB/PcrH, PopD/PcrH or an equimolar mixture of PopB/PcrH and PopD/PcrH (not shown). These results thus suggest that PcrV does not interact *in vitro* with heterodimeric or oligomeric PopB/PcrH or PopD/PcrH complexes.

#### **PopB and PopD oligomers generate ring-like structures only upon membrane contact**

In order to shed light on the pore assembly mechanism of the *P.aeruginosa* translocon, PopB and PopD lipoprotein samples, prepared by incubation of Pop oligomers purified at pH 5.3, incubated with liposomes (L1), and purified by sucrose gradients (fraction 3, Figure 5A; fraction 4,

Figure 5E; fraction 4, Figure 5G) were studied by negative-staining electron microscopy in uranyl acetate (Figure 7A, C and D). Rapid destruction of liposomal structure was evident after incubation with either Pop molecule, with PopD seeming less efficient (more intact liposomes were present in samples prepared uniquely with PopD proteoliposomes). In order to test whether this problem could be circumvented by analyzing the lipoprotein sample immediately after mixing protein and liposomes (and thus avoiding the sucrose gradient purification time lapse), L1 liposomes were mixed with a range of concentrations of PopB and immediately stained with sodium silicotungstate and analyzed by electron microscopy (Figure 7B). Several features are of note. Ring-like structures were evident upon analysis of PopB (pH 5.3) proteoliposomes after sucrose gradient purification (Figure 7A) or immediately following mixing of protein and liposomes (Figure 7B); in the latter samples, less liposomal destruction was observed, and ring-like structures were clearly detectable on the surface of the vesicles. Similar ring-like structures were observed for low pH PopD proteoliposomes purified through a sucrose gradient (Figure 7C) as well as for gradient-purified samples of an equimolar mixture of both Pops (Figure 7D). The features of the rings are more evident from the magnifications of individual rings shown next to Figure 7A, C and D. In addition, rings that were not directly attached to liposomes were also visible, suggesting that the proteinaceous structures could be surrounded or stabilized by micelles that resulted from liposomal destruction (see Figure 7A–D, highlighted by a white circle). Thus, experiments performed on proteoliposomes prepared with or without sucrose gradient purification, as well as with two distinct electron microscopy stains, confirmed the reproducibility of the results, since the same ring-like structures were observed for PopB (Figure 7A and B). In samples containing an equimolar mixture of PopB and PopD, no remaining intact liposomes could be detected, and rings of similar size and shape as the ones detected for the individual Pops were visualized concentrated in a region where a ‘ghost’ liposome could be seen (black arrow, Figure 7D). Cholesterol-free liposomes treated with equimolar amounts of both Pops at low pH (fraction 3, Figure 5H) were intact, and displayed similar ring-like structures as the ones seen in Figure 7A–D (not shown). Consequently, PopB and PopD are capable of forming ring-like pore structures with diameters of ~80 Å, surrounding indented holes of 40 Å in diameter ( $\pm 3.5$  Å). These results are in agreement with the observations of Dacheux *et al.* (2001), who determined by osmoprotection experiments that a PopB/PopD pore inserted into red blood cell membranes could have an internal diameter ranging between 28 and 35 Å.

In contrast, negative-staining studies performed with PopB/PcrH, PopD and an equimolar mixture of PopB/PopD oligomers in the absence of liposomes (directly after gel filtration at pH 5.3) did not identify any ring-like structures. Rather, PopB/PcrH grids contained a large number of Y-shaped molecules ~110 Å in length, with the distance between the arms of the Y measuring ~90 Å (Figure 7E); electron microscopic analyses of PopD and PopB/PopD mixtures did not yield identifiable homogeneous features. These results suggest that Pop

