Characterization of the Reconstituted and Native Pseudomonas aeruginosa Type III Secretion System Translocon

CHARACTERIZATION OF THE RECONSTITUTED AND NATIVE *PSEUDOMONAS AERUGINOSA* TYPE III SECRETION SYSTEM TRANSLOCON

A Thesis Presented

by

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ABSTRACT

CHARACTERIZATION OF THE RECONSTITUTED AND NATIVE *PSEUDOMONAS AERUGINOSA* TYPE III SECRETION SYSTEM TRANSLOCON

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The Type III Secretion (T3S) system is a system utilized by many pathogenic bacteria to inject proteins into host cells during an infection. Effector proteins enter the host cell by passing through the proteinaceous T3S translocon, which forms a pore on the host cell membrane. *Pseudomonas aeruginosa* is an opportunistic pathogen that utilizes the T3S system, and very little is known about how the *P. aeruginosa* translocon forms.

The proteins PopB and PopD are believed to assemble into the *P. aeruginosa* translocon. A pore-forming heterocomplex of PopB and PopD has been reconstituted in model membranes, however this heterocomplex has not been assessed in its relation to the translocon formed on the host cell. The interaction of this heterocomplex with other T3S system components was measured to determine if this complex acts similarly to the translocon. Initial assays that can be used to compare the molecular weight of the translocon isolated from eukaryotic cells after *P. aeruginosa* contact to the calculated molecular weight of the heterocomplex were developed as well. This study

provides insight into how the PopB:PopD heterocomplex formed in model membranes relates to the translocon formed during a *P. aeruginosa* infection.

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CHAPTER 1

INTRODUCTION

1.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a ubiquitous Gram-negative opportunistic pathogen that is one of the leading causes of nosocomial infections (Vincent, 2003). This bacterium colonizes a wide variety of niches including soil, plants, and animals. In humans, *P. aeruginosa* is found as a part of the skin flora (Cogen et al., 2008), however it commonly colonizes the respiratory, urinary, and gastrointestinal tracts causing severe infections in immunocompromised patients (Carmeli, 1999).

P. aeruginosa is the leading cause of infections in patients with chronic obstructive pulmonary disease, cystic fibrosis, and burns, as well as the number one cause of ventilator associated pneumonia; the latter two having mortality rates greater than 30% (Vincent, 2003; Oliver et al., 2008). Infections are difficult to treat as *P. aeruginosa* easily acquires antibiotic resistance (Tenover, 2006). Furthermore, this pathogen forms biofilms, which can allow development of antibiotic resistance up to 1,000 times that of planktonic cells (Hoyle and Costerton, 1991). In fact, biofilms occur in approximately 65% of all human *P. aeruginosa* infections (Filloux and Vallet, 2003; Chicurel, 2000).

Like other gram-negative pathogens, this bacterium uses the Type III Secretion (T3S) system to inject toxins into a host cell. The T3S system is closely tied with infection; in fact, immunization with T3S components successfully protects animals from *P. aeruginosa* pneumonia (Lyzak et al., 2000). This system

is common to many pathogenic bacteria including: *Chlamydia* species, *Shigella* species, *Salmonella* species, and *Yersinia* species (Coburn et al., 2007), and a greater understanding of this infection mechanism is necessary to develop effective infection therapies.

1.2 Type III Secretion system

The T3S system is used to inject toxins directly into the host cell cytoplasm. This system utilizes a syringe-like T3S apparatus. The T3S apparatus consists of an injectisome that spans both the inner and outer bacterial cell membranes, a needle that extends outward from the cell surface, and a protein oligomer that forms at the tip of the needle (Fig 1.1). Additionally, translocator proteins function to form a pore in the host cell membrane to allow effector proteins to pass into the host cell.

Type III Secretion remains inactive until the bacterium comes in contact with a host cell (Mueller, 2005; Matteï et al., 2011). The T3S apparatus is present at the bacterial cell surface, and contact between the needle tip and the host cell is hypothesized to activate Type III Secretion. Upon T3S activation, translocators are first translocated through the needle in a partially folded state, and assemble on the target membrane forming the translocon. The translocon is then believed to engage with the needle tip, allowing for efficient transport of effectors from the bacterial cytosol into the host cell cytosol (Fig 1.2).

1.3 Injectisome

The injectisome is a complex of proteins that spans the inner and outer membrane of the bacteria (Fig 1.1). The T3S system injectisome closely

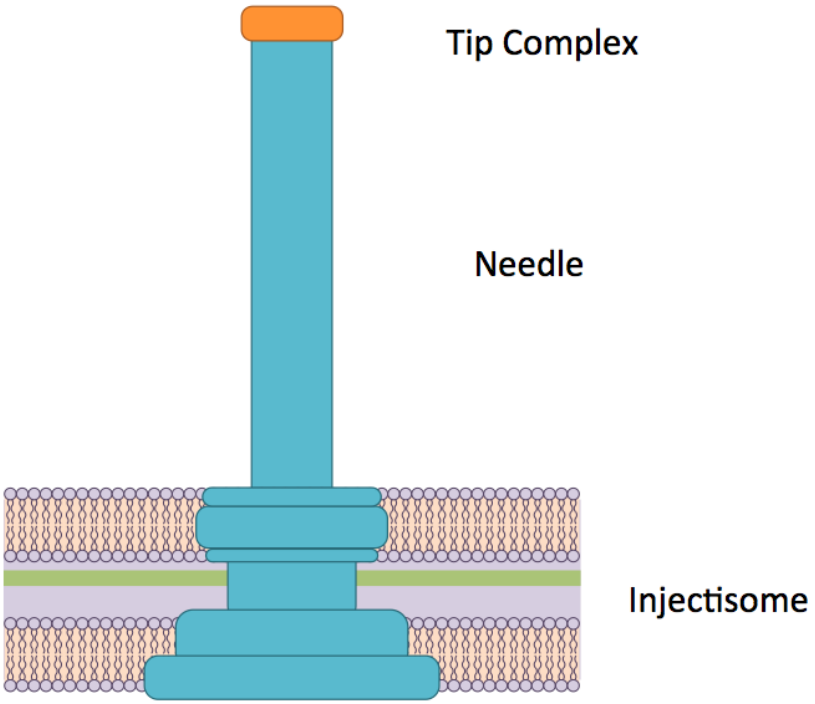

Figure 1.1. Ysc Family Type III Secretion Apparatus. *P. aeruginosa* utilizes the Ysc family T3S apparatus (Cornelis, 2006), which consists of an injectisome spanning the inner and outer bacterial membranes, a 50-80 nm needle that extends beyond the cell surface capped by a tip complex that interacts with the host cell membrane. Translocator protein effector proteins are secreted through this apparatus during an infection.

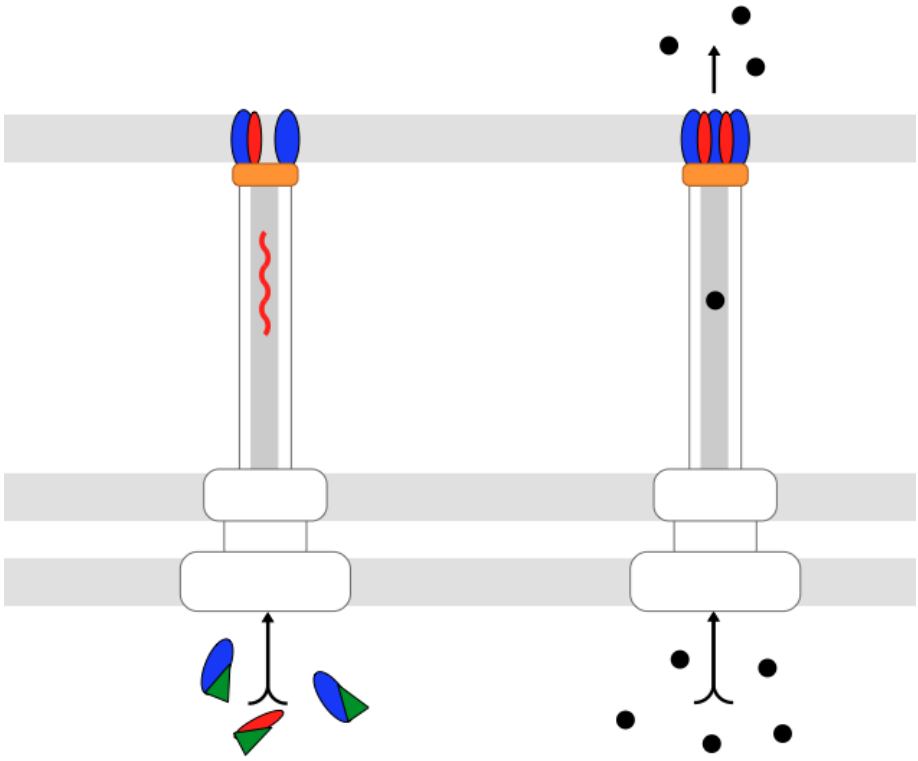

Figure 1.2. Translocon Hypothesis in *P. aeruginosa* T3S apparatus**.** Interaction with a host cell stimulates T3S and the translocators PopB (blue) and PopD (red), to be released from their chaperone PcrH (green) in the bacterial cytoplasm and be secreted through the needle in a partially unfolded state. The translocators insert into the host cell membrane and assemble into the translocon. The needle engages with the host cell membrane through the tip complex, PcrV (orange). Effectors (black) are secreted through the T3S apparatus and enter the host cell through the translocon pore.

resembles the flagellum (Cornelis, 2006). Based on cryo-EM of purified *Salmonella* T3S apparatuses, the injectisome consists of two rings anchored in the bacterial membranes with a hollow channel (~2 nm in diameter) traversing its length (Schraidt and Marlovitz, 2011). An ATPase component of the injectisome likely aids in unfolding secreted proteins to enable passage through the narrow channel (Akeda and Galán, 2005). The T3S needle extends from the injectisome, continuing the hollow channel that effectors pass through.

1.4 Needle

The T3S needle is an oligomer made up of repeating subunits of a small needle protein (PscF in *P. aeruginosa*) (Pastor et al., 2005). The T3S needle length is fixed within a species. In *P. aeruginosa* the needle is approximately 50 nm, with an outer and inner diameter of 8 and 2 nm respectively (Pastor et al., 2005). While *P. aeruginosa* needles have been isolated, their structure has not been well studied. Needles purified from *Shigella* showed that the needle protein oligomerizes into a helical structure with a 24 Å pitch with approximately 5.6 subunits per turn (Cordes et al., 2003). The needle is capped by a proteinaceous tip complex. PcrV forms the *P. aeruginosa* T3S needle tip complex (Mueller et al., 2005).

1.5 Translocators

While the injectisome and needle are sufficient to secrete effector proteins out of the bacterium, effectors need to translocate across the plasma membrane of the target cell. In *P. aeruginosa,* two proteins (PopB and PopD) secreted by the T3S system are required for translocation of effectors into the host cell

(Dacheux et al., 2001; Sundin et al; 2004; Gouer et al., 2004). These translocators form pores in model membranes (Schoen et al., 2003; Faudry et al., 2006; Romano et al., 2011), and are found on host cell membranes after contact with *P. aeruginosa* (Goure et al., 2004), supporting the hypothesis that these translocators assemble into the translocon.

PopB is the major translocator with a molecular weight of 40.3 kDa (Hauser et al., 1998). PopD is the minor translocator with a molecular weight of 31.3 kDa. The structures of PopB and PopD are not well characterized because these proteins have been difficult to crystalize due to their hydrophobicity. A more in-depth characterization of these proteins is necessary to understand how they interact with membranes.

It is hypothesized that the translocator proteins form the translocon spontaneously. This is an uncommon mechanism for membrane protein insertion, as most membrane proteins are inserted by complex cellular machinery involving the ribosome and chaperones (Cymer et al., 2014). The ability for these proteins to assemble on membranes spontaneously facilitates their study in model membranes. PopB and PopD translocator complexes can be reconstituted in liposomes (Romano et al., 2011).

These *P. aeruginosa* translocator proteins are important for infection of the host cell, and very little is known about how they assemble on the host cell membrane. Therefore it is important to study how PopB and PopD interact with the host cell to better understand how effectors are translocated in the T3S system.

1.6 Reconstituting translocators in membranes

PopB and PopD assemble on model membranes, forming pores individually as well as in complex (Romano et al., 2011; Schoen et al., 2003; Faudry et al., 2006). When incubated together, PopB and PopD form a heterocomplex of eight PopB and eight PopD (Romano et al., 2011). This complex is of particular interest because when in the complex, the pore formation and membrane insertion of PopD is enhanced (Romano et al., 2011; Monopoli, 2014). Formation of this complex in model membranes is irreversible (Monopoli, 2014).

To maximize heterocomplex assembly, translocators must be added simultaneously to model membranes (Monopoli, 2014). Heterocomplex assembly in model membranes is optimized at mildly acidic pH (Romano et al., 2011), however the heterocomplex remains inserted in membranes upon neutralizing the pH (Monopoli, 2014). This allows for study of this complex at neutral pH, allowing for study of this complex in relation to other proteins.

It is not known how the heterocomplex reconstituted in model membranes relates to the translocon inserted in host cells. Here the heterocomplex is studied to determine if it is functional for interaction with other components of the T3S system (specifically: *P. aeruginosa* and the T3S needle). Assessment of these interactions will provide insight into the relationship between the heterocomplex reconstituted in model membranes and the translocon formed in host cell membranes during *P. aeruginosa* infection.

CHAPTER 2

INTERACTION OF MEMBRANE-RECONSTITUTED TRANSLOCATORS WITH ISOLATED *P. AERUGINOSA* T3S COMPONENTS

2.1 Introduction

We have shown that the *P. aeruginosa* translocators PopB and PopD assemble a heterocomplex in model membranes (Romano et al., 2011), however this heterocomplex has not been assessed in relation to its interaction with the rest of the T3S system. One way to evaluate this is to determine if other components of the *P. aeruginosa* T3S system, that are predicted to interact with the translocon, interact with the reconstituted complexes. One of these components is the tip complex. The tip complex is believed to be composed of the protein PcrV, and located at the tip of the needle formed by the protein PscF (Mueller et al., 2005; Pastor et al., 2005). To assess their ability to interact with the heterocomplex, we purified recombinant PcrV protein and initiated attempts to purify needles formed by *P. aeruginosa*.

2.2 Experimental design

Translocator heterocomplexes were reconstituted in model membranes by diluting a premixed solution of PopB and PopD in 6 M urea, into buffer containing liposomes (see Methods 2.5.1) (Romano et al., 2011). This was performed at mildly acidic pH (pH 4.3) to maximize binding and insertion of translocators (Faudry et al., 2006; Romano et al., 2011). The pH was neutralized before adding other T3S components. Previous studies indicate that the heterocomplex, when inserted at pH 4.3, remains stably inserted in membranes upon pH

neutralization (Monopoli, 2014) (Fig 2.1). After incubating with the T3S components, a flotation assay was performed to isolate the membranes (see Methods 2.5.2). Components that bound to the heterocomplexes would be present in the membrane fraction isolated from the flotation assay. As a control, the binding of the added components to the liposomal membranes would be evaluated by exactly the same procedure without adding the translocators.

2.3 Results

2.3.1 Purification of unmodified recombinant PcrV

The needle tip complex in the *P. aeruginosa* T3S system is believed to be made up of PcrV (Mueller et al., 2005). It is hypothesized that PcrV interacts directly with the translocon. Using the protocol outlined in 2.5.3, unmodified recombinant PcrV was purified to approximate 88% homogeneity (C. Sanchez, unpublished data) (Fig 2.2).

