Structural Studies of Membrane-Assembled PopD and PopB, the Pseudomonas aeruginosa Type 3 Secretion Translocators

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Structural Studies of Membrane-Assembled PopD and PopB, the *Pseudomonas aeruginosa* Type 3 Secretion Translocators

A Dissertation Presented

by

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ABSTRACT

Structural Studies of Membrane-Assembled PopD and PopB, the Pseudomonas aeruginosa Type 3 Secretion Translocators

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Transport of proteins across membranes is essential during many stages of pathogen infection and colonization of human cells. Many Gram-negative pathogens use a Type 3 Secretion (T3S) system to inject proteins into the target cell during infection. Substantial genetic and biochemical evidence suggest that proteins are translocated across the host plasma membrane through a proteinaceous pore or translocon formed by two bacterial secreted proteins: the T3S translocators. Despite its key role in pathogenesis, virtually nothing is known about the assembly mechanism, structure, and composition of this critical transmembrane complex.

To this end, a cell-free system for the structural and functional characterization of Pseudomonas aeruginosa T3S translocators PopB and PopD was established. PopB and PopD assemble discrete sized pores in liposomal membranes. These pores are stable and heteromeric in nature. Combining this reconstitution methodology with single-molecule fluorescence microscopy methods, the stoichiometry of the membrane-assembled hetero-complex was determined: PopB and PopD assemble an hexadecameric complex at the membrane, with a calculated molecular weight of 601 kDa. The obtained stoichiometry is consistent with ex vivo estimations of the translocon
size, and represents the first report on the stoichiometric arrangement of a Type 3 Secretion System translocon.
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CHAPTER 1
INTRODUCTION

1.1 

P. aeruginosa is a Gram-negative rod shaped bacterium isolated by Carle Gessard in 1882. The name Pseudomonas occurred to professor Migula of the Karlsruhe Institute in Germany at the very end of the nineteenth century. It is believed that professor Migula had not traced directly the Greek ancestry of the name, but had simply used the name Pseudomonas because of resemblance of the bacterium to the nanogflagellate Monas, both in size and motility. Therefore Pseudomonas means “false Monas” (Pseudo=false) (Palleroni, 2010). The complete genome of P. aeruginosa strain PAO1 became available in the year 2000 (Stover et al., 2000). The P. aeruginosa PAO1 genome has a size of 6.3 Mbp (compared to 4.6 Mbp for E. coli), with 5570 predicted reading frames and one of greatest percent genes devoted to regulation networks (environment sensors and transcription factors).

P. aeruginosa has two distinct lifestyles: a planktonic one, where the bacterium moves through a flagellum and other appendages such as type IV pili; and a community lifestyle where, depending on conditions, bacteria regroup as a biofilm where they lose their mobility. Biofilms are specific organizations assembled on inanimate surfaces, or on living ones, making them very difficult to eradicate. Biofilms are present in approximately 65% of cases of human infection with P. aeruginosa, including tissues such as eyes, ears, urogenital tract and the lungs (Filloux and Vallet, 2003; Chicurel, 2000). Bacterial organization into biofilms confers increased resistance to antibiotics (up to 1000 fold) and protects against the host immune system. This resistance is possibly due to the slow metabolism and growth of bacteria within the biofilm, making the antibiotics
ineffective. In addition, the presence of an exopolysaccharide matrix slows the penetration of antibiotics (Kolter and Losick, 1998; Chicurel, 2000; Filloux and Vallet, 2003).

\textit{P. aeruginosa} is categorized as an opportunistic pathogen because in spite of its presence in our daily environment, it is only able to infect people with a weakened immune system, including patients with AIDS, diabetes, cancer, cystic fibrosis, burns, and intensive-care unit patients. Two types of infections are distinguished: the acute and the chronic type (Lyczak et al., 2000). Acute infections lead to severe sepsis. In particular skin burn patients are very susceptible to acute infection by \textit{P. aeruginosa}. Other traumas such as surgical procedures, endotracheal intubations and urinary catheters that produce wounds in mucous membranes result in susceptibility to \textit{P. aeruginosa} infection. Hospital equipment is a common source of contamination that leads to acute infections of the lung, digestive system, as well as urinary tract (Lyczak et al., 2000; Berthlelot et al., 2005). Another common type of acute infection is keratitis (corneal inflammation), which originates at a corneal injury that is used by \textit{P. aeruginosa} to reach the deep layers of the eye or originates by use of contaminated contact lenses which allows bacterial cells to adhere strongly to the cornea (Lyczak et al., 2000). \textit{P. aeruginosa} is also responsible for chronic lung infections in cystic fibrosis patients and is their leading cause of death. At the respiratory epithelium of these patients, the bacterium adopts a mucoid phenotype leading to the formation of a biofilm, which provides greater resistance to antibiotics and makes the infection difficult to treat (Lyczak et al., 2000). Infection persistence ultimately leads to accumulation of lung injuries and to an increase in mortality.

\textit{Pseudomonas aeruginosa} uses a complex Type III Secretion System (T3SS) to inject toxic effector proteins into animal cells. The T3SS plays an important role in
disease, as evidenced by animal models, and several studies link this system to unfavorable outcomes in human infections (Roy-Burman et al., 2001; Hauser et al., 2002; Zhuo et al., 2008; El-Solh et al., 2008). Given its key role in pathogenesis, the T3SS is an attractive target for therapeutic intervention. Despite rapid progress in understanding basic aspects of T3SS function, substantial gaps remain in our understanding of P. aeruginosa T3SS as well as T3SS from other pathogenic bacterial species. The efforts described in this dissertation address fundamental gaps in our understanding of the translocation machinery used the P. aeruginosa T3SS. The findings can be extended to evolutionary related T3SS from pathogenic bacteria such as Yersinia spp.

1.2 Origin and Evolution of the Type III Secretion System.

Genetic studies on secretion mechanisms present in Gram-negative bacteria initially identified two major highly conserved but functionally independent secretory pathways involving accessory apparatus: the Type I Secretion system (later identified as an ABC transporter family member that spans both bacterial membranes and can secrete proteins of various sizes), and a Type II Secretion System (a complex secretion machinery assembled by more than ten inner and outer membrane proteins, dependent on Sec and Tat secretory pathways for initial translocation into the bacterial periplasm). At the same time, data suggested the presence of a third conserved and independent secretory pathway: a Type III Secretion System (Salmond et al., 1993).

The T3SS is an ancient structure, probably acquired after the onset of eukaryotes. T3SS allows bacteria to secrete and translocate effectors into the cytosol of a target eukaryotic cell. It has a common ancestor with the flagellum and has evolved over many horizontal gene transfer events (Gophna et al., 2003). Thus, sequence
homology and conservation is observed between functional components of the T3SS and the flagellum (Blocker et al., 2003). They share a similar secretion apparatus and a similar assembly mechanism dependent in the bacterial Sec secretion machinery (Fig. 1.1). While the T3SS has a secretory function related to bacterial pathogenesis, the flagellum is necessary for bacterial mobility. Nevertheless the flagellum, in some cases, has also a secretory function (dependent on the base and the hook but not on the flagellar filament) that plays a role in pathogenesis. For example, Yersinia enterocolitica uses the flagellar secretion system to secrete YplA, a phospholipase with an important role in Y. enterocolitica pathogenesis (Young et al., 1999).

The T3SS resembles a “nanosyringe” which spans both bacterial membranes and extends outward the bacterial surface through a hollow needle structure. This apparatus is also known as “the injectisome” (Fig.1.1). Approximately ten proteins of the T3SS injectisome show strong similarity with components of the flagellum in regard to sequence identity and location of predicted transmembrane segments. This similarity is present for proteins that form the base of the T3SS and anchor it to both bacterial membranes crossing the periplasm, as well as appendices proteins such as the ATPase (Blocker et al., 2003; Yip and Strynadka, 2006). A similarity between proteins that assemble the hook of the flagellum and units that assemble the T3SS needle is also proposed (Blocker et al., 2003; Yip and Strynadka, 2006).
Figure 1.1. Schematic representation of the bacterial flagellum and the Ysc family Type III Secretion system. [Adapted from (A) Yip and Strynadka, 2006. Reproduced with permission from Elsevier, Copyright (2006); (B) Schraidt and Marlovitz, 2011. Reproduced with permission from the American Association for the Advancement of Science, Copyright (2011)].

Schematic representation of T3SS morphology and organization. A. The bacterial flagellum and the T3SS of Y. pestis and P. aeruginosa share a similar architecture. Selected protein components are indicated with their Yersinia family name and their corresponding homologue protein in the flagellum (N/A means not attributed). Each macromolecular assembly is composed of rings anchored to the inner membrane (green and brown) and to the outer membrane (orange), and ATPase (pink) and an extracellular helical structure (gray). The flagellum acts as a secretion system to export its extracellular components during assembly, and is used primarily for locomotion of the bacterium. The T3SS also secretes its own extracellular components during assembly (needle (gray), accessory needle protein (blue) and translocation pore proteins (yellow)). The main function of the T3SS is to translocate toxins or T3SS “effectors” into the cytosol of an adjacent host target cell. B. Sub-nanometer resolution structure of Salmonella tiphymurium “needle complex”. The outer ring (OR), inner ring 1 (IR1) and inner ring 2 (IR2) are indicated. The outer ring presents a 15-fold radial symmetry, while the inner rings present a 24-fold symmetry. The needle complex lacks a “C-ring” structure and the ATPase present in the functional T3SS.
1.3 Distribution and phylogeny of T3SS.

The T3SS is common to many pathogenic Gram-negative bacteria, including animal pathogens such as *Shigella* spp., *Salmonella* spp., *Yersinia* spp., *P. aeruginosa*, entheropathogenic EPEC and EHEC, *Clamidia* spp., *Burkholderia* spp., *Bordotella* spp., and plant pathogens such as *Erwinia amylovora*, *P. syringae*, *Ralstonia solanacearum* and *Xantomonas* spp. (Hueck, 1998).

The T3SS confers different biological advantages to bacteria depending on the species. For example, *Shigella* spp. and *Salmonella* typhimurium which are invasive bacteria (i.e. they require to gain intracellular access to survive in their host) use the T3SS to induce their internalization by macropynocitosis in intestinal epithelial cells. They also use a T3SS to escape their vacuole into the cell cytosol after bacterial replication (Finlay and Cossart, 1997). In the case of *Yersinia* spp and *P. aeruginosa* The T3SS confers a cytotoxic phenotype which serves these pathogens to counteract the action of macrophages (Cornelis, 1998). Interestingly, T3SS have also been involved in creating symbiosis with the host in the case of *Rhizobium* spp. and *Pseudomonas fluorescens* (Pallen et al., 2005).

Phylogenetic analysis based on T3SS proteins initially classified T3SS into five different families (Cornelis, 2002; Foulter et al., 2002). A later study based on conservation of the T3SS ATPase suggested the existence of seven families (Trosfontaines et al., 2005). Remarkably, it became evident that many pathogens harbor simultaneously two T3SS from two different families. For example, in *Salmonella*, these two T3SS are involved in different stages of the infection process. In this respect, *P. aeruginosa* offers an advantage as a model system for T3SS study as it only possess one type of T3SS belonging to the Ysc family. A phylogenetic tree based on 16S rRNA is completely different from the phylogenetic tree based on T3SS proteins. This divergence
between these two phylogenetic trees indicates T3SS have been acquired and have evolved through many horizontal gene transfer events (Cornelis, 2002; Foulquier et al., 2002; Gophna et al., 2003). We will focus here in two T3SS families which share very similar architectures.

**The Ysc family of T3SS.** The archetype of the Ysc family is the T3SS of *Yersinia spp.* encoded by the virulence plasmid pYV/pCD1. It includes in particular the single T3SS of *P. aeruginosa*, and the T3SS of *Aeromonas spp.* The Ysc T3SS displays the overall architecture described in Fig. 1.2. It allows bacteria to escape the immune system by killing immune cells and inhibiting phagocytosis. It also causes inflammatory reactions at the sites of infection.

**The Inv-Mxi-Spa family of T3SS.** It includes the Inv-Spa T3SS of *Salmonella enterica*, and the Inv-Mxi T3SS of *Shigella spp.* This system was first described in *Shigella spp.*, and the genomic region that encodes it was previously denominated *Shigella* pathogenicity island 1 (SPI-1). Newer examples of this family include the additions of the Ysa T3SS of *Y. enterocolitica*, the Eiv-Epa system of *E. coli* and the Inv-Spa system of *Burkholderia spp.*. The architecture of this T3SS family is very similar to that of the Ysc family (*including P. aeruginosa* T3SS) (Fig. 1.2). This T3SS is generally used by pathogens to trigger entry into host cells, specially into non-phagocytic cells.

Other T3SS families which display different architectures compared to the abovementioned families include: The SscA-Esc family (enteropathogenic and enterohemorrhagic *E. coli*); The Hrc-Hrp-1 and Hrc-Hrp-2 families (plant pathogens such as *Pseudomonas syringae*); The *rhizobiae* family (present in symbiotic bacterial species); The *chlamidia* family (present in obligate intracellular pathogens and required for intracellular survival) (Fig. 1.2).
Schemes of T3SS from four representative families based on electron microscopy studies.  

**A. Ysc Family**  
In the case of the Ysc family (Yersinia spp. and P. aeruginosa) and Inv-Mxi-Spa family (Shigella flexnerii and S. typhimurium) a hollow and stiff needle structure is observed. It has a well-defined length of about 60 nm, and is capped with a tip structure believed to serve as a platform for assembly of the translocon.  

**B. SscA-Esc Family**  
In injectisomes of the SscA-Esc family (EPEC and EHEC), the needle is very short and is topped with a long flexible filament of variable length (up to 600 nm).  

**C. Hrc-Hrp Family**  
The injectisomes of the Hrc-Hrp family from plant pathogens do not have a stiff needle structure, but rather a long and flexible Pilus structure with variable length (up to several µm long in the case of P. syringae). The dotted line represents the localization of a putative C-ring structure based on flagellum homology. IM. Inner membrane; OM, outer membrane; PG, peptidoglycan; D, diameter; L, length.
1.4 The injectisome

The injectisome is a complex system consisting of more than 25 different proteins that assemble into two rings anchored to both bacterial membranes and a continuous structure that traverses the bacterial periplasm. In addition, a hollow “needle” structure projects outward the bacterium (Cornelis, 2006) (Fig. 1.3). The injectisomes of *S. flexnerii* and EPEC have been successfully purified (Blocker et al., 2001; Sekiya et al., 2001). In the case of *S. flexnerii*, observations of injectisomes by cryo-EM led to a structural model with 17 Å resolution (Blocker et al., 2001) (Fig. 1.3). For a three-dimensional reconstruction of the injectisomes, cylindrical symmetry was assumed based on homology between the injectisome and the flagellum, since the flagellum has cylindrical symmetry. The resulting model included a triangular base, an upper ring doublet, and a needle. It also displays a hollow channel of about 2 to 3 nm in diameter across its full length (Fig. 1.3). After purification and observation by EM of injectisomes from *S. typhimurium* (Kubori et al., 1998), a three-dimensional model was also proposed at 17 Å resolution (Marlovitz et al., 2004). The model appears as a cylindrical structure 300 Å high and 240 Å wide and is comprised of the basal body consisting of two rings anchored to the inner membrane (assembled by PrgH and PrgK), and two rings anchored to the outer membrane (InvG). The model is also hollow with an inner channel (about 2.8 nm diameter) assembled by the protein PrgJ, which serves as an anchor for the injectisome needle assembled by PrgI subunits. Apart from PrgH, all other proteins are highly conserved in T3SS of a variety of bacterial species and their roles has been determined to be similar (Blocker et al., 2001; Sekiya et al., 2001). A recent study of S. typhimurium injectisomes revealed a cryo-EM model of the complex at sub-nanometer resolution indicating a 15-fold symmetry for the outer membrane rings, and 24-fold symmetry for the inner membrane rings (Schraidt and Marlovitz, 2011) (Fig. 1.3).
**Figure 1.3. Architecture of the T3SS injectisome.** [Adapted from (A) Cornelis, 2006. Reprinted with permission from Nature Publishing Group, Copyright (2006); (B) Blocker et al., 2001. Reprinted with permission from John Wiley and Sons, Copyright (2001); (C) and (E) Schraadt and Marlovitz, 2011. Reprinted with permission from the American Association for the Advancement of Science, Copyright (2011); (D) Marlovitz et al, 2004. Reprinted with permission from the American Association for the Advancement of Science, Copyright (2004)].

**A.** Transmission electron micrograph of *Y. enterocolitica* bacterium negatively stained. The arrow indicates a T3SS needle protruding from the cell surface. **B.** Electron micrographs of injectisomes purified from *S. flexnerii*. The base of the injectisome is clearly visible in these images. **C.** Average cryo-EM image of aligned injectisomes from *S. typhimurium*. Letters indicate the position of substructures (a, Outer Ring; b, Neck; c, Inner Ring 1; d, Inner Ring 2. The arrow indicate a particular electron density that is not present in all the injectisomes imaged. **D.** Cartoon representation of injectisome from *S. typhimurium* summarizing the location of major structural protein components. The asterisk indicates a region where the secretion tunnel markedly narrows, before the entry point to the needle. **E.** Cut away view of a 3D reconstruction of *S. typhimurium* injectisome based on cryo-EM images. The high resolution of this model allows to discern the conformation of structures such as the “cup” and the “socket” structures within the injectisome. The Outer Ring and Inner Ring 1 structures display 15-fold symmetry, while the Inner Ring 2 structure displays 24-fold symmetry.
1.5 The needle

The injectisome needle is assembled by polymerization of a small subunit (<10kDa), Prgl in *S. enterica* (Kubori et al., 1998), MxiH in *S. flexnerii* (Blocker et al., 2001), YscF in *Y. pestis* (Hoiczyk and Blobel, 2001), and PscF in *P. aeruginosa* (Pastor et al., 2005). This subunit is conserved among different animal-pathogenic bacterial species, with orthologous proteins sharing about 50% sequence identity. Conservation is high at the C-terminal domain which is critical to the subunit ability to polymerize (Deane et al., 2006; Quinaud et al., 2007). Structures formed by polymerization of PscF (*P. aeruginosa*) have a length of about 50 nm, and an outer diameter of 8 nm, with an internal channel of about 2 nm diameter (Pastor et al., 2005). The length of injectisome needles is invariable within a bacterial species, but varies between 50 and 80 nm depending on the species.

A ~ 1.6 nm resolution structure and helical properties for needles of *S. flexnerii* were determined by X-ray fiber diffraction and EM. These needles were about 50 nm long, with 7 nm outer diameter and a 2-3 nm inner channel diameter. They contained about 5.6 subunits per helix turn with a 24 Å pitch per turn (Cordes et al., 2003). These parameters were similar to those of helical structures forming the rod, hook and filament of the flagellum, which was surprising given the fact that Flagellin, the subunit component of the flagellum, has a molecular weight of about 45 kDa (4 to 5 times bigger than the T3SS needle subunit). This was explained by the fact that Flagellin consists of four domains, D0 to D3. D0 assembles the inner tube of the flagellum which has the same characteristics as the injectisome needle, while D1 assembles an outer tube and D2/D3 are exposed toward the filament outer space (Yonekura et al., 2003). Thus, the structure of the subunit forming the needle seems to be the minimum requirement for the assembly of such helical structure. Since the T3SS and the flagellum share a common
ancestor, it is proposed that Flagellin acquired additional domains specific to its function between its N and C terminal ends later during evolution (Cordes et al., 2003). The structure of the needle subunit MxiH S. flexnerii was solved by X-ray crystallography at 1.9 Å resolution (Deane et al., 2006). It consists of two extended α-helices connected by a short loop containing the sequence Pro-Ser-Asn-Pro (PSNP) (Fig. 1.4). The structure of MxiH provided the first detailed picture of a T3SS needle subunit, and revealed structural similarities to two other proteins that assemble similar polymeric helices: The D0 domain of flagellin (Yonekura et al., 2003), and the T3SS needle extension protein EspA from EPEC (Yip et al., 2005). The structure of MxiH was docked into the electron density map of the needle previously obtained by Cordes and colleagues (Cordes et al, 2003). The model obtained indicated that each monomer of MxiH is surrounded by seven other monomers, with the subunit’s major axis in line with the needle’s major axis (Fig. 1.4). The tail of the C-terminal part is buried in the needle core while the head points to the surface. This C-terminal region interacts with three neighbor subunits by hydrophobic interactions. This needle model suggested that the inner channel is only wide enough to accommodate single folded α-helices or proteins in a random coiled conformation (Fig. 1.4). Also, structures of MxiH were solved in two different conformations, suggesting the presence of a molecular hinge between its “head” and “tail” domains. On this basis, and also supported by mutagenesis analysis, a host sensing model was proposed where the MxiH subunit changes conformation upon host contact and signals this contact to the T3SS basal body, without affecting the helical parameters for the needle (Deane et al. 2006).

The needle protein YscF (Y. pestis) provides an antigenic protective effect upon immunization of animals. Studies have shown that active immunization of mice against YscF protects them partially (affective in about 60% of cases) against infection with Y.
*pestis* (Swietnicki et al., 2005). Thus the T3SS needle protein represents a potential vaccine target.

**Figure 1.4. Model of *S. flexnerii* T3SS needle assembly.** (Adapted from Deane et al., 2006).

The atomic model of MxiH was docked into the 16 Å resolution electron density map of the *S. flexnerii* needle. The model parameters are ~5.6 units per helix turn, and 24 Å pitch per turn. **A.** Ribbon representation of MxiH crystal structure shown as two views rotated by 90° about the long axis of the molecule. The N-terminal region of MxiH appears disordered in MxiH crystal structure despite strong α-helix sequence prediction. This segment is modeled as a cylinder in the shown structure. **B.** Bottom view of a 40 Å section of the docked model. MxiH subunits are colored differently starting with red, and ending with purple. The EM density is shown as a blue mesh. **C.** Side view of the assembled needle colored as shown in B. Note that panels B and C are not drawn to the same scale.
1.6 The T3SS tip complex

Interest in proteins that assemble the T3SS needle tip complex started more than fifty years ago, when the antigenic character of LcrV of *Y. pestis* was first discovered (Burrows, 1956, Burrows and et Bacon, 1958). Immunization of mice with LcrV induces an immune response that is protective against the plague (Burrows, 1956). Later on, orthologous proteins where shown to display the same antigenic properties. In studies designed to elucidate the functions of LcrV, two different teams demonstrated that LcrV was present on the surface of bacteria, before infection of eukaryotic cells (Fields et al., 1999; Pettersson et al., 1999). By immuno-fluorescence and by immuno-electron microscopy, LcrV was observed in clusters at the bacterium surface and was not injected into the cytoplasm of infected eukaryotic cells. This was later observed at higher resolution: LcrV assembled a structure at the tip of injectisome needles from *Y. enterocolitica* (Mueller et al., 2005). These structures had three characteristic parts, a globular “head” region, a slimmer “neck” region, and a globular “base” region (Fig. 1.5). In addition, the hollow channel of the secretion needle seemed to continue within this tip structure. A *Y. enterocolitica* strain not expressing LcrV, but instead complemented with expression of the homolog PcrV from *P. aeruginosa* features needles with a tip complex similar to that formed by LcrV (Fig. 1.5). However, it has a narrower base, which can be explained by the size difference between PcrV (32 kDa) and LcrV (37 kDa). This results indicated that needles from *P. aeruginosa* injectisomes bear a tip complex assembled by PcrV as well, even though a tip complex in *P. aeruginosa* needles has not yet been visualized directly (Mueller et al., 2005). The stoichiometry of the *Y. enterocolitica* tip complex was evaluated by quantitative immuno-blot analysis and scanning transmission electron microscopy (Broz et al., 2007). The analysis allowed to estimate the mass per unit length of *Y. enterocolitica* needles. Knowing the molecular weight of YscF, the
needle subunit, the authors estimated that the needle contains 139 ± 19 subunits. Next, by comparing known amounts of purified YscF and LcrV with the YscF and LcrV contents of purified needles, the authors estimated the molar ratio of LcrV and YscF in the injectisome needles at 1 LcrV per 32 ± 7 YscF monomers. This lead to an estimate of 4.3 ± 1.1 LcrV monomers per needle tip. Combining this result with the known X-ray structure of LcrV (Derewenda et al., 2004), and with the shape of the needle tip observed by electron microscopy (Mueller et al., 2005), the authors estimated a model that best fit their observations: a pentameric LcrV tip complex (Broz et al., 2007) (Fig. 1.5).

The tip proteins LcrV and PcrV are required for efficient translocation of T3SS effectors into target cells. Deletion of the tip proteins does not abolish effector secretion, but it impairs their translocation into the target cell cytosol (Goure et al., 2004). Interestingly, deletion of PcrV in *P. aeruginosa* does not abolish the secretion of hydrophobic translocators PopB and PopD, but notably, it impairs the proper insertion of PopD into the host cell membrane. Only PopB is found in the membranes of erythrocyte cells infected with PcrV deficient *P. aeruginosa*, while PopB and PopD are readily found inserted in the membranes of erythrocytes infected with wild type *P. aeruginosa* (Goure et al., 2004). This suggests a that PcrV may play a role in the assembly of translocators in their target cell membrane.
Figure 1.5. Modeling of T3SS tip complex. [Adapted from (C) Deane et al., 2006; (A), (B) and (D) Broz et al., 2007. Reproduced with permission from John Wiley and Sons, Copyright (2007)].

A tip complex is assembled by PcrV (*P. aeruginosa*) and LcrV (*Yersinia spp.*) at the top of *Y. enterocolitica* T3SS needles. **A.** Left, average projection EM image of tip complex assembled by PcrV. The cartoon represents the overall shape of a PcrV monomer and its proposed orientation in the complex. Right, PcrV was homology modeled for illustrative purposes (template LcrV, 41% sequence id)(Fabian B. Romano, unpublished). **B.** Left, average EM image of tip complex assembled by LcrV and cartoon representation of monomer. Right, crystal structure of LcrV (PDB id 1r6f; Derewenda et al., 2004). **C.** Pentameric model of T3SS tip complex assembled by LcrV (grey, surface representation) on *S. flexneri* needle model (colored structure). **D.** Top view of optimized pentameric LcrV model (surface representation).
How do effectors get into the target cell? The injectisome, as described here, does not allow the injection of toxins into the cytoplasm of host cells. This function requires the presence of two additional proteins, common to all T3SS described to date. These proteins were named, due to their function, “translocators”. These two proteins are hydrophobic (i.e. possess predicted transmembrane segments) and are believed to assemble a pore in the membrane of the host cell to assist the passage of toxins. In the case of \textit{P. aeruginosa}, these two translocators are PopB and PopD. As mentioned earlier, the needle tip protein PcrV is required for efficient translocation of effectors and it has been implicated in the proper insertion of translocators into their target membrane (Goure et al., 2004). Thereby, the needle tip protein is known as the “hydrophilic translocator”.

1.7 The translocon hypothesis

The translocon hypothesis states that the \textit{P. aeruginosa} T3S translocators, PopB and PopD, insert into the target membrane, engage with the tip of the T3S needle, and assist translocation of effector proteins into the host cell (Fig. 1.6). The hypothesis is based on the following experimental observations i) PcrV, PopB and PopD are not required for effector secretion, but are essential for effector translocation into the target cell (Dacheux et al., 2001; Sundin et al., 2004; Goure, et al., 2004), ii) only PopB and PopD are found inserted into the target membrane after pathogen-host contact (Goure et al., 2004), iii) PcrV, PopB and PopD are necessary to observe T3S dependent cell lysis (Goure et al., 2004; Vance et al., 2005), and iv) PopB and PopD can form pores in model membranes either individually or in combination (Schoen et al., 2003; Faudry et al., 2006).
The translocon hypothesis has been recently challenged by the observation that purified effectors, placed on the outer surface of *Yersinia* cells are efficiently translocated into target eukaryotic cells in a T3SS dependent manner (Akopyan et al., 2011), suggesting that at least *in vitro*, an extracellular translocation intermediate can be generated.