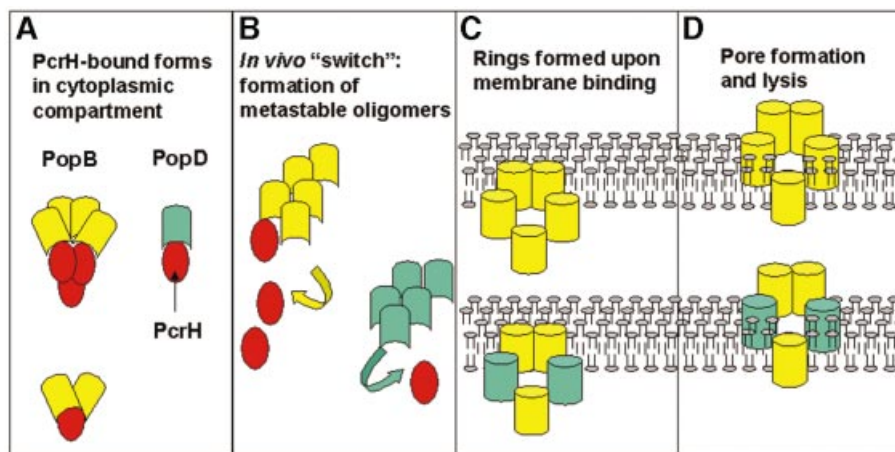
oligomerization may be a rate-determining, intermediate step in pore generation, and that membrane contact is the determining trigger for ring assembly and channel formation.

## Discussion

Formation of a proteinaceous pore of bacterial origin on the plasma membrane of target cells is an essential step for the injection of effectors via the TTSS mechanism for a variety of pathogenic bacteria. In this work, we have expressed *P.aeruginosa* pore-forming components PopB and PopD in complex with their common chaperone, PcrH, and have found that such complexes may be either dimeric and stable (as in the case of the high pH 1:1 PopD/PcrH complex) or oligomeric and metastable, as determined by circular dichroism (for PopB/PcrH and PcrH-free PopD). Only the metastable oligomers are able to recognize membranes, a behavior reminiscent of that observed for the channel-forming toxin colicin A, which must undergo a transition through a conformationally flexible intermediate (the ‘molten globule’) in order to become inserted into membranes (van der Goot *et al.*, 1991; Gouaux, 1997).

Co-expression of PopB and PopD in bicistronic systems with PcrH was necessary in order to render the proteins soluble and purifiable in high amounts. Thus, PcrH is a bifunctional chaperone, much like SycD (LcrH) of *Yersinia*, which is necessary for the stabilization and secretion of both YopB and YopD; SicA of *Salmonella typhimurium*, which is required for the stability of cognate effectors SipB and SipC; and IpgC, which binds to *Shigella* proteins IpaB and IpaC (Ménard *et al.*, 1994; Neyt and Cornelis, 1999a; Tucker and Galán, 2000). A key function of chaperones is to bind hydrophobic regions in their cognate proteins, shielding them from exposure and possible aggregation. Since PopB has two predicted transmembrane regions and PopD one (Figure 1), it is conceivable that an initial role for PcrH may be to mask the respective Pop hydrophobic domains; in its absence, the Pops may be trapped as aggregates, and thus be incapable of being secreted.

A second possible role for PcrH is revealed by the analysis of Pop/chaperone stoichiometries and stabilities at different pH values. The PopD/PcrH complex, purified at physiological pH, clearly exists in a 1:1 association, as shown by native mass spectrometry, and does not bind membranes efficiently. Although we were unable to identify a 1:1 PopB/PcrH complex, we propose that it may also exist as a defined complex, too unstable, however, to be detected after overexpression in an *E.coli* host, where it may spontaneously switch to the oligomeric form upon purification. In both PopD/PcrH and PopB/PcrH cases, a decrease in pH causes the membrane proteins to release PcrH, and in the extreme case of PopD, only the PcrH-free oligomeric protein is able to recognize membranes. Hence, *in vitro*, pH acts as a ‘switch’ (which probably mimics an *in vivo* signal), which promotes the release of the chaperone from the complexes. Since the release of PcrH seems to be related to the efficiency of membrane binding of Pop proteins, an additional function of PcrH could be to maintain PopB and PopD in ‘membrane-inactive’ conformations, thus preventing