2.3.2 Interaction of monomeric PcrV with heterocomplex

The interaction of monomeric PcrV with the membrane-bound translocators was measured using the assay described in 2.2. To control for nonspecific binding of PcrV to membranes, PcrV was incubated with membranes in the absence of the heterocomplex at neutral pH. Membrane-bound protein was separated on an SDS-PAGE and the presence of PcrV was detected with a western blot (Fig 2.3).

No binding of PcrV was seen after addition of monomeric PcrV (Fig 2.3). The absence of binding indicated that oligomeric PcrV or other T3S factors may be necessary for this interaction to occur. However, the addition of media

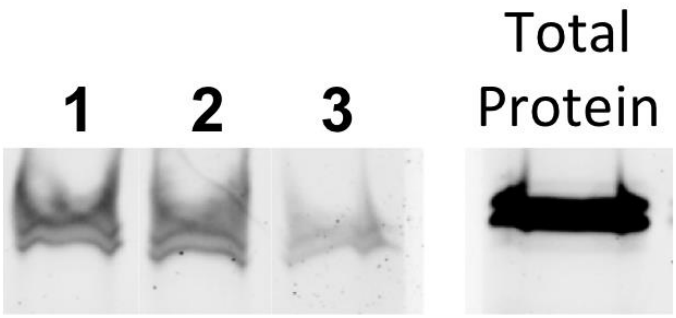

Figure 2.1. PopB in heterocomplex remains bound to membranes after pH neutralization. Heterocomplexes of $PopB^{V191CBodipy}$ and PopD were reconstituted in membranes at pH 4 (lanes 1 and 2) and pH 7 (lane 3) (essentially as in 2.5.1). The pH was neutralized to pH 7 with 2µ 2M Trizma, pH 9.0 (lane 2), or was unchanged (lanes 1 and 3), before performing a flotation assay to isolate membrane bound protein (see Methods 2.5.2). Samples were TCA precipitated and separated on a 10% acrylamide SDS-PAGE. $PopB^{V191CBodipy}$ was detected by scanning with a Typhoon FLA 7000 gel scanner exciting at 488 nm. A sample containing the total amount of $PopB^{V191CBodipy}$ present was included as a comparison (Total Protein). $PopB^{V191CBodipy}$ labeling efficiency was 55%.

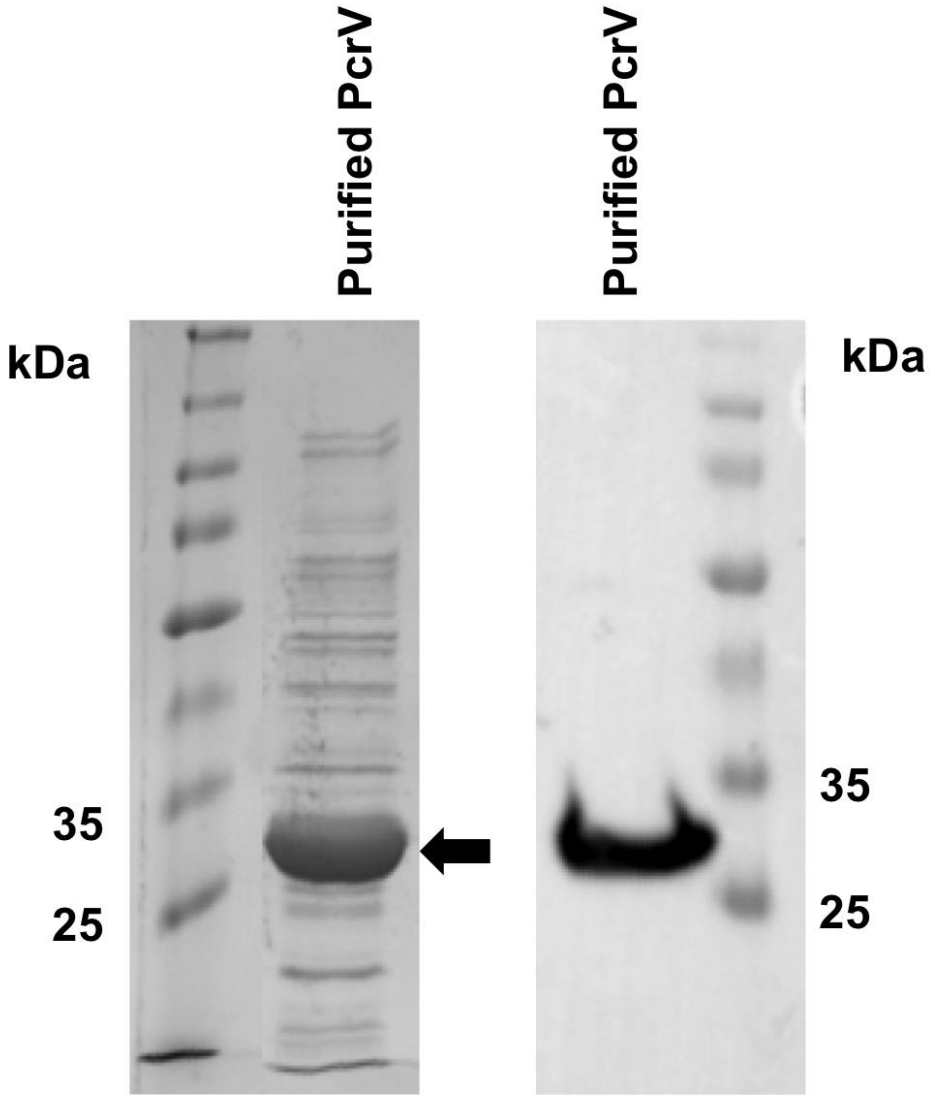

Figure 2.2. Purification of unmodified recombinant monomeric PcrV. Unmodified recombinant monomeric PcrV was purified (see Methods 2.5.3). The purified PcrV was separated on a 10% acrylamide SDS-PAGE and stained with Coomassie (left) or transferred to a polyvinylidene fluoride (PVDF) membrane and detected with polyclonal anti-PcrV antibodies (right). PcrV appears as the dominant band in the gel stained with Coomassie (arrow). Molecular weights are indicated, PcrV estimated molecular weight is 32 kDa.

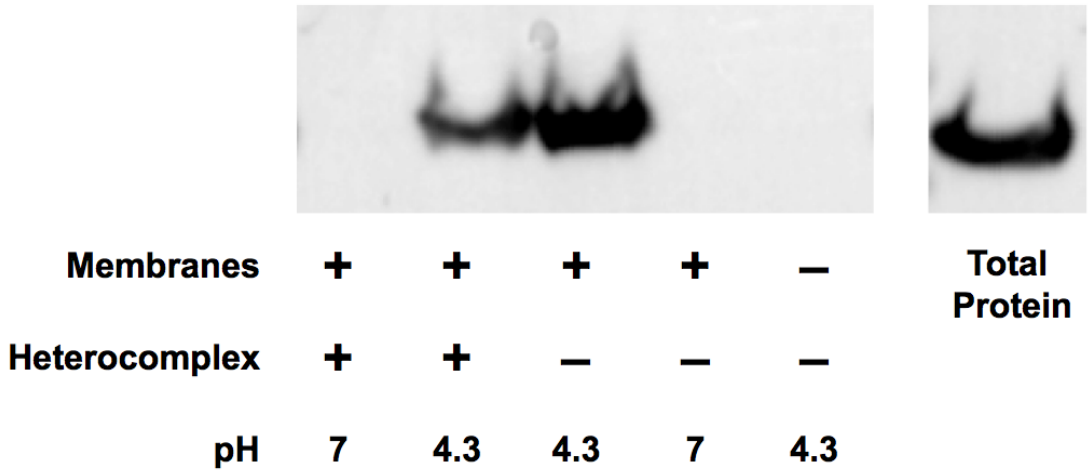

Figure 2.3. Monomeric PcrV does not bind heterocomplex. The translocator heterocomplex was reconstituted in membranes (see Methods 2.5.1) and incubated with monomeric PcrV. Membrane bound protein was isolated with a flotation assay (see Methods 2.5.2), and TCA precipitated before separating on a 10% acrylamide SDS-PAGE and transferring to a PVDF membrane. PcrV was detected with a polyclonal anti-PcrV antibody. Contents of the sample in each lane are indicated at the bottom. A sample containing the total amount of PcrV added was included as a comparison (Total Protein).

containing other factors secreted by *P. aeruginosa* did not affect monomeric PcrV binding (Fig 2.4). Purified oligomeric PcrV was not available, however we evaluated the possibility of using needles that contain the oligomers at the tip. T3S needles can be isolated from *P. aeruginosa* (Pastor et al., 2005; Gebus, 2008) as described below.

2.3.3 Purification of the T3S needle

Previous studies indicate that T3S needles from *P. aeruginosa* and other bacteria can be sheared from the surface of the bacteria mechanically (Gebus, 2008; Blaylock et al., 2010; Hoiczyk and Blobel, 2001). These needles can be further purified from other components using various methods. Two methods were adapted for purification of T3S needles from *P. aeruginosa* (see Methods 2.5.4). The proteins isolated by the needle purification procedures were analyzed initially by SDS-PAGE and Coomassie staining.

The needles are composed of the 9 kDa needle protein, PscF (Pastor et al., 2005). Therefore the presence of a dominant band at 9 kDa on an SDS-PAGE would denote the presence of needles. To resolve this lower molecular weight protein, a Tricine SDS-PAGE was used instead of the traditional Laemmli SDS-PAGE (see Methods 2.5.4.4). This method involves an alternative buffering system that allows for complete separation of proteins smaller than 20 kDa (Schägger and von Jagow, 1987). The Tricine SDS-PAGE provided satisfactory resolution of protein below 10 kDa (Fig 2.5).

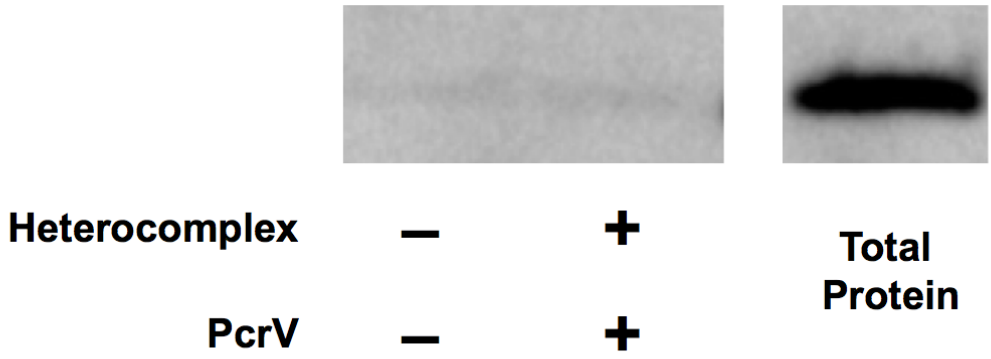

Figure 2.4. Components secreted by *P. aeruginosa* do not enhance PcrV binding to the heterocomplex. The translocator heterocomplex was reconstituted in membranes (see Methods 2.5.1) and incubated with monomeric PcrV along with growth media (2 µg protein). Growth media was obtained after culturing *P. aeruginosa* cultured under T3S inducing conditions (see Methods 2.5.4.1) until an OD_{600} of 0.8 was reached. Membrane bound protein was isolated with a flotation assay (see Methods 2.5.2), and TCA precipitated before separating on a 10% acrylamide SDS-PAGE and transferring to a PVDF membrane. PcrV was detected with polyclonal anti-PcrV antibodies. Contents of the sample in each lane are indicated at the bottom (growth media and membranes were present in both samples). A sample containing the total amount of PcrV added was included as a comparison (Total Protein).

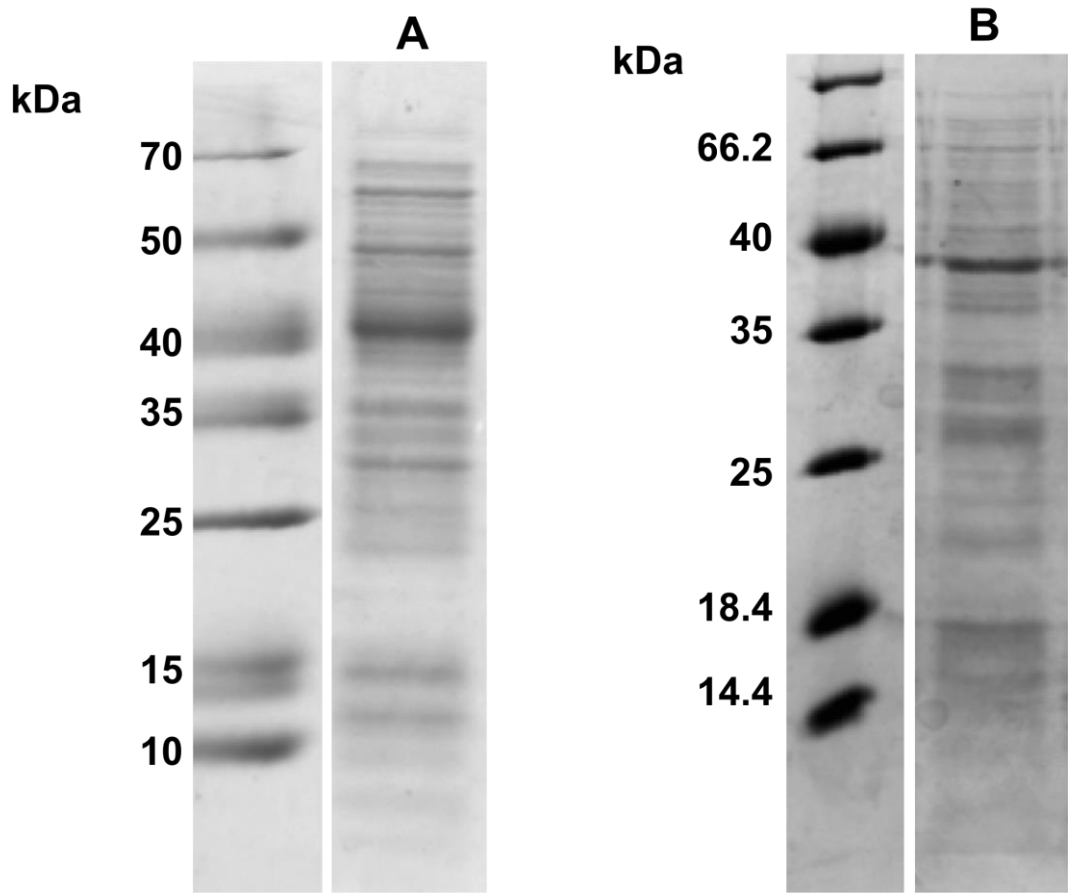

Figure 2.5. Tricine SDS-PAGE is superior to Laemmli SDS-PAGE in separating low molecular weight proteins. Partially purified *P. aeruginosa* T3S needles purified from PA103 using Method A (see Methods 2.5.4.1), were separated using (A) Tricine SDS-PAGE (see Methods 2.5.4.4) or (B) Laemmli SDS-PAGE and stained with Coomassie. Both gels contained 15% acrylamide. Molecular weights are indicated. The predicted molecular weight of the needle protein, PscF, is 9 kDa.