Regardless of the mechanism of effector translocation across the target eukaryotic membrane, hydrophobic translocators and their interaction with the membrane are essential. Investigating the basic aspects of this interaction is required in order to understand with molecular detail T3SS effector translocation.
Figure 1.6. The translocon hypothesis.

Protein effectors (up to four different proteins in *P. aeruginosa*) are secreted through the T3SS needle. The tip of the needle (formed by PcrV in *P. aeruginosa*) engages with the T3S translocon (PopD and PopB) to form a transmembrane channel by which protein effectors are translocated across the membrane. (Illustration by Kyle C. Rossi).
1.8 Genetic organization of the translocators, the translocation operon

The T3SS is encoded by about thirty genes generally organized in operons. These genes are grouped in chromosomal regions of 20 to 40 kb in size called “pathogenicity island”, or are carried by a plasmid named pYV in the case of *Yersinia* spp. (plasmid *Yersinia* Virulence). The proteins encoded by these have diverse functions and can be classified into five groups: i) The components of the secretion apparatus or “injectisome”, which allows the passage of secreted proteins across both bacterial membranes, including a membrane associated ATPase (homologous to the mitochondrial F0/F1 proton pump ATPase). ii) Dedicated T3SS chaperone proteins. iii) Effector proteins that are translocated into the cytoplasm of infected eukaryotic cells. iv) Protein components of the hypothetical transmembrane channel or translocon that allows the passage of cytotoxic effectors across the membrane of the eukaryotic target cell. v) Proteins involved in transcriptional regulation and activation of T3SS genes.

In all T3S systems described to date, the three translocators are encoded in the same operon (Fig. 1.7). This operon, *pcrGVH-popBD* in *P. aeruginosa* and *lcrGVH-yopBD* in *Y. pestis*, also encode a chaperone for the two hydrophobic translocators (*PcrH* in *P. aeruginosa* and *LcrH* in *Y. pestis*) and a bacterial cytosolic protein that interacts with the hydrophilic translocator (*PcrG* in *P. aeruginosa*, and *LcrG* in *Y. pestis*) (Bergman et al., 1991; Allmond et al., 2003). The genetic organization of the operon and the function of each translocator is conserved. In *Yersinia* spp., the translocation operon also codes for a protein involved in the regulation of T3SS effector secretion (*LcrG*). *LcrG* prevents the secretion of effectors in un-induced conditions. *LcrG* in its free form is located at the entrance of the secretion channel at the injectisome basal body. After induction, *LcrV* accumulates in the bacterial cytoplasm and interacts with *LcrG*. Once *LcrV* concentration raises enough to titrate *LcrG*, it removes *LcrG* from the secretion channel inlet, allowing
secretion of LcrV and effectors (Matson and Nilles, 2001). The LcrG homologue in *P. aeruginosa* is PcrG. It interacts with PcrV in the bacterial cytoplasm, but appears to lack the regulatory function observed for LcrG (Allmond et al., 2003; Nanao et al., 2003).

Importantly, the operons of *P. aeruginosa* and *Yersinia* spp. are functionally conserved and interchangeable between species. Expression of the *pcrGVHpopBD* operon in *Y. pseudotuberculosis* translocation-deficient mutants lacking YopB and YopD results in complete complementation of cell contact-dependent cytotoxicity and T3S effector translocation into HeLa cells (Frithz-Lindsten et al., 1998). The most evident interactions among proteins encoded by the operon are interactions between the hydrophobic translocators and their dedicated chaperone (Allmond et al., 2003). PcrH, the chaperone from *P. aeruginosa* and LcrH from *Y. pestis* interact with PopB/PopD and YopB/YopD respectively in the bacterial cytoplasm (Bergman et al., 1991; Allmond et al., 2003; Broms et al., 2003).

In *Y. pestis* LcrH, in addition to its role as chaperone, has a function in regulating the synthesis of T3SS effectors (Francis et al., 2001). *Y. pestis* strains lacking LcrH constitutively synthetize effectors, while in wild type *Y. pestis* effectors are synthetized only after an induction signal. Interestingly, a mutant not expressing YopD displays the same phenotype, while a mutant not expressing YopB retains a wild type phenotype. Therefore, LcrH is a negative regulator of effector synthesis when complexed with YopD. An induction signal leads to secretion of YopD, leaving free LcrH and removing the inhibitory signal (Francis et al., 2001). Unlike LcrH, PcrH in *P. aeruginosa* does not possess such a regulatory role (Broms et al., 2003).
The organization of translocation operons is substantially the same within injectisomes of different families (Mueller et al., 2008). The percent sequence identity between *Yersinia* and *Pseudomonas* genes is indicated. These two operons are functionally conserved and interchangeable between *Y. enterocolitica* and *P. aeruginosa* (Frithz-Lindsten et al., 1998). The two hydrophobic translocator genes (red) usually reside next to their dedicated chaperone (green). The hydrophilic translocator (purple) assembles a tip complex at the needle top. A regulatory protein (blue) interacts with the hydrophilic translocator in the bacterial cytoplasm. Although the genetic organization of translocation operons from *P. aeruginosa* and *Yersinia spp.* (Ysc T3SS family) is similar to that of *Salmonella spp.* and *Shigella spp.* (Inv-Mxi-Spa T3SS family) as well as the overall morphologies of both T3SS (Fig. 1.2), the hydrophobic translocators display sequence identity lower than 25%, substantially different molecular weight and primary structure predictions (Fig 1.8).
1.9 PcrH

PcrH (*P. aeruginosa*) is a small non-secreted protein with a molecular weight of 18.4 kDa, and an acidic theoretical isoelectric point of 4.6. PcrH was predicted early as the chaperone protein for translocators PopB and PopD (class II chaperone) (Yahr et al., 1997). PcrH interacts directly with PopB and PopD in the bacterial cytosol (Allmond et al., 2003; Broms et al., 2003; Schoen et al., 2003). This interaction is required for the efficient secretion of translocator proteins (Broms et al., 2003), and also stabilizes the translocators in the bacterial cytosol protecting them from degradation and aggregation. The PcrH-PopB and PcrH-PopD complexes were observed to dissociate in vitro as a result of pH decrease, which can result in interaction of translocators with liposomal membranes (Schoen et al., 2003). It was therefore proposed that PcrH interaction with PopB and PopD allowed retention of translocator proteins in a “membrane inactive” conformation, preventing an early and detrimental interaction of the translocators with the bacterial membrane (Schoen et al., 2003). Broms et al. (2003) showed, by using yeast two-hybrid experiments, that PcrH is able to interact strongly with *Yersinia spp.* YopD, and that this interaction can rescue the phenotype of a LcrH (PcrH homologue in *Yersinia spp.*) null mutant defective in YopD secretion. Interestingly, using the same assay, no interaction was detected between PcrH and YopB, or between LcrH and PopB (Broms et al., 2003). This suggested that although the structural determinants for interaction between the PcrH-PopD pair may be conserved in the LcrH-YopD pair, they may not be conserved for between the PcrH-PopD and LcrH-YopB pairs. Early analysis performed by us using bioinformatics indicated PcrH and other class II chaperones adopted a fold consisting of several repeats of a motif named “Tetra-trico-peptide repeat” (TPR), allowing to homology model these chaperones using existing structures of TPR domains as templates, such as the eukaryotic co-chaperone HOP (Heat shock protein...
Organizing Protein). The similitude suggested between class II T3SS chaperones and HOP was one of the first indications of structural similarity between eukaryotic and prokaryotic chaperones (Fig 5.1). TPR repeats consists of a 34 amino-acid sequence motif that folds into two antiparallel α-helices. TPR domains are found in numerous proteins, where they serve as interaction modules and multi-protein complex mediators. The crystal structures of three T3SS class II chaperones including PcrH later corroborated that these adopt a TPR fold (Büttner et al., 2008; Lunelli et al., 2009; Job et al., 2010). In addition to providing a crystal structure for IpgC, the *Shigella flexneri* class II chaperone, Lunelli et al also delimited a chaperone binding motif present in the *S. flexneri* translocator IpaB (PopB’s distant homologue). This binding motif was limited to IpaB residues 51-71 containing the sequence motif PELKAP. A short synthetic peptide carrying this sequence was able to bind IpgC with a dissociation constant of 72 µM. The authors solved a crystal structure of the class II chaperone IpgC bound to this short peptide providing the first molecular insight into how class II chaperones could recognize their translocator substrates. Moreover, Job et al recently solved a crystal structure of PcrH bound to a synthetic peptide carrying the homologue sequence motif VELNAP present in PopD. All the crystal structures of class II chaperones in complex with a peptide carrying the predicted chaperone binding motif to date display the class II chaperone binding the motif in the same fashion: Three hydrophobic pockets in the concave side of the TPR domain receive three hydrophobic residues within the CBM present in the synthetic peptide (Fig. 1.8). Although PopB also possess a CBM in its N-terminal region, not experimental data is currently available to corroborate its mode of interaction with PcrH.
Figure 1.8. PcrH adopts a TPR fold and recognizes a stretched sequence motif in PopD.

T3SS class II chaperones adopt a TPR fold and bind a stretched sequence motif in N-terminal region of translocators through hydrophobic interactions. **A.** Crystal structure of HOP TPR1 domain in complex with a seven residue peptide containing the C-terminal Hsp70 sequence GPTIEEVD (PDB id 1ELW; Scheufer et al, 2002). The C-terminal conserved Asp residue (red) provides a two-carboxylate anchor where critical electrostatic interactions take place. In addition, a conserved Val residue (orange) provides hydrophobic interactions. The rest of the peptide is bound through hydrogen bonding with backbone groups which is not sequence specific. **B.** The *P. aeruginosa* class II chaperone PcrH adopts a TPR fold and recognizes a stretched sequence motif present in the N-terminal region of PopD. PcrH was co-crystallized with a ten residue peptide containing the sequence VELNAP (PDB id 2XCB, Job et al., 2010). Conserved residues Val, Ley and Pro present in the binding motif are indicated in red. These residues fit into hydrophobic pockets in the concave face of the TRP. Binding motifs present in homologue translocators contain hydrophobic residues in the same positions.
1.10 PopD

PopD is a T3SS secreted protein with a molecular weight of 31.3 kDa, also known as the “minor” hydrophobic translocator due to its smaller size compared to PopB. PopD is required for T3SS dependent cytotoxicity in epithelial cells and for T3SS dependent hemolytic activity of *P. aeruginosa* (Hauser et al., 1998; Goure et al., 2004). The bioinformatics analysis of PopD protein sequence predicts the presence of a hydrophobic segment between residues 119 and 137, and an amphipathic helix in its C-terminal region between residues 267 and 281 (Frithz-Lindsten et al., 1998; Hauser et al., 1998) (Fig. 1.9). A similar structural organization is found in its *Yersinia* spp. homologue YopD (40% identity) (Fig. 1.9). Incubation of *P. aeruginosa* with red blood cells allows the detection of pores in the erythrocyte’s membranes, an activity that is dependent on the simultaneous presence of PcrV, PopD and PopB within the same bacterial cell (Dacheux et al., 2001). These membrane pores were estimated to have a diameter of 28 to 30 Å by osmoprotection assays (Dacheux et al., 2001). Schoen et al. (2003) conveniently discovered that co-expression of PcrH and PopD using a bi-cistronic vector allows for efficient expression of recombinant soluble PcrH-PopD complexes in *E. coli*. These complexes have a 1:1 stoichiometry. The PcrH-PopD complex dissociates as a result of dropping the solution pH to mildly acidic conditions (pH 5.3), and this results in aggregation of PopD into an oligomeric and heterogeneous soluble form. Incubation of liposomes with these PopD oligomers in high concentration resulted in liposome lysis. Therefore, it was proposed that oligomerization of PopD was required for membrane recognition and formation of membrane pores (Schoen et al., 2003) (Fig. 1.10). Interestingly, EM analysis of liposomal membranes after incubation with PopB, or a PopD:PopB mix at 1:1 molar ratio, suggested the presence of ring-like structures with an outer diameter of 80 Å and an inner diameter of 40 Å (Schoen et al., 2003). However,
the composition and stoichiometry of the observed structure could not be determined. PopD and PopB interaction in membranes has been detected by incubation of the oligomeric forms of PopD and PopB with liposomes, followed by liposome pelleting by ultracentrifugation, membrane solubilization with Triton X-100 and immunoprecipitation using anti-PopD antibodies (Goure et al., 2004). Nevertheless, this experiment does not preclude the possibility of unspecific interaction due to aggregation. In addition, the oligomeric soluble forms of PopD and PopB pellet together with liposomes after ultracentrifugation so this step does not eliminate the protein fraction that is not bound to liposomes (William Howes, unpublished results). Further in vitro functional studies of PopD performed by Faudry et al (2006) indicated that the oligomeric form of PopD had little pore forming activity in liposomal membranes at mildly acidic pH (~pH 5.3), and that co-incubation with PopB resulted in synergistic pore forming activity by both translocator proteins, providing the first in vitro indication for functional interaction between PopD and PopB. This study also measured, although not quantitatively, the membrane binding of translocators to liposomal membranes by FRET at distinct pH values. The authors concluded that translocators in their oligomeric form bound membranes regardless of pH, and that acidic pH was required for pore formation only (Faudry et al., 2006). A later study by the same group reported that PopD is present in a partially unfolded or molten globule state at neutral pH, either in its chaperone-free state or when bound to the PcrH chaperone (Faudry et al., 2007). Since molten globular states have been reported for a number of bacterial pore-forming toxins, and these have been shown to facilitate membrane interaction (van der Goot et al., 1991; Muga et al., 1993; Vécsey-Semjén et al., 1996), the authors interpreted that PopD remained in a “membrane-active” state even at neutral pH and that interaction with PcrH prevented neutral pH membrane interaction (Faudry et al., 2007).
PopD interaction with the chaperone PcrH has been initially characterized by co-expression of truncated versions of both proteins in order to map the smallest region of interaction. In the case of PcrH only 7 residues from its N-terminus and 30 residues from its C-terminus could be deleted without affecting interaction with PopD, indicating that integrity of PcrH TPR domain was essential for interaction (Faudry et al., 2007). In the case of PopD, deletion of a substantial portion still allowed for interaction with PcrH. The smallest truncated PopD version that still supported interaction with PcrH was a PopD fragment including residues 28-98 (full size PopD is 295 residues long). This suggested that essential portions in PopD required for interaction lied in its N-terminal region (Faudry et al., 2007). Later on, a keystone in understanding interaction between PopD and PcrH was provided by a PcrH X-ray crystal structure (Job et al., 2010). In this study, PcrH was co-crystallized with a seven aminoacid synthetic peptide containing the sequence VELNAP present in the N-terminal region of PopD. This peptide bound PcrH with high affinity and revealed atomic details of the PcrH-PopD interaction. The Val, Leu, and Pro residues present in the sequence are conserved within PopD homologues and represents a chaperone binding motif. The peptide binds PcrH in a extended conformation, and the three abovementioned hydrophobic residues fit into three conserved hydrophobic pockets present in the concave face of PcrH TPR domain (Fig. 1.8) (Job et al., 2010). In a similar study carried out earlier, the same chaperone binding characteristics and a similar chaperone binding motif had been observed for a chaperone-translocator pair of *Shigella spp.* by using X-ray crystallography combined with limited proteolysis and yeast-two hybrid screens (Lunelli et al, 2009). This earlier study also provided empirical support for a similar chaperone binding motif present in YopB and YopD (*Yersinia spp.*).
1.11 PopB

PopB is a secreted T3SS protein with a 40.3 kDa molecular weight and is also known as the “major” hydrophobic translocator protein. PopB is essential for T3SS dependent cytotoxicity of *P. aeruginosa* observed in epithelial cells, and for the contact-dependent hemolytic activity of *P. aeruginosa* (Hauser et al., 1998; Goure et al., 2004). Analysis of PopB primary structure using bioinformatics tools reveals the presence of two predicted transmembrane or hydrophobic segments at residue positions 170-188 and 232-250, and two predicted coiled-coil regions at positions 108-165 and 326-369 (Frithz-Lindsten et al., 1998; Hauser et al., 1998) (Fig 1.9). These properties are conserved in homologue translocators such as YopB (*Yersinia* spp.) (Fig. 1.9). YopB is also described as an essential protein for translocation of T3SS effectors into eukaryotic cells as well as T3SS dependent hemolytic activity of *Yersinia* spp. (Frithz-Lindsten et al., 1998; Hakansson et al., 1996). PopB presents pore-forming activity in model membranes, and also displays synergistic pore-forming activity when incubated together with PopD and liposomal membranes (Schoehn et al., 2003; Faudry et al., 2006). PopB has been reported to remain in a metastable oligomeric state when bound to the chaperone PcrH (Schoehn et al., 2003) and unlike PopD-PcrH, no monomeric PopB-PcrH form has been identified to date. Schoen et al suggested this oligomeric state was a pre-requisite for membrane interaction (Schoehn et al., 2003). A recent report indicated PopB remains in molten globular state in either its chaperone-bound or chaperone-free states (Dey et al., 2012). PopB contains a predicted PcrH interaction motif in its N-terminal region, but its interaction with the chaperone has not been studied. The sequence tag VALTPP present at residue positions 58-63 contains highly conserved Val, Leu, and Pro residues which may fit on conserved PcrH hydrophobic pockets in the same way as PopD chaperone binding motif (Fig. 1.8).
Figure 1.9. Scheme of translocators primary structure.

Structural organization of (A) hydrophobic major translocators and (B) hydrophobic minor translocators from *P. aeruginosa* (PopB, PopD) (Hauser et al., 1998), *Yersinia spp.* (YopB, YopD) (Frithz-Lindsten et al., 1998), *EPEC* (EspD, EspB) (Wachter et al., 1999), *S. flexnerii* (IpaB, IpaC) (Hume et al., 2003), and *Salmonella spp.* (SipB, SipC) (Hayward et al, 2000).
Figure 1.10. Model of translocon assembly by PopB and PopD proposed by Schoen et al. (2003).

A. The chaperone PcrH interacts with PopB and PopD in the bacterial cytoplasm. PcrH-PopB forms metastable oligomers, while PcrH-PopD assembles a simple dimer. B. The in vivo signal for translocator secretion is mimicked in vitro by a decrease in pH. This leads to chaperone release and secretion. The secreted translocators assemble metastable oligomers. C. Homo or Hetero-oligomers of PopB and PopD bind liposomal membranes and assemble ring structures that result in the formation of a translocation pore and liposome lysis in vitro (Schoen et al., 2003).
1.12 Spontaneous insertion of proteins into membranes

Initial studies about how hydrophobic proteins insert into lipid bilayers or traffic across them were split into two different views. One view was based in the observations that a hydrophobic leader or signal peptide was required for the insertion of membrane proteins, and also that ribosomes had been imaged bound to membranes. Thus, it was postulated that a specific protein machinery within the lipid bilayer assisted the membrane insertion of membrane proteins or protein traffic across the membrane (Blobel and Doberstein, 1975). The other view supported that protein insertion into lipid bilayers was a spontaneous process and did not require the assistance of a specific apparatus. This was based in evidence that a number of water-soluble proteins such as the complement system proteins, melitin, and α-toxin spontaneously assembled into membranes (Bretscher, 1973; Wickner, 1979). Engelman and Steitz (1981) proposed, based on energetics and considering the polypeptide’s tri-dimensional structure, that the initial event in the insertion of a membrane protein was the spontaneous penetration of the hydrophobic portion of the bilayer by a pre-folded helical hairpin (Engelman and Steitz, 1981). Thus, membrane insertion or membrane translocation would be initiated by the formation of a helical hairpin structure, which spontaneously partitions from the aqueous cytoplasm into the hydrophobic core of a bilayer. Later on, substantial evidence supported the fact that a protein machinery assists membrane protein insertion and traffic through the bilayer: A protein-conducting channel with an aqueous interior, present in the ER membrane or in the bacterial membrane, catalyzes the insertion of the membrane proteins, allowing hydrophilic portions to cross the membrane while hydrophobic segments leave laterally into the lipid phase (Simons and Blobel, 1991; Crowley et al., 1993; Crowley et al., 1994; van den Verg et al., 2004).
A number of proteins insert into lipid bilayers without the assistance of cellular protein machineries. Probably the best characterized group of proteins with these characteristics is the group of bacterial pore-forming toxins (Heuck and Johnson, 2005). This group of proteins has the ability to convert themselves from a water-soluble state into a membrane-inserted state. This insertion process, even though often depends on specific lipids or a membrane receptor, is spontaneous and is not assisted by other proteins or coupled to a chemical energy source such as ATP.

The translocator proteins PopB and PopD resemble bacterial pore-forming toxins in a number of ways. They remain water soluble after synthesis in the bacterial cytosol by association to their dedicated chaperone. In addition, both the chaperone-bound and chaperone-free states of recombinant PopB and PopD are water-soluble (Schoen et al., 2003). These water soluble states can bind to and assemble pores in liposomal membranes (Schoen et al., 2003; Faudry et al., 2006). Both translocators are readily found inserted in the plasma membrane of eukaryotic cells after bacterial infection, and abundant evidence indicates P. aeruginosa translocators and Yersinia spp. homologues form pores in the eukaryotic cell membrane to assist effector translocation (Hakkanson et al., 1996; Neyt and Cornelis, 1999; Tardy et al., 1999; Dacheux et al., 2001; Goure et al., 2004).

Generally speaking, pore-forming processes by bacterial toxins can be divided into well differentiated stages, some of which have been well characterized. Firstly, membrane recognition takes place. Pore-forming toxins recognize a particular characteristics in the target membrane which makes the toxin innocuous to the cell that produces it, but highly efficient against its target. These characteristics can be a particular lipid (i.e. Cholesterol in the case of Cholesterol dependent cytolysins from Gram-positive bacteria); a specific protein expressed on the surface of the target cell
(i.e. bacterial outer membrane proteins such as OmpF in the case Colicin); or a specific carbohydrate present on the target membrane or even a carbohydrate attached to a membrane-anchored protein (i.e. aerolysin targeting of GPI anchors) (Heuck and Johnson, 2005). In the case of T3SS translocators, their interaction with the specific class II chaperone prevents early interaction with the bacterial membrane, therefore is possible that no specific eukaryotic membrane component is required to trigger membrane insertion. Secondly, oligomerization on the membrane surface usually takes place after membrane recognition. This event is commonly triggered by conformational changes that result from membrane recognition, but in some cases proteolytic cleavage of the toxin is also required to trigger oligomerization (i.e. aerolysin and anthrax PA). Finally, membrane penetration and pore formation takes place. This event usually results from insertion of protein amphipathic structures into the membrane hydrophobic core. Examples have been described were either amphipathic α-helices are inserted (i.e. in the case of Actinoporins), or amphipathic β-hairpins are inserted (i.e. in the case of Cholesterol-dependent cytolysins). Nonetheless, exceptions to these generalizations exist. For example, is not clear whether Diphteria toxin oligomerizes in the membrane to assemble a pore, and Colocins can permeabilize the membrane by insertion of a hairpin of hydrophobic α-helices.

Since membrane insertion usually requires a large conformational change, for a number of bacterial pore-forming toxins it is proposed that membrane recognition and insertion happens through a molten globule intermediate state, which would lower the activation energy required (van der Goot et al., 1991). Interestingly, PopB and PopD have been reported to remain in molten globular states even in their chaperone-bound forms (Faudry et al., 2007; Dey et al., 2012). This dynamic configuration of translocators
may facilitate their secretion through the T3SS apparatus, and may also facilitate their association at the target membrane.

Acidic lipids and low pH have been extensively used to increase the membrane insertion efficiency of a number of toxins, including toxins that do not encounter low pH at their target membrane (i.e. Colicin A) (Wang et al., 1997; Chenal et al., 2002; Thuduppathy et al., 2006; Musse et al., 2006, Kyrichenko et al., 2009). In these cases, it is proposed that the acidic pH induces the previously mentioned molten globular state which lowers the activation energy for membrane insertion. Although a similar scenario seems likely for PopB and PopD, given that low pH and acidic lipids enhance their pore forming activity (Schoen et al., 2003; Faudry et al., 2006), PopB and PopD already remain in a partially unfolded or molten globular state at neutral pH (Faudry et al., 2007; Dey et al., 2012). Therefore, in the case of PopD and PopB it seems likely that in vitro low pH and negatively charged lipids are required for initial electrostatic interaction with the membrane which may lead to membrane insertion.

1.13 Membrane insertion of T3SS translocators.

Proteins that spontaneously insert into membranes, such as pore-forming toxins, are characterized by a water-soluble folded state that interacts with the membrane. The protein tertiary structure is usually involved in recognition of a specific membrane property such as a receptor, or a particular lipid constituent. Also, a number of these proteins have been crystallized in their monomeric water-soluble form, emphasizing the existence of a stably folded state. In contrast, membrane proteins that rely on the ER or bacterial translocation machinery to insert in membranes, reach it in an unfolded state (either through co-translational insertion, or by stabilization with chaperones prior to translocon interaction). Interestingly, T3SS translocators share properties of both
groups: They are secreted in an unfolded state and must recognize the target membrane and insert in a spontaneous way. Therefore, the membrane insertion of T3SS translocators represents a new paradigm in membrane protein integration (Fig 1.11).

The energy cost of inserting a pre-folded alpha helical structure into the membrane is much lower than the cost of forcing an unfolded and hence weakly hydrogen bonded polypeptide into the membrane where they would then form a helix (Engelman and Steitz, 1981). Although Engelman’s treatment originally aimed at explaining how an isolated membrane protein transfers from water into the membrane, it is probably more applicable to alpha-helical toxins, or in our case, to incorporation of T3SS translocators into membranes. At least three possible models can be proposed regarding translocator membrane insertion after secretion through the T3SS needle. I) The membrane insertion takes place while the protein is secreted through the needle and still unfolded. II) The protein is fully secreted and then undergoes a hydrophobic collapse assembling a molten globular state that later interacts with the membrane in a way similar to α-helical pore-forming toxins such as Colicin. III) The translocators interact with the membrane-water interface as they are secreted in an unfolded state. This triggers helix folding at the interface, which then results in insertion of pre-folded helical segments. IV) The translocators fold or assemble a structure at the needle tip after they are secreted, in a way similar to PcrV, and this structure later penetrates the target membrane. The first scenario is unlikely since the energy cost of inserting an unfolded polypeptide, which has a weak hydrogen bonding network, is very high. Helical protein segments are more prone to membrane insertion if a pre-folded alpha helix inserts in the membrane (Engelam and Steitz, 1981). The fourth scenario is possible, but to date, only PcrV and its homologs have been observed at the tip of the T3SS needle. Translocators from a different family have been observed at the tip, but they don’t share homology with
PopB and PopD. Therefore, most likely scenarios for PopB and PopD membrane integration are the second and third ones. Clearly, a significant gap remains in understanding translocator interactions with membranes. This gap needs to be addressed in order to fully understand T3SS effector translocation across the eukaryotic host membrane.
Figure 1.11. The membrane insertion of T3SS translocators represents a new paradigm in membrane protein insertion.

A. Membrane proteins are targeted to ER or bacterial inner membranes in an unfolded state where a complex machinery regulates and assists their proper insertion into membranes and folding. B. T3SS translocator proteins emerge from the needle in an unfolded state and must insert and fold into membranes in a spontaneous way. The cartoon represents the predicted topology of membrane inserted PopB (predicted using TMmod, Kahsay et al., 2005). C. Pore-forming toxins exist in a water-soluble folded state, and transition into a membrane inserted state in a spontaneous way. The cartoon represents the pore forming domain of Colicin A (PDB is 1col).
CHAPTER 2
RECONSTITUTION OF HETEROMERIC POPB-POPD COMPLEXES IN MODEL MEMBRANES

The majority of this chapter is the result of a collaboration with Sarah Kells, Kyle C. Rossi, Christos G. Savva, Andreas Holzenburg, Eugenia M. Clerico, and Alejandro P. Heuck (Romano et al., 2011).

