Development of a Site-Specific Labeling Assay to Study the Pseudomonas aeruginosa Type III Secretion Translocon in Native Membranes

Kyle A. Mahan
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Development of a site-specific labeling assay to study the *Pseudomonas aeruginosa* Type III secretion translocon in native membranes

A Thesis Presented

by

KYLE MAHAN

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

September 2021
Molecular and Cellular Biology
Development of a site-specific labeling assay to study the *Pseudomonas aeruginosa* Type III secretion translocon in native membranes

A Thesis Presented

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ABSTRACT

DEVELOPMENT OF A SITE SPECIFIC LABELING ASSAY TO STUDY THE PSEUDOMONAS AERUGINOSA TYPE III SECRETION TRANSLOCON IN NATIVE MEMBRANES

SEPTEMBER 2021

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The type III secretion system (T3SS) is employed by various Gram-negative pathogens to promote infection of host cells. Central to the function of the T3SS is the injectisome, a syringe-like membrane protein complex which allows direct translocation of cytotoxic effector proteins into the cytosol of a host cell. Contact between the needle tip and the plasma membrane of a host cell activates the Type III secretion system, promoting attachment of the bacteria to the host and secretion of T3SS substrates through the injectisome. In Pseudomonas aeruginosa, the proteins PopB and PopD are translocated through the injectisome to insert into the host membrane, forming a heterooligomeric, membrane-spanning translocon pore. Formation of the translocon provides a channel through which the attached bacteria can then translocate effectors into the host. Although the translocon pore is essential for T3SS-mediated infection, little is known about the structure and topology of PopB and PopD when associated with membranes in vivo.
In this work, I seek to develop a cysteine specific labeling assay to probe the transmembrane topology of PopD upon insertion into the membrane of a host cell. Several PopD variants were produced with single cysteines inserted within putative extracellular or intracellular loop domains. The transmembrane orientation of these cysteines was studied based on their accessibility to PEG maleimide, a cysteine specific, membrane impermeable labeling reagent.

By employing this method, I here characterize a loop domain of PopD that is accessible to host cytosol upon translocon formation. With optimization, the PEGylation assay may be used to further study the structure of PopD, as well as the other translocon protein PopB.
# ABSTRACT

Pseudomonas aeruginosa is an antibiotic resistant threat. P. aeruginosa employs a Type III secretion system to establish infections. T3SS infections are carried out through the injectisome needle. The Type III infection process is activated by host cell contact. The structure of the Type III translocon is not well characterized. The N terminus and a middle segment of PopD are exposed to host cell cytosol upon membrane insertion.

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INTRODUCTION

1.1 Pseudomonas aeruginosa is an antibiotic resistant threat

*Pseudomonas aeruginosa* is a Gram-negative bacteria commonly found in a wide variety of environmental niches. This versatile bacteria acts as an opportunistic pathogen, often infecting burn victims, individuals with open wounds, and immunocompromised patients in hospitals (Sadikot et al 2005). Outside of the hospital setting, *P. aeruginosa* has also been associated with severe skin rash symptoms and infections of the cornea which may cause blindness (Lutz and Lee 2011, Hilliam et al 2020).

For individuals with cystic fibrosis, *P. aeruginosa* infections are a leading cause of morbidity due to their association with severe pneumonia symptoms (Carratala et al. 1998). A 2018 report showed that over 75% of examined cystic fibrosis patients had cultured positive for *P. aeruginosa* within the past 12 months (Cystic Fibrosis Foundation, 2018 Annual Data Report). The ability of *P. aeruginosa* to form biofilms in the lung epithelium of infected patients adds another layer of protection by shielding the bacteria from immune cells, and allows chronic infections to be established (Maurice et al 2018). *P. aeruginosa* biofilms are also commonly found growing on hospital equipment such as catheters and ventilators. Formation of these biofilm aggregates enables the bacteria to survive shifting environmental conditions and treatment with many antiseptic agents, making them very difficult to eradicate (Whiteley et al 2001).
The multidrug resistance exhibited by *P. aeruginosa* provides another major challenge in treating these infections. Various innate and adaptive factors allow *P. aeruginosa* to resist treatment with many common antibiotics including the aminoglycoside, quinolone, and β-lactam families (Pang et al 2019). *P. aeruginosa* was listed as a serious multidrug resistant threat by the CDC, having caused 32600 cases and 2700 deaths in 2017 alone (CDC, 2019 Antibiotic Resistance Threats Report). The risks posed by *P. aeruginosa* infections and the challenges in treating them necessitate a better understanding of the infection mechanisms in order to identify novel therapeutic strategies.

### 1.2 *P. aeruginosa* employs a Type III secretion system to establish infections

Along with many other pathogenic Gram-negative bacteria, *P. aeruginosa* employs a Type III secretion system (T3SS) in order to carry out infections of host cells (Yahr et al 1996). The T3SS is a complex machine consisting of various secreted, cytosolic, and membrane-anchored protein components. It allows for establishment of infections by facilitating the translocation of several cytotoxic effector proteins across the plasma membrane of a host cell.

While different bacteria employ the T3SS for various purposes, in *P. aeruginosa* it is mainly used to disrupt the bronchial epithelium and attenuate the host immune response through attacks on macrophages, allowing the bacteria to establish a foothold during infection (Tsang et al 1994, Diaz et al 2008). Detection of antibodies against T3SS components in the lung epithelium of cystic fibrosis patients suggests the T3SS plays a role in infection of these individuals (Lee et al 2005).
1.3 T3SS infections are carried out through the injectisome needle

T3SS-mediated translocation of effectors into a host is achieved through the injectisome, a 3.5 MDa, syringe-like protein complex anchored across the inner and outer bacterial membranes and protruding into the extracellular space (Fig. 1A) (Puhar and Sansonetti 2014). Due to similarities between the T3SS and the flagellar machinery, the two are believed to be evolutionarily linked (Abby and Rocha 2012). The injectisome consists of several key structural domains including (1) a substrate sorting complex, (2) a membrane-anchored basal body, and (3) a hollow needle filament protruding into the extracellular space (Fig. 1B) (Deng et al 2017).

The substrate sorting complex consists of an ATPase and a “C-ring” platform to regulate the secretion of T3SS substrates (Fig. 1B). The P. aeruginosa ATPase is a hexameric ring of the protein PscN associated with the cytosolic face of the injectisome (Halder et al 2019). Energy generated by this complex through ATP hydrolysis allows T3SS substrates to be dissociated from their cytosolic chaperones and partially unfolded, facilitating export through the hollow needle of the injectisome (Kato et al 2015). The protein PscL acts as a regulator for this complex by dimerizing and interacting with the N-terminal region of PscN to attenuate ATPase activity (Halder et al. 2019). PscL also links the PscN ATPase with the C-ring, which is composed of 6 PscQ protein subunits and provides a platform for recruitment and loading of T3SS substrates (Deng et al. 2017).
The basal body is a series of concentric rings which anchor the injectisome complex across the inner and outer bacterial membranes (Fig. 1B). The inner membrane ring is a 24-subunit complex of the transmembrane proteins PscD and PscJ, connected to the cytosolic sorting platform by an adaptor protein PscK (Muthuramalingam et al 2020). The outer membrane ring consists of multiple subunits of the protein PscC (Deng et al 2017).