2.3.3.1 *P. aeruginosa* strain selection

The needles were isolated from the surface of *P. aeruginosa*, but selection of an appropriate strain was necessary to facilitate needle purification. Since needle purification involves sheering of the needle, it is necessary to limit contaminants that can be obtained in the sheered in the process. One major component is the flagella. To avoid contamination with flagella, the aflagellated strains PA103 and the mutant CHAΔFliC were selected. PA103 is an aflagellated clinical isolate strain (Liu and Hsieh, 1973; Montie et al., 1982). CHA is a cystic fibrosis clinical isolate strain (Toussaint et al., 1993). The CHAΔFliC mutant does not express the flagellin protein FliC (Pastor et al., 2005). After sheering protein from the cell surface, a greater amount of 9 kDa protein was obtained from CHAΔFliC than from PA103 (Fig 2.5), suggesting a greater amount of PscF, and therefore needles, were obtained from this strain. Therefore the CHAΔFliC strain was selected for further needle purifications. Additionally, needles have been purified from this strain previously (Pastor et al., 2005).

There was a large amount of contaminating protein present after purification of the needles (Fig 2.6). It would be ideal to utilize a mutant strain that produces more T3S apparatuses on the surface, to increase the amount of needles. It is possible to increase T3S apparatus production by stimulating T3S, which can be induced in *P. aeruginosa* by cultivating in a low calcium environment (Cornelis et al., 1987; Pettersson et al., 1996).

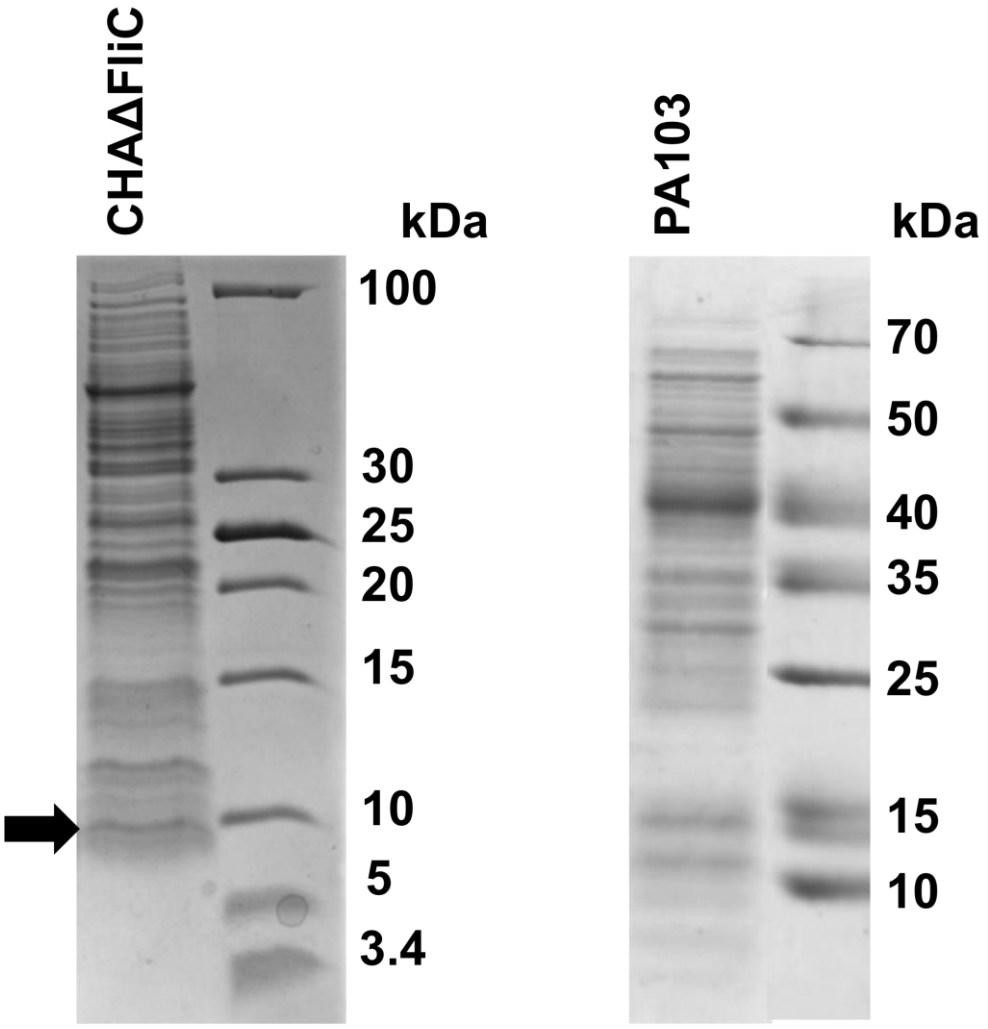

Figure 2.6. A greater amount of 9 kDa protein was obtained from the surface of CHAΔFliC than PA103. CHAΔFliC and PA103 were cultivated as described in 2.5.4.1. Proteins were isolated from the surfaces of the bacteria by vortexing for 5 minutes at 4°C. Twenty microliters of undiluted protein were run on a 15% acrylamide Tricine SDS-PAGE and stained with Coomassie (see Methods 2.5.4.4). The *P. aeruginosa* strain from which the sample was isolated is indicated at the top. A strong 9 kDa band indicating the presence of PscF is seen in proteins isolated from CHAΔFliC (arrow), but not from PA103. Molecular weights are indicated.

2.3.3.2 Shearing needles and purification

Previous studies indicate that T3S needles can be sheared by moderate agitation of the cells. These methods include resuspending the cells in buffer (Pastor et al., 2005; Baylock et al., 2010), passage through a needle (Espina et al., 2006), or vortexing (Journet et al., 2003). Other, more aggressive methods have been used including sonication and homogenization (Jessen et al., 2014; Hoiczyk and Blobel, 2001). Different needle shearing methods were examined (see Methods 2.5.4.3). The results from all methods except sonication are the same as those with gentle resuspension in buffer (Fig 2.7). Sonication increased the amount of protein (Fig 2.7, lane 5), but a similar protein distribution was observed by SDS-PAGE of proteins obtained by all shearing methods. A 9 kDa protein was obtained from all of the shearing methods. This suggests that simply washing the cells in buffer is sufficient to remove needles, and a more aggressive treatment is unnecessary and may only serve to increase contaminating protein.

The large amount of contaminants in the needle purification could not be removed. Type III Secretion was stimulated to induce greater needle production, but this also increases protein secretion. A similar protein distribution was observed by SDS-PAGE of proteins isolated from the growth media, as from the needle purification (although the growth media was less concentrated) (Fig 2.7, lane 6). Therefore it is likely that a large amount of the contaminating protein was secreted protein. This indicates that the method of purification was not sufficient to remove the secreted components. Washing the bacteria to remove the secreted components is not ideal as even gentle washing is likely to remove

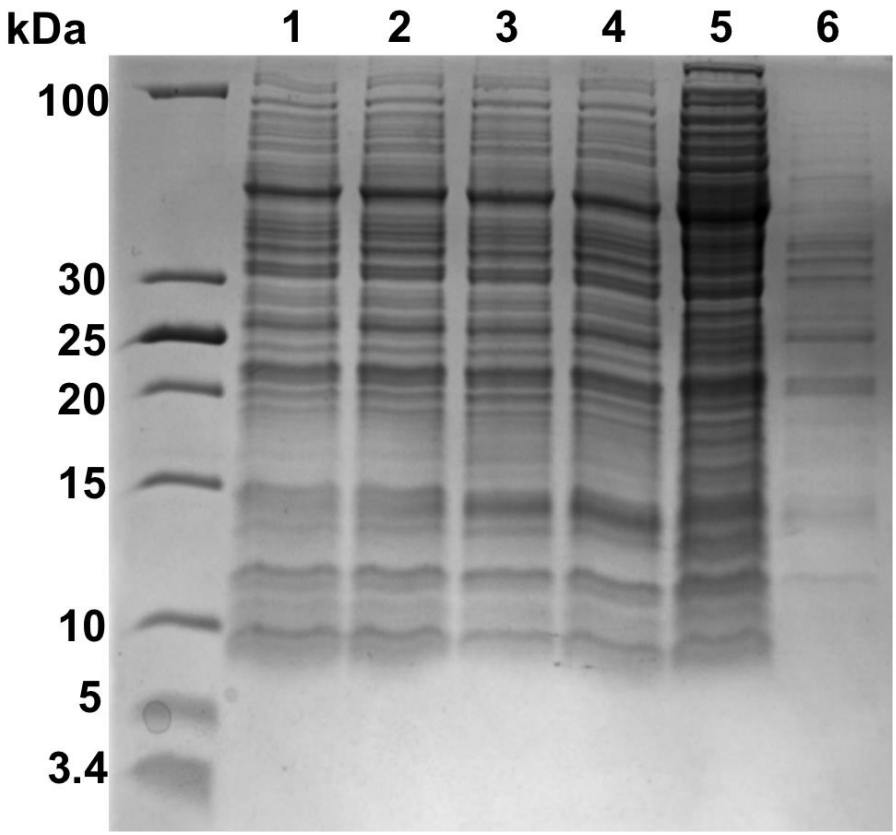

Figure 2.7. Alternative needle shearing methods did not affect protein obtained. CHAΔFliC was cultivated as stated in 2.5.4.1. Needles were sheared from the surfaces of bacteria by resuspension (lane 1), homogenization (lane 2), passing through an 18G needle (lane 3), vortexing (lane 4), or sonication (lane 5) (see Methods 2.5.4.3). Growth media isolated from the same cultivated cells was also analyzed (lane 6). Samples were run on a 15% acrylamide Tricine SDS-PAGE and stained with Coomassie (see Methods 2.5.4.4). Molecular weights are indicated.

needles. The large amount of contaminants also hindered the ability to identify needles by transmission electron microscopy.

The final step of the purification protocol involves centrifugation of the protein, after which the pellets containing the needles were resuspended in buffer. A similar protein distribution was observed by SDS-PAGE of the supernatant as the purified needles (Fig 2.8, lanes 2 and 3), suggesting these isolates contained the same proteins. Additionally, the needle tip component, PcrV, was not enriched, and it was present in equal amounts in the supernatant and pellet (Fig 2.9). Therefore it is likely that this step did not effectively remove contaminating proteins or concentrate the needles. No pellets were observed after centrifugation even when the starting culture volume was increased eight fold, so it is likely that the resuspended pellet was merely residual supernatant that was diluted. It is likely that the amount of needles present at the beginning of the purification was very low, so no pellets were obtained. Protein purified using Method B (see Methods 2.5.4.2) did not enter the SDS-PAGE gel, and it is therefore unclear whether needles were successfully purified using this alternative method.

2.4 Discussion and future research

While the unmodified recombinant PcrV purification was successful, it was difficult to purify the T3S needles. While a band at 9 kDa was present when the sample was separated on an SDS-PAGE, it was unclear if this protein was the needle protein, PscF. Transmission electron microscopy could be performed with the purified fractions, but the large amount of contaminating proteins would likely

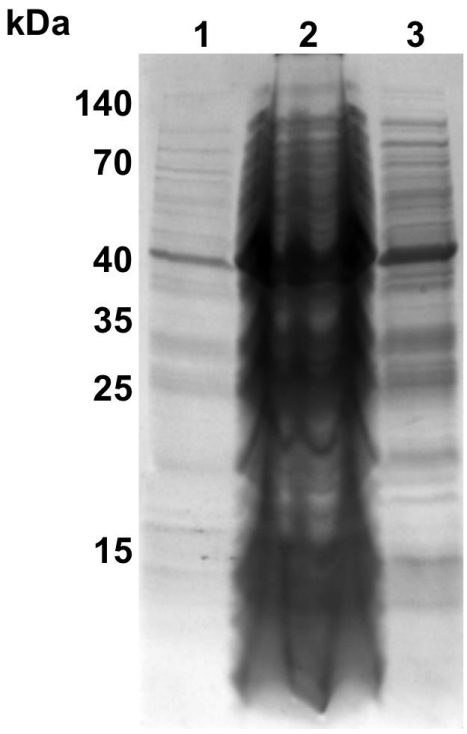

Figure 2.8. Needle precipitation by centrifugation. T3S needles were purified from CHAΔFliC using Method A (see Methods 2.5.4.1). Needles were sheared from the surfaces of bacteria by vortexing (see Methods 2.5.4.3). The needles were precipitated by centrifugation (20,000xg, 30 min, 4°C). The pellet containing the purified needles was resuspended and analyzed (lane 1). The supernatant after precipitation was also analyzed (lane 2). The supernatant contained a large amount of protein and was diluted 1:10 (lane 3). Samples were run on a 15% acrylamide Tricine SDS-PAGE and stained with Coomassie (see Methods 2.5.4.4). Molecular weights are indicated.

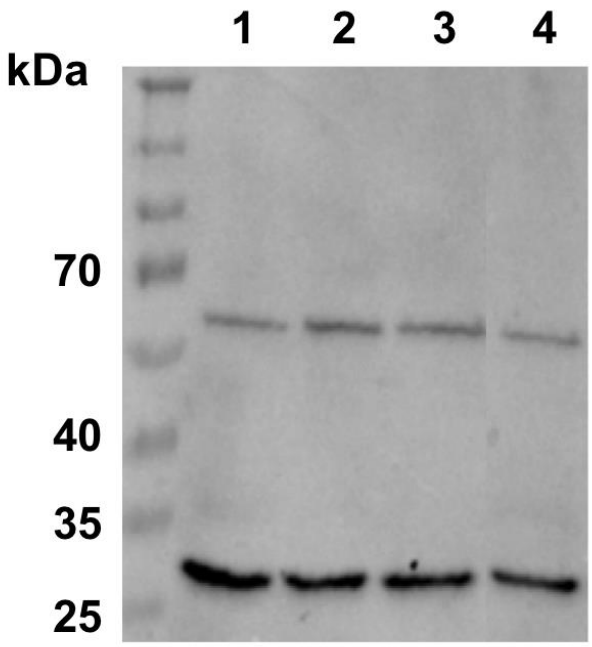

Figure 2.9. PcrV is present in needle fraction but not enriched with purification. T3S needle purification fraction was prepared from CHAΔFliC using Method A (see Methods 2.5.4.1). Needles were sheared from the surfaces of bacteria by vortexing (see Methods 2.5.4.3). Aliquots from the purification steps were analyzed before filtering (lane 1) and after filtering (lane 2). The supernatant after precipitation of the needles was analyzed (lane 3) along with the purified needle fraction (lane 4). Samples were run on a 10% acrylamide SDS-PAGE and transferred to a PVDF membrane. PcrV was detected with polyclonal anti-PcrV antibodies. Molecular weights are indicated at left. Interestingly, PcrV dimers were present in the needle purification fractions.

make it difficult to visualize any needles present. The purification method must be modified to decrease contaminants and concentrate PscF.

The failure to purify T3S needles suggests that needles may not be present at the start of the purification. This could be determined if a western blot were performed with an anti-PscF antibody, which is not available. No significant amount of protein was lost through the steps of the purification, so it is unlikely that the needles were lost. It is also unlikely that the bacteria were not producing needles. It is more likely that a low amount of needles were produced, because the cells were not grown for a long enough time. It is also possible that the needles were not sheared successfully from the bacteria, but unlikely. Washing the cells is sufficient to remove needles from *Yersinia* (Journet et al., 2003), and was the method used to shear needles from *P. aeruginosa* previously (Pastor et al., 2005). In fact, free needles are found in the culture media of *Shigella flexneri*, suggesting that T3S needles are quite fragile (Cordes et al., 2003; Olive et al., 2007). Gentler shearing methods, like washing the cells, yielded similar results to more aggressive methods like sonication and homogenization. This suggests that very little disruption is needed to remove proteins attached to the surface of *P. aeruginosa*. Furthermore, sonication and homogenization have potential to lyse the cells, increasing contaminants significantly.