2.1 Introduction

Current models for the P. aeruginosa translocon complex are quite rudimentary, and they are based in the following observations: i) the translocon proteins PopB and PopD are found associated with cell membranes after the interaction of P. aeruginosa with red blood cells (Goure et al., 2004); ii) PopB co-immunoprecipitates with PopD after Triton X-100 solubilization of membrane associated proteins (Goure et al., 2004); and iii) ring-like structures are observed using electron microscopy when the translocon proteins are incubated with model membranes (Schoehn et al., 2003). The topology of the translocon proteins is based only on the bioinformatic analysis of the primary structure for these proteins, which suggests that PopB possesses two potential transmembrane (TM) segments and PopD only one (Buttner et al., 2002), but no experimental data are available to corroborate such predictions. The non-polar character of the T3S translocators and their tendency to aggregate in solution has made the structural and functional characterization of these proteins very difficult (Tardy et al., 1999; Hume et al., 2003). Therefore, substantial gaps remain in understanding how translocator proteins interact with lipid bilayers to assemble the putative translocon and assist effector translocation. Translocators travel through the T3SS needle in a partially unfolded state. An in vitro reconstitution method that resembles this condition and efficiently
reconstitutes translocators in model membranes is a very desirable and tool. Moreover, many fundamental questions regarding translocator membrane-assembly still remain open. Why is acidic pH required for the *in vitro* pore forming activity of translocators? Do PopD and PopB interact directly and stably at the membrane, or does transient interaction result in their coupled function?

To this end, we have purified and characterized the *P. aeruginosa* translocators individually as homogeneous complexes with the cognate chaperone PcrH. After isolation, PopB and PopD were quantitatively separated from PcrH by combining immobilized metal ion affinity chromatography (IMAC) and elution with the chaotropic agent urea. The spectroscopic characterization of the urea-isolated PopD showed little secondary structure content, and that the two Trp residues were exposed to a polar environment. In contrast, the urea-isolated PopB presented more alpha helical content than PopD, and its single Trp residue was located in a non-polar environment. The pore forming activity of the urea-isolated translocators was very similar to the activity of the translocators separated from the chaperone by acidification (Faudry, Vernier, et al. 2006). Assembly and maximal pore formation occurred at pH below 5, indicating that protonation of acidic residues was critical for membrane insertion. Under these conditions, PopD, PopB, and their mixtures formed discrete and stable pores in lipids vesicles. Cryo-electron microscopy (EM) and dynamic light scatter (DLS) revealed that no aggregation or disruption of the integrity of the vesicles occurred after assembly of the transmembrane pores. Single Cys residues were introduced at specific locations in PopD and PopB, and these derivatives purified and labeled with the fluorescent probe Bodipy. Using both Bodipy excitation energy migration and self-quenching, we unambiguously showed that PopB interacts directly and stably with PopD on lipid membranes. We have therefore established an efficient procedure to purify, specifically
label, and assemble the \textit{P. aeruginosa} translocators and their derivatives into model membranes.

2.2 Results

2.2.1 Purification of homogeneous chaperone-translocator complexes containing native PopB and PopD

Co-expression of hisPcrH with native PopD (or PopB) was achieved by using the pETDuet-1 system (Merk4Biosciences) as described under the Methods sections 2.4.4 and 2.4.5. The translocators were therefore purified in their native state, without modifications (i.e., no affinity tags or amino acid additions/deletions resulting from the cloning procedure into the expression vector). The absence of polyHis tags or fusion-proteins (e.g., GST) is critical when electrostatic interactions and oligomerization of proteins are involved in the mechanism under investigation. Water-soluble hisPcrH-PopD and hisPcrH-PopB complexes were purified using IMAC and AEC. The IMAC step rendered a mixture of free hisPcrH together with hisPcrH-PopD (or hisPcrH-PopB) complexes. The AEC step separated the free hisPcrH chaperone and other minor contaminants from the hisPcrH-PopD (or from the hisPcrH–PopB) complex (Fig. 2.1A and B). A major peak containing the hisPcrH-PopD complex eluted when the concentration of NaCl was 0.21 M. The free hisPcrH chaperone eluted later when the concentration of NaCl was 0.35 M (Fig. 2.1A).

Size exclusion chromatography (SEC) analysis of the purified hisPcrH-PopD complex revealed a single symmetric peak (Fig. 2.1E). When compared to the elution times of molecular mass standards, hisPcrH-PopD eluted with an apparent molecular mass of $111 \pm 1$ kDa (expected 50.8 kDa), which suggested that hisPcrH-PopD may form 2:2 complexes. Similar SEC results have been described for \textit{Shigella flexneri} and \textit{Aeromonas hydrophila} translocators (Birket et al., 2007; Tan et al., 2009), however
analysis of these complexes have shown they adopt a 1:1 stoichiometry. Therefore it seems that purified hisPcrH-PopD forms an elongated 1:1 complex, as described for the P. aeruginosa CHA translocator and other related T3S proteins (Schoehn et al., 2003; Birket et al., 2007; Tan et al., 2009).
Figure 2.1. Purification of the hisPcrH–translocator complexes.

A. The fractions containing the hisPcrH–PopD complex isolated after the first IMAC purification step were dialyzed and loaded into a Q-Sepharose AEC column and eluted using a linear NaCl gradient. The peaks containing hisPcrH and the hisPcrH–PopD complex are indicated. B. The hisPcrH–PopB complex was purified as described for the hisPcrH–PopD complex. The first two large peaks that eluted from the AEC column contained the hisPcrH–PopB complexes, and the shoulder that eluted around 400 mL contained hisPcrH. C. SEC analysis of aliquots corresponding to the 1st peak and the 2nd peak illustrated in panel B. D. SDS–PAGE analysis of purified proteins: lane 1, molecular mass markers; lane 2, hisPcrH–PopB complex; lane 3, hisPcrH–PopD complex; lanes 4 and 5, urea-isolated PopB and PopD, respectively. E. SEC analysis of purified hisPcrH-PopD (solid line) and hisPcrH-PopB (dashed line) complexes (Superdex-200 GL 10/30 column).
In contrast to hisPcrH-PopD, the hisPcrH-PopB complex eluted in two peaks during the AEC step (Fig. 2.1B). The first peak eluted at 0.19 M NaCl and a second peak eluted at 0.25 M NaCl. A third peak corresponding to the free hisPcrH chaperone eluted at a higher NaCl concentration (~0.3 M). SEC analysis of isolated fractions from the peaks revealed that the first peak corresponded mainly to a 1:1 hisPcrH-PopB complex (eluted at 13.9 mL), while the second peak contained a larger proportion of aggregates (Fig. 2.1C). Interestingly, we noticed that the amount of hisPcrH-PopB appearing as aggregates was affected by the concentration of the proteins (Fig. 2.2). The higher the protein concentration of the sample, the larger the amount of non-specific hisPcrH-PopB aggregates observed, confirming the intrinsic tendency of the complex to aggregate in aqueous solution (Schoehn et al., 2003; Tan et al., 2009).

The hisPcrH-PopB peak corresponding to the 1:1 complex was isolated and re-analyzed by SEC. A main symmetric peak eluted in the second SEC run (Fig. 2.1E), indicating that hisPcrH-PopB was stable in solution and ran with a hydrodynamic radius equivalent to a globular protein of 95 ± 3 kDa. Since the expected molecular weight for a 1:1 complex is 59.6 kDa, this result suggested that hisPcrH-PopB adopted an elongated conformation rather than a globular shape, as shown previously for the homologue protein AopB (Tan et al., 2009).

We have therefore optimized procedures to obtain both hisPcrH-PopD and hisPcrH-PopB complexes purified to apparent homogeneity (Fig. 2.1D, lanes 2 and 3, and Fig. 2.1E).
A significant increase in the amount of large hisPcrH-PopB aggregates was observed by SEC analysis when the two AEC eluted fractions containing hisPcrH-PopB were pooled and concentrated to 10 mg/mL final total protein. While the formation of large heterogeneous hisPcrH-PopB aggregates is favored at high protein concentrations, homogeneous hisPcrH-PopB complexes can be isolated and stored at lower protein concentration (Fig. 2.1E). A. Elution of hisPcrH-PopB from Q-Sepharose AEC by linear NaCl gradient resulted in three main peaks, the first two contained hisPcrH-PopB and the last one contained hisPcrH. Fractions indicated (Pool) were combined and concentrated to 10 mg/mL. B. SEC analysis of hisPcrH-PopB (Pool) concentrated to 10 mg/mL. hisPcrH-PopB eluted mostly as an asymmetrical peak with elution volume corresponding to high molecular weight aggregates. SEC conditions were as described in Methods section 2.4.8.
Figure 2.3. Spectroscopic characterization of purified translocators.

A. Far-UV CD spectra of the SEC-isolated hisPcrH, hisPcrH–PopD complex, and hisPcrH–PopB complex recorded in buffer E, at a total protein concentration of 3.0 μM. B. Far-UV CD spectra of purified PopD and PopB recorded in 20 mM phosphate buffer (pH 7.5) supplemented with 6 M urea, with a protein concentration of 2.4 μM. C. Far-UV CD spectra of PopD and PopB inserted in liposomal membranes recorded in 50 mM sodium acetate buffer (pH 4.0) with a protein concentration of 2.4 μM and 1:3000 protein:lipid ratio. Lipid composition was POPC:cholesterol:POPS with molar ratio 65:20:15 (This unpublished result suggests PopB gains significant helical structure upon membrane insertion, compared to its chaperone bound state) D. Normalized fluorescence emission spectra of hisPcrH, the hisPcrH–PopD complex, and the hisPcrH–PopB complex recorded in buffer E. The excitation wavelength was 278 nm, and the total protein concentration was 2.4 μM. E. Normalized fluorescence emission spectra of PopD and PopB in 20 mM phosphate buffer (pH 7.5) supplemented with 6 M urea. The excitation wavelength was 278 nm, and the total protein concentration was 2.4 μM.
2.2.2 Spectroscopic and functional characterization of the purified chaperone associated proteins

The far-ultraviolet circular dichroism (UV CD) spectrum of the purified chaperone hisPcrH revealed a typical all-α protein, with double minima around 222 nm and 209 nm (Fig. 2.3A). These data correlated well with the recently solved three dimensional structure of the PcrH21-160 fragment, which consists of α-helical tetratricopeptide repeats (Faudry, Job, et al. 2007) (Job, et al. 2010). The far-UV CD spectrum of the hisPcrH-PopD complex suggested that PopD also contains a high content of α-helical structure (Fig. 2.3A) (Faudry et al., 2007). The band with minimum at 208 nm had a larger intensity than the one at 222 nm, suggesting the presence of other secondary structural elements in this complex (Manalavan and Johnson, 1983). The far-UV CD spectrum of the hisPcrH-PopB complex was similar to that of hisPcrH-PopD, but the intensity of the bands was lower, suggesting that PopB has less α-helical content than PopD when bound to the PcrH chaperone.

Additionally, we evaluated the local environment around the aromatic residues of the P. aeruginosa PAO1 purified proteins. The hisPcrH chaperone contains one Trp and nine Tyr residues, while PopD contains two Tyr and two Trp, and PopB contains one Tyr and one Trp. The emission fluorescence spectra of hisPcrH showed a peak with a maximum at 349 nm (Fig. 2.3B), indicating that the Trp residue is located in a polar environment, in agreement with the position observed in the X-ray solved structure (Job et al., 2010). The shoulder around 303 nm corresponds to the emission of the multiple Tyr residues. The hisPcrH-PopD complex presents an emission fluorescence spectrum similar to hisPcrH, with a maximum at 351 nm. This red-shifted maximum suggested that both the central and C-terminal Trp of PopD reside in a polar environment, assuming the environment around hisPcrH Trp residue does not change significantly upon PopD.
binding. In contrast, the emission fluorescence spectrum of hisPcrH-PopB complex showed a maximum at 337 nm, suggesting that the Trp residue of PopB was located in a non-polar environment (Fig. 2.3B).

The pore formation activity for both purified proteins was analyzed using a previously described florescent assay (Heuck et al., 2003). In this assay, a fluorescent marker was encapsulated into liposomes and a quencher was added to the external buffer solution. A high fluorescence intensity signal indicated that the membrane was intact, and the quencher could not contact the fluorophore. If a transmembrane pore is formed upon addition of protein, the fluorophore becomes accessible to the quencher and the fluorescent signal decreases. Both PopB and PopD dissociate from PcrH in vitro when the solution pH drops to 5.3, forming heterogeneous protein aggregates (Schoehn et al., 2003). These dissociated protein aggregates form pores in model membrane systems (Faudry et al., 2006), however, the translocators are presumably secreted as monomers in vivo and in the proximity of the target membrane. Under these circumstances binding to the membrane can precede any protein-protein association. We therefore reasoned that the dissociation of the translocators from the chaperone in the presence of liposomes would more accurately represent in vivo conditions.

When separated from PcrH by dropping the pH from 8.0 to 5.1 in the presence of lipid membranes, PopB, PopD, and the equimolar mixture of these proteins were able to form pores (Fig. 2.4A). Interestingly, in contrast to the results observed when protein aggregates were used, no synergy was observed between PopB and PopD (Faudry et al., 2006). It is therefore clear that the history (i.e., aggregation) of the proteins may affect the mechanism by which the translocators form a pore. Since the sequence of the events that leads to the assembly of a membrane-inserted translocon is far from
understood, an experimental procedure that replicates the *in vivo* scenario encountered by the proteins after being secreted through the T3S needle is desirable.
Figure 2.4. Pore forming activities at pH 5.1 of urea-isolated translocators and acid dissociated translocators are very similar.

Experimental conditions were similar to the conditions employed by Faudry et al (2006). The activity profiles obtained at pH 5.1 did not show apparent saturation at high protein concentration. These results suggested that only a fraction of the added translocators were forming pores at this pH when compared to the profiles obtained at pH 4.1 (Fig. 2.8). No synergism was observed between PopB and PopD at any of the analyzed concentrations. A. HisPcrH-PopB, HisPcrH-PopD or a 1:1 mixture of both complexes stored in buffer A were added at the indicated concentrations into quartz cuvettes containing liposomes loaded with Tb(DPA)$_3^{3+}$ (0.1 mM total lipids), buffer sodium acetate 30 mM, pH 5.1, and EDTA 5 mM. Pore formation was determined as indicated in experimental procedures. B. Urea-isolated PopB, PopD or an equimolar and additive mixture of both translocators (final concentration for each translocator is indicated) were assayed for pore forming activity as in A. Lipid composition in A and B was 80 mol % POPC, 20 mol % POPS.
2.2.3 An efficient procedure to isolate PopB and PopD derivatives

Insights into the mechanism of protein insertion into lipid bilayers and the interaction of proteins with membranes can be obtained by fluorescence spectroscopy and site-directed fluorescence labeling (Heuck and Johnson, 2002). A Cys residue is introduced by site-directed mutagenesis at a single site in the protein, and the unique Cys is specifically labeled with the fluorophore of choice. Protein-membrane association and protein-protein interactions can be therefore study using FRET, excitation energy migration (homo-FRET), or fluorescence quenching (Mutucumarana et al., 1992) (Hamman et al., 1996) (Johnson, 2005).

_P. aeruginosa_ PAO1 translocators do not contain Cys residues, and therefore they are optimal substrates for site-directed fluorescent labeling. However, the labeling of isolated translocators in solution is hampered by their intrinsic tendency to aggregate. Introduced Cys residues can be alternatively labeled while the translocator is still bound to the chaperone PcrH. We noticed that in the recently solved x-ray structure of PcrH the three Cys residues are not exposed to the solvent, (Job et al., 2010) , and therefore we reasoned that the specific labeling of the translocators may be possible even in the presence of the chaperone. However, our initial labeling reactions demonstrated that the chaperone was also efficiently labeled with thiol-specific probes.

Analysis of the solvent exposure for the native hisPcrH Cys revealed that on average, 2.20 ± 0.03 Cys residues reacted with the Ellman’s reagent (DTNB). Interestingly, incubation of hisPcrH with 6 M guanidinium chloride at 37 °C for 1 hr did not increase the reactivity of the hisPcrH Cys residues to DTNB, suggesting that a portion of those residues were forming intra- or inter-molecular disulfide bonds. SEC analysis of purified hisPcrH samples revealed a first small peak eluted at 14.3 mL and a large second peak eluted at 15.3 mL (Superdex 200 10/30 GL). Non-reducing SDS-
PAGE analysis of the two peaks revealed that hisPcrH formed intermolecular disulfide bonds in solution (Fig. 2.5). Hence, it is clear from these data that the structure of PcrH in solution is dynamic, and the side chains of the amino acids surrounding the Cys residues move and expose the sulfhydryl groups to the solvent.

Any attempts to replace all three Cys residues in PcrH with non-reactive residues (Ala or Ser) rendered a chaperone that no longer bound PopD. Therefore, the interpretation of the fluorescence signal and observed spectral changes derived from the single fluorescently labeled translocators would be veiled by the presence of labeled chaperone. The spectroscopic characterization of the translocon assembly mechanism, therefore, requires an efficient separation of the labeled translocators from the labeled hisPcrH chaperone prior to the analysis.

Initial attempts to isolate PopD from PcrH by dissociating the complex through acidification of the pH (Schoehn et al., 2003) indicated PcrH precipitated out of solution at acidic pH carrying considerable amounts of PopD (Fig. 2.5). Therefore, and efficient method for isolation of translocators was required.

We took advantage of the polyHis tag located at the N-terminus of the chaperone to isolate labeled translocators. After labeling, the translocators were separated from hisPcrH by binding hisPcrH-PopD (or hisPcrH-PopB) to a metal affinity column and a subsequently eluting PopD (or PopB) with buffer containing 6 M urea. Urea dissociated PopD or PopB from their cognate chaperone without affecting the interaction of hisPcrH with the IMAC column (Fig. 2.1D, lanes 4 and 5). Therefore, the single Cys translocator mutants can be labeled while still bound to hisPcrH, and separated from the chaperone in a subsequent step. This is a simple and efficient procedure to obtain the translocator derivatives required for the structural and functional characterization of the T3S translocon (e.g., single fluorescently-labeled translocators).
hisPcrH-PopD complexes (20.4 μM) were incubated for 5 min at the indicated pH values in buffer sodium phosphate 50 mM at ~20-23°C. After incubation, samples were centrifuged at 15,000×g for 15 min at 4°C to separate soluble and insoluble protein pellet fractions. Pellet fractions (P) containing the re-suspended precipitate, and supernatant fractions (S) containing the protein that remained in solution after acidification were analyzed by SDS-PAGE and stained with coomassie brilliant blue. SDS-PAGE analysis indicated hisPcrH precipitated at acidic pH carrying considerable amounts of PopD.
hisPcrH stored for more than 24 hr at 4°C in buffer A, was analyzed by SEC. hisPcrH distributed in two overlapping peaks centered at 14.3 mL and 15.3 mL elution volumes. SDS-PAGE analysis of the collected fractions using reducing and non-reducing conditions indicated the presence of intermolecular disulfide-bonds. A. hisPcrH stored under non reducing conditions analyzed by SEC as described in Methods section 2.4.8. B. SDS-PAGE analysis of SEC eluted fractions (fractions of 1 mL were collected). Samples were treated with 50mM DTT (reducing) or without DTT (non-reducing) before the SDS-PAGE run. Molecular weights in kDa for protein standards are indicated. The locations of the hisPcrH monomer (hisPcrH) and the dimer [(hisPcrH)₂] are indicated. (Data collected by Sarah Kells)
2.2.4 Spectroscopic and functional characterization of urea-isolated PopB and PopD

We analyzed the structural and functional properties of the urea-isolated translocators using mass spectrometry, far-UV CD, intrinsic protein fluorescence, and pore formation assays using model membranes. The molecular mass of urea-isolated PopB and PopD was determined using ESI-Ion trap mass spectrometry as detailed under Methods section 2.4.10. Only one species was detected for each protein sample with a molecular mass of 40,058 Da for PopB (expected value 40,061 Da), and 31,308 Da for PopD (expected value 31,309 Da).

The polarity around the Trp residues for the urea-isolated PopD and PopB was similar to the polarity observed for the proteins complexed with the hisPcrH chaperone (Fig. 2.3D). PopD and PopB in urea presented a fluorescence emission maximum at 358 nm and 334 nm, respectively. The far-UV CD negative band at 222 nm observed for PopB in urea 6 M suggested that PopB conserved a high proportion of its \( \alpha \)-helical structure even in the presence of the chaotropic agent (Fig. 2.3C). However, the far-UV CD spectrum of PopD in urea 6 M revealed that a sizeable portion of the secondary structure of this translocator was lost in the presence of the chaotropic agent.

Despite the differences observed in their secondary structure, both urea-isolated PopB and PopD conserved their pore forming abilities at a mildly acidic pH (Fig 2.4B). The pore formation activity profiles of urea-isolated translocators were very similar to the ones observed when the translocators were directly dissociated from the chaperone by reducing the pH of the medium (Fig 2.4B). Altogether, our data indicated that the purification procedure described above constitutes a simple and efficient alternative to obtain highly pure and active PopD, PopB, and their mutant derivatives.
2.2.5 Optimization of a model system to analyze the membrane inserted state of the translocons

Unambiguous interpretation of structural data requires that the components under investigation adopt a uniform conformational state. For the structural analysis of assembled T3S translocons, it is essential that the measured fluorescent signal come from probes located in the same conformation (i.e., membrane-inserted translocons). We therefore optimized our model system to maximize the assembly and insertion of the T3S translocons. Three factors affect the assembly of active transmembrane pores in our experimental system: i) the pH of the medium; ii) the presence of negatively charged phospholipids; and iii) the cholesterol concentration of the target membrane.

In membranes containing a mixture of the lipids commonly present in mammalian plasma membranes (i.e., POPC, POPE, POPS and sphingomyelin) and 15 mol% cholesterol, maximal activity for PopB (~60%) was observed at pH 5.1 or below (Fig. 2.7 A). For PopD, the maximal activity (~90%) was observed below pH 5. Interestingly, the activity of PopD was lower than the activity of PopB above pH 5.1, but it surpassed that observed for PopB at pH values below 4.8, suggesting that the insertion of PopD into membranes is more dependent on acidic residue protonation than the insertion of PopB.

When maintaining a constant POPC:POPE:POPS:sphingomyelin molar ratio in the absence or presence of high cholesterol (45 mol%), the pore forming activity of the translocators was less effective than that observed with 15 mol% cholesterol (See Appendix A). Since the activity of PopD and PopB plateau below pH 4.5 at intermediate cholesterol concentrations, we selected pH 4.0-4.3 and 20 mol% cholesterol for our assays to maximize the formation of uniform membrane-inserted translocons.

Negatively charged phospholipids affect the degree of insertion of T3S translocons (Schoehn et al., 2003) (Faudry et al., 2006). We therefore analyzed the effect of POPS on the activity of urea-isolated PopB and PopD in membranes containing
POPC and a fixed amount of cholesterol (20 mol%). Addition of 15 mol % POPS increased the activity of PopB two-fold, and more than three-fold the activity of PopD (Fig. 2.7 B). Doubling the amount of POPS to 30 mol % was not as effective as the addition of only 15 mol% POPS. Thus, to maximize the conformational homogeneity of membrane-inserted translocators and to minimize any effect caused by the variability of the lipid composition of the system, we chose the simplest membrane model system that maximized the formation of transmembrane pores: POPC:POPS:cholesterol at a molar ratio of (65:15:20).

The pore forming activity of urea-isolated PopD, PopB, and a 1:1 mixture of the translocators was studied using this model system. Maximal activity for PopB and PopD was observed below pH 4.8 and pH 4.3, respectively (Fig.2.8A). Interestingly, no additive or synergic effect was detected at higher pH, where the individual proteins showed intermediate pore forming activity. Maximal pore formation was observed by using these experimental conditions when the protein:lipid ratio was 1:3000 (Fig. 2.8B).
Figure 2.7. Effect of pH and lipid composition on the pore forming activity of the urea-isolated translocators.

Pore formation was determined as the percentage of the encapsulated Tb(DPA)$_3^{3-}$ that was quenched by EDTA as detailed in Methods section 2.4.17. A. Urea-isolated PopB or PopD was directly diluted into a solution of 50 mM sodium acetate buffered at the indicated pH, containing membranes. The pore forming activity of the urea-isolated translocators increased at acidic pH. The total lipid concentration was 0.1 mM, and the liposomes consisted of 35 mol % POPC, 15 mol % POPS, 20 mol % POPE, 15 mol % SM, and 15 mol % cholesterol. The protein:lipid ratio was 1:1000. B. Effect of POPS on the pore formation activity of urea-isolated translocators. The activity was measured as described for panel A; the pH was buffered at 4.3, and the lipid mixture consisted of POPC, 20 mol % cholesterol, and the indicated concentration of POPS. (Data collected by Sarah Kells).
Figure 2.8. Pore forming activity of the urea-isolated translocators.

Pore formation was determined as the percentage of the encapsulated Tb(DPA)\textsubscript{3}\textsuperscript{3−} that was quenched by EDTA as detailed in Methods section 2.4.17. A. Urea-isolated PopB, PopD, or an equimolar mixture of both proteins was diluted into a buffer solution containing membranes at the indicated pH. The total concentration of lipids was 0.15 mM. The total protein concentration was 60 nM for the PopB and PopD traces (protein:lipid ratio of 1:2500) and 120 nM when PopB and PopD were added together (protein:lipid ratio of 1:1250). B. Concentration-dependent pore formation by PopD, PopB (protein:lipid ratio ranged from 1:10\textsuperscript{5} to 1:100), or an equimolar mixture of both proteins diluted into buffer D containing 0.1 mM total lipids. When both proteins were added together, the sample contained twice as much total protein vs the concentration indicated in the graph (protein:lipid ratio ranged from 1:5 \times 10\textsuperscript{4} to 1:50). C. Cryoelectron micrographs of liposomes with and without PopB and PopD at the indicated protein:lipid ratios. Buffer B was added to the control sample with only liposomes to assess any effect of the residual urea concentration on membrane structure. The lipid concentration was 5 mM in all cases, and the total protein concentration was 1.5 or 15 μM. The POPC:cholesterol:POPS molar ratio was 65:20:15 in all panels. (cryo-EM data collected by Christos G, Savva). For additional cryoEM images see Appendix C.
2.2.6 High translocator-lipid ratios produced liposome aggregation.

Our initial attempts to visualize the membrane inserted PopD using transmission electron microscopy and uranyl acetate 2% as a contrast agent revealed that even when used at 1:1000 protein:lipid ratio, PopD disrupted the liposomes forming tubular membrane structures (see Appendix A). We therefore analyzed the aggregated state of the liposomes using DLS. Isolated liposomes, incubated at pH 4.0, showed a single particle size distribution with an average of 78 nm (expected ~100 nm, Fig 2.9). However, the incubation of the same liposomes with PopD, PopB, or an equimolar mixture of both translocators, using a 1:1000 protein:lipid molar ratio, shifted the average size distribution to 264 nm, 342 nm, and 164 nm, respectively. These data suggested that at this protein:lipid ratio the translocators caused aggregation of the vesicles, especially when added individually. Little or no aggregation was observed when lower protein lipid ratios were used (Fig 2.9). Therefore, when performing structural studies in model systems, it is important to confirm that the size of the vesicles is not altered by the addition of the proteins. If aggregation occurs, the structure adopted by the proteins when bound to membranes may differ from the structure adopted when forming discrete transmembrane pores.