The needle is a hollow structure composed of the protein PscF which protrudes 60-120 nm into the extracellular space and has a 6-10 nm internal diameter (Hauser 2009, Pastor et al 2005) (Fig. 1B). At the distal end of the needle is a tip complex formed from the protein PcrV (Caroline et al 2008, Sato and Frank 2011).

Regulation of T3SS gene transcription is coordinated by the interplay of four proteins: ExsA, ExsC, ExsD, and ExsE. ExsA is a transcriptional activator which binds to a consensus sequence in the promoter regions of several T3SS-related genes, including those for effector proteins and secretion machinery (Brutinel et al 2008). Another protein, ExsD, acts as an inhibitor for T3SS gene transcription by binding and sequestering ExsA (Brutinel et al 2010). ExsA may be released by the protein ExsC, which binds to and sequesters ExsD (Dasgupta et al 2004). However, ExsC is normally held in an inactive complex with a fourth protein ExsE, for which it has a higher affinity than ExsD (Rietsch et al 2005). The presence of ExsE in the cytosol keeps the T3SS dormant until the system is signaled to activate.
Figure 1: The Injectisome is a Needlelike Transmembrane Complex
The injectisome is organized into (i) a cytosolic ATPase ring and sorting complex (ii) a basal body (iii) a needle and tip complex. Each structure is highlighted here, with its protein components named below.
1.4 The Type III infection process is activated by host cell contact

The Type III secretion system infection process is activated when the PcrV tip complex detects contact with the surface of a host cell membrane. (Sato and Frank 2011) (Fig. 2A, 2B). Activation promotes release of ExsE through the needle to the extracellular space or cytoplasm of the host (Urbanowski et al 2007). Depletion of intracellular levels of ExsE frees ExsA to activate transcription of T3SS related genes in preparation for the infection process.

Activation of the Type III secretion system is followed by the secretion of two translocon proteins, PopB and PopD. In the aqueous environment of the cytosol, PopB and PopD adopt a molten globular structure that must be stabilized through association with a chaperone protein, PcrH (Broms et al 2003). The PscN ATPase facilitates dissociation of the translocators from PcrH and allows passage through the injectisome needle. PopB and PopD localize at the plasma membrane of the host, where they insert to form a heterooligomeric, membrane spanning translocon pore (Fig. 2B). Formation of the pore is critical because it serves as a channel through which the effector proteins gain access to the cytoplasm of the host cell (Hauser 2009). The *Shigella flexneri* translocator IpaC has been shown to interact with cytoskeletal filaments of the host cell, causing a conformational change in the pore which is required for docking of the needle tip complex onto the translocon (Russo et al 2019). Although these studies were done in *S. flexneri*, a similar mechanism may be conserved in the *P. aeruginosa* translocator PopD. However, the mechanisms of translocon insertion and assembly in the host membrane are generally not well characterized.
Formation of the translocon pore complex signals the attached bacteria to turn off translocator secretion and begin secretion of effector proteins through the translocon to the host cytosol (Fig. 2C). The signal may be generated by a conformational change in the PopD protein, which has been suggested to communicate with the injectisome through a linkage between its C-terminus and the PcrV needle tip complex (Armentrout and Rietsch 2016). The switch between secretion of translocators and effectors is accomplished by secretion of PopN, a “gatekeeper protein” which interacts with the cytosolic sorting platform of the injectisome (Sundin et al 2004, Lee et al 2010) (Fig. 2C).

*P. aeruginosa* employs four effector proteins as a part of its T3SS: ExoS, ExoT, ExoY, and ExoU. Different *Pseudomonas* strains only express three of the four, as the expression of ExoS and ExoU is mutually exclusive (Feltman et al 2001). Similar to the translocon proteins, the effectors are also complexed with chaperone proteins in the cytosol: the SpcS chaperone associates with ExoS and ExoT (Shen et al 2008), while SpcU is the chaperone for ExoU (Finck-Barbançon et al 1998) and the chaperone for ExoY is unknown (Hauser 2009). Putative signal sequences in the N-terminal regions of the effectors direct them to localize at the cytosolic sorting platform of the injectisome (Yahr et al 1996). After dissociation from their chaperones, they pass through the injectisome needle and translocon pore to the cytoplasm of the host (Fig. 2D). Here, the effectors interact with host cellular mechanisms in various ways to ultimately induce a cytotoxic response and help the bacteria establish infections.
ExoS and ExoT have 76% sequence similarity, and these proteins are believed to function similarly during infection as a result (Hauser 2009). Both are bifunctional proteins containing an N-terminal Rho-GAP domain and a C-terminal ADP-ribosyltransferase. The Rho-GAP domain inhibits the eukaryotic G proteins Rho, Rac, and CDC42, which are involved in maintaining the structural integrity of the cytoskeleton (Barbieri and Sun 2004). Inhibition of these targets ultimately leads to downstream collapse of the cytoskeleton and a distinct rounding phenotype in host cells (Lee et al 2005).

The ADP-ribosyltransferase domain is activated exclusively within the host cell by binding a eukaryotic cofactor termed “FAS” (factor activating ExoS), a member of the 14-3-3 protein family (Fu et al 1993). Through this domain ExoS induces cytotoxicity by inactivating the eukaryotic Ras protein (Henriksson et al 2000), an essential signaling component of cell differentiation and proliferation pathways (Crespo and Leon, 2000). ExoT ADP-ribosylates and inhibits proteins of the Crk family (Sun and Barbieri 2003) which play a role in the process of phagocytosis (Savill and Fadok 2000).

ExoU is a highly potent effector which rapidly disturbs host cell membranes to cause a necrosis-like cell death (Sato et al 2004, Finck Barbacon et al 1997). The N-terminal region of this effector contains a phospholipase A (PLA) domain which promotes hydrolysis of membrane phospholipids through use of a catalytic serine-aspartate dyad (Philips et al 2003). The C-terminal region of ExoU contains a domain which localizes it to the interior of the host plasma membrane upon translocation and is essential for full PLA activity (Veessenmeyer et al 2020). ExoU is exclusively activated upon binding to a eukaryotic cofactor, SOD1 (superoxide dismutase) (Sato et al 2006).
ExoY is an adenylyl cyclase which elevates host cell cAMP levels (Yahr et al 1998). This activity may contribute to cytoskeletal rearrangement, although the function this effector serves hasn’t been well characterized. Deletion of ExoY has little effect on the ability of PAK strains to induce cytotoxicity in CHO cells, suggesting it may only play a minor role in the infection process (Lee et al. 2005).
Figure 2: Contact with a host cell membrane activates the type III secretion system.
1.5 The structure of the Type III translocon is not well characterized.