Monomeric PcrV alone, and in the presence of putative T3S needles did not bind the PopB:PopD heterocomplex reconstituted in model membranes. If this interaction does occur, it is likely that oligomeric PcrV is necessary, as the tip complex is an oligomer of PcrV (Mueller et al., 2005). The T3S needles contain

the tip complex and therefore could provide the oligomeric PcrV necessary to test this hypothesis. In fact, PcrV dimers were detected in purified needle samples. Purification of the T3S needles was difficult and therefore, unless the needles are purified, an alternative source of oligomeric PcrV is needed to test this hypothesis. One possibility is to measure the interaction directly with *P. aeruginosa* by determining if the cells can interact with the heterocomplex through the T3S needle.

These results suggest that the heterocomplex formed in model membranes does not interact with the *P. aeruginosa* T3S system, but it is possible that these T3S components cannot bind the heterocomplex independently. The T3S system is a tightly regulated system, and the infection mechanism likely requires very precise interaction of the needle with the translocon, since no secreted protein is lost into the extracellular environment. Therefore it is likely that *P. aeruginosa* modulates the interaction with the translocon, and therefore must be present for the needle to engage with it.

2.5 Methods

2.5.1 Reconstituting the heterocomplex in model membranes

Binding reactions (75 µL) were prepared by equilibrating liposomes (65% POPC, 20% cholesterol, 15% POPS, Avanti Polar Lipids) to a final concentration of 5.94 mM in 50 mM sodium acetate, pH 4.3. The translocators were added to a final concentration of 900 nM PopB and 90 nM PopD from a premixed stock in 6 M urea, 20 mM Tris pH 8.0, 20 mM glycine, 20 mM NaCl. After a 15 minute

incubation at 23°C, the pH of the binding reactions were neutralized with 2 µL of 2 M Trizma, pH 9.0.

2.5.2 Isolating membrane-bound components with a flotation assay

Flotation assay was performed as in Romano et al., 2011. Purified PcrV was added to membrane bound heterocomplexes to a final concentration of 900 nM (in 75 µL). The sample was incubated for 15 minutes at 23°C before mixing with 225 µL of a 67% sucrose solution. A 40% sucrose solution was layered on top (360 µL), followed by a 4% sucrose solution (240 µL). All sucrose solutions were prepared in 30 mM sodium acetate, 30 mM 2-(N-morpholino)ethanesulfonic acid (MES) pH 7.0. The sample was centrifuged for 50 minutes at 90,000xg, 4°C. The sucrose gradient was separated into 300 µL aliquots, removing from the bottom layer. A trichloroacetic acid (TCA) precipitation was performed with the top layer containing the membrane-bound fraction using 15 µg of bovine serum albumin as a carrier protein. A western blot was performed to detect for the presence of PcrV in the membrane-bound fraction using a polyclonal anti-PcrV antibody.

2.5.3 Unmodified recombinant PcrV purification

Twenty milliliters of an overnight culture of the *E. coli* expression strain BL21(DE3)star (Invitrogen) expressing a pAH50-PcrV plasmid in Luria-Bertani (LB) broth was diluted in 1 L LB containing 100 µg/mL ampicillin. The culture was incubated at 37°C with shaking (150 rpm) until the optical density at 600 nm (OD_{600}) reached 0.60 (~3.5 hours), at which point Isopropyl α-D-thiogalactopyranoside (IPTG, Gold Biochemicals) was added to a final

concentration of 1 mM to induce PcrV expression. After an additional four hours of incubation, the cultures were harvested by centrifugation (4,400xg, 15 min, 4°C). The cell pellets were resuspended in 50 mM Tris-HCl, pH 8.8, and protease inhibitors were added (40 µg/mL PMSF and 170 µg/mL Benzamididine). The cells were lysed by passing through a Microfluidics M-110L microfluidizer two times at 18,000 psi. The cell lysate was centrifuged (20,000xg, 15 min, 4°C) retaining the supernatant.

An ammonium sulfate precipitation with 30-70% gradient was performed with the cleared cell lysate as follows. Ammonium sulfate was added with constant stirring to a final concentration of 30% w/v at 4°C. After stirring for 30 min, the solution was centrifuged (10,000xg, 10 min, 4°C), and the supernatant was retained. The precipitation was repeated with 70% ammonium sulfate, and the pellet was retained after centrifugation. The pellet was resuspended and dialyzed against 50 mM Tris-HCl, 100 mM NaCl, pH 8.0.

The sample was loaded onto a Q-Sepharose AEC column (GE Healthcare) equilibrated with 50 mM Tris-HCl, pH 8.0. A 50 mL gradient of 50-500 mM NaCl in 50 mM Tris-HCl, pH 8.0 was applied to elute PcrV. PcrV eluted in 150-300 mM NaCl. Peak fractions (detected by absorbance at 280 nm) were separated on an SDS-PAGE to confirm that PcrV was present, before pooling and dialyzing overnight against 50 mM Hepes, 150 mM NaCl, pH 7.5. The sample was concentrated with a Vivaspin 20 concentrator with a 10 kDa cutoff (GE Healthcare), and applied to a Superdex-200 GL 10/30 column (GE Healthcare). PcrV eluted in 11-14 mL. Peak fractions (detected by absorbance at

280 nm) were separated on an SDS-PAGE to confirm that PcrV was present, before pooling and dialyzing overnight against 20 mM MES, pH 6.5. The dialyzed sample was loaded onto a Hi-Trap Q-HP column (GE Healthcare) equilibrated in 20 mM MES, pH 6.5. A 50 mL gradient of 0-500 mM NaCl in 20 mM MES, pH 6.5 was applied to elute PcrV. PcrV eluted in 475-500 mM NaCl. Peak fractions (detected by absorbance at 280 nm) were separated on an SDS-PAGE to confirm that PcrV was present, before pooling and adding glycerol (to 10% v/v). The purified protein was aliquoted and flash frozen in liquid nitrogen before storage at -80°C.

2.5.4 Needle purification

2.5.4.1 Method A

Method A was adapted from Gebus, 2008. An overnight culture of *P. aeruginosa* (CHAΔFliC or PA103) in LB was diluted to an OD of 0.1 in 1 L LB. A low calcium environment was created to induce T3S by chelating calcium with EDTA (5 mM final concentration). The CHAΔFliC strain was graciously provided by Ina Attrée (Université Grenoble Alpes). After chelating the calcium, the media was supplemented with $MgCl_2$ (20 mM final concentration). Cells were cultivated at 37°C with shaking (150 rpm) until the cells reached an OD_{600} of 0.8 (~3 hours). The cells were pelleted (8,000xg, 10 min, 4°C). Cells were resuspended at 4°C in 10 mL 50 mM Tris-HCl, pH 7.5 and needles were sheared. Cells were removed by centrifuging (8,000xg, 10 min, 4°C) followed by passing the supernatant through a 0.45 µm acetate membrane (Whatman). Needles were precipitated by centrifugation (20,000xg, 30 min, 4°C). The pellet was resuspended in 1 mL 50

mM Tris-HCl, pH 7.5. Needles were aliquoted and flash frozen in liquid nitrogen before storing at -80°C.

2.5.4.2 Method B

Method B was adapted from Pastor et al., 2005. *P. aeruginosa* (CHAΔFliC or PA103) cells were cultivated as in 2.5.4.1. The cells were pelleted (5,700xg, 10 min, 4°C). Cells were resuspended with 30 mL 20 mM Tris-HCl, pH 7.5 and needles were sheared. Cells were removed by centrifuging (5,700xg, 10 min, 4°C) followed by passing the supernatant through a filter as in 2.5.4.1. Needles were pelleted by centrifugation (18,000xg, 30 min, 4°C). The pellet was resuspended in 15 mL Tris-HCl 20 mM, pH 7.5 with 0.1% (w/v) 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS). Insoluble protein was removed by centrifugation (50,000xg, 30 min, 4°C). The needles were precipitated on ice for 1 hour with 10% (w/v) polyethylene glycol (PEG) 6000 and 100 mM NaCl. Needles were collected by centrifugation (50,000xg, 30 min, 4°C). The pellet was resuspended in 1 mL 20 mM Tris-HCl, pH 7.5. Needles were aliquoted and flash frozen in liquid nitrogen before storing at -80°C.

2.5.4.3 Needle shearing methods

Several needle-shearing methods were attempted:

- **Washing:** Cells were resuspended gently in buffer (50 mM Tris-HCl, pH 7.5) by incubating on ice with shaking (100 rpm) until the pellets were completely resuspended (Pastor et al., 2005; Journet et al., 2003).
- **Vortexing:** Cells were resuspended in buffer (50 mM Tris-HCl, pH 7.5) and vortexed for 5 minutes at 4°C (in a cold room). Cells were vortexed for

five 1 minute intervals with a 1 minute incubation on ice between (Gebus, 2008; Blaylock et al., 2010)**.**

- **Sonication:** Cells were resuspended in buffer (50 mM Tris-HCl, pH 7.5) and the cell suspension was sonicated at maximum amplitude for one second on ice. This was repeated five times.
- **Homogenization:** Cells were resuspended in buffer (50 mM Tris-HCl, pH 7.5) and the cell suspension was transferred to a glass homogenizer and the sample was homogenized for fifty cycles (Jessen et al., 2014; Cordes et al., 2003).
- **Passing through a needle:** After resuspending cells in buffer, cells were passed through a 25G needle five times (Zhang et al., 2007; Espina et al., 2006; Mueller et al., 2005).

2.5.4.4 Tricine SDS-PAGE

The Tricine SDS-PAGE protocol was adapted from Schägger and von Jagow, 1987. The gel recipe is included in Table 2.1. A 15% acrylamide separating gel was poured and allowed to polymerized for 30 minutes before pouring a 4% stacking gel. Samples were prepared with sample buffer (180 mM Trizma, 15% v/v glycerol, 7.5 mM EDTA, 0.02% w/v bromophenol blue, 3.65% w/v SDS), heated to 95°C for 5 minutes, and loaded onto the gel. The gel was run in a Mini-Protean gel running apparatus (Bio-Rad). The cathode buffer contained 100 mM Tris, 100 mM Tricine, 0.1% SDS, pH 8.25. The anode buffer contained 100 mM Tris, 22.5 mM HCl, pH 8.9. Gels were run at 60 V until the dye

front entered the stacking gel after which the voltage was increased to 100 V until the dye front reached the bottom of the gel.

	4% Stacking	15% Separating
Acrylamide (30%)	0.83	5
0.5 M Tris 8.8	—	1.875
0.5 M Tris 6.8	1.26	—
Add water to final volume	2.77	2.925
SDS (10%)	0.05	0.1
Polymerize by adding:		
APS (10%)	0.05	0.1
TEMED	0.004	0.004

Table 2.1. Gel recipe for Tricine SDS-PAGE. Adapted from Schägger and von Jagow, 1987. The components of the stacking and separating gel are indicated along with the volume in mL. Recipe makes two 8.3 cm x 7.3 cm 15% acrylamide gels.

CHAPTER 3

INTERACTION OF *P. AERUGINOSA* WITH TRANSLOCATORS RECONSTITUTED IN MODEL MEMBRANES

3.1 Introduction

Monomeric PcrV did not interact with the translocator heterocomplex reconstituted in model membranes (see 2.3.2). It is likely that PcrV must be in an oligomeric state to interact with the heterocomplex, however oligomeric PcrV was difficult to isolate (C. Sanchez, unpublished data), and T3S needles (containing the PcrV tip complex) were also difficult to isolate. As an alternative method to study this interaction, live *P. aeruginosa* cells were utilized. By incubating *P. aeruginosa* with the translocator heterocomplexes reconstituted in model membranes, the functionality for interaction of the heterocomplex with other components of the T3S system was studied.

3.1.1 Experimental design

To measure *P. aeruginosa* interaction with the membrane-reconstituted heterocomplex, binding of the bacteria to liposomes was measured. The *P. aeruginosa* strain PA103ΩPopD was selected for this assay, because it does not produce the translocators PopB and PopD. This prevented any interactions between the bacteria and liposomes through translocators produced by the bacteria. To track liposomes, a fluorescent lipid was added.

3.1.1.1 Liposome composition

It has been shown that liposomes could interact with bacteria (Drulis-Kawa, and Dorotkiewicz-Jach, 2010). Therefore it was important to select a

liposome composition that minimized interactions between the liposomes and *P. aeruginosa* for binding through the translocator complexes to be accurately measured. Bucki et al. showed that inclusion of a charged fluorescent label increases interaction with *P. aeruginosa* (2004). Additionally, previous studies indicate *P. aeruginosa* binds to liposomes containing Rhodamine-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (DHPE) (F. Romano, unpublished results). This phospholipid contains a charged Rhodamine moiety on the head group such that it is accessible to the bacteria. NBD-cholesterol was selected for these experiments because the NBD label is buried in the lipid bilayer, and no charged residues are exposed on the membrane surface. This fluorophore selection will minimize any dye-bacteria interaction. The lipid composition POPC:cholesterol:POPS:NBD-cholesterol at molar ratio 65:19.8:15:0.2 was selected as it maximizes assembly of the translocator complexes in model membranes (Romano et al., 2011).

3.1.1.2 Heterocomplexes cannot be formed in the absence of homo-oligomers

The translocators were reconstituted in membranes before incubating with bacteria. The PopB:PopD heterocomplex is formed by incubating one translocator (PopB) in a ten times excess of the other (PopD); this ensures all of the PopD present is in a heterocomplex, so the amount of heterocomplex present in a sample is known (Romano et al., submitted). Since the heterocomplex is a complex of eight PopB and eight PopD, only 18% of the total protein present was in a heterocomplex, and the remaining 82% consisted of PopB homo-oligomers

(Fig 3.1, A). Therefore binding to the PopB homo-oligomers contributed to the signal obtained when measuring binding to the heterocomplex, so binding to PopB homo-oligomers (in the absence of PopD) was also measured. Binding to PopD homo-oligomers was measured as well for a comparison.

3.1.1.3 Assay to measure *P. aeruginosa*-liposome interactions

The binding of *P. aeruginosa* to liposomes was measured as follows. The translocators were reconstituted in liposomes (Fig 3.2, A), and the liposomes were then incubated with bacteria (Fig 3.2, B). Cells were pelleted along with any bound liposomes (Fig 3.2, C). The pellets were resuspended and liposome binding was measured by measuring the fluorescence emission intensity of the liposomes (Fig 3.2, D). Higher fluorescence emission intensity indicated greater liposome binding. These measurements were also performed with liposomes without membrane-bound translocators to measure nonspecific binding of the bacteria to liposomes.