We used cryo-EM to directly visualize the liposomes before and after incubation with the translocators, and to determine the optimal protein:lipid ratio to be used in our studies. Surprisingly, extruded liposomes prepared with POPC:POPS:cholesterol at a molar ratio of 65:15:20 were mostly multi-lamellar (Fig. 2.8C). Using different protein:lipid ratios, we found that addition of an equimolar mixture of both proteins at a protein:lipid ratio at or below 1:2500 did not alter the liposomal structure (Fig. 2.8C and Fig 2.9). Higher protein concentrations (e.g., 1:330), caused liposome-liposome interaction which
resulted into liposome clumps with µm size (Fig. 2.8 D and Fig 2.9), but no tubular structures were observed by cryoEM.
Liposome size distributions before and after incubation with the translocators was analyzed for different protein:lipid ratios using DLS. No significant aggregation was observed at protein:lipid ratios of up to 1:3300. In contrast, when a protein:lipid ratio of 1:330 was used large aggregates with average diameter of about 1.3 μm were observed. These data correlated well with the cryo-EM observations (Fig. 2.8 C and D). Distribution averages are indicated. Error bars correspond to ± standard deviations of 3 measurements. Urea-isolated PopB/PopD at 1:1 ratio was incubated with liposomes (200 μM total lipids) at the indicated protein:lipid ratios in buffer sodium acetate 50 mM pH 4 for 30 min at 21-23oC. The POPC:cholesterol:POPS molar ratio was 65:20:15. DLS measurements were carried out as described in Methods section 2.4.15.

Figure 2.9. High translocator-lipid ratios produced liposome aggregation.
2.2.7 PopB and PopD form discrete and stable membrane pores

We analyzed the ability of the urea-isolated translocators to form discrete and stable pores in liposomes encapsulating a streptavidin-Bodipy fluorescent marker (~14 nm diameter for tetrameric streptavidin). In contrast to fluorescein and fluorescein derivatives (e.g., calcein and Oregon green), the fluorescent properties of the Bodipy dye are stable at acidic pH (Karolin et al., 1994). Binding of biocytin (biotin moiety covalently attached to the amino acid Lys, ~1 nm in diameter) or biotin attached to the protein β-amylase (~ 4 nm in diameter) to streptavidin-Bodipy produces a two- or three-fold enhancement in the fluorescence intensity of the streptavidin-Bodipy marker (Nicol et al., 1999; Rosconi et al., 2004). When the biotin-labeled molecules were externally added to the sample containing intact liposomes, no fluorescence change was detected on the encapsulated streptavidin-Bodipy. Neither biocytin nor proteins like β-amylase or streptavidin-Bodipy can pass through the membrane unless a pore is formed. The size of the pore will dictate which molecules can cross the membrane. Addition of the translocators produced a fluorescence intensity increase only for the samples containing biocytin, and not for the ones containing biotin-β-amylase. These results indicated that the transmembrane pores formed by PopB and PopD were larger than 1 nm in diameter, but not large enough to allow the passage of the β-amylase protein or the encapsulated streptavidin-Bodipy (Fig. 2.10 A).

The stability of the formed pores was determined by adding the enhancer (biocytin) before or after the addition of the translocators. If the pores are stable, the enhancement of the fluorescence intensity of streptavidin-Bodipy will be observed when biocytin is added before or after the incubation with the translocators. In contrast, if the formed pores are not stable and close, the fluorescence will increase only when biocytin is present before the addition of the translocators, and not when added after the
incubation. Since the fluorescence intensity increased in both cases (Fig. 2.10 B), it is clear that the formed pores remained stably open for at least 1 hr in this model system. Control experiments without translocators did not show significant fluorescence change.

2.2.8 PopB and PopD binding to membranes also required acidic pH

To precisely determine the binding of the translocators to the target membrane we introduced single Cys residues in PopB at a location close to a predicted TM segment (S164), and in PopD at a location that was found to be exposed to the aqueous solvent in the membrane bound complex (F223, M. Buckner unpublished results). The Cys residues were labeled with Bodipy. As mentioned above, the emission properties of Bodipy are not sensitive to the polarity of the environment and are not affected by pH in the range used in this work (Karolin et al., 1994). Bodipy labeled translocators were isolated from the hisPcrH chaperone by elution with urea 6 M as described for the wild-type proteins (Fig. 2.1 D). Pore forming activity of the Bodipy-labeled translocators was similar to the activity of the wild-type proteins (not shown).

We first analyzed the binding of the translocators by using FRET. FRET is an effective method for identifying protein-membrane binding under equilibrium conditions (e.g., (Mutucumarana et al., 1992; Bazzi and Nelsestuen, 1987; Ramachandran et al., 2005; Posokhov and Ladokhin, 2006; Lovell et al, 2008). A typical FRET membrane binding experiment requires two fluorescent dyes, a donor (D) located at a specific site in the protein and an acceptor (A) distributed on the surface of the membrane. The Bodipy dye covalently attached to PopB$^{S164C}$ (or PopD$_{F223C}$) was used as the D dye in our FRET experiments, while Rh-PE incorporated into the membrane was the A dye. After excitation by the absorption of a photon, Bodipy can transfer its excited-state energy to Rh-PE. The efficiency of this transfer depends on, among other things, the extent of
overlap of the D emission and the A absorption spectra, the relative orientation of the transition dipoles of the D and the A, and more importantly, the distance between the D and the A dyes. The distance at which the efficiency of FRET from the D to the A is 50% is designated $R_0$ [the $R_0$ is ~52 Å for the Bodipy/Rh-PE pair, (Ramachandran et al., 2005)]. Since the FRET efficiency is strongly dependent upon the actual distance separating D and A, FRET will be detected if the dyes are within 95 Å of each other, and it will be maximal when the dyes are separated by less than 24 Å. While considerable FRET efficiency is expected for Bodipy dyes close to the membrane surface (i.e., membrane-bound translocators), no significant FRET is expected for unbound proteins since they will reside more than 100 Å away from the membrane surface. FRET efficiency was estimated using the amplitude weighted average lifetime of the D in the presence ($<\tau_{DA}>_a$) or the absence ($<\tau_D>_a$) of the A. The use of lifetime determinations over steady state measurements is advantageous because it minimize the problems associated with the quantification of the D in the presence and absence of the A (Wu and Brand, 1994). Two exponential discrete component were used to fit the lifetime data (Fig 2.12 and Table 2.2). The use of additional discrete components or lifetime distributions in the analysis did not improve data fit. Binding of the translocators was strongly dependent on the pH of the media (See also Chapter 3). We report here the FRET efficiency at binding saturation (pH 4) and at a point of low binding (pH 6). PopB FRET efficiency was 40% lower at pH 6.0 than at pH 4.0, while PopD FRET efficiency was more than 80% lower at pH 6.0 than at pH 4.0 (Fig. 2.11).
Figure 2.10. PopD and PopB form discrete and stable pores in model membranes.

A. The passage of biocytin (~10 Å diameter), biotinlabeled β-amylase (~50 Å diameter), or streptavidin<sup>Bodipy</sup> (~50 Å diameter) through the pores formed by PopD, PopB, or an equimolar mixture of the translocators was measured as detailed in Experimental Procedures. The total lipid concentration was 100 μM, and the protein concentration of the proteins was 100 nM (protein:lipid ratio of 1:1000 for individual proteins and 1:500 when added together). The fluorescence intensity of encapsulated streptavidin<sup>Bodipy</sup> was measured before (F<sub>0</sub>) or after the incubation for 60 min at 25 °C with the translocators (F). Only biocytin was able to diffuse through the formed pores. B. The stability of the formed pores was examined by measuring the increase in the fluorescence intensity of encapsulated streptavidin<sup>Bodipy</sup> when biocytin was present in the external buffer solution before the addition of the translocators, or added after incubation for 1 h with the translocators. The discrete pores formed by PopB, PopD, and an equimolar mixture of the proteins remained open after incubation for 1 h.(Data collected by Sarah Kells).
The pH dependent binding of the translocators was independently assessed by separation of the bound and unbound proteins fractions using a sucrose step gradient and ultracentrifugation as described under Methods section 2.4.14 (Schoehn et al., 2003; Dalton et al., 2005; Rivnay and Metzger, 1983). Given the different densities of proteoliposomes and unbound proteins, proteoliposomes containing the bound protein floats to the top of the sucrose cushion while unbound protein remains at the bottom (Dalton et al., 2005). Bodipy-labeled translocators were individually incubated with membranes at the pH of maximal pore formation activity (pH 4.0, see Fig. 2.5), and at pH 6.0, where the pore forming activity for PopB was significantly lower, and almost null for PopD. The amount of protein isolated in the top fraction (i.e., membrane bound translocator) was quantified using SDS-PAGE followed by fluorescence scanning with a phosphorimager, and reported as the fraction of the total protein bound to membranes (Fig. 2.11). At pH 4.0, more than 70 % of the added PopB\textsuperscript{S164C-Bodipy} and more than 90 % of the added PopD\textsuperscript{F223C-Bodipy} were isolated in the fraction containing the membranes. In contrast, membrane binding at pH 6.0 was only 40 % and 25 % for PopB and PopD, respectively. These data were consistent with the amount of protein binding observed by FRET. Therefore, we concluded from these complementary and independent approaches that the binding of PopB and PopD to the membrane was higher at the pH of maximal activity, and decreased when the pH was closer to neutral.
Figure 2.11. Acidic pH enhances translocator-membrane binding.

A. PopB$^{\text{S164C-Bodipy}}$ membrane binding was measured using the liposome flotation assay described in Methods Section. PopB$^{\text{S164C-Bodipy}}$ (0.4 μM) was incubated with membranes (2 mM total lipids) for 1 h at 20–23 °C. Typical SDS–PAGE analysis was conducted showing the amount of total protein added, and the amount of bound protein isolated after incubation with membranes at the indicated pH. The protein:lipid ratio was 1:5000. Gels were scanned for Bodipy fluorescence using a phosphoimager. B. Quantification of PopB$^{\text{S164C-Bodipy}}$ binding measured by the liposome flotation assay (fraction bound, black bars). Data were normalized against the total amount of protein used in the binding reaction. Each bar represents the average and range of at least two independent experiments. FRET efficiency (gray bars) for PopB$^{\text{S164C-Bodipy}}$ binding determined by time-resolved FRET as described in the Methods section 2.4.22. PopB$^{\text{S164C-Bodipy}}$ (120 nM) was incubated with membranes (total lipid concentration of 0.3 mM) as described for panel A (protein:lipid ratio of 1:2500). Each bar represents the average and range of two independent experiments. C and D. Analysis of PopD$^{\text{F223C-Bodipy}}$ binding as described for panels A and B, respectively, for PopB$^{\text{S164C-Bodipy}}$. The POPC:cholesterol:POPS molar ratio was 65:20:15 in all panels.
2.2.9 PopB interacts with PopD on model membranes

PopD and PopB have different optimal pH and lipid composition requirements to form pores in membranes (Fig. 2.7 and 2.8). Surprisingly, the pore forming properties of the translocators when added together were similar to the ones observed for PopB alone (Fig 2.8 A). No synergic effect between PopB and PopD was observed under our experimental conditions. Do the translocators interact with each other at all? We took advantage of the fluorescence properties of Bodipy to determine PopB-PopD association on membranes.

Bodipy dyes change their fluorescence properties significantly when brought into close proximity with one another. At distances of orbital-orbital contact the emission intensity and lifetime of the sample decreases significantly, presumably by the formation of non-fluorescent Bodipy dimers (Bergstrom et al., 2002). Proximity of Bodipy dyes at longer distances (i.e., between ~20-85 Å) is detected by the decrease in the anisotropy of the sample (depolarization) caused by energy migration (Bergstrom et al., 2002) (Mikhailov et al., 2002). Protein-protein association can be in principle determined by changes in the above mentioned fluorescence properties of the sample when Bodipy-labeled proteins are employed (Marushchak et al., 2006). We used a Bodipy-labeled derivative of Perfringolysin O (PFO) (see Methods section 2.4.6-7) as a control for how protein-protein association on membranes affects the fluorescence properties of Bodipy. PFO is well characterized cholesterol-dependent cytolysin that forms oligomeric complexes on lipid membranes (Heuck et al., 2010). These complexes are formed by association of up to 50 monomers in a circular ring-like oligomer on the membrane surface. The rings are ~300 Å in diameter (Czajkowsky et al., 2004; Dang et al., 2005). We compared a membrane assembled sample containing 100% labeled PFO, where the effects on the fluorescence properties will be maximal, with a sample containing only 10
mol% of the labeled protein (i.e., only 5 of 50 monomers will be labeled on average in each oligomeric complex). The diluted sample showed a 4.5 fold increase in the emission intensity and more than a two fold increase in the amplitude averaged fluorescence lifetime (Table 2.1), indicating that addition of unlabeled PFO separated the \( \text{PFO}^{\text{Bodipy}} \) molecules apart, increasing the distance between probes (Bergstrom et al., 2002). The increase on the distance between probes, caused by the intercalation of unlabeled proteins, also diminished the depolarization of the sample emission caused by Bodipy-Bodipy energy migration (i.e., the anisotropy increased, Table 2.1) (Jameson et al., 2003).

We assessed the interaction between PopB and PopD using \( \text{PopD}^{\text{Bodipy}} \) and analyzed the changes in the fluorescence properties of the sample when the labeled translocator was mixed with unlabeled PopB (and vice versa). As expected for the formation of mixed protein complexes containing both PopB and PopD in the membrane, dilution of \( \text{PopD}^{\text{Bodipy}} \) with unlabeled PopB increased the emission intensity and the amplitude averaged fluorescence lifetime of Bodipy (Table 2.1). When compared to the data obtained for the PFO oligomer, it is clear that unlabeled PopB intercalated between labeled PopD proteins when bound to the membrane. The significant but relatively smaller increase on the anisotropy for Bodipy labeled translocators suggested that PopD and PopB formed heteromeric complexes with smaller size than the large ~300 Å diameter rings formed by PFO, as Bodipy-Bodipy energy migration only occurs at distances shorter than 100 Å. Similar changes were detected when \( \text{PopB}^{\text{Bodipy}} \) was diluted with unlabeled PopD. The smaller changes in the fluorescence parameters observed for \( \text{PopB}^{\text{Bodipy}} \) reflected the lower percentage of labeling of this derivative (68% vs 96% for \( \text{PopD}^{\text{Bodipy}} \)). Thus, we concluded that when added together, PopB associated with PopD to assemble pore-forming complexes on model membranes.
Figure 2.12. Time-resolved lifetime measurements for protein-membrane FRET.

Bodipy labeled translocators (120 nM) were incubated with liposomes (3 mM total lipid concentration) at 25 °C with or without Rh-PE and at pH 4 or pH 6. Fluorescence lifetimes of Bodipy were measured using a frequency-domain fluorometer as described in Methods section 2.4.21. A. Phase (red) and modulation (blue) values as a function of frequency for PopD\textsuperscript{F223C-Bodipy} incubated with membranes at pH 4 containing (filled symbols) or not (open symbols) the FRET acceptor Rh-PE. Solid lines correspond to a two-exponential fit of the data with lifetimes and fractional amplitudes reported in table 2.2. B. Same as A but incubated at pH 6. C. Same as A but for the translocator PopB\textsuperscript{S164C-Bodipy}. D. Same as C but at pH 6. The POPC:cholesterol:POPS molar ratio was 65:20:15 in all panels.
Table 2.1: Bodipy Detected Protein–Protein Interaction in Intact Membranes$^a$.

$^a$Relative fractions of proteins added are indicated. Labeled and unlabeled proteins were mixed in buffer B supplemented with 6 M urea and 20 mM Glycine solution prior to dilution in buffer D in the presence of membranes. The final fluorescence parameters measured for the different samples are indicated: $I$, fluorescence emission intensity; $I_{10}/I_{100}$, ratio of the fluorescence emissions of diluted to undiluted labeled protein samples; $<\tau>_{\alpha}$, amplitude-weighted average fluorescence lifetime. The fluorescence lifetime of Bodipy in 50 mM sodium acetate (pH 4.0) was 5.85 ns. The fluorescence lifetime of monomeric PFOBodipy in buffer C was 5.51 ns. Labeling efficiencies were 100, 96, and 68% for PFO$^{Bodipy}$, PopDF223C-Bodipy, and PopB$^{S164C=Bodipy}$, respectively. The average and range of at least two determinations are shown. Protein:lipid ratios were 1:2500 for fluorescence lifetimes and 1:10000 for steady-state determinations. $^b$Time-resolved fluorescence data for 10% labeled PFO best fit to a single-exponential component. The remaining data best fit to a two-exponential component (see Table 2.2).
Table 2.2: Translocator-membrane FRET efficiencies calculated from donor fluorescence lifetimes.

Amplitude weighted average fluorescence lifetimes of Bodipy labeled translocators in membranes in the presence (\(<\tau_{DA}>_a\)) or in the absence of (\(<\tau_D>_a\)) of the acceptor dye Rh-PE. Time-resolved fluorescence data was acquired as described in Methods section 2.4.21. Data best fit to double discrete exponential decay models. Addition of a third component did not decrease the \(\chi^2\). The individual lifetimes and their pre-exponential factors are denoted as \(\tau_n\) and \(\alpha_n\) for the \(n^{th}\) component, respectively. FRET efficiencies (\(E\)) were calculated as described in Methods section 2.4.22.

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2.3 Discussion

Structural and functional characterization of the T3S translocators is inherently difficult given the non-polar nature of the proteins and their tendency to aggregate in solution. Co-expression of T3S recombinant translocators in the presence of their cognate chaperone renders soluble protein complexes and allows their purification in large quantities (Schoehn et al., 2003; Birket et al., 2007; Tan et al., 2009; Faudry et al., 2007). However, the analysis of individual T3S translocators requires their separation from the chaperone prior to their assembly into membranes. These separations have been achieved by reducing the pH of the medium (Schoehn et al., 2003; Birket et al., 2007), or by the inclusion of mild detergents (Birket et al., 2007). Unfortunately, upon dissociation from the PcrH chaperone, both PopB and PopD form large and non-uniform protein aggregates (Schoehn et al., 2003) that make it difficult to characterize the protein-membrane and protein-protein association processes. Furthermore, we observed that during the acidification of the medium to separate the translocators from the chaperone, PcrH precipitates carrying considerable amounts of the purified translocators (Fig. 2.5). Thus, the precise characterization of the T3S translocon assembly into membranes required a more efficient experimental approach to isolate active translocators.

We have found in these studies that PopB (as well as PopD) can be purified as a homogeneous complex with the cognate chaperone hisPcrH and be effectively dissociated and isolated from hisPcrH using urea. In contrast to PopD, which lost most of its secondary structure, PopB retained a high proportion of its secondary structure after solubilization in urea 6 M. The blue-shifted intrinsic fluorescence spectra of urea solubilized PopB, suggested that the Trp located at the N-terminus of a predicted TM
segment (residues 169-187) remained located in a non-polar environment even in the denatured state. As expected for proteins that are proposed to travel unfolded through the T3S needle and to be released in close proximity of the target membrane, both urea-isolated translocators formed discrete and stable TM pores upon dilution in the presence of lipid bilayers. The pore forming properties of urea-isolated PopB and PopD were consistent with the ones described previously for translocators isolated using low pH (Faudry et al., 2006). Interestingly, quantitative assessment of PopB and PopD binding to membranes showed that acidic pH was not only required for membrane insertion, but also for a stable protein-membrane interaction. In summary, we have established an efficient experimental procedure to isolate native and modified forms of PopB and PopD, and to specifically label the translocators with fluorescent or other type of probes (e.g., cross-linkers). Isolated translocators were assembled on model membranes where they form discrete and stable pores. Moreover, our spectroscopic examination of Bodipy-labeled translocators showed that when added together, PopB intercalates with PopD on membrane assembled complexes.

Purification of isolated recombinant translocators has shown to be a difficult task. PopD cloned in pET based vector systems produced large amounts of protein, but mostly as inclusion bodies. The yield of water-soluble PopD can be improved by expression at low temperature (i.e., ~18°C), but water-soluble PopD forms aggregates [(Schoehn et al., 2003) and data not shown]. PopB is toxic for Escherichia coli cells and cannot be stably produced (data not shown). Schoen et al elegantly solved these problems by co-expressing the translocators with their cognate chaperone PcrH. The water-soluble PcrH-PopD 1:1 complex was obtained in large amounts and the structural characterization of PopD showed it adopts a molten globular conformation (Faudry et al., 2007). Less is known about the structure of PopB, given that only minor amounts of a
homogeneous PcrH-PopB complex were obtained using this procedure (Schoehn et al., 2003; Tan et al., 2009).

We found that the purified hisPcrH-PopB complex can remain soluble and non-aggregated in aqueous solutions at relative low concentrations (up to ~ 2-3 µM) (Fig. 2.1). The structure of purified hisPcrH-PopB complex was analyzed using a combination of CD and fluorescence spectroscopy (Fig. 2.3). Far-UV CD analysis revealed that PopB had less α-helical content than PopD when bound to hisPcrH. However, in contrast to the low secondary structure content observed in urea-isolated PopD, urea-isolated PopB retained considerable amount of secondary structure. Furthermore, the only Trp of PopB was located in a non-polar environment when bound to the chaperone or when denatured in urea. These data suggested that PopB adopts a more compact conformation than the one observed for molten globular PopD (Faudry et al., 2007).

PopB and PopD remain stably bound to PcrH in the bacterial cytoplasm in the absence of secretion, most likely to avoid protein aggregation and to maintain a secretion-competent conformation (Mueller, 2005; Mattei et al., 2011). After bacterium contact with the target cell, PopB and PopD dissociate from PcrH and are presumably secreted through a narrow channel formed by the T3S needle (2-2.5 nm in diameter). Unfolded translocators are proposed to emerge from the tip of the T3S needle (formed by PcrV), in close proximity of the target membrane. PcrV is required to engage the membrane inserted translocon with the T3S needle (Goure et al., 2004; Sawa et al., 1999; Goure et al., 2005), however PcrV is not involved in the formation of the TM pore (Schoehn et al., 2003). Therefore, the use of urea-isolated translocators to reconstitute membrane-inserted protein complexes is reasonable since the dilution of urea-isolated PopB and PopD in the presence of membranes likely resembles the in vivo translocon assembly process.
While negatively charged phospholipids are crucial for the binding of translocators to membranes and formation of TM pores in model membranes, cholesterol seems not to play a central role in vitro (Faudry et al., 2006). We tested how different lipids affected the pore formation properties of PopB and PopD in our experimental system. The presence of cholesterol and POPS in the liposomal membranes facilitated the insertion of the urea-isolated PopB and PopD (Fig. 2.7). Other lipids like sphingomyelin and PE did not significantly altered the pore formation efficiency observed with liposomes composed of POPC:POPS:cho (65:15:20 molar ratio, data not shown). Therefore, we chose this composition as a simple and efficient model membrane system to study the assembly of PopB and PopD into lipid bilayers. Urea-isolated translocators formed discrete and stable pores in model membranes (Fig. 2.8 and 2.10). Taken together, our data showed that the treatment with urea 6 M did not significantly alter the pore formation properties of the translocators.

We found that urea-isolated PopB, PopD, or an equimolar mixture of both translocators formed pores very similar to the pores formed when the translocators are dissociated from PcrH at acidic pH in the presence of membranes (Fig. 2.4). Interestingly, the comparison of both experimental systems suggested that acidic pH was not only required to dissociate the translocators from the chaperone (Fig 2.5), but also to promote binding and insertion of translocators into membranes (Fig. 2.8 and 2.11). We employed two independent experimental approaches, time-resolved FRET and sucrose-step gradient ultracentrifugation, to analyze the binding of the translocators to membranes at different pHs (Fig. 2.11). Both assays clearly showed that binding at pH 4 was more efficient than at pH 6. Only a small fraction of the proteins was found associated with the membranes at pH 6. These results differ from the ones previously observed when heterogeneous aggregates of PopB and PopD were used (Faudry et al.,
The kinetics parameters used as a measure of membrane binding may represent the behavior of a small fraction of protein in the experimental system, and not necessarily the average behavior of all the protein in the sample (i.e., unbound protein does not generate a signal). Our data suggested that the decrease in the activity of the translocators at higher pH was, at least in part, caused by the inability of the proteins to bind to the lipid membrane.

A homogeneous sample is essential for the unambiguous interpretation of the spectroscopic signal obtained during the analysis of protein complexes assembled into membranes (Johnson, 2005). For this purpose, negatively charged phospholipid and low pH have been extensively used in the spectroscopic studies of pore-forming proteins like colicins, diphtheria toxin, Bcl-xL, etc. (Wang et al., 1997; Chenal et al., 2002; Thuduppathy et al., 2006; Musse and Merrill, 2003; Kyrychenko et al., 2009). The requirement of acidic pH for protein insertion into membranes has been associated with the presence of acidic molten globular states (van del Goot et al., 1991). While this pH dependent conformational change may be relevant to the more compact PopB conformation (see above), PopD was found to have a molten globule conformation even at neutral pH (Faudry et al., 2007). Therefore, the spontaneous insertion of PopD (and/or PopB) segments into lipid bilayers may require the protonation of acidic residues, as shown before for bacteriorhodopsin fragments (Hunt et al., 1997). In our model system, the use of acidic pH maximized the assembly of discrete and stable TM pores. Such a low pH is presumably not encountered by the proteins when interacting with the plasma membrane of the target cell in vivo. Clearly, other still unidentified components present in the bacterium, or in the target cell, may facilitate the assembly of the translocon but further studies are required in this area.
It has been widely assumed that assembled translocons contains both translocators forming a hetero-oligomer (Mattei et al., 2011). However, the structural arrangement of the membrane-inserted translocon and the stoichiometry of the complex are not known. By combining site directed fluorescence labeling with both excitation energy migration and fluorescence quenching, we have shown that fluorescently-labeled PopB interacted with native PopD in intact membrane-inserted complexes (Table 2.1). This interaction was confirmed by using the reverse combination of labeled PopD and native PopB. In both experiments, the intercalation of unlabeled molecules among the labeled ones produced the separation of the Bodipy dyes, and consequently an increase in the fluorescence intensity, lifetime, and anisotropy of the sample. These results also implied that when added individually, both PopD^{Bodipy} and PopB^{Bodipy} formed homo-oligomers. Self-interaction of the translocators in intact membrane-inserted complexes was confirmed by the relative increase of the fluorescence intensity, the lifetimes, and the anisotropy of the sample when native PopD (or PopB) was mixed with PopD^{Bodipy} (or PopB^{Bodipy}) and added to the membranes (data not shown).