The advent of powerful imaging techniques has made it possible to visualize many components of the Type III secretion system at high resolution and gain a deeper mechanistic understanding of these complex molecular machines. CryoEM structures have been generated for the injectisome needles of several species including *Salmonella typhimurium* (Hu et al 2017), *E. coli* (Majewski et al 2019), and *Shigella flexneri* (Lunelli et al 2020). Although the injectisome has been studied in great detail, little is known about the structure of the translocon pore formed by PopB and PopD during *in vivo* infections. The molten globular structure of these proteins makes them challenging to crystallize *in vitro*, and attempts to generate CryoEM structures of membrane-associated translocons have not been met with much success.

Studies *in vitro* have shown that PopB and PopD associate with liposomal membranes to form a hexadecameric pore with 1:1 stoichiometry of the translocators (Romano 2016). In addition to forming the heterooligomeric pore, both PopB and PopD can form distinct homooligomeric pores *in vitro* (Faudry et al 2006, Romano et al 2016). Homooligomeric pores are not formed during *in vivo* infections, however, and both PopB and PopD must be present for the formation of a functional translocon pore when delivered through the injectisome needle (Tang et al 2018, Goure et al 2004). PopB is predicted to play a role in facilitating the correct insertion of PopD into host membranes, perhaps by inducing a conformational change (Tang et al 2018, Romano et al 2016).
Both translocators have been found associated with the membranes of host cells after infection and removal of bacteria, suggesting either a peripheral or integral association with the host membrane. It has been demonstrated that membrane-bound translocators cannot be removed in the presence of harsh denaturing conditions or high salt concentrations, which suggests they act as integral membrane proteins (Goure et al 2004, Tang et al 2018). However, the transmembrane structure of the membrane-integrated translocons in *P. aeruginosa* has not been characterized. The PopD homologs IpaC (*Shigella flexneri*) and SipC (*Salmonella enterica*) have been shown to adopt an N-in/C-out topology in native membranes (Russo et al 2019). However, several key differences were also observed between the two which are believed to contribute to their unique roles in infection. Due to the structural variations observed between translocons of different species, we reasoned that PopD might adopt a distinct topology in native membranes.

1.6 The N-terminus and a middle segment of PopD are exposed to host cell cytosol upon membrane insertion.

Currently, we are working to build a topological map of PopD after insertion into host membranes *in vivo*. HeLa cells are employed as a model for infection with *Pseudomonas aeruginosa* strain PAK, which expresses the effectors ExoS, ExoT, and ExoY but is deficient in ExoU (Lee et al 2005). After infection, membrane-bound translocon structures can be isolated in their native conformation for study.
To study topology, we have already obtained some data through studies of PopD derivatives with a glycogen synthase kinase (GSK) tag inserted at key loci. The multi-subunit GSK protein is involved in regulation of various cellular pathways including glycogen synthesis (Welsh et al 1996), Wnt signalling and embryonic development (Nusse 1997), and turnover of cyclin D1 (Diehl et al 1998). It is also a downstream target of kinases in the eukaryotic Akt cell survival pathway, which inhibit GSK activity by phosphorylating it at Serine 9 of the beta subunit or Serine 21 of the alpha subunit (Cross et al 1995). The GSK tag (MSGPRRTTSFAES) is a short peptide containing the N-terminal Akt phosphorylation site from human GSK-3β (Torruellas Garcia et al 2020). If inserted at various loci within the primary sequence of a transmembrane protein, phosphorylation of the GSK tag can report the tagged region’s exposure to the eukaryotic cytosol.

Bioinformatics analysis of the PopD primary sequence reveals two putative membrane-spanning hydrophobic helices (Fig. 3A). Segment H1 was predicted under native conditions. The second transmembrane helix, H2, was detected when the analysis was run using protonated Asp and Glu residues to simulate the acidic pH necessary for PopD to successfully bind liposomal membranes in vitro. (Tang et al 2018).

Fluorescence quenching experiments with NBD-labeled PopD have revealed that both the H1 and H2 segments are exposed to the membrane interior upon in vitro translocon formation, supporting the identification of these regions as putative transmembrane segments (Tang et al 2018).
In addition to the two hydrophobic helices, a segment was identified in the C-terminus with the potential to form an amphipathic helix (A1). Due to the amphipathic nature of this segment, it may be involved in formation of the PopD pore interface. Deletion of the A1 segment attenuated the ability of PopD to associate with membranes, suggesting it plays an essential role in membrane anchoring (Tang 2018).

Sites for GSK-tag insertion were chosen based on the locations of putative cytosolic or extracellular loop domains within PopD (Fig. 3A). Areas permissible to tag insertion were identified based on the functional characterization of several segment deletions in YopD, a PopD homolog from the Yersinia pseudotuberculosis T3SS (Olsson et al 2004).

Notably, GSK tags inserted after residues 29 and 40 exhibited strong phosphorylation, indicating that the N-terminus of PopD is exposed to host cell cytosol (Fig. 3B). Phosphorylation was also observed for two tags (GSK157 and 178) inserted after the putative hydrophobic helix H2, suggesting a location within a cytosolic loop domain. Previously, a single-pass N-in/C-out topology for PopD was proposed with H1 as the sole membrane-spanning segment (Armentrout et al 2016). This does not account for the observed GSK157 and 178 phosphorylation, however, and a more complex PopD transmembrane structure seems likely.

Multiple models may be inferred from the bioinformatics analysis and GSK phosphorylation data (Fig. 3C). The C-terminus of translocon-associated PopD was previously shown to crosslink with the PcrV needle tip complex, suggesting accessibility
to the extracellular space and a C-out topology of the protein which is reflected in our models (Armentrout et al. 2016). All models also account for the cytosolic exposure of the N-terminal region indicated by the phosphorylation of PopDGSK29 and 40.

If A1, rather than H1, acts as the sole membrane spanning helix, then it is still possible to have a simple N-in/C-out topology with the majority of the PopD structure exposed to the cytosol (Fig. 3C, left panel). PopD could also adopt a triple pass-transmembrane structure with the N-terminus and H1-A1 loop domain exposed to the cytosol, while the C-terminus and H2-H1 loop are exposed to the extracellular space (Fig. 3C, middle panel). As the triple-pass transmembrane structure accounts for all the predicted helices and agrees with the GSK phosphorylation data, it appears to be the most likely representation of PopD topology.