3.2 Results and discussion

3.2.1 Binding of *P. aeruginosa* to liposomes is enhanced when the heterocomplex is present

The interaction between PA103ΩPopD and liposomes containing reconstituted translocator complexes was measured using the assay described in 3.1.1.3. Binding to the heterocomplex as well as both PopB and PopD homo-oligomers was measured. Binding was greatest when the heterocomplex was present (Fig 3.3, red). The relative fluorescence emission for the heterocomplex sample was 36% greater than that of the PopB homo-oligomer sample. Since

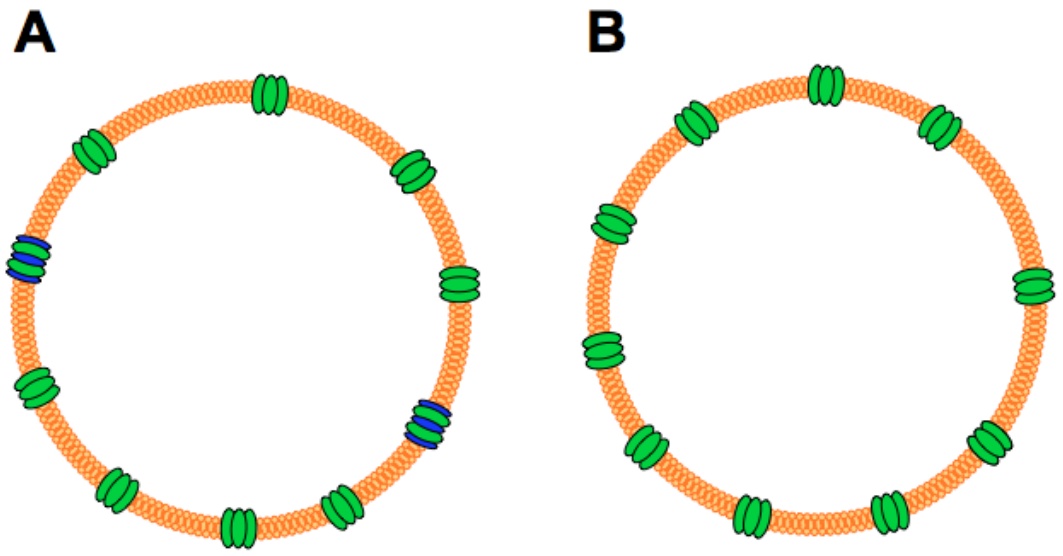

Figure 3.1. Heterocomplexes cannot be formed in the absence of homo-oligomers. PopB and PopD form homo-oligomers as well as heterocomplexes in model membranes (Romano et al., submitted). Therefore, to reconstitute the PopB:PopD heterocomplex in liposomes, PopB was added in a ten times excess of PopD. (A) When forming the heterocomplex, all of the PopD (blue) was incorporated into heterocomplexes while PopB (green) also formed homo-oligomers on the liposomes (yellow). Therefore there were more PopB homo-oligomers than heterocomplexes present, and the number of PopB homo-oligomers present was about the same as the number present in (B) the sample containing only PopB homo-oligomers.

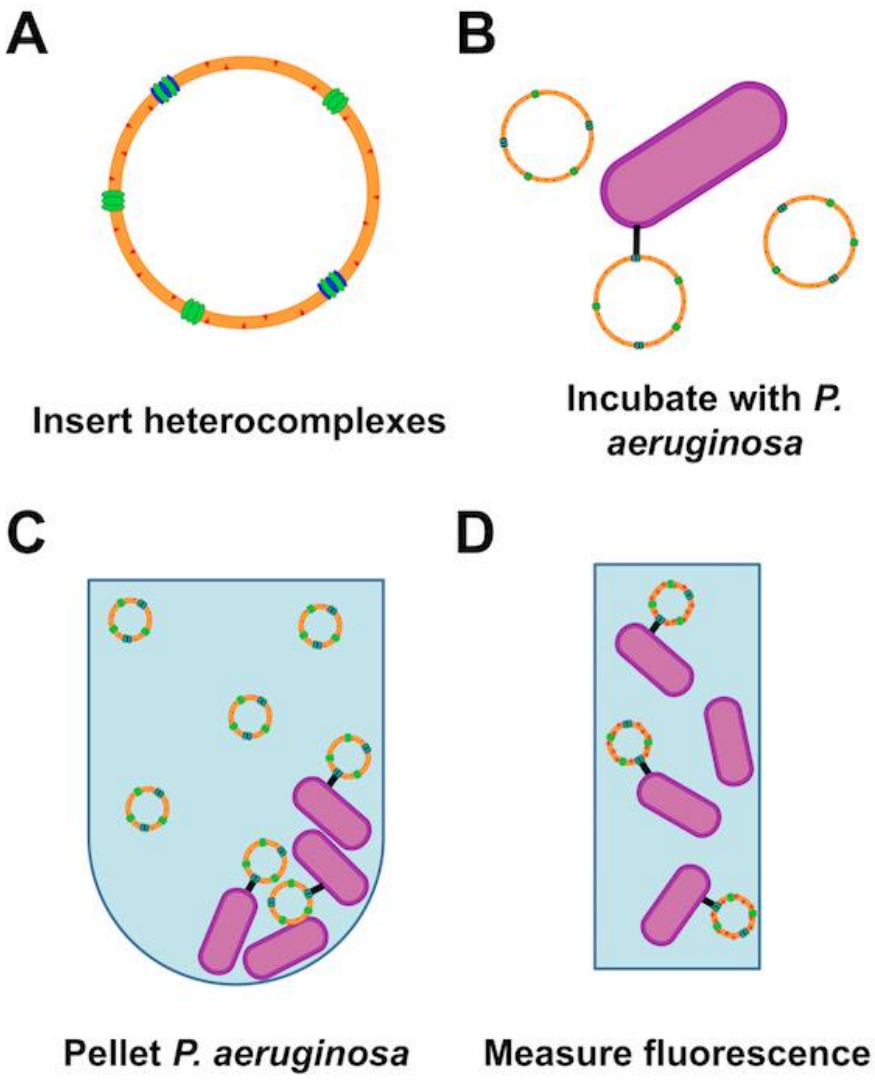

Figure 3.2. Measuring the interaction between *P. aeruginosa* and the heterocomplex. (A) Translocator complexes of PopB (green) and PopD (blue) were reconstituted in liposomes (yellow) labeled with NBD-cholesterol (red dots). (B) *P. aeruginosa* (purple) was incubated with liposomes to allow for interaction with the T3S needle (black). (C) Cells were pelleted along with bound liposomes. (D) The pellet was resuspended and fluorescence emission of NBD-cholesterol was measured to measure the amount of liposome binding. Binding to liposomes without translocators was measured in the same way. Model not drawn to scale.

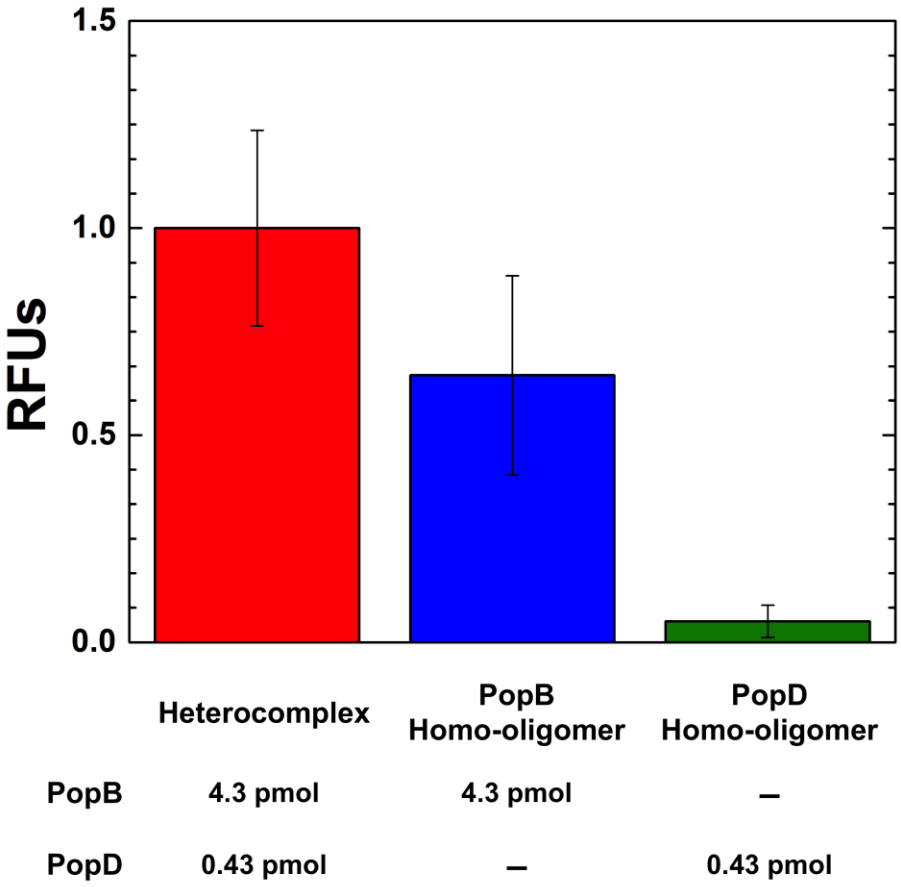

Figure 3.3. *P. aeruginosa* binds to Liposomes with membrane-bound translocators. *P. aeruginosa* interactions with fluorescently-labeled liposomes containing translocator heterocomplexes (red) or homo-oligomers of PopB (blue) or PopD (red) were measured with fluorescence spectroscopy (Methods section 3.4.1). Translocator complexes (homo-oligomers and heterocomplexes) were reconstituted on liposomes (amounts in each sample indicated at bottom). Protein:lipid ratio was 1:6000, with lipid composition POPC:cholesterol:POPS:NBD-cholesterol at molar ratio 65:19.8:15:0.2. PA103ΩPopD was grown to OD_{600} of 1.2 under conditions that induced T3S (see Methods 3.5.4.1). Cells were washed and diluted in PBS to OD_{600} of 0.25 before incubating with the liposomes. Fluorescence emission intensity was normalized to the maximum fluorescence value. Binding to liposomes lacking translocators was subtracted. Error bars indicate the standard deviation between at least three independent measurements.

only 18% of the heterocomplex sample consists of heterocomplexes, this large increase in fluorescence was due to a small amount of protein.

Binding to PopB and PopD homo-oligomers appears to be dependent on the amount of protein present. The amount of PopB was ten times greater than PopD in their respective homo-oligomer samples, and the relative fluorescence emission of the sample containing PopD homo-oligomers was 8% of that of the PopB homo-oligomer sample (Fig 3.3, blue and green). This dependence on protein amount was not true for the sample containing heterocomplexes, which only contained 10% more protein than the PopB homo-oligomer sample. These results indicate that binding to of *P. aeruginosa* to liposomes is enhanced in the presence of the heterocomplex compared to the homo-oligomers.

3.2.2 BSA blocked *P. aeruginosa* interactions with PopB and PopD independently of their oligomeric arrangement

It was possible that the interactions seen between bacteria and liposomes were due to nonspecific protein-protein interactions. Bovine serum albumin (BSA) can be added to cells to block nonspecific protein-protein interactions (Deng et al., 2007). BSA was added to *P. aeruginosa* to a final concentration of 1% before incubating with liposomes. BSA did not affect binding to liposomes without translocators (data not shown). When BSA was present, the relative fluorescence emission of the sample containing heterocomplexes decreased to 0.20 ± 0.03 RFUs (Fig 3.4, purple). The relative fluorescence emission of the PopB homo-oligomer sample decreased similarly, to 0.25 ± 0.034RFUs (Fig 3.4, orange). In both cases, binding was not blocked entirely.

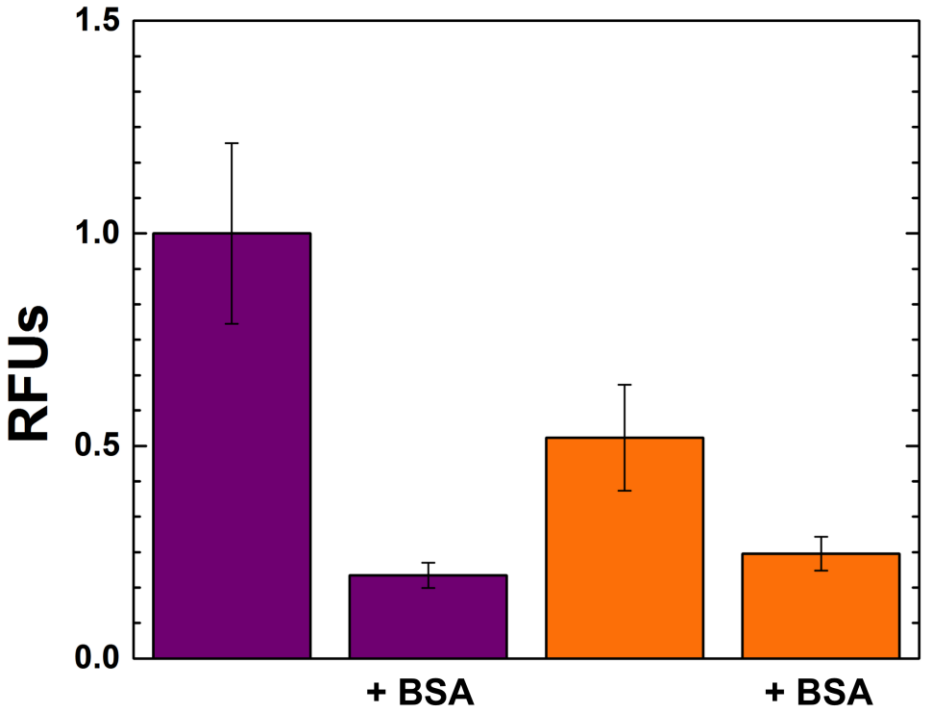

Figure 3.4. Adding BSA to *P. aeruginosa* blocked binding to translocators. *P. aeruginosa* interactions with liposomes with membrane-bound translocator complexes after blocking cells with 1% BSA (+BSA) or not, were measured using fluorescently labeled liposomes (see Methods 3.4.1). Heterocomplexes (purple) and PopB homo-oligomers (orange) were reconstituted on liposomes (heterocomplex: 4.3 pmol PopB and 0.43 pmol PopD), (homo-oligomer: 4.3 pmol PopB). Protein:lipid ratio was 1:6000, with lipid composition POPC:cholesterol:POPS:NBD-cholesterol at molar ratio 65:19.8:15:0.2. PA103ΩPopD was grown to OD_{600} of 1.2 under conditions that induced T3S, cells were washed and diluted in PBS with or without 1% BSA to OD_{600} of 0.25 before incubating with proteoliposomes. Fluorescence emission intensity was normalized to the maximum fluorescence value. Nonspecific binding to liposomes lacking translocators was subtracted. Error bars indicate the range between at two independent measurements.

These results do not show any specificity for *P. aeruginosa* binding to the heterocomplex compared to the PopB homo-oligomer, as the binding enhancement seen in the presence of the heterocomplex was lost upon addition of BSA. One percent BSA blocks a large amount of protein-protein interactions between *P. aeruginosa* and the translocators in either oligomeric state, suggesting that at least some of interactions between are nonspecific.

It is possible that BSA blocked specific interactions with complexes reconstituted in liposomes. BSA is commonly used to block nonspecific interactions when host cells are present (Raybourne and Bunning, 1994). In the presence of a host cell, bacterial adhesins can recognize the host and aid in the bacterial-host interaction. In this study, model membranes were selected in place of eukaryotic cells. Therefore factors that normally would aid in the cellular interactions are not present, and therefore BSA could have a more significant effect on binding, such as interfering with specific protein-protein interactions.

3.2.3 *P. aeruginosa* bound translocator complexes without the T3S apparatus

It is possible to determine if binding to the membrane-reconstituted heterocomplex is dependent on the T3S system, by measuring the interactions with a mutant that does not produce the T3S apparatus. The PAKΔPscC mutant was selected for these experiments because it lacks the secretin component of the T3S apparatus and therefore does not produce the apparatus or secrete any effectors (Lee et al., 2005). Wild-type PAK was selected as a control.