In summary, we have established a cell-free system to analyze the structural assembly of the *P. aeruginosa* T3S translocators. Using this system, we found that PopB interacts with PopD when forming discrete and stable pores in intact membranes. Cell-free systems have been extremely useful to examine interactions among relevant protein components in membrane protein complexes and the study of their assembly and their structure (Ramachandran et al., 2005; Lovell et al., 2008; Wang et al., 1997; Kyrychenko et al., 2009; Shatursky et al., 1999; Musse and Merrill, 2003; Zakharov et al., 2008). The identified mechanism and structural information obtained using cell-free system are very valuable to design *in vivo* experiments and evaluate the contribution of other known or unknown factors to the mechanism of T3S effector translocation.
2.4 Methods

2.4.1 Buffers

**Buffer A.** 20 mM 2-amino-2-(hydroxymethyl)propane-1,3-diol (Tris-HCl) (pH 8.0) and 100 mM NaCl.

**Buffer B.** 20 mM Tris-HCl (pH 8.0).

**Buffer C.** 50 mM Hepes (pH 8.0) and 100 mM NaCl.

**Buffer D.** 50 mM sodium acetate (pH 4.0) and 5 mM EDTA.

**Buffer E.** 10 mM sodium phosphate (pH 7.5).

2.4.2 Cloning, expression and purification of PcrH derivatives. The DNA sequence coding PcrH was obtained by standard PCR procedures from *P. aeruginosa* PA01 genomic DNA using the following primers: forward 5’- CCA TGA AGG ATC CGA CCC CTT CCG ACA CCG ACC -3’, reverse 5’- GCA GTC CGA AGC TTT CAA GCG TTA TCG GAT TCA TAT GC -3’. The PCR product was cloned into the multiple cloning site 1 (MCS1) of the pETDuet1 vector (Novagen, Madison, WI) using the BamHI and HindIII restriction sites. The first twenty N-terminal amino acids of the cloned PcrH derivative (hereafter called hisPcrH) were MGSSHHHHHHHSQDP4TPSDTD, with the underlined native PcrH sequence starting at Pro number four. The thirteen non-native amino acids included a poly-(His)6 tag used for affinity purification. Single amino acid substitutions to the hisPcrH coding DNA sequence were made using the Quikchange procedure (Stratagene, Santa Clara, CA). The E. coli expression strain BL21(DE3) star (Invitrogen) was transformed with the pETDuet1-hisPcrH plasmid by electroporation. Growth of the E. coli strain was initiated by inoculating 1 L of sterile Luria-Bertani (LB) broth (containing 50 μg/mL ampicillin) with 20 mL inoculum of an overnight culture grown at 37°C in LB broth (containing 100 μg/mL ampicillin). The 1 L culture was incubated at 37°C with constant agitation until turbidity of the culture reached ~0.6-0.8 at 600 nm. The
expression of hisPcrH was induced by the addition of isopropyl α-D-thiogalactopyranoside (IPTG, Gold Biochemicals, St. Louis, MO) to a final concentration of 1 mM. Cells were incubated with constant agitation at this temperature for ~4 hrs and harvested by centrifugation (4,400×g, 15 min, 4°C). Cell pellets were suspended in a total of 150 mL buffer A. The protease inhibitors PMSF and Benzamidine were added to a final concentration of 40 μg/mL and 170 μg/mL, respectively. Cells were lysed using a Microfluidics M-110L microfluidizer (Newton, MA) at 18,000 psi. The cell lysate was cleared by centrifugation (20,000×g, 15 min, 4°C) and the hisPcrH containing supernatant was loaded onto a Chelating Sepharose HP (GE Healthcare, Piscataway, NJ) IMAC column (1.6 cm I.D. × 6 cm) preloaded with Co2+ ions and equilibrated with buffer A at ~20-23°C. The column was washed with 120 mL of buffer A (5 mL/min), followed by a step wash with 50 mL of 5% buffer F (imidazole 0.5 M, pH 8.0) to remove additional contaminating proteins. The bound hisPcrH was eluted with 50 mL of 60% buffer F. The fractions containing the bulk of the protein were pooled, and dialyzed overnight at 4°C against 4 L of buffer B supplemented with 5 mM EDTA and 5 mM 2-hydroxy-1-ethanethiol, and then loaded onto a Q-Sepharose fast flow (GE Healthcare, Piscataway, NJ) AEC column (1.6 cm I.D. × 13 cm L) previously equilibrated with buffer B. The column was washed with 60 mL of buffer B (5 mL/min), and eluted with a 400 mL linear gradient (7 mL/min) from 0.1 to 0.5 M NaCl in buffer B. The pooled fractions containing the hisPcrH (single peak centered around 290 mM NaCl) were concentrated using a Millipore Ultragel-10K centrifugal filter unit and then dialyzed against 4L of buffer A. Protein was made 10% (v/v) in glycerol, fractionated, quick-frozen in liquid nitrogen, and stored at -80°C until use. Protein concentration was estimated using molar absorptivity of 18910 M⁻¹ cm⁻¹ for hisPcrH (Pace et al., 1995).
2.4.3 Cloning of PopD into the pETDuet1-PcrH vector. The DNA sequence coding for PopD was obtained by standard PCR procedures from *P. aeruginosa* PAO1 genomic DNA using the following primers: forward 5’- CGA TAA CAA TGG TAT CGA CACGCA ATA TTC CCT GGC GGC-3’, reverse 5’- GCT TCC TCG AGT CAG ACC ACT CCG GCC GCC GCA CGC C -3’. The PCR product was cloned into the MCS2 of the pETDuet1-hisPcrH vector (Novagen) using the MfeI and XhoI restriction sites and the resulting plasmid named pETDuet1-hisPcrH/NcysPopD. The first ten N-terminal amino acids of the expressed NcysPopD protein were MADLNC12DTQ, with the underlined native PopD sequence starting at Ile number two. Subsequently, the DNA coding for NcysPopD was mutated to code wild type PopD by using the Quick Change procedure (Stratagene). The resulting plasmid was named pETDuet1-hisPcrHPopD.

2.4.4 Expression and Purification of hisPcrH-PopD and derivatives. The expression and purification of the hisPcrH-PopD and derivatives were done as described for the hisPcrH protein with the following modifications. A 1 L culture was incubated at 37°C
with constant agitation until turbidity of the culture at 600 nm reached ~1.2. The culture was equilibrated to 18°C and the expression of hisPcrH-PopD was induced for 10 hrs by the addition of IPTG to a final concentration of 1 mM. Two well-resolved peaks were obtained during the elution of the AEC column. The first peak contained the hisPcrH-PopD complex and eluted when the gradient was ~0.19 M NaCl, and the second peak contained the free hisPcrH (Fig. 2.1A). Pooled fractions containing the hisPcrH-PopD were concentrated, made 10% (v/v) in glycerol, separated into small aliquots, quick-frozen in liquid nitrogen, and stored at -80°C. Protein concentration was estimated using molar absorptivity of 13,980 M⁻¹cm⁻¹ for PopD and assuming a 1:1 hisPcrH-PopD complex (Pace et al., 1995).

2.4.5 Expression and Purification of hisPcrH-PopB derivatives. The expression and purification of the hisPcrH-PopB derivatives were done as described above for hisPcrH with the following modifications. The expression of hisPcrH-PopB was induced by the addition of IPTG to a final concentration of 1 mM when the culture turbidity at 600 nm reached 1.8, and induction continued at 37°C for an additional 6 hrs. Two peaks and a shoulder were observed during the elution of the AEC column. The first peak contained the hisPcrH-PopB complex, the second peak contained hisPcrH-PopB aggregates, and the shoulder after the second peak contained the free hisPcrH (Fig. 2.1 B and C). Pooled fractions containing the hisPcrH-PopB complex were concentrated, made 10% (v/v) in glycerol, separated into small aliquots, quick-frozen in liquid nitrogen, and stored at 80°C. Protein concentration was estimated using molar absorptivity of 6,990 M⁻¹cm⁻¹ for PopB and assuming a 1:1 hisPcrH-PopB complex (Pace et al., 1995).

2.4.6 PopB and PopD derivatives. The mutants PopB^{S164C} (introduced mutations are indicated using a superscript text where the substituted amino acid and the introduced amino acid are indicated at the left and the right side of the number, respectively),
PopD\textsuperscript{F223C}, and the non-lytic, pre-pore former Perfringolysin O derivative (PFO\textsuperscript{E167C-F181A-F318A-C459A} or PFO) were obtained using the Quickchange reverse PCR method (Stratagene) and using the template expression plasmids pETDuet1-hisPcrHPopB, pETDuet1-hisPcrHPopD, and pAH21 (Heuck et al., 2003). PopB\textsuperscript{S164C} and PopD\textsuperscript{F223C} were expressed and purified as described for wild type PopB and PopD. PopB\textsuperscript{S164C-Bodipy} and PopD\textsuperscript{F223C-Bodipy} showed pore forming activities similar to wild type proteins. and pPFO was contributed by Benjamin B. Johnson from our laboratory and was expressed and purified as previously described (Heuck et al., 2003).

2.4.7 Fluorescent protein labeling. PopB\textsuperscript{S164C} and PopD\textsuperscript{F223C} were labeled using N-(4,4-difluoro-5,7-dimethyl-4a-diaza-s-indacene-3-yl)methyl)iodoacetamide (Bodipy FL C\textsubscript{1}-IA or BodipyFL, Invitrogen) as follows. Two mg of PopB\textsuperscript{S164C} or PopD\textsuperscript{F223C} complexed with hisPcrH were first incubated in buffer A supplemented with 5mM DTT for 1 hr, then run through a Sephadex G-25 column (1.5 cm I.D. x 20 cm) pre-equilibrated with buffer C. Given the relatively low water solubility of BodipyFL, dye dissolved in dymethyl sulfoxide was added in four consecutive steps to the protein solution with a 5:1 dye:protein ratio each. The first two additions were followed by 1 hr incubation at 20-23\degree C in the dark with gentle shaking, while the last two additions were followed by a 30 min incubation in same conditions. Then, any precipitated dye and protein were cleared out by centrifugation and excess soluble fluorophore was removed by SEC using Sephadex G-25 resin pre-equilibrated with buffer Tris 20 mM pH 7.5, 100mM NaCl. PopB\textsuperscript{S164C-Bodipy} and PopD\textsuperscript{F223C-Bodipy} were isolated from hisPcrH using urea 6 M as described for wild type translocators. pPFO was labeled with Bodipy as indicated above for the translocators. Labeling efficiencies were calculated as 96\% for PopD\textsuperscript{F223C-Bodipy}, 68\% for PopB\textsuperscript{S164C-Bodipy}, and 100\% for PFO\textsuperscript{Bodipy} by using the molar absorbtivities at 280nm for PopB and PopD (see above), PFO (Moe et al., 2010) and Bodipy (55,000 cm\textsuperscript{-1}).
1 M⁻¹ at 502 nm in 6M urea). The absorbance of Bodipy at 280 nm was ~4% of its absorbance at 502 nm.

2.4.8 Size exclusion chromatography analysis of translocators. Purified proteins were analyzed using an ÄKTA FPLC system (GE Healthcare) equipped with a Superdex 200 10/300GL analytic size exclusion chromatography column (GE Healthcare). Elution took place using 0.5 mL/min flow rate at ~20-23°C using buffer A. For column calibration purposes, protein standards were analyzed using the same experimental conditions. Bovine Thyroglobulin (669 kDa); Horse Spleen Apoferritin (443 kDa); Sweet Potato β-Amylase (200 kDa); Yeast Alcohol Dehydrogenase (150 kDa), Bovine Serum Albumin (66 kDa); Ovalbumin (45 kDa), and Bovine Carbonic Anhydrase (29 kDa). Void volume of the column was determined by using Blue Dextran.

2.4.9 Isolation of PopB and PopD using 6 M urea. 2-4 mg of purified hisPcrH-PopD (or hisPcrH-PopB) were loaded onto spinTrap IMAC columns (GE Healthcare) packed with 300 µl each of Quelating Sepharose Fast Flow (GE Healthcare) resin slurry, previously loaded with Co²⁺ and equilibrated with buffer A. [20 mM 2-Amino-2-hydroxymethyl-propane-1,3-diol (Tris-HCl), pH 8.0, 100 mM NaCl]. Elution of PopB or PopD through the resin took place by spinning dry the columns for 30 sec at 2000xg. 450 µl of buffer B (Tris-HCl 20mM pH 8.0) supplemented with 6 M urea and 20 mM Gly, was added and columns were incubated for 30 min at 4°C on a rocking platform. Dissociated PopD (or PopB) were eluted by spinning the columns for 30 sec at 2000xg. Samples were fractionated, frozen in liquid N₂ and storage at -80°C until use.

2.4.10 Mass Spectrometry. Purified PopB and PopD were analyzed using an Esquire Mass spectrometer (Brucker Daltonics, Billerica, MA) equipped with electro-spray ionization source and ion-trap mass detector. 0.1 mg of protein was dialyzed extensively against pure water at 4°C. Then, protein was diluted to 50% v/v methanol, 3% acetic
acid and sprayed into the ionization source at a 120µl/hr rate. Mass/charge data was collected, averaged, and protein molecular mass was calculated from deconvolution of the average mass spectra.

2.4.11 Liposome Preparation. All non-sterol lipids were obtained from Avanti Polar Lipids (Alabaster, AL). Cholesterol was obtained from Steraloids (Newport, RI). Liposomes were generated using an Avanti® Mini-Extruder (Alabaster, AL) and polycarbonate filters with a 0.1µm pore size (Whatman) as described previously (Shepard, et al. 1998). Briefly, a mixture of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), Cholesterol, and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (POPS) (65:20:15 mole %, respectively) in chloroform was dried at 20-23°C under N₂ and then kept under vacuum for at least 3 hrs. Lipids were hydrated by adding buffer C to a 10-30 mM final concentration of total lipids, and incubated for 30 min at 20-23°C with vortexing at 5 min intervals. The suspended phospholipid/sterol mixture was frozen in liquid N₂ and thawed at 37°C a total of three cycles to reduce the number of multilamellar liposomes and to enhance the trapped volumes of the vesicles. Hydrated lipids were extruded 21 times through a 0.1µm pore size polycarbonate filter. All liposome preparations were analyzed as monodisperse with an average particle diameter of about 100 nm +/- 5 nm by using DLS. The resultant liposomes were stored at 4 °C and used within 2 weeks of production. Liposomes used in FRET experiments were prepared similarly, except that 0.5 mol% of the total lipid was replaced with rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (Rh-PE), triethylammonium salt (Invitrogen). The pore forming activity of translocators was measured using liposomes containing Tb(DPA)₃³⁻. The Tb(DPA)₃³⁻ loaded liposomes were prepared as described previously (Heuck et al., 2003).
2.4.12 Preparation of liposomes containing streptavidin-Bodipy. Streptavidin (GenScript) was dissolved in buffer G (sodium bicarbonate 100 mM pH 8.3) at 1.3 mg/mL final concentration. The amine reactive dye Bodipy Fl-SE (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, succinimidyl ester) (Invitrogen) was dissolved in dimethyl sulfoxide and added to the streptavidin solution up to a final concentration of 150 μM (4:1 dye:protein ratio). Sample was incubated for 1 hr at ~20-23°C while shaking gently. Labeled protein was buffer exchanged and purified from unreacted Bodipy by using a Sephadex G-25 SEC in buffer B. Labeling efficiency was 65% as estimated using the BodipyFl-SE molar absorptivity of 68,000 cm-1M-1 at 504 nm, and the streptavidin molar absorptivity of 41,326 cm-1M-1 at 280 nm. Liposomes encapsulating strepavidinBodipy were made as described previously for other proteins (Heuck et al., 2003) with some modifications. The dried lipid film was hydrated with buffer B containing 6 μM streptavidinBodipy, and the non-encapsulated protein was separated from the liposomes using a Sephacryl S-500 HR column.

2.4.13 β-Amylase biotinylation. β-Amylase (Sigma) was dissolved in 0.5 mL of 100 mM buffer G up to 10 mg/mL final concentration. The amine reactive label biotin-XX SSE (6-((6-((biotinoyl)amino)hexanoyl)amino)hexanoic acid, sulfosuccinimidyl ester) (Invitrogen) was added to the β-Amylase solution to a final concentration of 0.4 mg/mL. Sample was incubated at ~20-23 oC for 1 hr with constant shaking and then biotinylated β-Amylase was separated from unreacted biotin using a Sephacryl S-500 HR column and running buffer C.

2.4.14 Liposome Flotation –Membrane Binding Assay. Binding reactions (75 μl) containing liposomes (2 mM total lipids) and Bodipy-labeled PopB or PopD (400 nM total protein) were established in ultracentrifuge tubes and incubated at 20-23°C for 30 min. Binding reaction buffer was a mixture of sodium acetate 30 mM and 2-(N-
morpholino)ethanesulfonic acid 30 mM regulated at pH 4.0 or 6.0. Liposomes were equilibrated with the buffer prior to protein addition. Liposome-bound and unbound proteins were separated by flotation of proteoliposomes through a sucrose gradient as follows. A 225 µl aliquot of 67% sucrose was thoroughly mixed with each binding reaction and the samples were overlaid with 360 µl of 40% sucrose, followed by 240 µl of 4% sucrose. Samples were centrifuged for 50 min at 90,000 × g at 4 °C (23). Three 300 µl fractions (upper fraction containing proteoliposomes, middle fraction empty, and bottom fraction containing free protein) were collected from the gradient. After trichloroacetic acid precipitation and resuspension in SDS denaturalization buffer, samples were analyzed by SDS-PAGE followed by fluorescence scan using a FLA-500 phosphorimager (Fujifilm Corporation, Japan). Protein bands corresponding to liposome bound protein were quantified by gel densitometry using Genetools 4.01 software (Syngene, UK).

2.4.15 Dynamic light scattering. The average size of the liposomes was determined by dynamic light scattering. DLS measurements were carried out at 25°C using a Zen 3600 instrument (Malvern Instruments, UK), equipped with 633 nm laser diode and avalanche photodiode detector at 90° angle. Autocorrelation data was fit using Dispersion Technologies Software® version 5.1 and using the cumulant method. Hydrodynamic radius were derived from the obtained decay rates (Koppel, 1972).

2.4.16 Cryo-EM. Samples were prepared for cryo-EM by applying 3 µl of the liposome-protein mixture to freshly glow-discharged holey carbon films (C-Flat, Protochips Inc.) and plunge freezing them in liquid ethane using an FEI Vitrobot. Specimens were observed on an FEI Tecnai F20 transmission electron microscope operating at 200kV. Images were acquired under low-dose and zero-loss imaging conditions on a Gatan Ultrascan 1000 attached to the end of a Gatan Tridiem post-column energy filter.
2.4.17 Pore Formation Assay. Liposomes were suspended to a final concentration of 0.15-0.30 mM total lipids in 300 µl of buffer D (sodium acetate 50 mM pH 4.0, 5 mM EDTA). The net initial emission intensity (F₀) was determined after equilibration of the sample at 25°C for 5 min. Aliquots of PopB or PopD were added to the liposome suspension at a 30-60 nM concentration and samples were incubated 15 min at ~23°C. After re-equilibration to 25°C, the final net emission intensity (Fᵢ) of the sample was determined (i.e., after blank subtraction and dilution correction) and the fraction of Tb(DPA)₃³⁻ quenched was estimated using (F₀-Fᵢ)/F₀ (Heuck et al., 2003). For the analysis of the pore forming activity at different pHs, an equimolar mixture of sodium acetate 30 mM and 2-(N-morpholino)ethanesulfonic acid 30 mM was used.

2.4.18 Analysis of formed pores. The presence of discrete size membrane pores formed by PopB, PopD, or an additive equimolar mixture of both was estimated by measuring the ability of biocytin (~1.5 nm size) or biotin-β-amylase (~4 nm size) to diffuse through the pores formed by translocators. Liposomes encapsulating streptavidinBodipy were treated with the translocator/s and diffusion of the biotin markers through the pores was detected as an increase in streptavidinBodipy fluorescence as follows: liposomes loaded with streptavidinBodipy (100 µM total lipids) were suspended in buffer sodium acetate 50 mM pH 4.3, 0.5 mM DTT, containing 1 µM biocytin or 100 nM biotin-β-amylase. The net initial emission intensity (F₀) was determined after equilibration of the sample at 25°C for 5 min. Translocators were added individually or together at a final concentration of 100 nM each and samples were incubated at 20-23°C for 15 min (protein:lipid ratio 1/1000 for individual proteins, and 1/500 when both proteins were added together). After re-equilibration at 25°C the final net emission intensity (F) of the sample was determined (i.e., after blank subtraction and dilution correction) and the enhancement of streptavidinBodipy fluorescence emission was estimated using (F/ F₀).
which is proportional to the amount of biotinylated marker able to diffuse through membrane pores and bind to streptavidin\textsuperscript{Bodipy}. As a control, samples containing biotin-\(\beta\)-amylase were treated with Triton X-100 after recording \(F\) in order to disrupt membranes and corroborate the binding activity between biotin-\(\beta\)-Amylase and Streptavidin\textsuperscript{Bodipy} (not shown).

**2.4.19 Circular dichroism spectroscopy.** Measurements were performed at 25°C on a Jasco J-715 spectropolarimeter (Jasco Corporation, Japan) equipped with a Peltier-effect device for temperature control. Scan speed was set to 20 nm/min with a 1 s response time, 0.5 nm data pitch and 1 nm bandwidth. Far-UV spectra were collected using 0.2 cm cells and protein concentration 2-3 \(\mu\)M in buffer E (sodium phosphate 10 mM, pH 7.5). Six spectra were recorded and averaged for each sample.

**2.4.20 Steady-State Fluorescence Spectroscopy.** Steady-state fluorescence measurements were collected using a Fluorolog-3 photon-counting spectrofluorimeter equipped with a double monochromator in the excitation light path, a single emission monochromator, cooled photo multiplier tube housing, and a 450 W xenon lamp and temperature controlled sample holder (Moe and Heuck, 2010). For pore formation activity assays employing Tb(DPA)\textsubscript{3}\textsuperscript{3+} liposomes, excitation/emission wavelengths were set to 278/544 nm and a 385 nm long pass filter was placed in the emission channel in order to block second-order harmonic light from passing through the emission monochromator.

**2.4.21 Time-Resolved Fluorescence Spectroscopy.** Time-resolved fluorescence measurements were carried out in a Chronos multifrequency cross-correlation phase and modulation fluorometer equipped with a three chamber cuvette holder for background subtraction from ISS (Champaign, IL). Samples were excited with a 470 nm laser diode (HBW 4 nm) filtered through a 472 nm interference filter (% transmittance
HBW 10 nm) to eliminate spurious light. Emitted light was collected through a Melles Griot GG 495 sharp cutoff glass filter to eliminate scattered light and a Melles Griot 03SWP608 dielectric shortpass filter at 550 nm to minimize the contribution of direct excitation of Rh-PE. To avoid any polarization artifacts, measurements were done under magic angle conditions using Glan-Thompson Prism Polarizers (10x10 mm aperture in the excitation, and 14x14 mm aperture in the emission, set at 0° and 55° relative to the lab vertical axis, respectively). Fluorescence lifetimes were calculated by measuring the phase delay and modulation ratio spectra of samples in the 10 to 200 MHz frequency modulation range selecting 25 frequencies (25°C). Blank subtraction was carried out using an equivalent sample without the fluorophore and using a subtraction algorithm (Reinhart et al., 1991) incorporated into the acquisition software. A solution of fluorescein (Invitrogen) in NaOH 0.1 M was used as a reference lifetime with a value of 4.05 ns (Shepard et al., 1998; Crowley et al., 1994) and a total intensity similar to the one of the measured sample (±10 %) (vandeVen et al., 2005). One single exponential lifetime of 4.05 ± 0.1 ns was obtained for this reference sample when measured against Rhodamine B in methanol [lifetime 2.5 ns, (Boens et al., 2007)]. The lifetime data were analyzed assuming different models including monoexponential, multiexponential, or continuous lifetime distribution decay models (Alcala et al., 1987). The goodness of the fit was determined by using the reduced $\chi^2$ values.

**2.4.22 Membrane binding FRET measurements.** The binding of PopB$^{S164C-Bodipy}$ or PopD$^{F223C-Bodipy}$ to membranes under equilibrium conditions was measured by Förster resonance energy transfer (FRET) between a Bodipy-labeled translocator (donor or D), and Rh-PE as acceptor (A), randomly distributed at the lipid bilayer. Four biochemically equivalent samples were prepared in parallel: sample D0 (D only) contained 60 nM total translocator (an equimolar mixture of Bodipy-labeled translocator mutant and wild type
translocator was used to minimize Bodipy self-quenching), and POPC:POPS:cho membranes (65:15:20 molar ratio) lacking Rh-PE; sample DA (D plus A) contained the same protein mixture as in D₀ and membranes containing Rh-PE (0.5% of the total lipids); sample A₀ (A only) contained 60 nM wild type translocator and vesicles containing Rh-PE; and the blank (B) sample contained 60 nM wild type translocator and vesicles lacking Rh-PE. In all four samples, the total lipid concentration of the membranes was 150 µM. All samples were incubated at 25°C for 30 min to permit complete insertion of translocator derivatives to the model membranes before spectral measurements at 25°C. FRET efficiency ($E$) was calculated as describe by Wu & Brand (1994).

$$E = 1 - \frac{\langle \tau_{D} \rangle_a}{\langle \tau_D \rangle_a}$$

Where $\tau_{D_M}$ and $\tau_D$ are the average amplitude weighted lifetimes of the D in the presence and absence of A, respectively. The $\tau_{D_M}$ and $\tau_D$ for DA and D₀ samples were determined as described in the time-resolved fluorescence spectroscopy section using the A₀ and B samples for blank subtraction, respectively. Phase-delay and modulation-ratio data best fit to double discrete lifetime models.
3.1 Introduction

Very little structural information is currently available for the membrane-assembled complexes formed by T3S translocators. PopB and PopD complexes have been investigated by negative-staining EM after their insertion in model membranes (Schoehn et al., 2003). The obtained electron micrographs revealed ring-like structures but the nature and stoichiometry of the proteins forming the rings remains uncertain. Interestingly, the same ring dimensions were observed in cases when either PopD alone or a 1:1 stoichiometric ratio of PopB:PopD was incubated with liposomes, suggesting that ring structures corresponded to homo-oligomeric protein. A similar ring-like complex has been reported for a related T3S translocon from EPEC using atomic force microscopy (Ide et al., 2001). These putative translocon complexes were obtained by incubation of concentrated supernatants EPEC cultures with erythrocytes. However, we should note that no direct evidence of which protein/s was/were forming the rings was provided (Mueller et al., 2008). Clearly, no detailed information is currently available regarding membrane assembled T3SS translocators. This gap needs to be addressed in order to further understand how T3SS effectors reach their eukaryotic cell targets.

P. aeruginosa strains lacking either PopB or PopD display deficient effector translocation into HeLa cells. Interestingly, infecting cells simultaneously with both strains does not restore efficient translocation, even though both PopB and PopD are readily secreted (Goure et al., 2004). Moreover, PopB and PopD are secreted in a partially unfolded state given the narrow diameter of the needle conduit. Therefore, it
seems likely that simultaneous refolding and assembly of PopB and PopD as they emerge from the T3SS needle may be required for building a functional translocation machinery. We have recently reported that purified PopB and PopD can be simultaneously refolded and inserted into model membranes where they interact with each other forming discrete size and stable pores (Chapter 2). Here, we established the experimental conditions to maximize membrane association and hetero-oligomerization of the translocators, and provide specific information about the stoichiometric arrangement of complexes assembled by PopB and PopD in membranes. We found that interaction of the translocators with the membrane is favored at pH below 4.5, and that the presence of PopB facilitates the association of PopD with the membrane at higher pH values. Homo-FRET and fluorescence quenching data indicated PopB and PopD intercalate with each other assembling hetero oligomeric structures in model membranes. Using single-molecule fluorescence imaging of translocators in supported lipid bilayers, we established the stoichiometry of these membrane inserted hetero-complexes. Strikingly, when PopB and PopD formed hetero-oligomers they arranged mostly hexadecameric complexes containing eight PopB and eight PopD molecules. Control experiments carried out with individual translocators indicated that they also assemble oligomeric structures but with a different stoichiometric arrangement. The PopB/PopD hexadecameric complex has an expected molecular mass of 570 kDa. The molecular mass of homologue translocators YopB-YopD, which are functionally conserved and interchangeable with PopB-PopD (Frithz-Lindsten et al., 1998), is expected to be 601 kDa with the same stoichiometry as PopB-PopD. The molecular mass estimated for YopB-YopD hetero-complexes inserted into erythrocytes membranes after incubations with *Y. pseudotuberculosis* suggests hetero-complexes assembled in model membranes resemble the ones inserted *in vivo* (Montagner et al., 2011). This
experimental data provides the first report for the stoichiometry of a membrane inserted T3S translocon complex.