In order to distinguish between these models, however, further characterization of the region surrounding GSK tags 104 and 115 is required. These residues could be in an extracellular loop domain between segments H2-H1 (Fig. 3C, middle panel), but could also be partially inserted into the membrane (Fig. 3C, right panel) or exposed to the cytosolic face of the membrane but hindered from interaction with Akt enzymes (Fig. 3C, left panel). We also need to confirm the location of the GSK208 variant, which wasn’t phosphorylated despite phosphorylation of the adjacent GSK178 tag and a predicted location within the cytosolic H1-A1 loop.
Despite the useful data generated by the GSK phosphorylation assay, there are several limitations to the method which need to be addressed as well. The GSK assay can only conclusively report cytosolic exposure of a loop domain due to its reliance on eukaryotic kinases. Lack of phosphorylation could indicate a tag’s exposure to the transmembrane or extracellular space, or simply mean that it is sterically hindered from interaction with the kinase. Additionally, GSK tag insertion in certain locations precluded the formation of functional translocons. Despite being a small tag, insertion within functional regions can be detrimental to PopD structure by disturbing its interactions with other proteins or disrupting the transmembrane structure. The PopDGSK60 variant was unable to be secreted, likely due to disruption of the PopD-PcrH binding interface, and it was not further characterized (Tang 2018). PopDGSK287 showed a reduced ability to associate with membranes, possibly due to disruption of the A1 transmembrane segment. Insertion of GSK tags after residues 104, 115, and 237 disrupted the ability of the translocon to traffic effectors into the cytosol, suggesting a structural disturbance. To fill in the gaps in knowledge left by the GSK phosphorylation results and generate a more complete topological map of PopD, development of another assay was required.
Figure 3: The GSK-tag phosphorylation reveals possible novel transmembrane topologies for PopD. (A) Bioinformatics analysis reveals two putative, membrane-spanning hydrophobic helices (H1 and H2) and a C-terminal amphipathic helix (A1). (B) HeLa cells were infected at an MOI of 30 with PAK ΔPopDΔExsEΔExoSTY strains complemented with plasmids expressing the indicated GSK-tagged PopD derivatives. Membrane proteins were obtained by Triton X-100 solubilization and analyzed by western blotting with anti-phosphoGSK antibodies (1:1000). Images in (A) and (B) were generated by Yuzhou Tang. (C) The bioinformatics analysis in combination with the GSK-tag labeling data reveal multiple possible transmembrane topologies for the PopD protein. Black numbers represent the sites of phosphorylated GSK tags, while red-numbered tags weren’t phosphorylated. Blue numbers denote the starting and ending residues of the putative transmembrane segments.
CHAPTER TWO

DEVELOPMENT OF A CYSTEINE-SPECIFIC LABELING ASSAY TO PROBE THE TRANSMEMBRANE TOPOLOGY OF PopD

2.1: Introduction

Conclusive interpretation of the GSK-tag analysis required an assay which could confirm the exposure of a loop region to the extracellular space. The assay also needed to be minimally invasive to the PopD structure in order to study the topology of regions that weren’t permissible to GSK tag insertion. We here employ a site-specific, single cysteine labeling assay with the reagent PEG maleimide. PEGylation has been employed by other groups as a method for defining the topology of transmembrane proteins (Davis et al 2019, Russo et al 2019) and may translate well to topological study of the T3SS translocon of \emph{P. aeruginosa}. This assay only requires the use of a single cysteine as a reporter group and will allow us to confirm the locations of extracellular loop domains. Therefore, the PEGylation assay will allow us to fill in the gaps left by the GSK labeling assay and generate a more comprehensive roadmap of the PopD transmembrane topology in native membranes.
2.1.1 PEG maleimide can specifically label accessible cysteine residues.

PEG maleimide is a ~5 kDa, membrane impermeable polyethylene glycol polymer linked with a terminal maleimide group (Fig. 4A). Maleimide specifically reacts with the thiol group of cysteines, forming a covalent thioether linkage between the target protein and the polymer. Successful labeling will lead to a 5 kDa increase in the molecular weight of the target protein which is readily detectable on a western blot (Fig. 4B).

The specificity of the maleimide labeling reaction is pH dependent, but it has been shown to react exclusively with cysteines between pH 6.5-7.5 (Hermanson 2013). Reactions carried out at higher pH may lead to cross-reactivity with amines (Brewer and Riehm 1967), but at pH 7.0 the reaction is 1000 times faster with thiol side chains than with amines (Hermanson 2013). As such, carrying out these reactions around physiological pH will enable specific labeling of cysteines while avoiding off target binding.
Figure 4: PEG maleimide can label accessible cysteines on a protein to cause a ~5 kDa increase in the molecular weight. (A) PEG maleimide is a large (5 kDa) polyethylene glycol polymer linked with a terminal maleimide group. (B) PEG maleimide can covalently link to an accessible cysteine on a target protein, increasing the molecular weight by 5 kDa.
2.1.2 PEG maleimide is a useful reporter of transmembrane topology due to its membrane impermeability

Due to its polar character and membrane impermeability, PEG maleimide is a useful tool for mapping the transmembrane topology of proteins. Similar to the GSK assay, HeLa cells are here employed as a model for infection with PAK. After infection and thorough washing of bacteria, we can obtain HeLa cells with functional PopB:PopD translocons inserted into their membranes. Cells will then be incubated with PEG maleimide in the presence or absence of membrane-permeabilizing conditions in order to probe topology.

If the inserted residue is located in a cytosolic domain, PEG maleimide labeling will only be observed after permeabilization of the host membrane provides the reagent with access to the cytosolic space (Fig. 5A). Cysteine residues located in extracellular loop domains will be successfully labeled regardless of membrane permeabilization (Fig. 5B). Failure to label a residue after membrane permeabilization indicates that it may not be accessible e.g., in a transmembrane domain, interface with PopB in the hetero-complex, or interacting with another protein such as the PcrV needle tip.

While this assay would generally require removal of all native cysteines from the target protein before addition of the reporter cysteines, PopD is naturally cysteine-free and as such is an ideal candidate for this method (Bröms et al 2003).
Figure 5: Site-specific labeling with PEG maleimide can be used to probe transmembrane protein topology. (A) If an inserted cysteine is located in a cytosolic loop domain, it can only be labeled with PEG maleimide after permeabilization of the cell membrane. (B) Externally located cysteine residues can be PEGylated while the cell membrane remains intact.
2.1.3 rPFO selectively permeabilizes eukaryotic cell membranes.