Binding of *P. aeruginosa* to liposomes with and without the heterocomplex was compared. In the previous experiments (3.2.1 and 3.2.2) nonspecific binding to liposomes was subtracted from the measurements. Here the binding of the T3S apparatus was in question, not the translocator complexes present, and therefore liposome binding was included in the comparison. The relative fluorescence of the sample containing PAK plus liposomes with or without translocators was similar (Fig 3.5, blue), suggesting PAK bound liposomes with and without translocators equally. The relative fluorescence of the sample containing PAKΔPscC plus liposomes was lower when the heterocomplexes were absent (Fig 3.5, red). This suggests that binding of the PAKΔPscC mutant increased when heterocomplexes were present (Fig 3.5, red). These results suggest that *P. aeruginosa* does not bind to the heterocomplex through the T3S apparatus.

The heterocomplex sample contained PopB homo-oligomers, since PopB was added in excess to force all of the PopD into heterocomplexes (Fig 3.1, A). In some bacterial species, T3S translocators, when in a different oligomeric state, perform alternative functions in addition to translocation. For example, in the Shigella T3S system, IpaC functions to translocate effectors when in complex with IpaB, however in its homo-oligomeric state it performs a different role (Osiecki et al., 2001; Mounier et al., 2009). It is therefore possible that PopB homo-oligomers and PopB:PopD heterocomplexes both play a role physiologically. If the PopB homo-oligomers interact with the bacteria through a structure other than the T3S apparatus, then binding would still occur in the

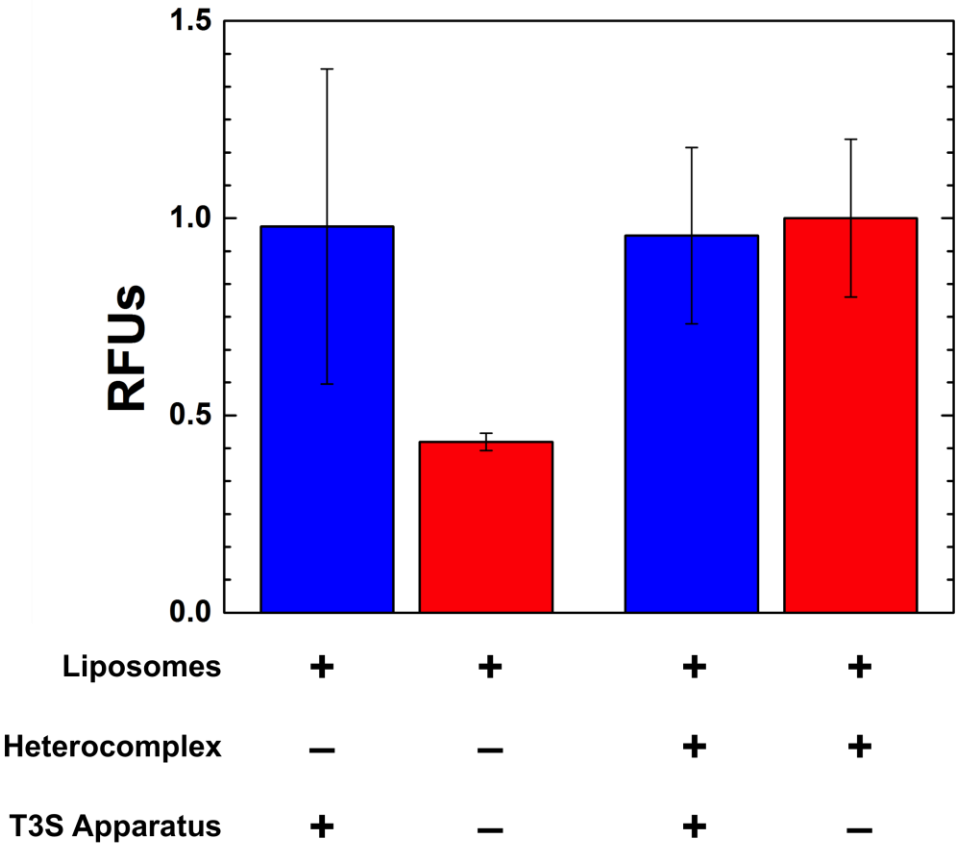

Figure 3.5. *P. aeruginosa* binds to the heterocomplex in the absence of the T3S apparatus. *P. aeruginosa* interactions with fluorescently-labeled liposomes with and without translocators bound were measured when the T3S apparatus was present (blue) or absent (red) (see Methods 3.4.1). Translocator heterocomplexes were reconstituted on liposomes (4.3 pmol PopB and 0.43 pmol PopD). Composition of each sample is indicated at bottom. Protein:lipid ratio was 1:6000, with lipid composition POPC:cholesterol:POPS:NBD-cholesterol at molar ratio 65:19.8:15:0.2. PAK or PAKΔPscC (no T3S apparatus) was grown to OD_{600} of 1.2 under conditions that induced T3S; cells were washed and diluted in PBS to OD_{600} of 0.25 before incubating with liposomes. Fluorescence emission intensity was normalized to the maximum fluorescence value. Error bars indicate the range between two independent measurements.

ΔPscC mutant. Binding to PopB homo-oligomers alone was not measured, however, so it is not clear how much this contributed to the signal. Therefore it is not clear if one translocator oligomer was active to interacting with the bacteria when the T3S apparatus was absent.

Wild-type PAK produced the translocators PopB and PopD, and secreted them into the media (data not shown), however is unclear whether these translocators are inserted into liposomes. The hydrophobic translocators aggregate in solution (Tardy et al., 1999; Hume et al., 2003), so even if PAK did not insert these translocators, secreted translocator aggregates likely stuck to the liposomes. The liposomes may have become saturated with translocators. Binding to these translocators (even if nonspecific) may have made the reconstituted translocator complexes inaccessible, explaining why the fluorescence signal did not increase when the heterocomplex was present (Fig 3.5, blue). These results exemplify the importance of using a mutant strain that does not produce the translocators to measure interactions with the translocator complexes in liposomes.

3.3 Conclusion

The use of fluorescently labeled liposomes to assay *P. aeruginosa* binding allowed for bacteria-liposome interactions to be measured efficiently. Measuring interactions between the bacteria and the membrane-reconstituted translocators was possible, however it was difficult to determine if these interactions occurred through the T3S apparatus. There was a broad distribution of the binding data obtained. The assay used is an innovative method developed to allow for a more

high-throughput measurement of liposome-bacteria interactions than fluorescence microscopy. While Luisi et al. used a similar method to measure liposome binding, the application of this method to identify bacterial-protein complex interactions is novel and likely needs to be modified to increase precision and reproducibility of measurements (Luisi et al., 1998).

Despite possible limitations in the assay, it is clear that *P. aeruginosa* more readily binds the reconstituted heterocomplex than homo-oligomers of either translocator. The interactions may be weak, but entropically favored when the translocon is inserted in close proximity of the needle *in vivo* when the bacteria are attached to the host cell. Further research is necessary to determine if the heterocomplex reconstituted in model membranes is functional for this type of interaction.

3.4 Methods

3.4.1 Measuring *P. aeruginosa* binding to translocator complexes in liposomes

Homo-oligomers and heterocomplexes were reconstituted in liposomes (37.5 µM) (65% POPC, 19.8% cholesterol, 15% POPS, 0.2% NBD-Cholesterol, Avanti Polar Lipids) as described in 2.5.1, with the final concentration of PopB and PopD being 56.8 nM and 5.68 nM respectively. The pH was neutralized with 2 µL of 2 M Trizma pH 9.00. Homo-oligomers were formed with either 56.8 nM PopB or 5.68 nM PopD in 37.5 µM liposomes. PA103ΩPopD was cultivated as in 2.5.4.1 to induce T3S. Cells were grown until they reached an OD_{600} of 1.2. Cells were washed in PBS, pH 7.2 and collected by centrifugation (600xg, 10 min,

4°C). The cell pellet was resuspended in 10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.2 (PBS) and diluted to OD_{600} of 0.25. Four hundred and fifty microliters of cells were transferred to a glass culture tube. Liposome-bacteria interactions were measured using a protocol adapted from Luisi et al., 1998. To the glass culture tube, 75 µL of proteoliposomes were added. Cells and liposomes were incubated for 15 min with shaking (150 rpm), 23°C. Cells and bound liposomes were pelleted (1,200xg, 10 min, 23°C). Pellets were resuspended in 300 µL PBS, pH 7.2 and the fluorescence reading was taken by exciting the sample at 485 nm (bandpass 5) and measuring emitted light at 550 nm (bandpass 5).

CHAPTER 4

CHARACTERIZATION OF THE *P. AERUGINOSA* T3SS TRANSLOCON ISOLATED FROM INFECTED RBCS

4.1 Introduction

The heterocomplex reconstituted in model membranes is a hexadecamer of eight PopB and eight PopD (Romano et al., submitted). Since the molecular weights of PopB and PopD are 40.3 kDa and 31.3 kDa respectively, the *P. aeruginosa* heterocomplex is predicted to have a molecular weight of 601 kDa (Romano et al., submitted). The molecular weight of the native *P. aeruginosa* translocon has not been determined. The *Y. enterocolitica* T3S translocon formed by contact with red blood cells (RBCs) has been isolated, and the molecular weight of the complex was estimated to be between 500 and 700 kDa using Blue Native (BN)-PAGE (Montagner et al., 2011). A BN-PAGE can be used to roughly estimate the molecular weight of the *P. aeruginosa* translocon, which will give insight into how it relates to the heterocomplex formed in model membranes.

The heterocomplex reconstituted in model membranes has a 1:1 stoichiometry of PopB to PopD (Romano et al., submitted). This diverges from the 5:1 translocator stoichiometry of the *Shigella* translocon (Dickenson et al., 2013). The stoichiometry of the native *P. aeruginosa* translocon is not known. The translocon, isolated by BN-PAGE, can be further analyzed in a second dimension by a denaturing SDS-PAGE (Fiala et al., 2011). This will separate the translocators present in the translocon, allowing for their relative amounts to be

compared. If the translocon forms a 1:1 complex, an equal amount of PopB and PopD will be detected. This will provide greater insight into the structure of the *P. aeruginosa* translocon and will determine how the translocon formed in cell membranes relates to the heterocomplex formed in model membranes.

4.2 Experimental Design

4.2.1 Inducing *P. aeruginosa* contact with RBCs

The translocon was to be isolated from mammalian cells. To perform the analysis by BN-PAGE, a large amount of translocon complexes need to be isolated (about 60 µg total protein per lane) (Montagner et al., 2011). This would require a large amount of cultured cells. A good alternative is to use mammalian RBCs. RBCs can be obtained in high amounts and lysis can easily be assessed. T3S translocons have been isolated from RBC membranes after contact with *Y. enterocolitica* (Montagner et al., 2011).

P. aeruginosa was incubated with RBCs at a multiplicity of infection (MOI) of 3 (Fig 4.1, 1). Bacteria-RBC contact was inducted by centrifugation (Fig 4.1, 2, B). The pelleted cells were incubated at 37°C to allow for the translocon formation to proceed (Fig 4.1, 2, C). The cells were then resuspended (Fig 4.1, 2, A). This process was repeated four times to increase cell contact, which increased the number of translocons formed (Broz et al., 2007).

4.2.2 Isolating RBC membranes

After contact with *P. aeruginosa*, RBCs lyse (Goure, 2005) (Fig 4.1, 3). The lysed RBCs were separated from bacteria by centrifugation (Fig 4.1, 4). RBC membranes containing the translocon complexes were isolated from unbound

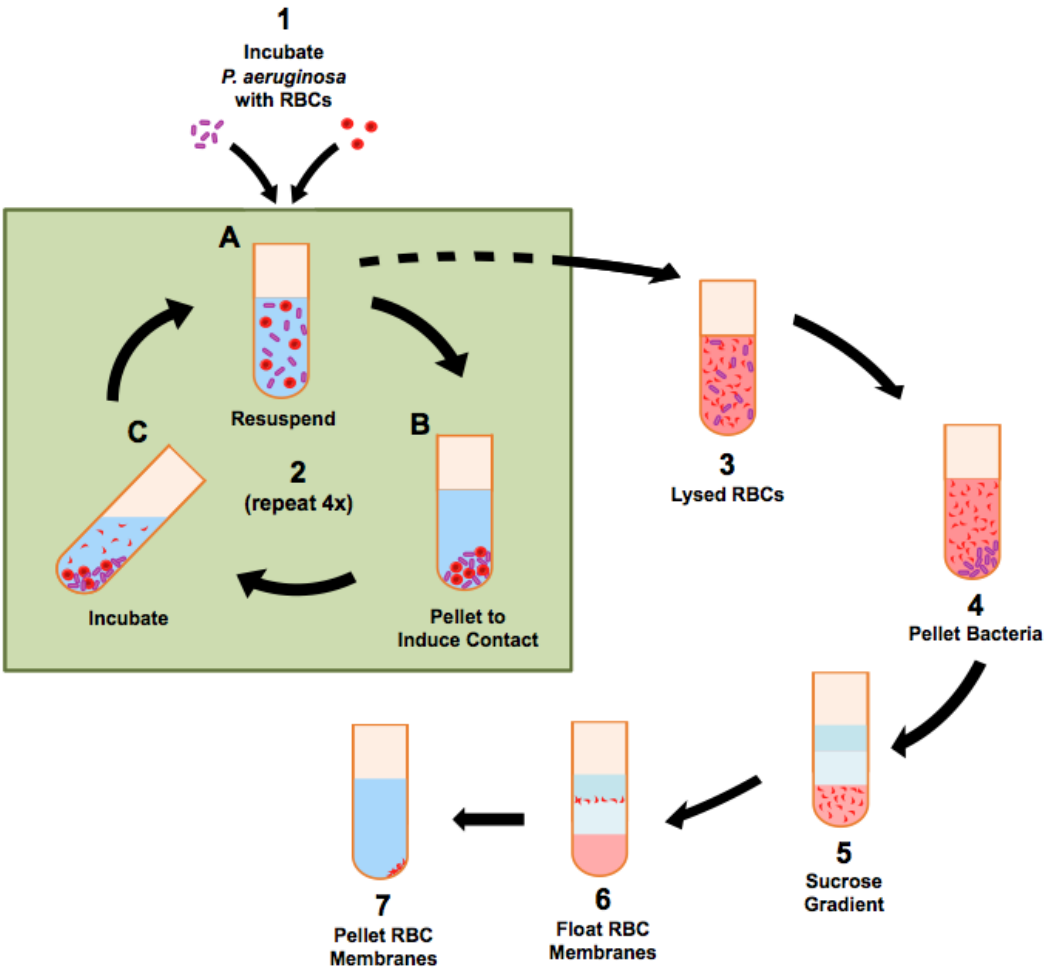

Figure 4.1. Schematic of method used to induce *P. aeruginosa* contact with RBCs. (1) *P. aeruginosa* (purple) was mixed with RBCs (red) at an MOI of 3 in a 3 to one ratio of 30 mM Tris-HCl, 150 mM NaCl, pH 7.4. (2) Translocons were formed on RBCs by bacteria-RBC contact, which was facilitated by (B) pelleting the cells (1,000xg, 10 min, 37°C). (C) Pellets were incubated for 10 minutes at 37°C and (A) resuspended by shaking (100 rpm, 2 min, 37°C). This procedure was repeated three more times to maximize the number of translocons inserted. (3) T3S protein injection induced RBC lysis. (4) Bacteria were removed by centrifugation (1,500xg, 10 min, 4°C). (5) Lysed RBCs were mixed with sucrose and a sucrose gradient was established. (6) RBC membranes were floated by centrifugation. (7) RBC membranes were harvested by centrifugation.

proteins and other cellular components by floating on a sucrose gradient (Fig 4.1, 5 and 6). The RBC membranes were pelleted and stored at -20°C (Fig 4.1, 7).