**Fig. 8.** Model of translocon formation by PopB and PopD in *P.aeruginosa*. (A) PcrH associates to both PopB and PopD while they remain in the bacterial cytosol, preventing aggregation and/or activation. (B) The ‘*in vivo* switch’, which *in vitro* is mimicked by a decrease in pH, may involve recognition of the TTSS secretion or transport through the needle, and renders Pops in metastable oligomeric forms. (C) PopB and PopD may associate into a homomeric and/or heteromeric structures which recognize microdomain lipid rafts on the plasma membrane of target cells, forming rings with outer and inner diameters of 80 Å and 40 Å, respectively. (D) The PopB/PopD translocon forms a channel on the target membrane, allowing for other components of the *P.aeruginosa* TTSS infection machinery to be transported into the cellular cytoplasm. Indication of pentameric molecules is for schematic purposes only.

early activation of the Pops whilst in the bacterial cytoplasm (an event that could be detrimental for the bacterial membrane itself).

It is of note that the PopB/PcrH complex is able to bind to and lyse artificial membranes only at pH values <7.0. This behavior is reminiscent of that of the pore-forming toxin aerolysin, which does not display membrane-disrupting activity at pH values above 7.4 (Buckley *et al.*, 1995). In the case of aerolysin, the pH effect on activity is linked to the deprotonation of a single histidine residue, which at basic pH causes a reduction in the toxin’s ability to oligomerize, and thus form channels. Although in our hands, PopB/PcrH remains oligomeric at all pH values tested (albeit the clear release of PcrH at low pH), it is conceivable that protonation of key residues may also play a role in the processes of activation or PcrH release.

PcrV is a 30 kDa protein that has been shown to be essential for translocation of effectors into the host cytosol (Sawa *et al.*, 1999), and has been suggested as being able to integrate itself into membranes and determine the translocon pore size (Holmström *et al.*, 2001). LcrV, the homolog of PcrV in the *Yersinia* system, has been shown by affinity and immunoblotting experiments to associate to YopB and YopD, the potential components of the *Yersinia* translocon pore, and to be necessary for their secretion from the bacterium (Nilles *et al.*, 1998; Sarker *et al.*, 1998). In our hands, however, PcrV did not associate to artificial membranes (prepared in five distinct lipidic compositions) either on its own or in the presence of the Pop molecules, and was not able to associate to Pops in the presence or absence of a membrane environment. Thus, the functionality of PcrV in *P.aeruginosa* cytotoxicity and translocation is still unclear.

PopB and PopD are capable of binding, but not lysing, liposomes that do not contain cholesterol. In addition, both molecules require a negatively charged phospholipidic environment for membrane recognition. Interestingly, *P.aeruginosa* and *Shigella flexneri* have been recently reported to require the presence of cholesterol/sphingo-

lipid lipid raft microdomains on plasma membranes for efficient infection (Lafont *et al.*, 2002; Grassmé *et al.*, 2003). In this work, liposomes that were prepared in the presence of sphingomyelin and cholesterol (which are usually enriched in lipid raft microdomains; Simons and Toomre, 2000) were efficiently recognized by both Pops. These results suggest that PopB and PopD are able to associate to lipid rafts on plasma membranes, and although cholesterol is not an absolute requirement for binding, it may play a pivotal role in structural rearrangements required for pore formation and membrane disruption.

Although the individual steps leading to pore formation for a variety of toxins have been extensively debated in the literature, two main models are presently accepted. Tweten *et al.* (2001) propose that complete oligomerization must occur prior to channel formation, a theory supported by the crystal structure of pneumolysin from *Streptococcus pneumoniae*, which reveals that formation of the pore requires domain rearrangements within preformed oligomers (Gilbert *et al.*, 1999). The model proposed by Palmer *et al.* (1998), based on studies of the assembly mechanism of streptolysin O, suggests that the complete oligomer is only formed upon contact with the membrane. Although such molecules interact with membranes through a  $\beta$ -sheet conformation and become more stable upon oligomerization (Gilbert, 2002), a behavior that differs from the mostly  $\alpha$ -helical PopB and PopD whose oligomers display low thermostability, several observations point to a potential channel-forming mechanism. PopB and PopD are capable of binding to and lysing membranes individually, and such an effect is observed uniquely when they are present as metastable oligomers. Particles of PopB and/or PopD in acidic solution in the absence of liposomes did not exhibit ring-like structures in electron microscopy analyses; rather, the PopB/PcrH complex could be seen as an elongated, Y-shaped molecule. Evidence for the formation of rings by Pops was only obtainable once the proteins had been in contact with membranes. Hence, much like the  $\alpha$ -helical