The PEGylation assay requires a method to selectively permeabilize eukaryotic cell membranes while leaving the bacteria intact. Lysis of the bacteria will release any PopD-PcrH complexes present in the bacterial cytosol, allowing the PopD to get PEGylated in solution and giving a false positive result. To achieve this, we are employing a recombinant form of the *C. perfringens* cholesterol-dependent cytolysin Perfringolysin O (PFO), which perforates cell membranes forming a 250-300Å diameter pore complex (Verherstraeten et al 2015).

The pore-forming ability of PFO is dependent on surface-accessible cholesterol, which is abundant in eukaryotic membranes (Heuck et al 2007) but lacking in bacteria. This protein therefore allows for selective permeabilization of HeLa cell membranes in our assay, which will release the cytosolic fraction (Fig. 6) and allow the passage of PEG maleimide across the membrane to access intracellular loop domains of PopD. In these assays we employ a recombinant form of PFO (rPFO) with the native cysteine 459 residue mutated to an alanine (C459A). Removal of this cysteine does not abolish the pore-forming activity of rPFO (Johnson et al 2017), but it will prevent any blockage that may occur due to PEG maleimide labeling the pore complex as it crosses the membrane.
Figure 6: Permeabilization of HeLa cell membranes with rPFO effectively releases the cytosolic fraction. HeLa cells were incubated with 2 µM rPFO for 30 minutes at room temperature (~22-25°C), then pelleted to separate the soluble cytosolic components from the membrane fraction. The membrane and cytosolic fractions were chloroform-methanol precipitated and resuspended in 1X western blot loading buffer. Samples were analyzed with anti-phosphoGSK3β 1:1000 and anti-rabbit HRP 1:20,000. GSK3β is a protein intrinsic to the mammalian cell cytosol.
2.1.4 Sites for cysteine insertion were chosen based on conservation analysis between Ysc-family homologs of PopD.

In order to choose sites for cysteine substitution, it was necessary to identify residues that are not well-conserved between PopD and homologous translocators from closely related T3SS. Based on sequence conservation between the injectisome ATPase machinery, the T3SS of different species have been organized into various families (Troisfontaines and Cornelis 2005). The *P. aeruginosa* T3SS belongs to the Ysc family, which is named after *Yersinia* spp. and encompasses many diverse pathogens (Troisfontaines and Cornelis 2005). A multisequence alignment was set up between PopD and its homologs from the Ysc family: YopD (*Yersinia pseudotuberculosis*), AopD (*Aeromonas hydrophila*), LopD (*Photobacterium luminescens*) and VopD (*Vibrio parahaemolyticus*) (Fig. 7A). Residues located within the predicted loop domains, N terminus, or C terminus, and not well conserved between homologs were identified as candidate sites for cysteine insertion. Serines and alanines were also preferentially chosen due to their structural similarities with cysteine.

The N-terminal A43C cysteine is expected to be located in the host cytosol based on the phosphorylation seen for the GSK29 and GSK40 tags (Fig. 7B). The A212C cysteine is located in the predicted cytosolic loop between segments H1 and A2 (Fig. 7B). The unexpected failure of the GSK208 tag to get phosphorylated could indicate location in a transmembrane domain or steric hindrance preventing accessibility to the Akt enzymes. The A212C variant will therefore be essential for distinguishing between these two possibilities. We expect to see PEGylation of the PopD\textsuperscript{A43C} and PopD\textsuperscript{A212C} variants only
upon membrane permeabilization due to the predicted cytosolic exposure of these regions.

The A84C cysteine is located in the putative extracellular loop domain between segments H2 and H1 (Fig. 7B). Since GSK tags located in this region were not phosphorylated or functional for translocation, this residue will be vital to confirm if this loop is exposed to the extracellular space. The A292C cysteine is located in the C-terminus of the protein, which is predicted to be extracellular as described previously (Fig. 7B). As such, we expect to see PEGylation of the PopD$^{A84C}$ and PopD$^{A292C}$ variants without permeabilization of the host plasma membrane.

In addition to the inserted cysteine, all constructs except for the PopD$^{A43C}$ variant include the N-terminal GSK40 tag. This tag is only phosphorylated when PopD adopts a native topology in the membrane and can be used to confirm that the PopD variants are properly inserted into active translocons. (Tang et al 2018).

A combination of point mutagenesis PCR and Gibson assembly was used to generate various pUCP18 plasmid constructs expressing the PopD single cysteine variants. Constructs were transformed into *P. aeruginosa* strain PAK through an electroporation method as described by Choi KH et al, 2005.
Figure 7. Sequence alignment of PopD with other homologous proteins in the Ysc family.
(A) Multisequence alignment between PopD (AAO91774.1) and homologous Ysc family translocon proteins: YopD of *Yersinia pseudotuberculosis* (AAA72322.1), AopD of *Aeromonas hydrophila* (AAV30236.1), LopD of *Photobacterium luminescens* (AAO18056.1), and VopD of *Vibrio parahaemolyticus* (EDM61192.1). Putative hydrophobic and amphipathic helices are indicated as H1, H2, and A1. Candidate sites for cysteine mutations are indicated with red boxes and the numbers of the residues. Multisequence alignments were generated using Clustal Omega and then exported to Jalview for annotation. (B) Model of the proposed PopD transmembrane structure with cysteine mutations indicated within putative loop domains.
2.2 Results

2.2.1 The four PopD single cysteine variants were secreted to the media and partially labeled with PEG maleimide.

It was first necessary to ensure all PopD variants could be effectively secreted and PEGylated \textit{in vitro}. Failure to be secreted could reflect a disruption in the region of PopD expected to interact with the PcrH chaperone. Because PcrH is essential for stabilizing the molten globular structure of PopD in an aqueous environment as well as localizing the translocator to the basal body of the injectisome, disruption of the binding interface will prevent PopD exit from the cytosol. It was also necessary to ensure that all of the variants could be successfully labeled with PEG maleimide upon secretion. This validates that the constructs contain the required cysteine, and ensures that PEG maleimide functions to label the translocators.