The proteins present on the RBC membranes were separated on an SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. PopB and PopD were detected with polyclonal antibodies. The presence of the translocators would suggest that the translocon was present on the RBC membrane.

4.2.3 Isolating the translocon from RBC membranes

The translocon was isolated from RBC membranes. The RBC membranes needed to be solubilized with mild detergents to maintain the quaternary structure of the translocon. Selecting detergents to dissolve the membrane without disrupting the protein-protein interactions within the translocon complex was not trivial. The ideal detergent for isolating membrane protein complexes varies depending on the complex size and stability, as well as the charges and hydrophobicity of the proteins in the complex (Linke, 2009). Since a BN-PAGE was to be performed, a nonionic detergent needed to be selected (see 4.2.4). Three nonionic detergents were selected for these experiments: Triton X-100, digitonin, and n-Dodecyl β-D-maltoside (DDM).

The detergent concentration also has a significant impact on the solubilization of a membrane protein complex. When detergents are added to membranes, they are first incorporated into the membrane bilayer. As more detergent is added, the membrane becomes saturated. The critical micellization concentration (CMC) of a detergent is the concentration at which micelles begin

to form (Linke, 2009). Once the membrane is saturated, if enough additional detergent is added for the concentration of free detergent to rise above the CMC, the membrane begins to solubilize (Lichtenberg, 2013). Therefore to fully solubilize the membrane, the detergent concentration must not only be high enough to saturate the membrane, but also high enough that the free detergent concentration is above the CMC. The CMC is a property of a detergent that does not change if the temperature remains constant (Linke, 2009). The amount of detergent needed to saturate the membrane depends not only on the amount of membrane present, but also on the membrane composition (Linke, 2009). Therefore several different detergent concentrations were tested in their ability to solubilize the RBC membranes to isolate the translocon.

4.2.4 Estimating the molecular weight of the translocon using BN-PAGE

After solubilization, a BN-PAGE could be performed to estimate the molecular weight of the translocon. The BN-PAGE is a method that separates membrane proteins using PAGE in non-denaturing (native) conditions. This method is similar to SDS-PAGE in that a reagent is added to negatively charge the proteins, a factor that distinguishes BN-PAGE from Native-PAGE. Coomassie brilliant blue G250 binds to the hydrophobic regions of solubilized membrane proteins, giving them a negative charge without denaturing the proteins (Schägger et al., 1994). This causes all proteins to migrate in the same direction in an electric field. For this reason, nonionic detergents must be used to solubilize membrane protein complexes. Unlike SDS-PAGE, BN-PAGE cannot be used to accurately calculate the molecular weight of a protein complex, but it

can be useful to estimate oligomerization states.

The solubilized translocon isolated from RBC membranes was separated by BN-PAGE. A gradient acrylamide gel was used to allow for the separation of complexes with a broad range of molecular weights. A western blot was performed to detect for the presence of the translocator proteins.

4.3 Results and Discussion

4.3.1 Translocators were found on RBC membranes after contact with *P. aeruginosa*

The proteins present on the RBC membranes were separated on a 10% acrylamide SDS-PAGE and transferred to a PVDF membrane. PopB and PopD were detected with polyclonal antibodies. Lysed *P. aeruginosa* as well as RBC membranes that did not come in contact with *P. aeruginosa* were included in the gel as a control (Fig 4.2, A and B, lanes 2 and 3). RBCs membranes that did not come in contact with *P. aeruginosa* were prepared by lysing RBCs with a hypotonic solution. These RBC membranes were isolated from other cellular components using a flotation assay (Fig 4.1, 5 and 6).

PopD was detected in the RBC membranes following contact with *P. aeruginosa.* A band at the predicted molecular weight of PopD (31.3 kDa) was detected (Fig 4.2, A, lane 1, arrow), however the anti-PopD antibodies detected several other proteins in this sample. The ~37 kDa and ~80 kDa bands were also present in the lysed bacteria (Fig 4.2, A, lane 2), suggesting this was nonspecific binding of the antibody to a bacterial protein that was also found on RBC membranes after contact with *P. aeruginosa.* The other bands were not detected

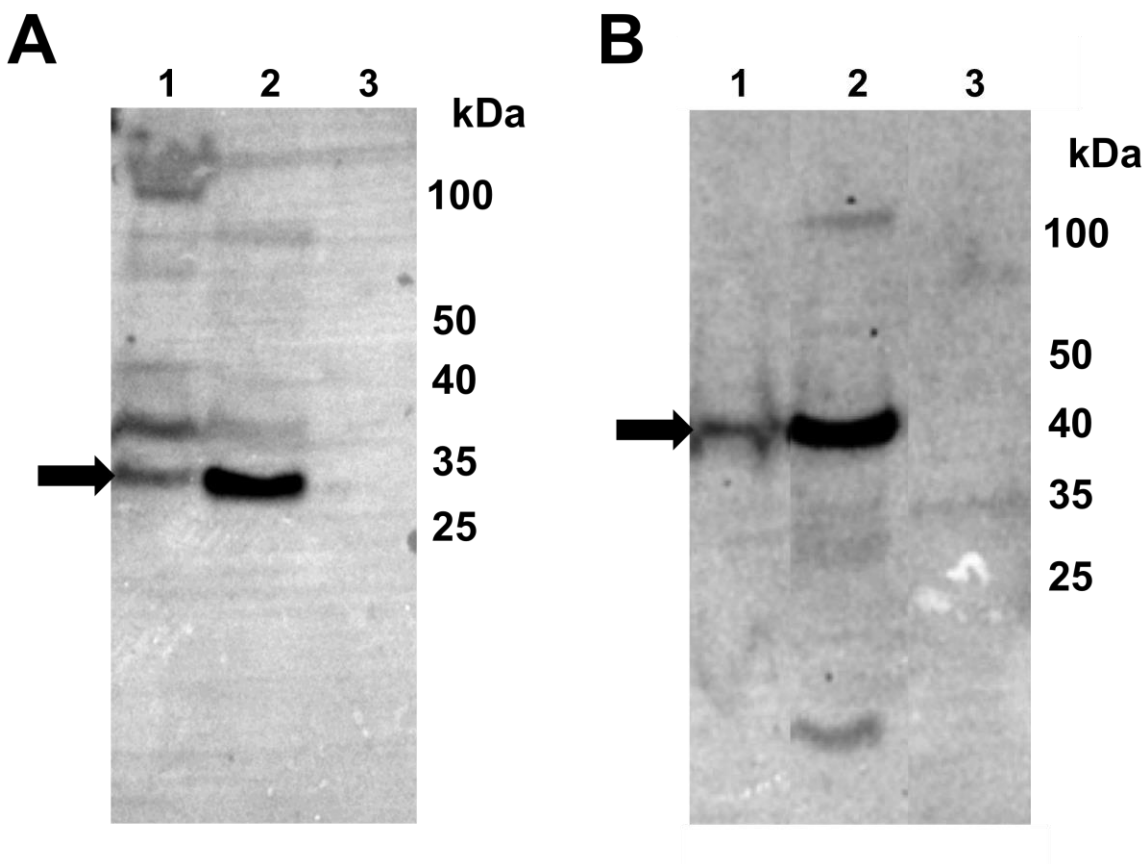

Figure 4.2. Translocators were found on RBC membranes after contact with *P. aeruginosa*. Translocators (A) PopD and (B) PopB were detected after separation on a 10% acrylamide SDS-PAGE and transferring to a PVDF membrane. (1) RBC membranes isolated by flotation assay after contact with *P. aeruginosa*. (2) *P. aeruginosa* cell lysate after sonication. (3) RBC membranes that did not come in contact with *P. aeruginosa.* Arrow indicates the predicted migration of PopD in A, and PopB in B. Translocators were detected with polyclonal anti-PopB and anti-PopD antibodies.

in the RBC membranes that did not come in contact with *P. aeruginosa* (Fig 4.2, A, lane 3), so they are not mammalian proteins. These other bands could be secreted bacterial proteins that were not obtained when the bacteria were lysed.

A single band was detected at 40 kDa by the anti-PopB antibodies in the RBC membranes following contact with *P. aeruginosa* (Fig 4.2, B, lane 1, arrow). The anti-PopB antibodies detected several other proteins in the lysed bacteria that were not present in the RBC membranes after contact with *P. aeruginosa* (Fig 4.2, B, lane 2). This was likely due to nonspecific binding of the antibodies to bacterial proteins. No proteins were detected in the sample containing RBC membranes that did not come in contact with *P. aeruginosa* (Fig 4.2, B, lane 3).

The antibodies used for detection were polyclonal and were developed using recombinant protein. These antibodies could be binding nonspecifically to other proteins. This is likely why, in some samples, other bands were detected in addition to the bands detected at the predicted molecular weights of PopB and PopD. Using monoclonal antibodies developed against the native translocators would minimize this nonspecific binding.

PopD and PopB were detected on RBC membranes only after contact with the bacteria. These results suggest that *P. aeruginosa* inserted the translocon into the RBC membranes.

4.3.2 Detection of translocon obtained from RBC membranes on BN-PAGE

The translocon on RBC membranes was solubilized in detergent (DDM) and separated on a BN-PAGE. A sample solubilized in 1% SDS was included as a control, since 1% SDS is predicted to completely dissociate the translocon

complex into monomers (Montagner et al., 2011). Several bands were stained with Coomassie (Fig 4.3), since RBC membranes contain many membrane proteins that were likely isolated along with the translocons. The 3-20% gradient gels could only separate protein complexes with molecular weights lower than ~500 kDa. The predicted molecular weight of the heterocomplex is 601 kDa. This could explain why a lot of protein remained in the wells.

A western blot was performed to detect the translocator proteins PopB and PopD (Fig 4.4). PopD was detected in RBC membranes solubilized in 3.5% DDM and 1% SDS (Fig 4.4, A, lanes 1 and 7). A very faint band of PopB was detected in the lane containing RBC membranes solubilized 1% SDS (Fig 4.4, B, lane 7), however no PopB was detected in the lane containing RBC membranes solubilized in 3.5% DDM (Fig 4.4, B, lane 1). It is possible that these bands do not contain the translocator proteins, since nonspecific binding of both anti-PopB and anti-PopD antibodies was seen in the previous western blots performed with these antibodies (Fig 4.2),

The 3-20% gradient could only separate proteins up to 500 kDa; therefore the bands detected were likely dissociated translocon complexes. Furthermore, PopB and PopD bands did not migrate similarly, indicating that the translocon complex quaternary structure was not maintained. These results suggest that 3.5% is too high of a concentration of DDM to maintain the quaternary structure of the translocon. It also is possible that the molecular weight of the translocon is less than predicted, and could therefore be separated by the 3-20% gradient gel used. Unfortunately, the molecular weight marker did not stain with Ponceau

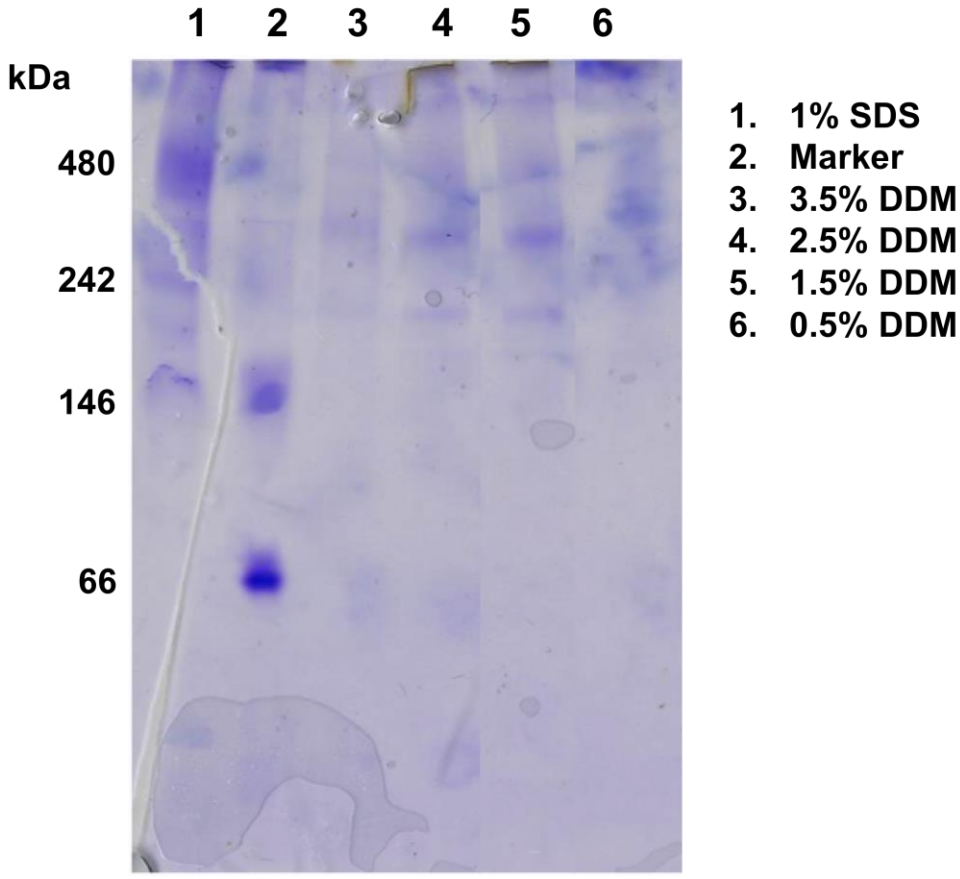

Figure 4.3. Separation of translocon isolated from infected RBCs on BN-PAGE with a 3-20% acrylamide gradient. After solubilizing in the indicated detergent (right), samples containing isolated translocons were run on a 4-20% gradient acrylamide (see Methods 4.4.3). Protein was detected by staining with Coomassie. All lanes contain 30 µg of protein. Approximate molecular weights are indicated.