3.2 Results

3.2.1 PopB modified the membrane binding properties of PopD.

The spectroscopic characterization of membrane proteins requires a homogeneous population of membrane inserted/interacting proteins in order to unambiguously interpret the generated signal. Negatively charged lipids and acidic pH have been often used to optimize the interaction of pore forming toxins and other membrane interacting proteins with membranes (Wang et al., 1997; Chenal et al., 2002; Thuduppathy et al., 2006; Musse et al., 2006). Molten globules are intermediates of protein folding. In some cases, acidic pH can induce a molten globular state in proteins due to electrostatic charge changes and this intermediate facilitates membrane insertion (van der Goot et al., 1991). While the exposure of hydrophobic surfaces by molten globular states may favor protein aggregation in solution, the increase of the net positive charge of the protein at low pH will increase the electrostatic repulsion between proteins monomers, and favor the electrostatic interaction with negatively charged membranes. It is therefore not surprising that the association of purified T3S translocators to model membranes requires acidic pH and is facilitated by the presence of anionic lipids (Chapter 2, Fig. 2.7; Faudry et al., 2006). We showed that maximal pore formation for urea-purified PopD occurred at pH lower than 4.5, while maximal pore formation for urea purified PopB occurred at slightly higher pH (Chapter 2, Fig 2.8A). Surprisingly, the addition of an equimolar amount of both proteins showed a pore forming activity profile similar to the profile observed for PopB alone (Chapter 2, Fig 2.8A).
A variety of spectroscopic techniques including electron paramagnetic resonance (Fanucci and Cafiso, 2006; McHaourab et al., 2011), Fourier transform infrared spectroscopy (Tatulian, 2003), and fluorescence spectroscopy (Heuck and Johnson, 2002) have been successfully used to gain insights into the structural arrangement of membrane proteins and how they interact with lipid membranes. Interpretation of the data recorded using these spectroscopic techniques requires a precise biochemical characterization of the system (Johnson, 2005). We therefore quantified the association of PopB and PopD with model membranes and determined experimental conditions that maximized protein-membrane association and PopB-PopD interaction. Association of PopB and PopD to lipid membranes was determined using a liposome flotation assay followed by SDS-PAGE analysis and quantification of the proteins by gel densitometry (Fig. 3.1A). In order to detect any cooperative interaction between PopB and PopD while binding to membranes, protein membrane association was firstly quantified for individual translocators, and secondly for a 1:1 combination of both translocators. Any difference in the binding properties when incubated alone or in combination with membranes will indicate potential interaction between PopB and PopD. A membrane binding curve spanning the 4.0-7.0 pH range was obtained for these experiments (Fig. 3.1B, C).

PopB membrane association was maximal at pH 4.75, with 50% association at pH 5.75 (Fig. 3.1A). Membrane association for individual PopD was maximal at pH 4.0 with 50% of total association taking place at pH 5.5 (Fig. 3.1B). PopB membrane association was not affected by pre-incubation with PopD (Fig. 3.1C). Therefore, maximum membrane association conditions (pH 4.0 for PopD alone, pH 4.5 for PopB alone or PopB:PopD) were used in our following experiments. Surprisingly, when PopD and PopB were mixed in equimolar amounts previous to incubation with membranes, PopD membrane association was significantly enhanced in the 5.0-6.0 pH range (Fig. 3.1C).
3.1B). Interestingly, this effect also occurred within the same pH range where PopB was reported to enhance the pore forming activity of PopD, or synergistic pore formation occurred (Faudry et al., 2006). This suggests that the previously reported synergistic effect may be limited to a narrow range of pH in test-tube experiments.
Figure 3.1. PopB enhances PopD membrane binding.

Translocator membrane binding was assayed using the liposome floatation assay described in Methods section 2.4.14. PopDF223C-BpyFL, PopBS164C-BpyFL, or an equimolar mixture of both proteins in 6 M urea buffer solution was diluted and incubated with liposomes at the indicated pH values. Protein:lipid ratio was 1:5000, with lipid composition POPC:cholesterol:POPS at molar ratio 65:20:15. PopD membrane binding was enhanced in the 5.0-6.0 pH range when PopB was present in equimolar amounts. 

A. Typical SDS-PAGE analysis of membrane bound protein fractions recovered from the liposome floatation assay. B. Protein bands in SDS-PAGE gels were quantified by gel densitometry. The amount of membrane bound protein was normalized using the total amount of protein added to the liposomes (left lane), and the membrane bound fraction was plotted as a function of pH. Membrane bound fractions for PopD incubated alone with liposomes (red), and PopD incubated with an equimolar amount of PopB (blue) are indicated. Each data point represents the average of at least two independent assays, and error bars indicate the data range. C. Same experiments as B, but for PopB alone (black) and PopB plus an equimolar amount of PopD (green).
3.2.2 PopD and PopB formed multimers in lipid membranes.

It is difficult to assess the interaction of proteins in membranes or on cell surfaces. FRET is a convenient approach to determine molecular proximity between donor-labeled and acceptor-labeled proteins in membrane. FRET efficient depends on \( R_0 \), the Förster critical distance, and the inverse sixth power of the separation between dyes. With a typical sensitivity in the 20-80 Å distance range, FRET is a particularly sensitive technique to assess protein oligomerization in membranes using fluorescently tagged proteins. Homo-FRET, particularly energy migration FRET (Bergstrom et al., 2002; Mikhalyov et al., 2002), is a simpler variant of energy transfer because it occurs between like chromophores and hence requires only one type of fluorescent label. Homo-FRET is manifested by the presence of depolarized fluorescence emission, hence homo-FRET is readily measured by changes in the sample steady-state or time-resolved fluorescence anisotropy (Hamman et al., 1996). FRET and homo-FRET measurements as a function of fractional fluorescent labeling can be used to estimate the size of oligomeric membrane protein arrangements (Runnels and Scarlata, 1995; Yeow and Clayton, 2007). In terms of experimental implementation, a simple approach is to measure the steady-state fluorescence anisotropy of the sample. Under certain conditions, the steady-state fluorescence anisotropy is inversely proportional to the number of subunits in the protein oligomer (Runnels and Scarlata, 1995). However, the fluorescence anisotropy can also be influenced by changes in rotational diffusion of the labeled protein. Conveniently, these sources of ambiguity can be overcome by analyzing the sample steady-state fluorescence anisotropy as a function of fractional labeling (i.e. diluting labeled protein with unlabeled one). Under these conditions, changes in fluorescence anisotropy are due to differential separation between the fluorescent probes and reflect on the size of the protein oligomer.
The size and possibly the stoichiometry of complexes assembled by PopD and PopB can be investigated by analyzing the homo-FRET and fluorescence self-quenching between BodipyFL probes located either in PopD or PopB as a function of fractional labeling. Figure 3.2 indicates the steady-state anisotropy data obtained for PopD$^{\text{BpyFL}}$ assembled in liposomal membrane and as a function of fractional PopD labeling. Both the steady-state anisotropy and the fluorescence intensity ($F/F_o$) were recovered as the fractional labeling of PopD decreased, indicating an oligomeric arrangement of PopD in membranes (Fig. 3.2A and B). When the same experiment was carried out but using a mixture of a constant amount of PopD$^{\text{BpyFL}}$ and increasing amounts of PopB so as to obtain the same fractional labeling decrease in PopD, the same final recovery of the fluorescent intensity was observed, indicating PopB associated with PopD as efficiently as PopD with itself (Fig. 3.2B). In contrast, a much lower increase of the fluorescence anisotropy was observed (Fig. 3.3A). This observation indicated that even at high ratios of PopD$^{\text{BpyFL}}$ dilution with PopB, some of the longer range homo-FRET interaction remained between the dyes. Therefore, a specific stoichiometry of PopB/PopD hetero-complexes must be present under these conditions. Given that PopD$^{\text{BpyFL}}$ interacts efficiently with PopB at the dilution ratio assayed (Fig 3.2A), the low increase in anisotropy would only be observed if homo-FRET between dyes PopD is still significant, indicating the presence of at least two or more PopD monomers per PopB/PopD hetero-complex (Fig. 3.2B). The oligomerization state of individual PopD molecules in membranes was estimated based on the anisotropy vs fractional labeling curves obtained and fitting the data to existing models (Runnels and Scarlata, 1995). The best fit of PopD homo-FRET data suggested a composition of 5 to 6 monomers per complex, but the fit was poor probably due to PopD assembling complexes with a size distribution rather than a simple discrete size population of complexes (not shown). From these
results, it was clear that PopD can assemble homo-oligomeric structures in membranes, but in the presence of PopB it forms hetero-oligomeric structures of yet undetermined size and stoichiometry.
Figure 3.2. PopD assembled homo or hetero oligomeric structures in membranes.

PopD\textsuperscript{F223C-BpyFL} fluorescence emission intensity and steady-state anisotropy were analyzed as a function of PopD fractional labeling as described in Methods section 3.4.7. Proteins were reconstituted in membranes following the optimized procedure described in chapter 2. PopD\textsuperscript{F223C-BpyFL} was premixed with PopD or PopB in 6 M urea buffer prior to dilution and incubation with membranes. The solution pH was 4.0 and the protein:lipid ratio was kept constant at 1:1000. A. Steady-state fluorescence intensity of PopD\textsuperscript{F223C-BpyFL} at the indicated percent PopD labeling in the case of pre-dilution with PopD (blue) or PopB (red). (F/F\textsubscript{0}) indicates to the ratio between F, the fluorescence intensity recorded at the indicated percent labeling, and F\textsubscript{0}, the fluorescence intensity at 100% labeling. B. Steady-state anisotropy of PopD\textsuperscript{F223C-BpyFL} at the indicated percent PopD labeling in the case of pre-dilution with PopD (blue) or PopB (red).
3.2.3 PopB and PopD assembled hexadecameric hetero complexes in membranes.

Steady-state fluorescence data clearly indicated PopB and PopD formed hetero-oligomeric structures when incubated together with membranes (Fig. 3.2). To effectively determine the stoichiometry of membrane inserted hetero-complexes, we applied a technique that allows quantifying the number of protein molecules within a protein complex. Single-molecule fluorescence was used to quantify the oligomerization state of membrane-assembled translocators. In practice, fluorescence detection with single-molecule efficiency allows visualization of single photobleaching events as a step-like decrease in the fluorescence signal. This high sensitivity, coupled to the space resolution of optical microscopy and imaging of single protein complexes allowed us to quantify the number of photobleaching events occurring in a single protein complex. The number of photobleaching events is equivalent to the number of fluorophores attached to a protein complex, or in the case of singly-labeled polypeptides, the number of polypeptides forming part of the protein complex. We adapted the single-molecule photobleaching technique to allow the quantification of translocators inserted in a native lipid bilayer environment by using supported lipid bilayers (SLB). PopDF^{223C} and PopB^{S164C} were labeled at ~100% efficiency with the red emitting fluorescent probe BodipyTMR (PopD_{TMR}^B) and the green emitting probe BodipyFL (PopB_{FL}^B).

Labeled translocators were reconstituted into liposomes using our previously optimized conditions for membrane association and formation of hetero-complexes (Chapter 2). The resulting proteoliposomes were diluted with excess liposomes in order to later achieve the optimal low protein density required for single-molecule imaging. This sample was used to assemble supported lipid bilayers on glass coverslips by the vesicle fusion method (Tamm and McConnell, 1985). Bilayers were imaged using dual color single-molecule TIRF microscopy as described in the methods section. Assembly
of a continuous and fluid lipid bilayer was confirmed in a parallel experiment by including a fluorescently labeled phospholipid in the membranes, NBD-DHPE, and observing fluorescence recovery after photobleaching (FRAP) of a membrane patch. FRAP under our experimental conditions occurred in a timescale consistent with phospholipid diffusion in a fluid lipid bilayer (calculated $2.4 \times 10^{-12}$ m$^2$/s lateral diffusion coefficient for NBD-DHPE, not shown). This approach allowed us to visualize singly fluorescently labeled PopD or PopB present in hetero-complexes inserted in membranes (Fig. 3.3 A and C). Importantly, this technique allows the analysis of hetero-complexes regardless of the presence of co-existing homo-oligomeric forms of PopB and PopD, as only hetero-complexes display co-localization of green (PopB) and red (PopD) fluorescence (Fig. 3.3).

Membrane protein complexes remained immobile during the time scale of visualization before complete photobleaching of the attached probes (i.e. no significant drifting of the fluorescence complex was observed). The same characteristics have been observed for integral membrane proteins inserted in lipid bilayers, presumably due to interaction or absorption of the protein to the glass surface through protein segments facing the glass side of the membrane (Wagner and Tamm, 2000). Photobleaching was performed by constant illumination from a 532nm laser line in order to photobleach BodipyTMR, followed by constant illumination with a 488nm laser line in order to photobleach BodipyFL. This consecutive photobleaching of BodipyTMR followed by BodipyFL eliminated any problems associated with possible FRET between the two dyes and possible direct excitation of BodipyTMR at 488nm wavelength. Time point image stacks were independently acquired for PopD$^{\text{TMR}}$ and PopB$^{\text{FL}}$ and florescence vs time curves of individual protein complexes were derived from these as detailed in Methods section (Fig 3.3A).
A quantitative analysis of photobleaching steps from individual protein complexes revealed the stoichiometry of hetero-complexes assembled by PopD and PopB. Remarkably, histograms of photobleaching counts indicated PopB:PopD complexes contained mostly eight photobleaching counts for PopD\textsuperscript{BpyTMR}, as well as eight photobleaching counts for PopB\textsuperscript{BpyFL} (Fig. 3.2B), consistent with an hexadecameric arrangement of translocators containing eight PopD molecules and eight PopB molecules with an expected molecular weight of 570 kDa. Interestingly, assuming a similar arrangement for homologs YopD and YopB yields an expected molecular weight of 601 kDa. This is very similar to the recently determined molecular weight of YopD-YopB hetero-complexes inserted in erythrocyte membranes after \textit{Yersinia pseudotuberculosis} infection (600 ± 100 kDa) (Montagner et al., 2011), although the stoichiometric arrangement of YopB and YopD in this case was not determined.
Figure 3.3. PopB and PopD assemble hexadecameric hetero complexes in membranes

PopD$^{\text{BpyTMR}}$ and PopB$^{\text{BpyFL}}$ at a 1:1 stoichiometric ratio were reconstituted in liposomes using the method described in Chapter 2. Protein lipid ratio was 1:5000 and buffer pH was 4.5. Resulting proteoliposomes were diluted with liposomes in order to achieve a 30 fold lipid dilution and then further diluted with buffer at 0.2 mM final lipid concentration and sub-nanomolar protein concentration. SLBs were assembled as described in Methods section 3.4.4. SLBs containing the hetero-complexes were imaged using dual color single-molecule TIRF microscopy, and photobleaching data was analyzed as described in Methods section 3.4.6. A. Merge image of single molecule PopD$^{\text{BpyTMR}}$ (red) and PopB$^{\text{BpyFL}}$ (green) fluorescence. Hetero-complexes appear yellow due to colocalization of the fluorescence emissions. B. Typical fluorescence intensity time traces of single protein complexes. Photobleaching events appear as step-wise decrease in the intensity. C. Histogram of dual-color photobleaching counts obtained from time-traces of PopD$^{\text{BpyTMR}}$ and PopB$^{\text{BpyFL}}$ present within the same hetero-complex. The observed distribution is centered at eight photobleaching counts for PopD and eight photobleaching counts for PopB.
3.2.4 PopD and PopB adopt mostly hexameric arrangements when assembled individually in membranes.

Does oligomerization of individual translocators precede the assembly of hetero complexes? If so, interaction with its partner translocator should not affect the oligomerization state of either PopD or PopB assembled individually in membranes. Strikingly, independent single-molecule photobleaching experiments using PopD or PopB reconstituted individually in membranes indicated PopD arranged mostly into hexameric structures (Fig. 3.3C), while PopB showed a broader size distribution dominated by hexameric and dodecameric forms (Fig. 3.4F). These structures, while still consistent in size with the formation of membrane pores, differed significantly with the arrangement observed when PopB and PopD were allowed to hetero-oligomerize in membranes. PopB and PopD must emerge in a partially unfolded state from the same bacterium in order to observe T3SS dependent hemolytic activity by *P. aeruginosa* (Goure et al., 2004). Therefore, the simultaneous reconstitution of PopB and PopD in membranes from 6 M urea solution seems to better represent this scenario, resulting in a stoichiometric arrangement different from the one observed when translocators are reconstituted individually in membranes (Fig 3.3).

Steady-state fluorescence intensity data indicated PopD intercalated with PopB efficiently at high dilution ratios (i.e. 1:10 ratio PopD\(^{BpyFL}\) : PopB) (Fig 3.2A). Therefore, it is expected that under these conditions PopD\(^{BpyFL}\) will form mostly PopD/PopB hetero-complexes, and a minimal amount of homo-complexes. Consequently, a single-molecule photobleaching experiment performed in the same conditions should yield a size distribution centered at eight monomers, rather than the size distribution centered at 6 monomers obtained when PopD is assembled individually in membranes. This hypothesis was tested by measuring the distribution in photobleaching counts for PopD\(^{BpyFL}\) samples reconstituted in membranes in the presence of a 10 fold excess of
non-fluorescent PopB. Remarkably, the photobleaching counts distribution obtained was centered at eight monomers, indicating that under the specified conditions PopD is indeed mostly part of hetero-complexes and that these contain mostly eight PopD molecules (Fig 3.5). When the same experiment was carried out but using Pop$\text{B}_{\text{BpyFL}}$ and a 10 fold excess of non-fluorescent PopD, the size distribution also centered at 8 PopB molecules per complex, although a small percent of PopB remained as higher order oligomeric species centered at 12 and 16 subunits in size (not shown). This finding not only corroborated the stoichiometric arrangement found by using a 1:1 stoichiometric rations of PopB:PopD and dual color single-molecule photobleaching (Fig. 3.3), but also indicated an optimal translocator ratio that can be used to study labeled translocators in hetero-complexes through bulk steady-state and time-resolved spectroscopic approaches.
Figure 3.4. PopD and PopB adopt mostly hexameric arrangements when assembled individually in membranes.

PopD$_{\text{BpyFL}}$ and PopB$_{\text{BpyFL}}$ were individually reconstituted in liposomes using the method described in Chapter 2. Protein lipid ratio was 1:5000 and buffer pH 4.0. Resulting proteoliposomes were diluted 30 fold with liposomes and used to assemble SLBs as described in Methods section (final protein concentration was in the sub-nanomolar range). SLBs containing the PopD or PopB membrane complexes were imaged using TIRF microscopy, and photobleaching data was analyzed as described in Methods section. A. Single-molecule TIRF microscopy image of PopD$_{\text{BpyFL}}$ complexes in SLB. B. Intensity time-trace obtained from a single membrane-assembled PopD$_{\text{BpyFL}}$ complex. C. Histogram of photobleaching counts obtained from time-traces of single PopD$_{\text{BpyFL}}$ complexes (a total of 100 protein complexes were analyzed). D, E and F. Same as A, B, and C but for PopB$_{\text{BodipyFL}}$ individually assembled in membranes (a total of 155 proteins complexes were analyzed).
Figure 3.5. PopD size distribution shifts to 8 subunits per complex in the presence of 10 fold excess PopB.

PopD\textsuperscript{Bpy\textsuperscript{Ft}} was pre-mixed with a 10 fold molar excess of PopB and then reconstituted in liposomes using the optimized method described in Chapter 2. Protein lipid ratio was 1:5000 and buffer pH was 4.5. The experimental conditions and imaging procedures were the same as Fig. 3.4 (a total of 100 single protein complexes were analyzed). The histogram of photobleaching counts (red) shifted to 8 PopD molecules per protein complex. The data obtained in Fig. 3.4C (blue) for PopD alone is reproduced for comparison. Lines indicate Gaussian fits to the histogram data.
3.3 Discussion

Our analysis of T3S translocator assembly in membranes provided detailed information regarding the final state of membrane inserted translocators. Acidic pH was required for the efficient association of translocators to membranes and PopB enhanced the membrane binding of PopD at pH values closer to neutral. Steady-state fluorescence measurements, and dual color single-molecule photobleaching experiments indicated that co-assembly of PopD and PopB in membranes, using the optimized method described in Chapter 2, results in hetero oligomeric PopD/PopB complexes composed mostly of eight PopD subunits and eight PopB subunits (Fig 3.2; Fig 3.3). The size of this complex is very similar to the recently determined size of hetero complexes assembled by Y. pseudotuberculosis YopB and YopD in erythrocyte membranes after bacterial infection (Montagner et al., 2011). This oligomerization process taking place at the membrane surface after membrane binding resulted mostly in hexameric PopD structures (Fig. 3.4C). Complexes assembled by PopB alone or PopD alone had sizes consistent with the formation of membrane pores, but they differed significantly from hetero-complexes assembled by both translocators together. Individual translocators adopted mostly hexadecameric arrangements in membranes (Fig 3.4).

The following information can be compiled into a model for the T3SS translocon: i) Although PcrH has a globular shape (Job et al., 2010), hisPcrH-PopD and hisPcrH-PopB seem to have elongated shapes, as purified complexes have hydrodynamic radius about double as expected for globular proteins with same molecular weight (Chapter 2, Fig 2.1E). The same is observed for homologue translocators (Birket et al., 2007; Tan et al., 2009). Also, the crystal structure of a protease resistant fragment of IpaB was recently solved which revealed an elongated coiled coil structure very similar to Colicin Ia (Barta et al., 2012); ii) PopB and PopD assemble a hetero-complexes in membranes
(Chapter 2). PopB and PopD intercalate with each other at these hetero-oligomeric complexes (Fig 3.2A and B); iii) Translocators assemble hexadecameric complexes with eight PopB molecules and eight PopD molecules (Fig. 3.3). These considerations are compiled in the following drawn to scale model of a T3SS translocon. Although different arrangements are conceivable considering the abovementioned observations, it is possible to arrange translocators in a hexadecameric complex consistent in size with the needle tip assembled by PcrV (Broz et al., 2007). Moreover, other T3SS components are likely to assemble similar size structures. For example, the S. typhimurium protein InvG, which assembles a ring in the outer bacterial membrane and around the inner rod of the T3SS injectisome, is a 66 kDa protein that oligomerizes into pentadecameric rings with an expected molecular weight of 930 kDa (Schraußt and Marlovitz, 2011).
Figure 3.6. Model of the T3SS translocon assembled by PopB and PopD

PopB and PopD assemble hexadecameric structures at the membrane (8 PopB: 8 PopD). This one of many possible models (drawn to scale) suggests a size compatible with the T3SS needle tip assembled by PcrV (top EM image). PopB (red) and PopD (blue) were approximated as cylinders with constant volume relative to their molecular weight, and arranged intercalated based on steady-state fluorescence data (Fig. 3.2A) and into an hexadecameric ring, based on single-molecule photobleaching data (Fig 3.3). [EM image adapted from Mueller et al.(2005). Reproduced with permission from the American Association for the Advancement of Science, Copyright (2005)].
3.4 Methods

3.4.1 Fluorescent protein labeling.

PopDF223C was labeled with 6-((4,4-difluoro-1,3-dimethyl-5-(4-Methoxyphenyl)-4-bora-3a,4a-diaza-s-indacene-2 propionyl)amino)C5-maleimide (BODIPY® TMR C5-Maleimide, Invitrogen) or BodipyTMR (PopDBpyTMR) as described in Chapter 2 Methods section 2.4.7 for BodipyFL. PopBS164C was labeled to ~100% efficiency using BodipyFL (PopBBpyFL) as described in Chapter 2 Methods section 2.4.7 with the following modification. Monomeric hisPcrH-PopB$^{S164C}$ was isolated by AEC as described in Chapter 2 Methods section 2.4.5, and the labeling reaction was initiated immediately after in order to avoid aggregation of hisPcrH-PopB$^{S164C}$.

3.4.2 Liposome Preparation.

Liposomes were prepared as described in the Chapter 2, Methods section 2.4.11, and using the optimal lipid composition determined in Chapter 2 (65% POPS, 20% Cholesterol, 15% POPS).

3.4.3 Preparation of glass coverslips for supported lipid bilayers.

Glass coverslips were placed in ceramic holders and boiled for 20 min in a 20% vol solution of 7X™ detergent (MP Biochemicals). Coverslips were allowed to cool and then rinsed extensively with tap water, followed by extensive rinse with pure water. Coverslips were then submerged in pure water and boiled for 10 min. Dried coverslips were then heated for 4 hours at 400°C in a kiln oven, allowed to cool and stored until use.

3.4.4 Supported lipid bilayers.

Supported lipid bilayers were assembled as follows: 500 µl of liposome or proteoliposomes solution in the appropriate buffer (50 mM sodium acetate buffer pH 4.0 or pH 4.5) with 0.2 mM final lipid concentration was supplemented with 5 mM CaCl$_2$ and placed on a Petri dish between two stripes of 1 mm thick aluminum foil. A clean glass
coverslip was suspended on top of the sample and aluminum foil stripes and incubated for 30min to allow SLB formation. After this, a glass slide containing two spaced stripes of double sided adhesive tape in its upper face was placed next to the coverslip and the Petri dish was filled with buffer (50mM Sodium Acetate buffer pH 4.0 or pH 4.5). While submerged in buffer, the coverslip was mounted on the glass slide spaced by adhering it to the double sided adhesive tape, assembling a liquid chamber between the glass slide and the glass coverslip that contained the SLB.

3.4.5 Single-molecule microscopy.

Single molecule images were acquired using an electron multiplier CCD (EM-CCD) Cascade II camera (Photometrics,Tucson, AZ). Total internal reflection fluorescence (TIRF) microscopy used a home built laser system around a Nikon Eclipse Ti microscope and a high numerical aperture objective (60X, NA ¼ 1.49) (Nikon, Melville, NY). TIRF excitation was achieved using a 488-nm Argon-ion air laser for PopD<sub>BPyFL</sub> and PopB<sub>BPyFL</sub>, and a 537nm laser for PopD<sub>BPyTMR</sub>. Neutral density filters were employed to attenuate laser light to the desired level and increase the average photobleaching time. The standard exposure and acquisition interval time was 70 ms for BodipyFL and 100 ms for BodipyTMR probes.

3.4.6 Single-molecule photobleaching and data analysis.

Photobleaching of PopD<sub>F223C-BpyFL</sub> and PopB<sub>S164C-BpyFL</sub> complexes was performed in the same chambers described previously for SLB imaging, except that the SLB contained membrane inserted PopD<sub>F223C-BpyFL</sub> or PopB<sub>S164C-BpyFL</sub> complexes. Movies were taken at constant laser excitation. Photobleaching data was inspected and analyzed manually. Time-resolved fluorescence intensity plots of single protein complexes were generated by analyzing movies as image stacks using ImageJ (http://rsb.info.nih.gov/ij/) and exporting the measured fluorescence intensities as a function of time into Origin.
(OriginLab, Northampton, MA). The time traces were inspected manually, and the number of fluorescent molecules in each complex was obtained by dividing the initial background subtracted fluorescence intensity at time zero by the average intensity contribution of a single dye revealed by the single-photobleaching events (Ross and Dixit, 2010; Hendricks et al., 2010; Diaz-Valencia et al., 2011).

3.4.7 Steady-State Fluorescence Spectroscopy.

Steady-state fluorescence measurements were collected as described in Chapter 2 Methods section 2.4.20. Multiple measurements were carried out at varying BodipyFL fractional labeling. In order to achieve decreasing fractional labeling of PopD^{BpyFL}, the fluorescently labeled PopD was diluted by pre-mixing increasing amounts of either unlabeled PopD or unlabeled PopB in 6 M urea buffer. These were used to reconstitute translocators in liposomes using the optimized method described in Chapter 2.
CHAPTER 4
CONCLUSIONS

4.1 Summary

A cell-free system for the structural and functional characterization of T3SS translocators is required to study with molecular detail how T3S effectors translocate across target membranes. The hydrophobic translocators PopB and PopD from *Pseudomonas aeruginosa* were produced in *E. Coli* and protocols for efficient purification of active translocators in high amounts were devised. PopB was for the first time obtained in a monomeric native state in complex with the chaperone PcrH. This allowed spectroscopic characterization of PopB, and most importantly, fluorescent labeling of a PopB derivative with 100% efficiency.