Although secretion of T3SS translocon proteins is normally induced by contact with the membrane of a host cell, it can also be induced non-specifically through depletion of extracellular calcium ions (Sawa et al 1999). The chelating agent EGTA is ideal for generating the required low calcium condition due to its high affinity for Ca$^{2+}$ ions and a lower affinity for other ions such as Mg$^{2+}$. For secretion experiments, the media is also complemented with magnesium chloride to maintain a high extracellular concentration of Mg$^{2+}$. 
Cultures of PAK were grown to mid-logarithmic phase, and secretion was induced through addition of 5 mM EGTA and 20 mM MgCl₂. The culture media containing the secreted proteins was separated from bacteria by centrifugation, PEG maleimide was added to a final concentration of 5 mM, and samples were incubated for 30 minutes at 37°C. Excess of PEG maleimide was quenched through addition of a molar excess of DTT, a reducing agent containing two thiol groups. Samples were then TCA precipitated and analyzed by western blotting with anti-PopD antibodies. These results indicate that all four cysteine variants were effectively secreted, and were successfully PEGylated in vitro (Fig. 8). The PopD⁴³C variant appears as a smaller molecular weight band than the other cysteine mutants because this variant lacks the GSK40 tag. PopD forms water-soluble aggregates when secreted non-specifically to the extracellular media, which may shield some cysteines from accessibility to PEG maleimide. (Romano et al 2011, Schoen et al 2003). Formation of these aggregates could explain why the translocators were not labeled with 100% efficiency upon non-specific secretion.
Figure 8: All single cysteine PopD variants generated can be effectively secreted, and secreted PopD can be PEGylated. Cultures of the indicated PAK strains were grown to the middle of the logarithmic phase, and secretion was induced through chelation of extracellular Ca²⁺ ions with EGTA. After an additional incubation under the low calcium condition, bacteria were centrifuged to separate from secreted proteins. For PEGylation experiments, PEG maleimide was added to the supernatant to a final concentration of 5 mM, and samples were incubated at 37°C for 30 minutes. PEGylation was quenched by adding DTT to a final concentration of 10 mM and incubating for 5 minutes at room temperature. Samples were TCA precipitated and resuspended in 32 µL of 1x loading buffer, then 15 µL of sample were analyzed by western blotting with anti-PopD 1:1000 (G3284, Thermo Fisher) and anti-rabbit HRP 1:20,000 (Cell Signaling, 7074P2). Results are representative of at least two experimental replicates per variant.
2.2.2 After infection, the four PopD single cysteine variants are found associated with HeLa cell membranes.

Introduction of cysteines within the PopD primary sequence has the potential to preclude translocon formation by affecting PopD structure and interactions with other proteins. To rule out this possibility, the next step was to establish that all of the cysteine variants were able to associate with the membrane of the host cell after infection.

To isolate membrane-bound PopD, HeLa cells were first infected with PAK strains deficient in wild-type PopD, the effectors ExoS, ExoT, and ExoY, and the regulator ExsE (PAKΔPopDΔExsEDΔExoSTY), and expressing the indicated PopD variant. (Tang et al 2018, Tang et al 2021). Deletion of the ExsE gene allows for overexpression of the T3SS and insertion of more translocators into the membrane, while deletion of the effectors ExoS, ExoT, and ExoY attenuates cytotoxicity in the HeLa cells post-infection. After infection, bacteria were washed off and the HeLa cells, now containing the membrane-bound translocators, were permeabilized with rPFO to release the cytosol. Permeabilized cells were separated from the cytosolic fraction by centrifugation followed by washes in DPBS. Samples were then incubated at 4°C with 0.1% Triton X-100. At this concentration and temperature, Triton X-100 has been shown to exclusively solubilize eukaryotic cell membranes and associated proteins while leaving bacterial membranes intact (Tang et al 2021).
All of the cysteine variants were found associated with the host membrane, indicating that cysteine substitutions at the indicated residues did not preclude membrane association (Fig. 9). While the PopDA\textsuperscript{A43C} mutant appeared to insert with lowered efficiency here, it was shown to insert with a similar efficiency to the other variants in future experiments (Fig. 11B).
**Figure 9:** All of the PopD variants can be found associated with HeLa cell membranes. HeLa cells were infected with PAK ΔPopDΔExsEΔExoSTY strains expressing the indicated PopD cysteine variants for 1 hr at an MOI of 30. Cells were washed and then permeabilized with 2 μM rPFO for 30 minutes at room temperature (~22-25°C). Permeabilized cells were pelleted to separate the cytosolic and membrane fraction. Supernatant was discarded and pellets were washed, then incubated 30 minutes in 0.1% Triton X-100 to solubilize HeLa cell membranes and associated proteins. Samples were pelleted to separate out insoluble components, then the supernatant was chloroform-methanol precipitated and analyzed through western blotting with anti-PopD 1:1000 and anti-rabbit HRP 1:20,000.
2.2.3 All generated PopD variants were functional for translocation of effector proteins into HeLa cells.

It was next necessary to validate that the PopD variants were able to form functional translocon structures in the host cell membranes. Non-functional translocons may represent nonnative topologies of PopD in the membrane, and therefore cannot provide useful information about the transmembrane structure.

The functionality of a translocon pore can be assessed through a HeLa cell cytotoxicity assay. In this assay, we can use the actin depolymerization and host cell rounding phenotype caused by the effectors ExoS and ExoT as a reporter of successful translocation (Lee et al 2005). Observation of the rounding phenotype indicates the effector proteins are able to access the host cytosol, which can only happen through a functional translocon pore (Fig. 10A).

HeLa cells are infected with PAK strains that are deficient in wild-type PopD protein (PAK ΔPopD) and expressing the indicated PopD variant. Infected cells are screened for the rounding phenotype over two hours, and compared against cells infected with PAK ΔPopD::pUCP18_PopD<sup>WT</sup> as a positive control, and cells infected with PAK ΔPopD::pUCP18 (empty plasmid) as a negative control. After 2 hours, all cysteine variants tested were able to induce rounding at a level comparable to that of the wild-type PopD protein, indicating formation of functional translocons (Fig. 10B). Cell rounding was not observed in the HeLa cells infected with the PAK strain carrying an empty
plasmid. The ability of all single cysteine variants to form a functional translocon confirmed that none of the introduced mutations were detrimental to the structure and assembly of PopD. Therefore, they will all be useful reporters of native PopD topology.
Figure 10: All PopD cysteine variants formed functional translocon pores in host membranes. (A) The rounding assay allows monitoring of the formation of a functional translocon. If the PopD variant can form a functional translocon, the effectors ExoS and ExoT are able to access the host cytoplasm and induce a rounding phenotype. If variants are nonfunctional, no rounding is observed. (B) HeLa cells were infected at an MOI of 10 with PAK ΔPopD strains complemented with plasmids carrying the various PopD cysteine derivatives, the wild-type PopD gene, or an empty plasmid (pUCP18). Cells were imaged after 2 h of infection with indicated PAK strains.
2.2.4 When inserted into the host cell membrane, the PopD cysteine variants were not effectively PEGylated under the conditions tested.

Now that I was confident the variant constructs were functional, it was next necessary to confirm that the single-cysteine PopD variants could be effectively labeled in membranes. HeLa cells were washed and then infected with PAK ΔPopDΔExsΔExoSTY strains expressing the indicated PopD cysteine variants. Infection was carried out at an MOI (multiplicity of infection) of 30. At this MOI, we were able to maximize the insertion of translocons into membranes while also maintaining viability of the host cells (Tang et al 2021). Although the PAK strains used don’t express effectors, insertion of translocons alone or secretion of other toxins can cause cell death at a high MOI (Tang et al 2021).