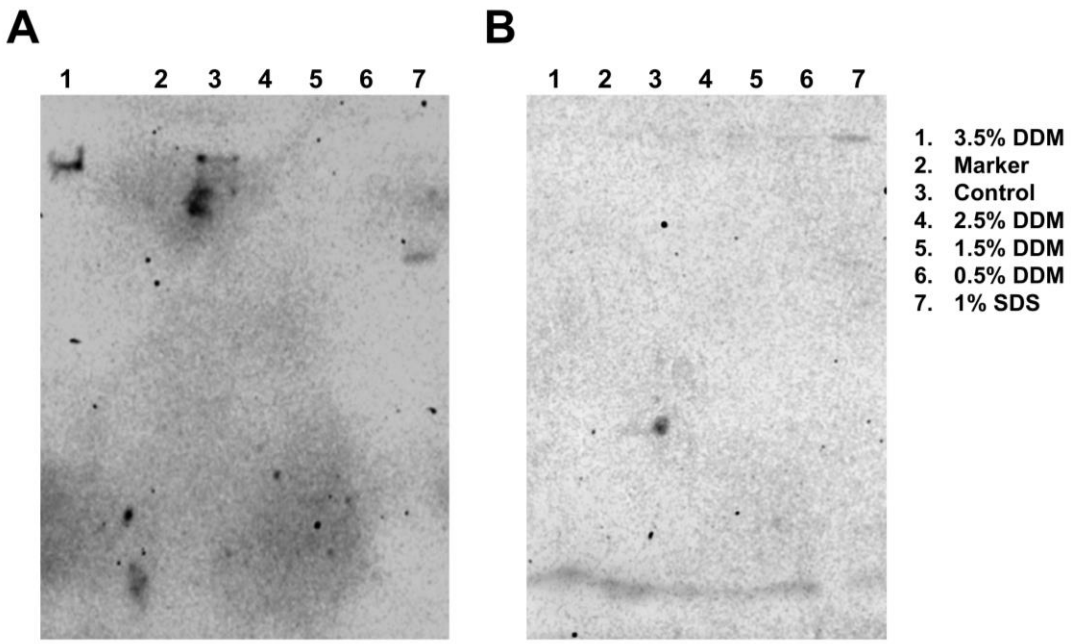

Figure 4.4. Detecting PopB and PopD in the translocon isolated from RBCs after separating on a BN-PAGE. After solubilizing in the indicated detergent (right), samples containing isolated translocons were run on a 4-20% gradient acrylamide gel (see Methods 4.4.3). A western blot was performed to detect (A) PopD and (B) PopB. All lanes (excluding the control, lane 3) contain 30 µg of protein. Lane 3 contains a control sample containing 10 times the minimal amount of protein detected by the antibodies (A) 100 ng PopD, or (B) 10 ng PopB. Translocators were detected with polyclonal anti-PopB and anti-PopD antibodies.

after transferring to the PVDF membrane, so it was not possible to determine the molecular weights of these bands.

Purified PopB and PopD were both detected in their respective control lanes, however the translocators migrated very differently (Fig 4.4, A and B, lane 3). The BN-PAGE separates proteins by size, and no denaturing agent was added (Wittig et al., 2006). PopD likely aggregated and this aggregate migrated differently from monomeric PopD. In the future, purified translocator complexes should be reconstituted in membranes and solubilized with detergents before running on a BN-PAGE.

The BN-PAGE was repeated with a 3-12% acrylamide gradient gel to allow for higher molecular weight complexes to be separated. Alternative detergents and detergent concentrations were selected to solubilize the membrane proteins to determine optimal conditions for solubilizing the translocon. The BN-PAGE was stained with silver rather than Coomassie. Silver staining is up to 100 times more sensitive than Coomassie (Chevallet et al., 2006), and the marker was more easily visualized using this staining method. A western blot was performed to detect PopD.

Unfortunately, there was a problem with the gel, which caused the inner lanes to run much faster than the outer lanes. Many proteins were detected by silver staining, indicating that proteins were entering the gel (Fig 4.5, A). The molecular weight marker ran differently in the 3-12% gradient gel, separating complexes as large as ~750 kDa. PopD was detected in all lanes at the top of the wells, indicating the translocon was not fully solubilized (Fig 4.5, B). PopD

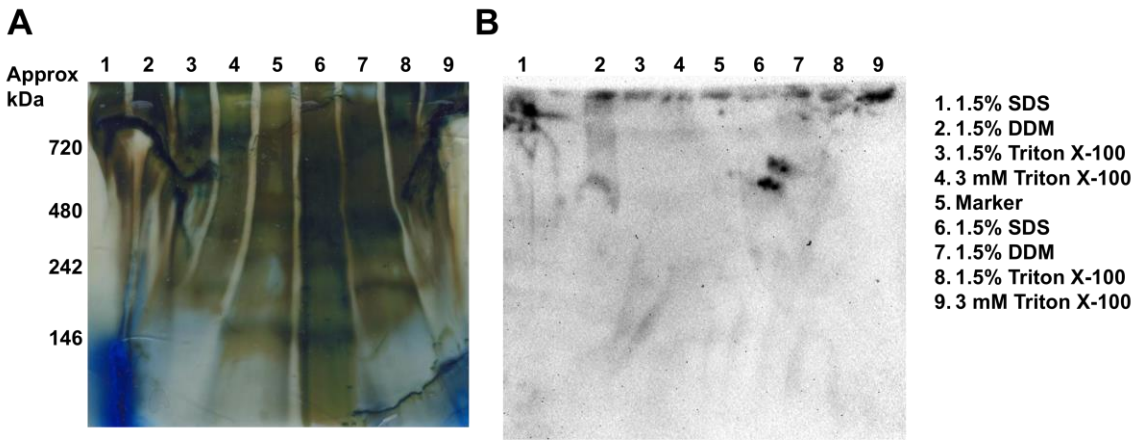

Figure 4.5. Separation of translocon isolated from infected RBCs on BN-PAGE with a 4-12% acrylamide gradient. After solubilizing in the indicated detergent (right), samples containing isolated translocon were run on a 4-12% gradient acrylamide gel (see Methods 4.4.3) Lanes 1-4 contain 30 µg of protein. Lanes 6-9 contain 60 µg of protein. Protein was detected by (A) staining with silver, or (B) transferring to a PVDF membrane and detecting for PopD. Approximate molecular weights are indicated at left. PopD was detected with a polyclonal anti-PopD antibody.

solubilized with DDM and SDS entered the gel (Fig 4.5, B, lanes 2 and 6), however these samples were run in duplicate on the gel and their duplicate samples did not enter the gel. This was likely due to the gel not running properly. Few conclusions can be drawn from this gel because it did not run properly, however the results indicate that the 3-12% acrylamide gradient is appropriate for isolating a 601 kDa complex, the predicted molecular weight of the membrane-reconstituted heterocomplex.

4.4 Methods

4.4.1 Inducing *P. aeruginosa*-RBC contact

An overnight culture of PAK was performed in 100 mL LB. Four 500 mL LB cultures were inoculated with 25 mL of the overnight culture. The cells were cultivated until they reached an OD_{600} of 1.10. Cultures were pooled before splitting into four tubes and centrifuging to pellet cells (3,220xg, 10 min, 37°C). Cell pellets were washed in 50 mL LB. Cell pellets were resuspended in a minimum volume of LB (~1 mL). The number of bacteria present in each pellet was estimated using the formula OD_{600} 1.0 = $6x10^8$ cells/mL (C. Rossi, unpublished data).

RBCs were isolated from sheep blood with sodium heparin as an anticoagulant (Innovative Research) by washing. Two hundred milliliters of sheep blood was split into 20 mL aliquots in 50 mL centrifuge tubes. To each aliquot, 30 mM Tris-HCl, 150 mM NaCl, pH 7.4 was added to bring the volume to 45 mL. The solution was resuspended gently by pipetting and then centrifuged to pellet RBCs (500xg, 15 min, 4°C). This process was repeated three times or until the

supernatant appeared clear. Washed RBCs were pooled and counted using a cell counter. Protease inhibitors (Roche) were added to the RBCs.

An appropriate amount of RBCs were added to the bacteria to attain an MOI of two. Bacteria were diluted in LB to maintain a 3:1 ratio by volume of RBCs to bacteria. Cell contact was induced using a protocol modified from Montagner et al., 2011. Cells were centrifuged (1,000xg, 10 min, 37°C) to induce cell contact. The pellet was incubated at 37°C for 10 minutes before resuspending by shaking (100 rpm) for 2 min at 37°C. This was repeated four times.

The reaction was transferred to a 50 mL tube and centrifuged (1,500xg, 10 min, 4°C) to pellet bacteria. A flotation assay was performed with the supernatant containing the lysed RBCs to isolate the RBC membranes. Flotation assays were set up in 50 mL tubes to which 7.5 mL lysed RBCs were added along with 7.5 g sucrose. The solution was vortexed gently to dissolve the sucrose, generating a 60% sucrose solution. The solution was overlaid with 12 mL 50% sucrose in 50 mM Tris-HCl, pH 7.4, followed by 10 mL of 25% sucrose solution in the same buffer. The samples were centrifuged at 15,000xg, 16 hours, 4°C. The RBC membranes were collected between the 25% and 50% sucrose layers.

The RBC membranes were pelleted (45,000xg, 60 min, 4°C). The pellets were washed two times in 35 mL BN-PAGE sample buffer (50 mM Bis-Tris, 50 mM 6-aminohexanoic acid, 1 mM EDTA, pH 7.2). The pellet was resuspended in a minimal volume of BN-PAGE sample buffer and the protein concentration was determined using a bicinchoninic acid (BCA) assay (Pierce). The membranes

were split into aliquots containing 400 µg of protein, and 10% glycerol was added before pelleting (100,000xg, 30 min, 4°C). Pellets were stored at -20°C for several weeks.

4.4.2 Isolating the translocon from RBCs after contact with *P. aeruginosa*

Infected RBC membrane pellets were thawed on ice. Detergent in BN-PAGE sample buffer was added to the pellet. The pellet was resuspended gently by pipetting up and down. Pellets were solubilized on ice on a shaker (50 rpm), for one hour. When SDS or digitonin was used, pellets were solubilized at 4°C to prevent precipitation of the detergent. Insoluble material was pelleted (20,000xg, 1 hour, 4°C). The supernatant contained the solubilized translocon as well as other solubilized membrane proteins from the RBCs.

4.4.3 Separation of the translocon isolated from RBCs by BN-PAGE

Solubilized translocons were prepared for BN-PAGE by adding Coomassie brilliant blue G250 in MilliQ water to a final concentration of 25% of that of the detergent (Schägger et al., 1994). Ten percent glycerol was added to the samples. A Bis-Tris 4-12% gradient gel (Genscript) was set up in a Bio-Rad Mini Proteon electrophoresis apparatus. Initially a Bis-Tris 3-20% gradient gel (Genscript) was used with poor separation of the complexes. Samples were loaded onto the gel along with 5 µL of the NativeMark molecular weight marker (Invitrogen). The cathode buffer contained 50 mM Bis-Tris, 50 mM Tricine, 0.02% Coomassie brilliant blue G250, pH 6.8, while the anode buffer contained 50 mM Bis-Tris, pH 6.8. The gel was run at 100 V at 4°C until the dye front reached one third of the way down the gel, after which the cathode buffer was switched to 50

mM Bis-Tris, 50 mM Tricine, 0.002% Coomassie brilliant blue G250, pH 6.8. The gel was run at 30 mA until the dye front reached the bottom of the gel.

The gel was soaked in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 10% methanol, 0.01% SDS) for 20 minutes at 4°C to coat proteins in the gel with SDS (Montagner et al., 2011). The gel was then transferred to a PVDF membrane for 90 minutes at 350 mA using a Trans-Blot Mini Electrophoretic Transfer Cell (Bio-Rad). The membrane was blocked with 5% milk, 50 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.2 (TBST) overnight. The membrane was then incubated with double the normal concentration of primary antibody (1:500 anti-PopD or 1:37,500 anti-PopB in 2.5% milk TBST) for one hour on a shaker (50 rpm), 23°C. The membrane was then washed three times with TBST and incubated on a shaker as before. A 1:30,000 dilution of secondary antibody (anti-rabbit conjugated to alkaline phosphatase) in 2.5% milk TBST was incubated with the membrane for 1 hour as before. The membrane was washed as before but with an additional wash in TBS to remove the Tween 20 detergent. Immun-Star AP Substrate (Bio-Rad) was added to the membrane to develop. The western blot was visualized using a Gel Doc XR+ System (Bio-Rad).

CHAPTER 5

CONCLUSIONS

5.1 Summary

While *P. aeruginosa* translocators have been successfully reconstituted in model membranes (Romano et al., 2011), no studies have been performed to assess these complexes in relation to those formed during infection. A study of the reconstituted complexes along with those formed in host cell membranes was conducted.

Initially the membrane complexes were assessed in relation to the *P. aeruginosa* T3S complex protein, monomeric PcrV. It is likely that the oligomeric form is necessary to interact with the membrane bound translocator complex. Ideally, interaction with the oligomeric PcrV complex would be assayed, however PcrV oligomers were not available. The needle contains the PcrV complex and therefore was considered as an alternative source of PcrV oligomers, however the needles were difficult to purify. Further interaction studies were performed with live *P. aeruginosa* cells. Results from these experiments indicate that *P. aeruginosa* interacts more readily with the reconstituted heterocomplex than homo-oligomers of either translocator, however it is unclear whether this binding occurs through the T3S apparatus. From these studies, it is not clear whether the heterocomplex reconstituted in model membranes is functional for interaction with other components of the T3S system.

The translocon formed during infection was isolated. RBCs were successfully infected by induced contact with *P. aeruginosa*. The RBC

membranes were dissolved with detergent, and the translocator complexes were solubilized. While the size of the translocon isolated from RBC membranes could not be estimated, appropriate conditions for forming the translocons on RBCs, and appropriate conditions for solubilizing the translocon from RBC membranes were assessed.

5.2 Future directions

5.2.1 Does *P. aeruginosa* bind specifically to heterocomplexes reconstituted in model membranes?

While it is clear that *P. aeruginosa* binds more readily to the heterocomplex, it is unclear whether the binding of *P. aeruginosa* to PopB and PopD homo-oligomers or heterocomplexes in model membranes is specific. Further experimentation comparing binding to different amounts of the heterocomplex and different amounts of both homo-oligomers, would help determine if homo-oligomer binding is specific. Furthermore, reconstituting other irrelevant membrane proteins in the membranes would determine if P. aeruginosa binds nonspecifically to protein on membranes.

5.2.2 Does *P. aeruginosa* binding to heterocomplexes reconstituted in model membranes occur through the T3S apparatus?

To confirm interactions are specific to the T3S apparatus, the experiment described in 3.2.3 must be repeated with a PAKΩPopD strain in place of PAK. This would allow for the comparison of binding between cells that do not produce the T3S apparatus, and those that do, without secreting additional translocators that affect binding to liposomes. Furthermore, this experiment should also include

a sample containing only PopB homo-oligomers. This would allow for comparison to binding with the heterocomplex, since homo-oligomers are present when forming the heterocomplex (Romano et al., submitted).

5.2.3 Utilizing a *P. aeruginosa* mutant that does not secrete phospholipases

P. aeruginosa secretes effector proteins through the T3S system. These effectors are translocated through the translocon pore into the host cell. One of the effectors (ExoU) functions as a phospholipase (Sato and Frank, 2004). It is possible that this effector is secreted, degrading the liposomes. If a specific interaction occurred between the cell and the heterocomplex, this could stimulate secretion of this component. It is also possible that induction of T3S by low calcium could stimulate secretion of this phospholipase. By not stimulating T3S, secretion of this phospholipase would be minimized, however fewer T3S apparatuses will be produced (Mueller et al., 2005). To overcome this, a mutant strain that does not produce this effector could be used for this experiment.

5.2.4 Isolate the translocon from infected epithelial cells

Translocators have been detected on epithelial cells following infection with *P. aeruginosa* (Y. Tang, unpublished data). Since epithelial cells are the targets of *P. aeruginosa* infections in humans, it would be interesting to compare these translocons to those isolated from RBCs. The translocon could be isolated from the cell membranes using methods similar to those used for isolation from RBCs. Less translocons will be obtained, as infections cannot be performed

easily with a large amount of epithelial cells as can be done with RBCs. To overcome this, the translocons could be purified as described below.

5.2.4.1 Purification of the translocon from membranes

After solubilizing the membranes, the translocon could be isolated from other solubilized membrane proteins using chromatography. It is possible to add a tag to isolate the translocon from other complexes efficiently (Montagner et al., 2011). This would allow for translocons to be concentrated, allowing the translocon to be more easily analyzed by BN-PAGE.

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