toxin colicin A, Pop molecules may undergo a transition from an ordered structure to a more dynamic arrangement (i.e. through loss of PcrH) prior to membrane insertion, namely the 'molten globule intermediate', and it is this form, upon interacting with membranes, that is capable of forming rings. Thus, we suggest that the assembly mechanism of the PopB/PopD translocon follows a modified Tweten model, in which components must be fully assembled into an oligomeric, metastable conformation prior to membrane recognition, ring formation and pore generation. Although the stoichiometry of the PopB/PopD channel is still unclear, it is evident that each Pop can form a homooligomeric pore on its own; if PopB and PopD do form a heteromeric pore, which is probably the case, the metastability of both oligomers could allow for proper stoichiometry to be achieved prior to membrane insertion.

PopB and PopD molecules, prior to pore formation, must initially be transported through the TTSS needle, which displays an internal diameter of ~25 Å (Blocker *et al.*, 2001; Cordes *et al.*, 2003). If such transport occurred in the form of the ring-like structures identified in this work (which have an external diameter of 80 Å), this would require a considerably larger internal TTSS needle radius. From the dimensions of the Y-shaped PopB/PcrH oligomer at low pH (90 Å wide, 110 Å long) and based on the sizes determined by gel filtration of oligomeric Pop molecules, it is also improbable that such intermediate oligomeric forms would be able to be transported through the needle. Consequently, it is conceivable that Pops could travel through the needle complexed in 1:1 fashion with PcrH, as in the PopD/PcrH complex isolated in this work, although PcrH would probably not be secreted. This would also imply that a 1:1 PopB/PcrH complex could also exist in the bacterial cytoplasm.

Based on our results, we propose a model for TTSS translocon formation, shown in Figure 8. PcrH forms a heterodimeric complex with PopD and a heterooligomeric (and/or possibly heterodimeric) complex with PopB upon co-expression in the bacterial cytoplasm (Figure 8A). Following the action of an as yet undetermined 'in vivo switch', PopB and PopD enter their active, metastable states, releasing PcrH (Figure 8B). In their oligomeric conformations, both PopB and PopD recognize target cell plasma membranes enriched in lipid raft microdomains (Figure 8C), leading to the final step that inserts ring-like complexes into membranes, lysing them (Figure 8D). Such ring-like molecules may be heteromeric, being composed of both PopB and PopD molecules. We propose that this model, involving intermediate, oligomeric conformations of pore forming proteins, is likely to be common to other pathogens which employ a type III secretion mechanism to deliver effector molecules into target host cells.

## Materials and methods

### Construction of expression vectors

DNA fragments encoding PopB and PopD were amplified by PCR using QBioTaq polymerase (QBiogene), cloned in pGEM-T (Promega) and verified by double-stranded sequencing. The template plasmid for all PCRs was pIA60, a pUC18 derivative containing operons *pcrGVHpopBD* and *exsCBA* on an 8 kb *EcoRI* fragment isolated from the *P. aeruginosa* strain CHA (Dacheux *et al.*, 2000). Isolated fragments were cloned into

pET15b (Novagene) generating pET/*popB* and pET/*popD* harboring a His<sub>6</sub> tag upstream from the coding region of both genes.

The *pcrH* gene was amplified by PCR methods and cloned into pCR-Script Amp SK+ (Stratagene), generating pSK/*pcrH*. A *Bam*HI fragment from pSK/*pcrH* was introduced into pET15b, resulting in plasmid pET/*pcrH*. Subsequently, the bicistronic vector, pET/*popBpcrH* was obtained by ligating a blunt-ended *Xba*I–*Hind*III His<sub>6</sub>PcrH-encoding fragment from pET/*pcrH* into a blunt-ended *Bam*HI site of pET/*popB*, allowing the co-expression of His<sub>6</sub>-PopB and His<sub>6</sub>-PcrH from the same plasmid.