After infection, bacteria were washed off and HeLa cells were incubated with 2 μM rPFO protein to permeabilize the membranes and allow release of cytosolic components. The rPFO was also supplemented with 5 mM TCEP and 1 mM EDTA to reduce any potential disulfide bonds formed by the PopD cysteine variants. PopD has been shown to crosslink to form homodimers upon cysteine substitution in certain locations (Armentrout et al 2016), so it is essential to reduce disulfides in order to facilitate labeling with PEG maleimide. TCEP is a reducing agent which doesn’t contain thiols, and will reduce disulfide bonds without inactivating the PEG maleimide reagent (Rüegg et al 1977). EDTA will prevent re-oxidation of disulfides by chelating excess metal ions in the media. After a 30 minute incubation with 5 mM PEG maleimide, excess PEG reagent was
quenched by addition of a molar excess of DTT, and membrane-associated proteins were separated from attached bacteria through incubation with 0.1% Triton X-100 at 4°C.

Because we expected all of the substituted cysteines to be located in loop domains or the termini of the protein, all of the PopD variants should have been effectively PEGylated after membrane permeabilization. However, successful labeling was not observed for any of the variants tested (Fig. 11B). While there was a band seen around the expected size of PEGylated protein, this band also appears after membranes are infected with wild-type PopD (Fig. 11B), and it likely represents non-specific binding of the antibody. The anti-PopD antibodies are polyclonal and therefore may bind to off-target bands when a low amount of total protein is present on the membrane. Additional bands could be removed by additional TBST washes in between incubation of the PVDF membranes with primary and secondary antibodies (Fig. 11B, middle panel). However, a band corresponding to PEGylated PopD was still not detected after this additional treatment.

The failure of the cysteine variants to get labeled after membrane permeabilization was surprising. I expected the PopD^{A43C} variant in particular to be highly accessible for labeling due to the phosphorylation of GSK29 and GSK40 tags which indicated the N-terminus was accessible to the cytosol. The observed labeling failure indicated that the conditions for the assay were suboptimal and needed to be modified in order for successful PEGylation to occur.
**Fig. 11: PopD cysteine variants were not effectively PEGylated when inserted into membranes.**

(A) Workflow of the PEGylation assay. (B) After rPFO permeabilization and PEGylation HeLa cells were infected with PAK ΔPopDAΔExsΔExoSTY strains expressing the indicated cysteine variants at an MOI of 30. Bacteria were removed and infected cells were permeabilized with 2 μM rPFO. Cells were pelleted to remove cytosolic components and resuspended in 230 μL DPBS with 5 mM PEG maleimide, then incubated for 30 minutes at 37°C. Membrane-associated proteins were obtained by solubilization in 0.1% Triton X-100 and analyzed by western blotting with anti-PopD 1:1000 and anti-rabbit HRP 1:20,000. Results are representative of at least two replicates per cysteine variant.
2.2.5 Residue 212 of PopD is exposed to host cell cytosol upon membrane insertion.

One reason the detection of PEGylated PopD failed could be due to a low amount of total PopD protein. Even when the PEGylation was tested with secreted proteins, PEGylation efficiency was not very high and only a small proportion of the total PopD protein was labeled (Fig. 8). When testing PopD insertion in vivo, numerous wash steps are required to remove bacteria and the HeLa cell cytosolic fraction. While this ensures that we obtain only membrane-associated translocators, it also causes a low yield of total PopD protein when compared to what can be obtained from the secretion assay. Therefore, it could be possible that PEGylation is occurring in the membrane system but is not observed because the band corresponding to labeled protein is below the detection threshold.

To obtain more total PopD protein for analysis, I scaled up the method to include a higher number of HeLa cells (2.8x10^6 cells vs 1.2x10^6 cells for prior experiments). The amount of bacteria added was also scaled up appropriately to maintain an MOI of 30, which increased the total amount of translocon proteins that could be obtained during the experiment. Increasing the amount of total protein could also prevent detection of background bands (Fig. 11B).

I chose to work with one variant at a time from this point on and optimize the conditions as best I could, then apply the improved protocol to the other variants. I chose the PopD^{A212C} variant for these purposes because it was predicted to be exposed to the cytosol
upon membrane insertion. Determining the location of this cysteine would also fill in a gap in knowledge left from the GSK assay, in which the GSK208 tag was not phosphorylated but was predicted to be cytosolically exposed.

HeLa cells were infected with PAK\DeltaPopD\DeltaExsE\DeltaExoSTY::pUCP18_PopD_A212C as described previously. PEGylation was carried out in the presence or absence of 2 \mu M rPFO (Fig. 12, lanes 2 and 3). Membrane-bound proteins were isolated as described previously and analyzed by immunoblotting.

Labeling of the PopD^{A212C} variant was seen only upon cell permeabilization with rPFO, indicating that this residue is in a cytosolic loop domain (Fig. 12, lane 2). This indicates that the GSK208 tag located nearby was also located in the cytosolic domain. Therefore, the lack of phosphorylation seen for the GSK208 tag was likely due to steric hindrance preventing interaction with Akt enzymes rather than location of the tag within a transmembrane domain.

Although labeling was successful in this experiment, the efficiency was low and similar results could not be obtained for other PopD variants. As such, the method required further optimization in order to improve PEGylation.
**Figure 12: Residue 212 of PopD is exposed to the cytosolic face of the host membrane upon translocon formation.** HeLa cells (2.8x10⁶ cells per flask) were infected with PAK ΔPopDΔExsΔExoSTY::pUCP18_PopD_A212C at an MOI of 30 for 1 hr. Sample in the middle lane was permeabilized with 2 μM rPFO in DPBS for 30 minutes at room temperature, others were incubated in DPBS alone under the same conditions. To indicated samples, PEG maleimide was then added to a final concentration of 5 mM and labeling was carried out for 30 minutes at 37°C. Labeling was stopped through addition of 10 mM DTT. Samples were pelleted and resuspended in 0.1% Triton X-100 in DPBS to selectively solubilize bacterial membranes, and analyzed by western blotting with anti-PopD 1:1000 and anti-rabbit HRP 1:20,000.
2.2.6 Solubilization of secreted proteins with SDS prior to labeling causes a minor increase in labeling efficiency

The low labeling efficiency of PopD variants in vivo may be a result of steric hindrance shielding cysteine residues from PEG maleimide. To explore this possibility, I next examined the effect that pre-treatment of membrane complexes with 2% SDS would have on the efficiency of PEGylation in native membranes. Unlike 0.1% Triton X-100, which will solubilize membrane-bound translocons while preserving secondary structure and protein-protein interactions (Tang et al 2018), solubilization with 2% SDS should break up the translocon complexes more thoroughly and render more cysteines accessible for labeling. If labeling of translocon-associated PopD variants is only possible after SDS solubilization, this could indicate that protein-protein interactions or the host cell membrane are shielding the cysteines from accessibility to PEG maleimide.