A cell-free system for reconstitution of PopB and PopD in model membranes was established based on solubilization of PopB and PopD in 6 M urea buffer followed by dilution in the presence of liposomes. Experimental conditions such as pH and lipid composition for PopB and PopD membrane insertion and pore were optimized. Pore formation was highly efficient at pH values lower than 5.0, suggesting protonation of acidic residues was required. Acidic pH was shown to promote efficient membrane binding by translocators. The membrane pores assembled by PopB and PopD using this system are discrete in size, stable over time, and heteromeric (i.e. composed by both PopB and PopD).

A method for single-molecule fluorescence imaging of T3S translocators inserted in model membranes was devised. This methodology allowed the quantification of stoichiometries for PopB and PopD homo and hetero-complexes inserted in model membranes. PopB and PopD assembled mostly hexadecameric structures containing...
eight PopB molecules and eight PopD molecules, with an expected molecular weight of 570 kDa. This value is similar to the recently reported molecular weight of *Yersinia* homologue translocators YopB and YopD hetero-complexes inserted in erythrocytes membranes after *Yersinia* infection (Montagner, et al.2011). A model can be build considering the experimental data that has a size consistent with interaction with the T3SS needle tip (Fig. 3.6).

Fluorescence techniques (Heuck and Johnson, 2002) (Johnson 2005) were used to analyze the membrane interaction of PopD’s hydrophobic segment, and PopD N-terminal amphipathic segment. Mutation of acidic residues in the amphipathic segment allowed PopD to form pores at pH values closer to neutral, suggesting a role of acidic residues in membrane binding and/or membrane insertion. PopD hydrophobic segment and PopD n-terminal amphipathic segment interacted with the bilayer non polar core, but remained in the water/membrane interface.
4.2. Future Research.

4.2.1 Single-molecule studies of T3SS components.

Our studies on the stoichiometry of membrane-assembled PopB and PopD using single-molecule techniques has provided the first report on the stoichiometric arrangement of a reconstituted T3SS translocon. A next step will be to push these techniques to corroborate the stoichiometric information of T3SS components in vivo.

Current models of *P. aeruginosa* and *Yersinia* spp T3SS, members of the Ysc T3SS family (Fig. 1.2), are mostly based on studies carried out in organisms of related T3SS families, mostly the Inv-Mxi-Spi family which includes the T3SS of *Shigella* spp. and *S. enterica* spp. In particular, the *P. aeruginosa* T3SS has been understudied. One important property of bacterial T3SS is the low density they present in the cell surface. This low density allows imaging of GFP labeled components of T3SS with single particle resolution in living bacteria or during infection of cultured human cells (Fig. 4.1). Coupling this approach to the single-molecule techniques described in Chapter 3 could corroborate *in vivo* the PopB/PopD translocon arrangement provided in Chapter 3, and could also provide *in vivo* stoichiometric information of other T3SS components, improving on current models of the Ysc family T3SS. A similar approach has been successfully utilized *in vivo* to study the variable oligomerization state of single Tat transporters in the cytoplasmic membrane of *E. coli* cells (Leake et al., 2008). Moreover, although no stoichiometric information is provided, the research group of Guy Cornelis has reported that T3SS components of *Yersinia* spp. fused to fluorescent proteins are functional and allow T3SS effector secretion, while they co-localize at the bacterial surface displaying bright distinguishable foci (Diepold et al., 2010; Diepold et al., 2011) (Fig. 4.1). This study indicated fusion of T3SS components to GFP did not impair their secretion through the Sec secretory machinery prior to injectisome assembly, and is
therefore not likely to affect the secretion of T3SS components such as PopB/PopD, also coupled to ATP hydrolysis. One possible caveat of using GFP for *in vivo* estimation of protein stoichiometry is the fact that GFP maturation is relatively slow (eGFP mutation half-time is 65 min; Evdokimov et al., 2006) and therefore full maturation of GFP is required before conducting photobleaching experiments because every protein molecule must emit a fluorescence signal for accurate estimation. Fortunately, T3SS components are pre-synthesized and stored in the bacterial cytosol prior to T3SS induction and secretion. Thus, GFP fused T3SS components such as PopB/PopD can mature in the bacterial cytosol prior to eukaryotic cell infection. In addition, GFP homologues with fast maturation kinetics are available, such as the *Arthropoda* GFP homologue TurboGFP, which matures with a half-time of 23 min. (Evdokimov et al., 2006). Our laboratory also counts with *P. aeruginosa* strains and an optimized cellular infection assay that can be used to test the functionality of strains carrying GFP-fused T3SS components.
Figure 4.1. *Yersinia enterocolitica* strains with T3SS components fused to fluorescent proteins display bright foci at the bacterial surface. [Adapted from Diepold et al, 2010. Reproduced with permission from Nature Publishing Group, Copyright (2010)].

Various T3SS components (YscC, YscD, YscN, and YscQ) when fused to fluorescent proteins display fluorescent foci in the bacterial surface that co-localize with other T3SS components. The strains carrying these fluorescent T3SS proteins display functional T3SS and can secrete effectors. A similar study carried out with *P. aeruginosa* strains, coupled to single-molecule photobleaching as described in Chapter 3, can potentially yield a model of the *P. aeruginosa* T3SS with stoichiometric detail. **A.** Fluorescence and contrast phase images of *Y. enterocolitica* strains carrying a T3SS with the indicated component fused to a fluorescent protein. Discrete particles are visible in the bacterial surface. **B.** Model of *Yersinia spp.* T3SS indicating the location of different T3SS components (Diepold et al., 2010).
4.2.2 Does the PopB/PopD hetero-complex assemble a membrane pore or transmembrane channel?

It is assumed that the hetero-complex assembled by PopB/PopD or homologue T3SS translocators contains a membrane pore or transmembrane channel. Although substantial evidence indicates PopB and PopD can individually assemble a membrane pore (Chapter 2; Schoehn et al., 2003; Faudry et al., 2006), all the translocator ratios tested to date contain an heterogeneous mixture of homo and hetero-oligomeric forms of PopD and PopB (Chapter 3). Therefore, it is still not clear whether the hetero-complex assembled by T3SS translocators indeed transverses the membrane with an aqueous pore structure (the pores observed could be assembled by homo-oligomeric forms of the proteins). This question needs to be addressed in order to unambiguously model how translocation of T3SS effectors takes place at the eukaryotic cell membrane.

The PopB/PopD hetero-complex will need to be purified after it is assembled in membranes using the method described in Chapter 2. Fluorescence self-quenching data with BodipyFL labeled translocators suggest the membrane assembled oligomeric forms of PopB and PopD do not change significantly upon solubilization with mild detergents such as Triton X-100 or NP-40 (Kyle Rossi, unpublished results). Therefore, including an affinity tag in PopD such as a His-tag should facilitate the purification of the PopD/PopB hetero-complex from homo-complexes after their assembly in model membranes. PopD can be assembled into liposomes using a 1:10 ratio of PopD:PopB. This results in a sample where PopD is mostly inserted into hetero-complexes (Fig. 3.5). Therefore, solubilization of such sample with a mild detergent such as Triton X-100 or Dodecyl-Maltoside (DDM), followed by affinity purification, will yield pure PopD-PopB hetero-complexes. The stoichiometry of solubilized hetero-complexes will be analyzed by absorbing BodipyFL labeled hetero-complexes to glass coverslips and performing
the same single-molecule photobleaching analysis described in Chapter 3. Translocator hetero-complexes purified this way could also be visualized by EM.

Testing the presence of a channel on purified translocator hetero-complexes can be challenging. Options to study this could include cryo-EM in case resolution and channel diameter allow direct visualization. Alternatively, the micelle-solubilized protein complex could be reconstituted into planar lipid bilayers and a channel detected by electrophysiological measurements. A different approach to detect the presence of a pore could use the strategy described in Chapter 2 to detect the presence of discrete pores and study pores stability (Methods Section 2.4.18). Hetero-complexes could be reconstituted into liposomes encapsulating Streptavidin-BodipyFL, and the presence of discrete pores detected by addition of biocytin or β-Amylase-Biotin. A similar approach has been recently used to study the effect in liposome membrane permeability produced by reconstituted SNARE and Rab proteins (Zucchi and Zick, 2011).

4.2.3 Possible models for PopB and PopD assembly.

The hexadecameric model described in Chapter 3 represents the first model with stoichiometric detail presented to date for any T3SS translocon. In order to further understand how this machinery works, it is required to study the topological arrangement of PopB and PopD in membranes and how they interact together to assemble such a structure.

Considering T3SS translocators insert into membranes spontaneously from an unfolded or partially unfolded state (Fig. 1.10), PopB membrane insertion mechanism and topology is more predictable than that of PopD. PopD (295 aa) possess a single potential α-helical transmembrane segment (residues 119-137) (Fig. 1.9). Therefore, is hard to imagine how a single TM segment located close to the center of the protein can
insert in the membrane in a spontaneous way. Insertion of this segment in a transmembrane configuration requires a big portion of PopD to be translocated across the membrane. This represents an enormous activation energy for a spontaneous process. Indeed, structural studies performed on this segment using homo-oligomeric forms of PopD assembled in membranes indicated the segment does not adopt a transmembrane conformation (Appendix A). Nevertheless, it may be possible that PopB assists PopD in its insertion process. In fact, we observed that the presence of PopB enhanced the membrane association of PopD in the 5.0-6.0 pH range (Chapter 3, Fig. 3.1).

In contrast to PopD, PopB membrane topology can be predicted and the energetics and geometric restrains involved in inserting such a structure in a membrane are compatible with a spontaneous membrane insertion process (see section 4.2.3). PopB has two potential α-helical transmembrane segments located within residues 170-202 and 238-255. Prediction of PopB membrane topology using TMmod (Kahsai et al., 2005) indicates these two segments insert in the membrane forming a transmembrane hairpin (Fig. 5.2).

PopB possess two coiled-coil regions predicted with probability higher than 0.98 [residues 108-164 and 335-370, predicted using COILS server (Lupas et al., 1991)]. Interestingly, these two predicted coiled-coil regions flank the predicted transmembrane hairpin, further suggesting how PopB may arrange in its membrane inserted state or associate with neighboring PopB molecules (Fig. 4.2A). These two predicted coiled-coil segments could assemble a coiled-coil region right above the membrane inserted hairpin, or could participate in PopB-PopB oligomerization or ring-formation (Fig 4.2). The C-terminal coiled-coil region of a PopB monomer may form a coiled-coil structure with the equivalent C-terminal region of a neighbor PopB monomer, and the same may
take place for the N-terminal coiled-coil region (Fig 4.2B). An alternative model is also possible where C-terminal coiled-coil regions associate with N-terminal coiled-coil regions, but this model is less probable given that N-terminal and C-terminal predicted coiled-coil regions differ significantly in size. N-terminal coiled-coil regions have a predicted length of approximately one hundred residues, while C-terminal regions have a predicted length of only forty residues (Fig 4.2).

The predicted model for monomeric PopB suggests in may be possible to obtain a highly soluble mutant form of PopB by trimming the hydrophobic hairpin and connecting the coiled coil regions with a short 5 residue linker, producing a “cytosolic” (or in this case “extracellular”) version of PopB. This deletion should not interfere with PcrH interaction as the PcrH binding motif resides in the N-terminal region of PopB. This may allow to perform a crystallization screening using this mutant version of PopB. Protease-stable fragments of the distantly related translocators IpaB from S. flexnerii and SipB from Salmonella enterica have been crystallized and their structures solved. The structures displays a large coiled-coil region with good similarity to the bacterial pore forming toxin Colicin 1a (Barta et al., 2012).
Figure 4.2. Possible PopB membrane topology and oligomerization interface.

PopB is predicted to insert a hairpin of hydrophobic helices in the membrane. **A.** Prediction of PopB topology in its membrane inserted state (top region, extracellular space; bottom region, intracellular space). The indicated topology is the most probable considering PopB inserts in the membrane from the extracellular space. The numbers indicate residue positions flanking the predicted transmembrane regions and the predicted N-terminal and C-terminal coiled-coil regions. **B.** Coiled-coil regions in PopB may interact with coiled-coil regions in neighboring PopB molecules providing an interface for oligomerization. Given the different length of the predicted N and C-terminal coiled-coil regions, the most likely interaction is the one indicated in the cartoons, with N-terminal coiled-coil regions interacting with each other, and C-terminal coiled-coil regions interacting with each other.
4.2.4 PopB membrane insertion.

Hydrogen bonding networks that result from protein secondary structure are highly stabilized within the low dielectric, water-less membrane environment. Unlike β-strands, α-helices can fold as independent units. Their hydrogen bonding network is self-contained within the protein segment. Therefore, membrane proteins can be stabilized by a single transmembrane hydrophobic helix crossing the membrane (Type I membrane proteins), while in the case of β-transmembrane structures the minimum stable configuration that satisfies its hydrogen bonding network is a β-barrel, usually assembled by a protein domain or in the case of β pore-forming toxins usually assembled by β-hairpin contributions from seven or more toxin monomers (Heuck and Johnson, 2005). Therefore, the concept of a pre-pore complex where monomers assemble a ring arrangement before membrane insertion is very common in the case of β-pore forming toxins. This is not the case for α-pore forming toxins, where monomers can insert in the membrane, and even small pores with ionic conductance can be assembled by a single membrane monomer as in the case of Colicin K and E1 family members (Schein et al., 1978; Slatin 1988). Considering PopB contains a predicted hairpin of transmembrane helices, and that it is secreted in an unfolded state, the most probable model for its membrane insertion is that where an unfolded monomer binds the membrane, folding of the hydrophobic hairpin takes place, and is followed by hairpin insertion in a transmembrane conformation. This is the helical hairpin hypothesis described by Engelman and Steitz (1981). Although this hypothesis was initially developed to describe how helical membrane proteins may insert spontaneously into cellular membranes, before available evidence for a translocation machinery, it is in its treatment most applicable to α-pore forming toxins, or in this case PopB. Interestingly, we have observed that the membrane inserted state of PopB in comparison to its
monomeric chaperone-bound state presents a big increase in α-helical content (Fig. 2.2C). It has been recently reported that PopB remains in a molten globular state in its chaperone-bound form (Dey et al., 2012), however our observations that PopB α-helical content increases significantly upon membrane insertion may suggest soluble PopB remains in a pre-molten globular state or even in a intrinsically disordered state before membrane interaction (Uversky, 2002). In addition to the observed increase in helical content, monomeric PopB bound to the globular chaperone PcrH presents an overall hydrodynamic radius that is 1.7 fold higher than expected for its molecular weight and assuming globular shape (Fig. 2.1E). We initially interpreted this as PopB having an elongated shape, but this could also be attributed to PopB presenting a disordered or unfolded region.

Consistent with a TM helical hairpin insertion model, the loop connecting both helices in the hydrophobic hairpin contains three Asp residues. These acidic residues would need to protonate in order for the hairpin loop to translocate across the membrane hydrophobic core. We observed in vitro that the membrane interaction and pore-forming activity of PopB was significantly enhanced by acidic pH and negatively charged lipids, which may raise the pKa values of nearby Asp or Glu residues and also reduce the local pH at the membrane-water interface (Chapter 2, Fig. 2.8A, Fig 2.11A).

Also consistent with the mentioned mechanism for membrane insertion, we observed by FRET that PopB membrane binding is a fast process taking full completion in the order of seconds (Appendix B), while the kinetics for membrane permeabilization and therefore membrane insertion is slower taking place in the order of minutes. Considering this, it may result interesting to study the kinetics of PopB folding upon membrane interaction by using stopped-flow CD spectroscopy. The abovementioned
predictions and observations can be summarized in the following model for PopB membrane insertion after secretion from the T3SS needle (Fig. 4.3).
Figure 4.3. A model for PopB spontaneous membrane insertion.

Considering PopB is secreted in an unfolded state from the T3SS needle, its predicted membrane topology, and various experimental observations, the energetically most favorable model for its membrane interaction is described by the helical hairpin hypothesis (Engelman and Steitz, 1981). In addition to the predicted transmembrane hairpin, the connecting loop contain acidic residues that need to be protonated for membrane insertion. A putative subsequent oligomerization mechanism by coiled-coil regions is also proposed.
As mentioned earlier, a membrane topology for PopD is hard to envision given it has a single predicted TM segment and spontaneous insertion of this segment in a transmembrane conformation would require a big activation energy (a substantial portion of PopD would need to be translocated across the membrane). Our initial studies of PopD topology in model membranes (Appendix A) indicated this segment, although it does interact with the membrane hydrophobic core, does not adopt a transmembrane conformation. This was assayed in the same conditions where PopD efficiently inserts in the membrane and forms pores (Chapter 2) while assembling mostly into hexameric structures (Chapter 3). Unfortunately, the same study carried out in the presence of PopB was done using a 1:1 stoichiometric ratio of both translocators where they assemble an heterogeneous population of homo and hetero-oligomers (Chapter 3). These studies need to be assayed again using a 1:10 stoichiometric ratio of PopD:PopB where PopD forms part mostly of hetero-complexes (Chapter 3).

The predicted transmembrane segments in PopB and PopD display the highest sequence conservation within the protein. Since hydrophobicity and proper length are the only energetic requirements for helix-membrane interaction in the case of transmembrane segments, this sequence conservation suggests the segments may also be involved in helix-helix packing interaction that may drive protein-protein interaction. Indeed, analysis of PopB predicted transmembrane segments using bioinformatics tools indicates the presence of at least five high probability contacts between them. Since PopD’s single hydrophobic segment also has high sequence conservation, it is possible that packing of this segment with PopB hydrophobic segment takes place within the membrane environment. In this regard, it may be interesting to test the presence of correlated mutations between PopD and PopB hydrophobic segments which may suggest they drive PopB-PopD interaction within the membrane.
We have prepared over the last few years, in collaboration with undergraduate students (Kyle Rossi, Michael Buckner, and John Holt), a collection of more than 50 single Cys PopD mutants that span all tentative membrane-interacting segments in PopD including PopD’s predicted transmembrane segment, both N-terminal and C-terminal amphipathic segments and a charged amphipathic segment present between the C-terminal amphipathic and predicted TM segment. Again, these mutants need be assays in the optimal conditions described in Chapter 2 and Chapter 3. These studies should provide further insight into how PopD inserts in membranes, forms a pore, and interact with PopB to assemble the arrangement described in Chapter 3.

5.2.5 Observation about cryoEM studies

cryoEM imaging was performed on liposomal samples containing PopB/PopD at protein:lipid ratios where maximum pore formation took place, and protein:lipid ratios as much 10 times higher (Chapter 2, Fig. 2.8C and D; Appendix C). DLS analysis of samples with high protein:lipid ratios indicated a big increase in the average particle size (Chapter 2, Fig. 2.9), which was interpreted as “liposome aggregation”.

A close analysis of these “aggregates” suggests a complex phenomenon takes place at the membrane interfaces when protein reaches a critical concentration. The membranes are maintained in close proximity and in a flat configuration, presumably by the packing of PopB/PopD from adjacent membranes. These structures are very evident at high protein concentrations (protein:lipid ratios of 1:330), but the same structures are also visible, although with lower frequency, at lower protein concentrations (protein:lipid ratio 1:3300) (Fig 5.4).
cryoEM images were obtained as described in Chapter 2, Methods section 2.4.16. A. Part of Figure C.2B, protein lipid ratio was 1:3300 (appendix C). B. Part of Figure C.3A, printein lipid ratio was 1:330 (appendix C).

Figure 4.4. cryoEM images of liposomes incubated with PopB and PopD
The area between the membranes indicated by the arrow in Fig. 4.2.4 has the
darkest contrast in the image, and therefore the highest electron density. It may be
possible that PopB and PopD arrange in a crystalline lattice under these conditions,
aided by protein-protein packing from adjacent membranes. The same type of packing
takes place for membrane proteins that crystallize in lipidic cubic phases, with the
difference that membrane cubic phases provide a continuous stream of protein by lateral
diffusion into the growing lattice. In the case of liposomes, lattice growth would be
limited by the amount of protein present in each liposome. It may result interesting
therefore to assemble lipid cubic phases containing membrane inserted PopB/PopD by
using PopB/PopD proteoliposomes prepared as described in Chapter 2, and screen
these for crystallization. Unlike most membrane proteins, protein production yield is not a
limiting factor in the case of PopB and PopD.
APPENDIX A

PUTATIVE MEMBRANE INTERACTING SEGMENTS IN POPD INTERACT WITH THE MEMBRANE HYDROPHOBIC CORE BUT DO NOT ADOPT A TRANSMEMBRANE CONFORMATION

A.1 Introduction

Despite considerable data available regarding characterization of T3SS components, fundamental questions still remain to be elucidated. Only limited structural information is available regarding the membrane associated state of translocators. PopB and PopD are able to form pores in model membranes, but this only happens efficiently at acidic pH (Faudry et al., 2006; Chapter 2 Fig. 2.8A). Moreover, PopB and PopD are able to bind efficiently to membranes only at acidic pH (Chapter 2, Fig. 2.10; Chapter 3, Fig. 3.1). This observation partially explains the acidic pH dependence in the pore forming activity of translocators, as membrane binding is required prior to membrane insertion and pore formation. The pore forming activity of translocators in liposomes is greatly enhanced by the presence of negatively charged phospholipid heads such as Phosphatidylserine (Faudry et al., 2006; and Chapter 2 Fig. 2.7B). Therefore, it is possible that membrane binding is at least partially triggered by ionic interactions that become favorable at low pH. Alternatively, pH induced unfolding can potentially promote membrane interaction by inducing a partially unfolded state exposing a hydrophobic surface. At least in the case of PopD, this is not likely the case since PopD remains in a partially unfolded or molten globule state regardless of pH (Faudry et al., 2007). PopD possesses one hydrophobic segment that is predicted as transmembrane by hydropathy analysis algorithms, and two predicted amphipathic segments based on hydrophobic moment calculations (assuming α-helical conformation) as calculated using Membrane
Protein Explorer (MPEx) (Snider et al., 2009). PopD’s amphipathic segments are located in its N-terminal region, and in its C-terminal region (Faudry et al., 2007). Interestingly, we found that the amphipathic character of PopD’s N-terminal segment is greatly induced by the presence of three acidic residues (Fig. 4.1). In addition, protonation of these residues followed by hydropathy plot analysis indicates this segment becomes hydrophobic enough to be predicted as transmembrane (Fig 4.1). Acidic residues in this segment were mutated to non-charged ones and the resulting mutant PopD was able to assemble membrane pores at pH values closer to neutral. The topology of this segment in wild type PopD was analyzed using multiple fluorescence techniques (Heuck and Johnson, 2002; Johnson, 2005), and found this segment interacts with the lipid bilayer but does not adopt a transmembrane conformation. PopD’s predicted transmembrane segment was also analyzed in the same way and we found it did not adopt a transmembrane conformation, at least when PopD was incubated with liposomes individually in the absence of PopB. These studies were performed previous to single-molecule experiments (Chapter 3). Unfortunately, a 1:1 ratio of PopD:PopB was employed when analyzing these PopD segments in PopD/PopB hetero-complexes. We learned from single-molecule experiments (Chapter 3) that at a 1:1 PopD:PopB ratio only about 20% of the protein sample remained as hetero-complex, hence the protein sample was not homogeneous enough for proper interpretation of the fluorescence data (Johnson, 2005). Therefore, only findings using PopD assembled individually in membranes are reported here. Experiments using a 1:10 ratio of PopD:PopB where PopD is mostly part of hetero-complexes (Chapter 3 Fig. 3.5) are currently underway in our laboratory.

A.2 Results
A.2.1 PopD's C-terminal amphipathic segment is predicted as transmembrane when acidic residues are protonated

Acidic pH is required for efficient translocator binding to model membranes (Chapter 2, Fig. 2.10; Chapter 3, Fig. 3.1). A close analysis of PopD sequence using hydrophobicity scales indicated that protonation of acidic residues in PopD induced the appearance of a new transmembrane segment. In addition, this segment was predicted as amphipathic in the non-protonated form and assuming α-helical structure by using MPEx (Snider et al., 2009). This property was conserved among PopD homologues of the T3SS Ysc family such as YopD, AopD and VopD (Fig A.1). Interestingly, well characterized pore forming proteins where acidic pH is required for membrane insertion such as Colicin A and Diphteria toxin also display a similar change in the hydropathy plot when acidic residues are protonated (not shown).
Figure A.1. PopD C-terminal amphipathic segment displays hydropathy increase when acidic residues are protonated.

A. Hydropathy plots of PopD and YopD at neutral and acidic pH. The arrow indicates the n-terminal amphipathic segment position. Segments with a positive score are predicted as transmembrane. B. Helical wheel representation of PopD N-terminal amphipathic segment at neutral pH (left) and acidic pH (right). Negatively charged acidic residues are indicated in red color. The segment’s overall vectorial hydrophobic moment is indicated by the blue arrow in the center of the representation. Sequence analysis and helical wheel representations were performed using MPEx (Snider et al., 2009).
A.2.2 Mutation of acidic residues in PopD 63-81 segment facilitates pores formation at pH closer to neutral

Acidic pH requirement for membrane insertion and pore formation has been attributed to protonation of His residues in cases such as Diphtheria toxin (Perier et al., 2007). In other cases, such as Colicin family members, it is postulated that acidic pH promotes a partially unfolded or molten globular state in the protein causing exposure of hydrophobic surfaces and membrane interaction (van der Goot et al., 1992). At least in the case of PopD, these two scenarios are not likely since: 1) PopD membrane interaction and pore formation happen efficiently at pH values lower than 5.0, consistent with protonation of Glu and Asp residues rather than His; and 2) PopD already exists in a molten globular conformational assembly regardless of pH (Faudry et al., 2007). To test whether PopD acidic residues in the C-terminal amphipathic segment play a role in the pH dependent membrane interaction, three mutations that eliminate acidic residues we introduced in this segment (E69A, D71A, and E75C). Interestingly, the mutant PopD<sup>E69A D71A E75C</sup> was able to form pores at pH values closer to neutral when compared with wild type PopD on its ability to form pores at varying pH conditions (Fig. A.2).
Figure A.2. Mutation of acidic residues in PopD C-terminal amphipathic segment facilitates pores formation at pH closer to neutral.

The pore forming activity of wild type PopD and PopD^{E69A-D71A-E75C} was measured at the indicated pH values using the Tb(OPA)$_3^{3+}$ quenching method described in Methods section 2.4.17. The reaction buffer was supplemented with 0.5mM DTT to prevent PopD disulfide formation given one of the acidic residues was mutated to Cys. Protein:lipid ratio was 1:1000 and lipid composition was POPC:cholesterol:POPS at molar ratio 65:20:15. (Fluorescence data was collected by Sarah Kells).
**A.2.3 PopD hydrophobic segment interacts with the membrane in a non-transmembrane conformation**

It has been widely assumed that PopD’s predicted transmembrane segment inserts into the lipid bilayer upon translocon assembly but to date there is no experimental data to support this hypothesis. No structural data is currently available regarding which segment/s in PopD (or homologues) insert in the membrane to assemble a pore.