For the construction of the pET/*popDpcrH* bicistronic vector, the *popD* gene was extracted from pET/*popD* (described above) and cloned into pET30b (Novagen), leading to the construct pET30b/*popD* in which the His<sub>6</sub> tag located upstream from the *popD* coding region was deleted. The *pcrH* gene was cloned into pET30b/*popD*, leading to the bicistronic construct pET30b/*popDpcrH*, which allowed the co-expression of PopD and His<sub>6</sub>-PcrH from the same plasmid.

In order to verify whether Pop complexes expressed from the *P. aeruginosa pcrGVHpopBD* operon behaved in a similar fashion to the ones expressed with the bicistronic constructs, a PCR fragment containing *pcrHpopBD* was amplified using pIA60 as template and Vent polymerase (BioLabs). The fragment was cloned into pCR-Blunt II-TOPO (Invitrogen); an *Nde*I–*Bam*HI fragment was extracted by partial digestion and cloned into pET15b, yielding a construct which codes for a His<sub>6</sub> tag uniquely at the N-terminus of PcrH.

### Expression and purification of the PopB/PcrH and PopD/PcrH complexes

For the PopB/PcrH complex, expression was induced in *E. coli* strain BL21(DE3) in terrific broth with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h at 37°C. Cells were harvested by centrifugation and lysed by sonication in 25 mM Tris–HCl pH 8.0, 0.2 M NaCl, 2% glycerol. The supernatant was cleared by centrifugation and applied to a Ni-Sepharose column, pre-equilibrated in lysis buffer. The PopB/PcrH complex was eluted with a step imidazole gradient and dialyzed overnight in 25 mM Tris pH 8.0, 20 mM NaCl. Subsequently, the protein was loaded onto a MonoQ column which had been previously equilibrated in 25 mM Tris pH 8.0 and 1 mM EDTA, and was eluted by employing a gradient to 0.5 M NaCl in the same buffer. Further purification was performed by gel filtration chromatography (Amersham HR10/60 column). For the PopD/PcrH complex, most purification steps were similar, with the exception that protein expression was induced by adding 1 mM IPTG to bacterial cultures at 18°C for an overnight period.

Expression of PcrH, PopB and PopD proteins in *E. coli* BL21(DE3) from the tricistronic vector was performed as described for the PopB/PcrH complex; purification of the bacterial lysate over a Ni-Sepharose column yielded isolated PopD/PcrH and PopB/PcrH complexes which displayed the same biochemical characteristics as the ones purified from the bicistronic expression systems.

### Expression and purification of PcrV

The DNA fragment encoding full-length PcrV was generated by standard PCR techniques and subsequently cloned into the expression vector pET15b (Novagen) downstream from a His-tag-encoding, thrombin-cleavable region. Expression in *E. coli* strain BL21(DE3) was induced in LB medium with 1 mM IPTG for 3 h at 37°C. Cells were harvested by centrifugation and lysed by sonication in 25 mM Tris–HCl pH 8.0, 0.2 M NaCl, after which the cleared lysate was loaded onto a Ni-Sepharose column. PcrV was eluted with a step imidazole gradient and dialyzed overnight in 25 mM Tris pH 8.0, 50 mM NaCl. The protein was loaded onto a MonoQ column in dialysis buffer, from which it eluted with a linear NaCl gradient, and was subsequently loaded onto a gel filtration column (Amersham HR10/60) in 25 mM Tris pH 8.0, 0.1 M NaCl, 1 mM EDTA. His-tag free PcrV was prepared by adding thrombin to the purified protein mixture at a final ratio of 1:400, stopping the reaction with 2 mM phenylmethylsulfonyl fluoride, and loading the cleaved material onto a column containing a mixture of Ni-Sepharose and benzamide-Sepharose resins (Amersham), in 25 mM HEPES pH 7.0, 0.1 M NaCl. Purified protein was stable for many weeks at 4°C.

### Liposome preparation and membrane flotation experiments

Liposomes containing five distinct ratios of lipids (w/w) were produced: (L1) 60% L-α-phosphatidylcholine (PC), 25% PS, 15% cholesterol; (L2) 25% PS, 25% PC, 25% sphingomyelin and 25% cholesterol; (L3) 60% PC, 25% PE, 15% cholesterol; (L4) 60% PC, 40% PE; (L5) 60% PC, 40% PS. Briefly, a lipid detergent film was generated by lyophilization of a mixture of the indicated lipid composition (a total of 10 mg each) and 200 μg of N-octyl β-D-glucopyranoside solubilized in an ethanol:

chloroform solution (ratio depended on lipid composition). The lipid-detergent film was resuspended in 5 ml phosphate-buffered saline (PBS) and dialyzed against PBS using a liposomat (Dianorm). Detergent-free liposome formation was completed after ~3 h of dialysis. One hundred and fifty micrograms of PopB/PcrH complex, PopD/PcrH complex, PopD and/or PcrV were incubated with 250 µl of liposomes for 6 h at room temperature. Sucrose gradients were generated in PBS in a total volume of 4.5 ml, initially by adjusting the proteoliposomes to 60% sucrose, and subsequently by overlaying sucrose solutions ranging from 45 to 5% onto the proteoliposome–sucrose mixture. Centrifugation was performed in a Beckman SW55 rotor at 40 000 r.p.m. for 16 h at 10°C. Fractions (400 µl) were collected from the gradients and aliquots were separated by 12.5% SDS–PAGE; bands were detected with Coomassie Blue staining.

#### Affinity column interaction experimentation

Four 1 ml Ni-NTA columns were washed in 25 mM HEPES pH 7.0, 0.1 M NaCl. Each column was loaded with: (A) 700 µg PcrV; (B) 700 µg PopD/PcrH complex; (C) 700 µg PopB/PcrH complex; and (D) 700 µg PopB/PcrH complex and 700 µg PopD/PcrH complex; all protein samples were in the same HEPES buffer. Subsequently, columns B, C and D were loaded with 700 µg PcrV each, after which all columns were washed with 4 ml HEPES buffer. Elution was performed with 3 ml of 25 mM HEPES pH 7.0 + 0.1 M NaCl + 400 mM imidazole. Fractions (1 ml) were collected during all steps, and were analyzed by 12.5% SDS–PAGE; bands were detected with Coomassie Blue staining.

#### Circular dichroism measurements

Circular dichroism spectra were measured on a Jasco J-810 spectropolarimeter at 22°C in a 1 mm cell in either 10 mM sodium phosphate pH 7.4, 100 mM NaCl or 10 mM Na acetate pH 5.0, 100 mM NaCl at a protein concentration of 0.1 mg/ml for all complexes indicated. Subsequently, the thermodynamic stability was recorded at 222 nm by monitoring the circular dichroism signal in the range of 4–95°C with a scan rate of 1°/min. The spectra were corrected against the spectra of the buffer reference. The measured ellipticity was adjusted to the same starting value for better visualization of the curves.

#### Native mass spectrometry

Non-covalent mass spectrometry measurements were performed by using a Q-TOF Micromass spectrometer (Micromass, Manchester, UK) equipped with an electrospray ion source operating with a needle voltage of 3 kV and sample cone voltage of 30 V. Samples of 10 µM were prepared in 15 mM ammonium bicarbonate pH 7.8 or 15 mM ammonium acetate pH 5.0, and were continuously infused at a flow rate of 10 µl/min. Mass spectra were recorded in the 1500–5000 mass-to-charge (*m/z*) range. Data were acquired in the positive mode and calibration was performed using the multiply charged states produced by a separate injection of heart horse myoglobin dissolved in water/acetonitrile (1/1, v/v) with 0.2% formic acid. Data were processed with MassLynx 3.5 (Micromass).

#### Electron microscopy

Samples were applied to the clean side of carbon on mica (carbon/mica interface) and negatively stained with 1% uranyl acetate or 1% sodium silicotungstate. A grid was placed on top of the carbon film, which was subsequently air-dried. Micrographs were taken under low-dose conditions with a JEOL 1200 EX II microscope at 100 kV and a calibrated magnification of 39 750× (using the tobacco mosaic virus particle). Selected negatives were then digitized on a Zeiss scanner (Photoscan TD) with a pixel size of 14 µm (3.5 Å at the sample scale).

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