To directly analyze the particular environment of amino acid side chains within PopD C-terminal amphipathic segment and in membrane assembled PopD, single Cys residues were introduced in PopD, and the sulfhydryl group was reacted with the environment sensitive fluorophore NBD. The fluorescently labeled PopD derivatives were assembled in liposomal membranes as described in Chapter 2, and their spectral properties were examined. NBD spectral properties change drastically in the presence of water making it a preferred probe for protein-membrane interaction studies (Heuck et al., 2002; Johnson, 2005). NBD fluorescence lifetime and quantum yield increase several fold when the probe moves from a water exposed environment into the non-polar core of the bilayer (Crowley et al., 1993 and 1994). In addition, NBD is relatively small, non-charged, and its N and O atoms provide it with enough water solubility. This last property is important because the probe must not bias the location of the amino acid side-chain being studied. Interaction of a labeled residue with the lipid bilayer can be therefore assessed by measuring collisional quenching of the fluorescence by membrane attached quenchers such as spin-labels, or soluble quenchers such as iodide. Also, properties such as the probe quantum yield, the fluorescence excited-state lifetime, and its wavelength of maximum emission report on the environment of the probe.

A collection of singly NBD9 labeled PopD mutants was obtained spanning both its N-terminal amphipathic segment (in collaboration with Kyle C. Rossi) and its central
predicted transmembrane segment. These mutants were reconstituted in liposomes as described in Chapter 2, and the following fluorescence properties of NBD were analyzed: Wavelength of maximum emission; Fluorescence lifetimes; Collisional quenching by water soluble iodide ions; Quenching by the membrane restricted spin label 10-doxyll-nonadecane (Musse, 2006; Heuck et al., 2002).

The collisional quenching pattern obtained from the abovementioned experiments clearly indicated that PopD central hydrophobic segment, although it was exposed to the bilayer hydrophobic core, did not adopt a transmembrane conformation, at least in the absence of PopB (Fig A.4B). None of the NBD probes displayed fluorescence quenching compared to NBD-Cholesterol, which positions the probe at the center of the bilayer core (Fig A.4 B). In a similar way, PopD N-terminal amphipathic segment interacted with the bilayer in a seemingly non transmembrane conformation, although since this segment is amphipathic, the possibility that this segment lines a lipidic type of pore in a transmembrane conformation cannot be discarded (Fig A.4A). Other fluorescence properties such as NBD fluorescence lifetimes and wavelength of maximum emission indicated the same pattern (not shown).
Figure A.3. C-terminal amphipathic and hydrophobic segments in PopD are located in the water/membrane interface.

The orientation of the membrane interacting segments was analyzed using collisional quenchers. Exposure of the NBD dyes to water-soluble quenchers (iodide ions) or quenchers restricted to the non-polar core of the membrane (10-doxyl-nonadecane) was analyzed as described in the Methods sections A.4.2 and A.4.4. Quenching values obtained for Cholesterol-NBD are indicated (red lines). Protein lipid ratio was 1:3300, membrane lipid composition was POPC:cholesterol:POPS at molar ratio 65:20:15 in panels without membrane quencher. Reaction buffer was 50mM Sodium Acetate pH 4.0 in all panels. A. Quenching of PopD NBD probes in C-terminal amphipathic segment by a membrane restricted quencher (left, 10-doxyl-nonadecane), and a water soluble quencher (iodide ions). Measurements we recorded at 12 °C in the case of membrane restricted quencher, and 25 °C in the case of water soluble quencher. B. Same experimental data as in A but for PopD predicted transmembrane segment. Data in A collected by Kyle C. Rossi.
A.2.3 PopD hydrophobic segment membrane insertion and pore formation display the same pH dependence.

A single Cys mutant PopD was used to investigate how PopD hydrophobic segment interacts with the membrane as a function of pH. Cys residue at position 130, located in the center of the hydrophobic segment, was labeled with the environment sensitive fluorescent probe NBD. The fluorescence intensity of the probe located in this segment increased with decreasing pH (Fig. A.5B). Also the probe fluorescence lifetime and collisional quenching by 10-doxylnonadecane increased at acidic pH (not shown). This indicated the segment became increasingly exposed to the membrane hydrophobic core with decreasing pH. Interestingly, the pH dependence of this membrane interaction correlated with the pH dependence of PopD’s pore-forming activity (Chapter 2, Fig. 2.8A). PopD binding to membranes had also been measured using the same pH Buffers (Chapter 3, Fig. 3.1A and B). Comparing the curves of PopD membrane binding and pore-formation, as a function of pH, indicated PopD membrane binding occurred efficiently at pH values higher than the pH required for membrane insertion and pore formation (Fig. A.3A and B). Altogether, this data suggests that PopD membrane binding is not the only event triggered by low pH, as membrane binding is almost complete at pH values around 5.0 where no significant pore formation is yet detected (Fig. 2.8A). It is clear that PopD pore formation has a pH dependence different from that of membrane binding, and occur at lower pH values.
Figure A.4. PopD hydrophobic segment membrane insertion and PopD pore formation display the same pH dependency.

Curves of PopD pore formation and membrane binding as a function of pH were compared. Membrane binding took place at pH values closer to neutral. A. PopD total membrane binding was measured using the liposome floatation assay described in Methods section 2.4.14. Protein lipid ratio was 1:5000. Pore formation was measured using the Tb(DPA)$_3^{3-}$ quenching method described in Methods section 2.4.17, protein lipid ratio was 1:2500. B. Data in A is superimposed to a curve of PopD$_{G130C-NBD}$ fluorescence intensity as a function of pH. PopD$_{G130C-NBD}$ was incubated with membranes at the indicated pH values and the final fluorescence intensity was divided by the value obtained at pH 7.0. The Y-axis therefore represents the fold increase in fluorescence intensity relative to pH 7.0 (i.e. where minimal membrane interaction takes place). Protein lipid ratio was 1:3300.
A.3 Discussion

In summary, we have identified important features of PopD pH dependent membrane interaction. PopD N-terminal amphipathic segment seems to play a role in binding and/or insertion. Eliminating charged acidic residues in this segment tuned the pH dependence of PopD pore formation towards more neutral values. Analysis of the segment membrane interaction through multiple fluorescence techniques (Heuck and Johnson, 2002) indicated the segment remained in a water/membrane interface. This segment could lie parallel to the membrane, as is the case in some protein segments of pore forming toxins of the Colicin family (Musse et al., 2006), or could lie perpendicular to the membrane in the case PopD assembles a toroidal or lipidic type of pore, in a way similar to Actinoporin’s amphipathic α-helix (Kristan et al., 2009).

PopD predicted transmembrane segment did not adopt a transmembrane conformation, at least in the absence of PopB. Nevertheless, residues in this segment did become exposed to the bilayer core upon protein interaction with membranes. In the case of residue 130, located in the center of the segment, exposure of this residue position to the membrane hydrophobic core had approximately the same pH dependency as the pore forming activity, and this happened at pH values more acidic than the ones required for PopD membrane binding. It seems possible that further changes in the protein triggered by pH, after membrane binding, are required for efficient pore formation. Protein oligomerization prior to membrane insertion and pore formation is a hallmark of pore forming toxins. We have also shown that PopD oligomerizes at the membrane forming mostly hexameric structures (Chapter 3, Fig. 3.4A, B and C). Thus, it is possible that lower pH is required for efficient protein oligomerization and/or insertion after membrane binding. Mutation of acidic residues in PopD’s N-terminal amphipathic segment allows PopD to assemble pores at pH closer to neutral values. It may result
interesting to investigate what parameters are affected in this mutant PopD. Membrane
binding could affected, or membrane oligomerization/insertion could be affected.

The interaction of PopB and PopD with model membranes requires acidic pH, but the translocators do not likely find such acidic environment in vivo prior to membrane insertion. Nevertheless, translocators are secreted in close proximity to the membrane through the T3SS needle, presumably in a partially unfolded state (the T3S needle channel has a 2.5 nm diameter). Interestingly, it has been reported that the interior of the needle channel is likely highly negatively charged, and a repulsive electrostatic mechanism for the export of T3S effectors across the T3S needle was proposed (Rathinavelan et al., 2010). Therefore, it could be hypothesized that this negative potential in the needle channel affects the pKa of acidic residues of secreted proteins inducing their protonation prior to membrane engagement. In addition, other protein factors absent in our in vitro studies could play a role in keeping translocators in a protonated state, or simply chaperone translocators into the membrane. For example PcrV, the T3S needle tip protein is essential for T3S translocation, and a P. aeruginosa strain lacking PcrV is able to secrete PopD, but PopD fails to insert into the target cell membrane (Goure et al., 2004).

A.4 Methods

A.4.1 Protein Labeling. Single Cys mutants were expressed and purified according to the Methods section in Chapter 2. The protein sulfhydryl group was labeled in the same way described in Chapter 2 for BodipyFL, with the exception that a single reaction step was carried out by adding a 10 fold molar excess of N,N’-dimethyl-N-(iodoacetyl)-N’-(7-nitrobenz-2-oxa-1,3-diazolyl)ethylenediamin (IANBD, Invitrogen). The labeling reaction took place for 2 hours at 23 °C with gentle agitation.
A.4.2 Quenching of NBD Intensity by Iodide Ions. PopD-NBD fluorescence intensity was measured at six different concentrations of iodide ranging from 0 mM to 90 mM. PopD-NBD was incubated with liposomes at 1:3300 protein:lipid ratio for 30 min at 23°C. The reaction buffer was 50mM Sodium Acetate pH 4.0. After incubation, a mixture of potassium chloride and potassium iodide was added to each one of the six samples in order to obtain increasing iodide concentrations ranging from 0 mM iodide (only potassium chloride was added) to 90 mM iodide (only potassium iodide was added) while maintaining the same ionic strength in all samples. The final fluorescence emission intensity of each sample was measured at 25°C as described in Methods section 2.4.20. Stern-Volmer plots were generated using the acquired data and the Stern-Volmer constant (Ksv) taken from the curve slope. Data was fit to the Stern-Volmer equation: 

\[
\frac{F_0}{F} - 1 = K_{sv}[I^-],
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where \(F_0\) is the NBD fluorescence intensity in the absence of quencher (i.e. at 0 mM iodide concentration), \(F\) is the NBD intensity in the presence of the iodide quencher, and \(K_{sv}\) is Stern-Volmer quenching constant. \(K_{sv}\) is equal to \(k_q \times \tau_o\), where \(k_q\) is the biomolecular quenching constant and \(\tau_o\) is the intensity weighted average fluorescence lifetime in the absence of quencher. The average fluorescence lifetime of each mutant PopD-NBD was previously measured as described below in section A.4.3.

A.4.3 NBD Fluorescence Lifetime Measurements. The average fluorescence lifetime of each PopD-NBD mutant was measured using the same experimental setup described in Chapter 2 Methods section 2.4.21 for PopD-BodipyFL mutants. Phase-delay and Frequency-modulation data points were fit to a model of two discrete lifetime components to calculate an average fluorescence lifetime weighted by the intensity contributions of each component.
A.4.4 Fluorescence quenching of NBD by Spin-Labels. PopD-NBD mutants were reconstituted in liposomes using the method described in Chapter 2 and using two parallel samples each incubated with a different liposome preparation, one had lipid composition POPC:cholesterol:POPS with molar ratio 65:20:15, and the other contained the membrane quencher 10-doxylo-nonadecane (Avanti Polar Lipids) with lipid composition 10-doxylo-nonadecane:POPC:cholesterol:POPS at molar ratio 10:55:20:15. The protein:lipid ratio was 1:3300, and the reaction buffer was 50mM Sodium Acetate pH 4.0. After 30 min incubation at 23 °C, the sample was equilibrated at 12 °C and final emission intensity of NBD was measured as described in Methods section 2.4.20. The blank subtracted final fluorescence intensity was used to calculate the % fluorescence quenching using the equation % quenching = 100% x (1 – F/F0) where F is the fluorescence intensity of the sample containing 10-doxylo-nonadecane, and F0 is the fluorescence intensity of the control sample without quencher.
APPENDIX B.

KINETICS OF POPB/POPD-MEMBRANE AND POPB-POPD INTERACTION

B.1 Membrane binding preceded the re-organization of the translocators at the membrane surface

PopB and PopD isolated using native conditions exist as soluble aggregates, and these conserve the ability to bind membranes and form pores (Schoehn et al., 2003). These PopB and PopD aggregates were reported to form pores synergistically when incubated together with membranes (Faudry et al., 2006; Chapter 2). It was therefore suggested that protein oligomerization in solution was a requirement for membrane interaction (Schoehn et al., 2003). However, urea isolated PopD and PopB form pores in model membranes as efficiently as translocators isolated in native conditions (Chapter 2, Fig. 2.4). This data suggests different possible pathways for translocon assembly in membranes which are not necessarily exclusive. It is therefore important to determine the order of protein-protein and protein-membrane interactions that lead to translocator assembly in membranes. A convenient way to study this is to determine the kinetics of membrane binding as well as the kinetics of translocator changes in oligomerization state. An excellent method for recording membrane binding kinetics is FRET (Mutucumarana et al., 1992; Ramachandran et al., 2005). Since FRET occurs efficiently at distances lower than ~80 Å, only membrane associated FRET donor molecules originate a signal. We measured FRET between PopB$^{\text{BpyFL}}$ or PopD$^{\text{BpyFL}}$ as donor molecules, and liposomal membranes containing randomly distributed Rhodamine-DHPE as the FRET acceptor molecule. This approach has been successfully used by our group as well as other groups to measure protein-membrane interactions (Mutucumarana et al., 1992; Bazzi and Nelsestuen, 1987; Ramachandran et al., 2005; Posokov et al., 2006; Lovell et al., 2008). Remarkably, PopB and PopD bound to
membranes very rapidly after dilution from a 6 M urea buffer solution into a liposome sample buffered at pH 4.0 (i.e. pH of maximum membrane interaction). The membrane binding reached completion in the order of seconds (Fig. B.1).
Figure B.1: Membrane binding preceded the re-organization of the translocators at the membrane surface.

The kinetics of translocator membrane binding was measured by FRET as described in Methods section B.4.1. BodipyFL self quenching was measured as a function of time as described in Chapter 2. BodipyFL increase in self-quenching was interpreted as oligomerization, and BodipyFL decrease in self-quenching (i.e. increase in fluorescence intensity) was interpreted as protein dissagregation or de-oligomerization. The translocator protein was added to liposomes at time zero. A. FRET signal (blue) for PopD\textsuperscript{bpy} membrane interaction, and BodipyFL fluorescence decrease (oligomerization) (open squares). FRET and BodipyFL fluorescence intensity experiments were carried out independently. The arrow indicates the addiction of 5mM Triton X-100 to the FRET sample. Detergent dissolved the membranes and eliminated the FRET signal. B. FRET signal (red) for PopBBodipyFL membrane interaction, and BodipyFL fluorescence increase (de-oligomerization). The arrow indicates the addition of Triton X-100 to FRET curves only.
Complementary to membrane-binding FRET experiments, an efficient way of recording changes in the oligomeric state of translocators is to monitor the fluorescence intensity of BodipyFL dyes over time, as close proximity between dyes resulting from protein-protein association reduces the probe’s quantum yield and fluorescence lifetime, which can be monitored as fluorescence quenching using steady-state fluorescence measurements (Bergstrom et al., 2002; Mikhalov et al., 2002; Chapter 2, Table 2.1). PopD\textsubscript{BpyFL} or PopB\textsubscript{BpyFL} purified in 6M urea solution were incubated in buffer alone or buffer containing liposomal membranes, and the kinetics of BodipyFL fluorescence intensity was recorded (Fig. B.1, and not shown). PopD\textsubscript{BpyFL} fluorescence intensity curves revealed that PopD oligomerized subsequent to membrane binding, as BodipyFL fluorescence decreased over time (Fig. B.1A). Interestingly, incubation of PopD with buffer alone resulted in a slower decrease in BodipyFL fluorescence intensity (not shown), suggesting PopD self-association occurs faster in membranes. This was not surprising considering membrane binding increases the local concentration of protein molecules.

In contrast, when PopB\textsubscript{BpyFL} was incubated with liposomal membranes, the fluorescence intensity of BodipyFL increased over time instead of decreasing. This indicated that either pre-existent PopB oligomers re-arranged upon membrane binding, or that a conformational change produced by membrane binding separated BodipyFL dyes apart, diminishing the self-quenching of the fluorescence (Fig. B.1B). Consistent with the formation of PopB:PopD hetero-complexes, when samples containing 1:1 ratios of 6M urea PopD\textsubscript{BpyFL}:PopB or PopD: PopB\textsubscript{BpyFL} were incubated with membranes, similar changes in fluorescence intensity were recorded, but the final fluorescence intensity was higher in both cases presumably due to intercalation of unlabeled translocator with labeled ones (not shown). Altogether, this data indicated that changes in oligomeric
state that result in the assembly of PopB:PopD hetero-complexes involve membrane-bound translocators.

**B.2. PopB remained oligomeric in 6 M urea.**

In order to determine changes in protein oligomerization that lead to translocon assembly in model membranes, it is important to determine the initial oligomerization state of protein samples. PopB in 6M urea solution retains some secondary structure and its single Trp residue remains in a hydrophobic environment (Chapter 2, Fig. 2.3). This raises the question of whether PopB remains oligomeric in 6M urea solution. To test this, two independent experiments were carried out. Firstly, a FRET experiment between PopB\(^{S164C}\) molecules solubilized in 6M urea buffer was carried out. To test the oligomerization state of PopB in 6M urea solution, monomeric hisPcrH-PopB\(^{S164C}\) was labeled with a 1:1 mixture of Cys reactive BodipyFL (donor) and Rhodamine (acceptor), and then labeled PopB\(^{S164C}\) was eluted from PcrH using 6M urea as described in Methods section 2.4.9. This labeling approach resulted in a PopB\(^{S164C}\) sample where the single Cys residues in PopB were about 50% labeled with BodipyFL and about 50% labeled with Rhodamine. If PopB molecules associate together bringing probes within a FRET efficient range, FRET is detected. FRET efficiency within a PopB\(^{BpyFL/Rho}\) sample was measured using time-resolved fluorescence. The fluorescence lifetime of the donor molecule was measured in the presence or absence of acceptor using PopB\(^{BpyFL/Rho}\) or PopB\(^{BpyFL}\) samples. Interestingly, the fluorescence lifetime of donor molecules in the presence of acceptor was shorter than in the absence or acceptor, indicating FRET occurred within PopB molecules. In contrast, a same FRET experiment carried out using PopD\(^{F223C}\) resulted in no detectable FRET efficiency between PopD molecules in 6M urea buffer (not shown). Secondly, we used single-molecule fluorescence
photobleaching in 6 M urea solution to quantify the oligomerization state of glass absorbed \( \text{PopB}^{\text{BpyFL}} \). \( \text{PopB}^{\text{BpyFL}} \) in 6M urea was diluted to nanomolar amounts and the protein was flowed into a chamber containing a glass coverslip and allowed to absorb to the glass surface. Single protein complexes were imaged using Total Internal Reflection Fluorescence (TIRF) microscopy, and their fluorescence emission was recorded over time in order to detect single photobleaching events. This experiment revealed that in 6M urea PopB remained as a heterogeneous mixture of soluble protein aggregates containing between 8 and 20 PopB subunits (Fig. B.2). Experiments carried out in the same way but using a \( \text{PopD}^{\text{BpyFL}} \) sample indicated PopD remained mostly monomeric in 6M urea solution (not shown). Altogether, PopD remained mostly monomeric in 6M urea solution, prior to association with membranes and pore formation, while PopB remained associated in clusters of heterogeneous size.
Figure B.2. PopB remains oligomeric in 6 M urea solution.

The oligomerization state of PopB$^{BpyFL}$ in 6 M urea solution was determined using single-molecule photobleaching. PopB$^{BpyFL}$ in 6M urea solution was allowed to absorb to a glass coverslip at sub-nanomolar concentration and then analyzed as described in Chapter 3, Methods sections 3.4.5 and 3.4.6. **A.** TIRF microscopy image of PopB$^{BpyFL}$ absorbed to a glass coverslip in 6 M urea solution. **B.** The histogram of photobleaching counts obtained indicates PopB remains as a heterogeneous oligomeric mixture in 6 M urea solution.
B.3 Kinetics of PopD-PopB interaction

A FRET assay similar to the one described for measuring PopB-PopB interaction in 6 M urea solution was performed using PopD as FRET donor, and PopB as FRET acceptor in 6 M urea solution. This assay suggested some PopD-PopB interaction took place even in 6 M urea solution (not shown). In our experiments, PopB and PopD are usually pre-mixed in 6 M Urea solution prior to addition to lipoomes, so we therefore studied the kinetics of PopD-PopB association in 6 M urea solution. This was carried out by using PopD$^{H104C}$ labeled with the Cys reactive blue emitting dye [N-(7-Dimethylamino-4-methylcoumarin-3-yl)maleimide] (DAC, Anaspec) (PopD$^{DAC}$). The FRET acceptor in this case was the PopB$^{BpyFL}$ described in Chapters 2 and 3. To maximize the FRET donor-acceptor ratio a molar ratio of 1:10 PopD$^{DAC}$: PopB$^{BpyFL}$ was used. This experiment indicated that PopD and PopB associated together rapidly upon incubation in 6 M urea solution (Fig. B.3).
Figure B.3. Kinetics of PopD-PopB association in 6 M Urea solution.

FRET between Pop$^{DAC}$ (donor) and Pop$^{BpyFL}$ (acceptor) was measured as a function of time as described in Methods section B.4.1 and at 25 °C. Labeled translocators stored in 6M urea buffer solution, pH 8.0, were mixed together at time zero in the same buffer solution. Final protein concentration was 30nM Pop$^{DAC}$, and 300 nM Pop$^{BpyFL}$. Red and blue data points indicate the results of two independent experiments.
The kinetics of PopD and PopB association to membranes is rapid and takes place in the order of seconds. Moreover, re-arrangements in protein oligomerization state seem to take place after membrane binding (Fig. B.1). We directly investigated the kinetics of association between PopD and PopB after membrane binding by using FRET. Again, the FRET molecules used for this experiment were PopD\textsuperscript{DAC} (donor) and PopBBpyFL (acceptor) at a 1:10 PopD\textsuperscript{DAC}:PopB\textsuperscript{BpyFL} ratio in order to maximize the number of donor molecules paired to an acceptor molecule and hence optimize the FRET signal. Since we observed PopD-PopB association already took place in 6 M solution when translocators were pre-incubated together, the kinetics of PopD-PopB association in membranes was studies in two different conditions: I) When PopD and PopB were premixed in 6 M urea solution prior to addition to the membranes; and II) When PopD and PopB were added individually to the same membrane sample. As expected, in the case of the first conditions, FRET efficiency at time zero was already significant. Nevertheless, the FRET efficiency decreased over time and reached a plateau after about five min incubation with membranes. This suggested a re-arrangement of PopB-PopD interaction triggered by membrane binding (Fig. B.4A). In the second conditions tested, the FRET efficiency increased over time after addition of translocators to membranes, indicating increasing PopD-PopB interaction, and reached a plateau after about 20 min incubation with membranes (Fig. B.4B). Interestingly, the final FRET efficiencies measured in both cases was very similar, suggesting that even though PopD-PopB interaction takes place in 6 M urea solution, re-arrangements after membrane binding may yield the same type of final protein arrangement in both cases.
Figure B.4. Kinetics of PopD-PopB interaction after membrane binding.

FRET efficiency between PopD$^{DAC}$ (donor) and PopB$^{BpyFL}$ (acceptor) was measured as a function of time as described in Methods section B.4.1 and at 25 °C. Protein:lipid ratio was 1:7000 in all panels (final protein concentration was 30nM PopD$^{DAC}$, 300nM PopB$^{BpyFL}$), reaction buffer was 50mM Sodium Acetate pH 4.5. Membrane composition was POPC:cholesterol:POPS at molar ratio 65:20:15. Translocators were diluted into the liposome sample from 6 M urea solution at time zero. A. FRET kinetics in the case were PopD$^{DAC}$ and PopB$^{BpyFL}$ were pre-mixed in 6 M urea solution prior to addition to liposomes. B. FRET kinetics in the case were PopD$^{DAC}$ and PopB$^{BpyFL}$. 

\[ \text{FRET Efficiency} \]

\[ \text{Time, min} \]
B.4. Methods

B.4.1 FRET measurements.

The binding of PopBS164C-Bodipy or PopDF223C-Bodipy to membranes under equilibrium conditions was measured by Förster resonance energy transfer (FRET) between a Bodipy-labeled translocator (donor or D), and Rh-PE as acceptor (A), randomly distributed at the lipid bilayer. Four biochemically equivalent samples were prepared in parallel: sample Do (D only) contained 60 nM total translocator (an equimolar mixture of Bodipy-labeled translocator mutant and wild type translocator was used to minimize Bodipy self-quenching), and POPC:POPS:cho membranes (65:15:20 molar ratio) lacking Rh-PE; sample DA (D plus A) contained the same protein mixture as in Do and membranes containing Rh-PE (0.5% of the total lipids); sample A (A only) contained 60 nM wild type translocator and vesicles containing Rh-PE; and the blank (B) sample contained 60 nM wild type translocator and vesicles lacking Rh-PE. In all four samples, the total lipid concentration of the membranes was 150 µM. Liposome samples were equilibrated at 25°C and protein was added at t=0.

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E = 1 - \frac{\langle F_{DA} \rangle}{\langle F_D \rangle}
\]

FRET efficiency (E) over time was calculated as

where \( F_{DA} \) and \( F_D \) are the steady-state fluorescence intensities of the donor in the presence and absence of acceptor, respectively.

B.4.2 Protein Labeling

The mutant derivative PopD-H104C was obtained and purified as described in Chapter 2, Methods sections 2.4.4 and 2.4.6. The single Cys residue was labeled with [N-(7-Dimethylamino-4-methylcoumarin-3-yl)maleimide] (DACM, Anaspec), as described
for BodipyFL labeling of Cys PopD mutants (Chapter 2, section 2.4.7), with the exception that a single dye addition was performed using a 10:1 dye:protein molar ratio, and the labeling reaction took place for 3 h at 23 °C with gentle agitation.
APPENDIX C

CRYO-EM AND NEGATIVE-STAINING TEM IMAGES OF LIPOSOMES INCUBATED WITH TRANSLOCATORS

cryoEM images were acquired in collaboration with Christos G. Savva and Andreas Holzenburg.

Methods

Liposomes were prepared at 30mM final lipid concentration as described in Methods section 2.4.11. Lipid composition in all samples was POPC:cholesterol:POPS at 65:20:15 molar ratio. Liposomes were diluted in Buffer 50mM Sodium Acetate pH 4.0 to the indicated lipid concentrations. cryoEM imaging was performed as described in Methods section 2.4.16.

Figure C.1. cryoEM images of liposomes alone and in the presence of urea.

A, B and C. Samples contained 0.5mM final total lipids. Individual monolayers in the lipid bilayers are distinguishable. D. Sample contained 5mM final total lipids. E. Sample contained 5mM final lipids and was supplemented with 1.3M Urea (same amount of Urea added to liposomes when translocators are diluted with liposomes at the highest concentration assayed during cryoEM imaging).
Figure C.1C
Figure C.2. cryoEM micrographs of liposomes incubated with PopB and PopD.

A, B and C. Samples were prepared by pre-incubating PopB and PopD at 1:1 molar ratio with liposomes prior to cryoEM imaging. Protein:lipid ratio was 1:3300 (75nM PopB, 75nM PopD, 0.5mM total lipids).
Figure C.3. cryoEM micrographs of liposomes incubated with excess PopB and PopD.

A, B and C. Samples were prepared by pre-incubating PopB and PopD at 1:1 molar ratio with liposomes prior to cryoEM imaging. Protein:l lipid ratio was 1:330 (750nM PopB, 750nM PopD, 0.5mM total lipids).
**Figure C.4. Negative-staining TEM micrographs of liposomes incubated with PopD.**

**A and B.** Samples were prepared by incubating PopD with liposomes at pH 4.0 prior to staining with Uranyl Acetate and TEM imaging. Protein:l lipid ratio was 1:2500 and lipid composition was POPC:cholesterol:POPS with molar ratio 65:20:15. Membrane tubules were observed in this case upon incubation with PopD.


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