Before testing the effects of SDS solubilization on membrane-inserted translocons, I first observed if it would improve the labeling of PopD variants that were secreted non-specifically to the media. As PopD forms water-soluble aggregates in solution, we hypothesized that SDS would dissociate these complexes and improve the PEGylation efficiency by rendering more cysteines available for labeling. By observing the maximum labeling possible for secreted proteins, we could get an idea of how much labeling we could expect to see for membrane-bound translocons as well.
Secretion was induced in a culture of PAKΔPopDΔExsEΔExoSTY::pUCP18_PopD_A212C through chelation of extracellular calcium, as described previously (Section 2.2.1). Secreted proteins were incubated with DPBS buffer alone (Fig. 13, Lane 1), 5 mM PEG maleimide (Fig. 13, Lane 2), 5 mM PEG maleimide with 0.1% Triton X-100 (Fig. 13, Lane 3), or 5 mM PEG maleimide with 2% SDS (Fig. 13, Lane 4). Although the PopD12A variant was employed for this experiment, I expect that results will be similar for the other PopD variants if subjected to the same conditions after secretion. While pre-treatment with 2% SDS did improve the efficiency of labeling, the secreted protein was only partially PEGylated under these conditions (Fig. 13). This indicates that other conditions of the assay may also play a role in decreasing labeling efficiency.
Fig. 13: Solubilization of secreted PopD aggregates with SDS prior to labeling led to an increase in PEGylation efficiency. Cultures of PAKΔPopDΔExsEΔExoSTY were grown to mid-logarithmic phase, and T3SS secretion was induced through addition of EGTA and MgCl₂. Secreted proteins were labeled with PEG maleimide after incubation with either 0.1% Triton X-100 or 2% SDS, or incubation with DPBS buffer alone. A non-PEGylated sample was included as a negative control.
2.2.7 Solubilization of membrane-bound translocon complexes with SDS prior to PEGylation improves the efficiency of PopD labeling.

After observing the effect of SDS solubilization on PopD labeling in vitro, the next goal was to observe the increase in labeling efficiency when membrane-inserted PopD was solubilized under similar conditions.

This was first tested with the PopD\textsuperscript{A212C} variant. HeLa cells were infected as described previously. Membranes were permeabilized with rPFO and solubilized with Triton X-100 to separate the membrane fractions from attached bacteria. Membrane fractions were then incubated with 5 mM PEG maleimide in the presence of either 2% SDS (Fig. 14A, Lane 1) or Triton X-100 alone (Fig. 14A, Lane 2) for 30 minutes at 37°C. A faint PEGylation band was seen upon treatment with Triton alone, while the proportion of labeled PopD was increased upon pretreatment with 2% SDS. The same experiment was set up for the PopD\textsuperscript{A43C} variant (Fig. 14B), and a similar increase in labeling efficiency after SDS solubilization was observed. This indicates that steric hindrance plays a role in reducing the efficiency of the PEGylation assay, and SDS solubilization can effectively counteract it to make more cysteines available for labeling.
Fig. 14: Membrane-associated PopD could be PEGylated with higher efficiency after solubilization with 2% SDS. (A) Membrane-bound PopDA212C was incubated with 5 mM PEG maleimide in the presence or absence of 2% SDS. (B) PopDA43C labeling efficiency was examined using the same conditions as in (A).
2.3 Materials and Methods

2.3.1 Generation of Single Cysteine PopD Constructs

Point mutagenesis PCR was used to generate cysteine substitutions at various loci within the PopD gene sequence. The pUCP18_PopD_GSK40 plasmid was used as a PCR template to generate all variants except for PopD^{A43C}, which was generated with a pUCP18_PopD^{WT} template plasmid. Primers used in this study are listed in Table 1. To generate the backbone for Gibson assembly, a pUCP18 template plasmid was transformed into a dam' strain of E.coli for propagation (gift of the Sandler lab) and miniprepped following the procedure laid out in the Zymo Research Quick DNA Miniprep Plus Kit (Zymo Research, 11-397). The backbone plasmid was double digested with EcoRI-HF and NruI-HF (New England Biolabs, R3101S and R3192S respectively) for 1 hr at 37°C. Purified backbone was obtained by separation on an 0.9% agarose gel followed by recovery with the Zymoclean Gel DNA Recovery Kit (Zymo Research, 11-301). For Gibson assembly, the PCR inserts and vector backbone were mixed together in a 5:1 molar ratio and incubated with Gibson Assembly master mix (NEB) for 1 hr at 50°C. The ligated plasmid was subsequently transformed into chemically competent E. coli DH5α by 45 seconds of heat shock at 42°C followed by 1 hr of incubation at 37°C, shaking at 225 rpm. Cultures were plated onto 100 ug/mL ampicillin plates and screened for transformants.

2.3.2 Preparation and Transformation of Electrocompetent PAK Cells

Protocol is adapted from Choi KH et al 2005. The desired PAK strain was grown overnight from a frozen glycerol stock overnight in LB media with 100 ug/mL
ampicillin, shaking at 225 rpm in a 37°C incubator. After approximately 15 hrs of incubation, cultures were split into 1.5 mL aliquots and pelleted by centrifugation 1 minute at 16,000 x g, room temperature. Supernatant was removed and the pellet was washed twice by resuspension in 1 mL of 300 mM sterile sucrose followed by a 30 second centrifugation at 16000 x g, room temperature. The pellet was then resuspended in 100 μL of 300 mM sucrose and mixed with 50 ng of desired pUCP18 plasmid for electroporation. Competent cells were electroporated at 1640 V and then 1 mL of sterile LB was promptly added. Cells were then transferred to a sterile microfuge tube and incubated 1 hr at 37°C shaking at 225 rpm. After incubation, cells were pelleted again at 16,000 x g for 1 minute and resuspended in 100 μL of LB media. Resuspended cells were plated on agar plates supplemented with 200 ug/mL carbenicillin and incubated 15 h at 37°C.

2.3.3 Type III Translocon Protein Secretion Assay

PAKΔPopDΔExsEΔExoSTY expressing the indicated PopD single cysteine variants was grown overnight in 4 mL LB media (Fisher) supplemented with 300 ug/mL carbenicillin (GoldBio). After approximately 15 hours of growth, bacteria were diluted to an OD$_{600}$ of 0.1 in fresh LB and grown at 37°C shaking at 225 rpm until the mid-logarithmic phase was reached (OD$_{600}$ 0.8-1.0, approx. 2.5 hr incubation). Secretion of the T3SS translocators was induced by adding MgCl$_2$ (Fisher) to a final concentration of 20 mM and EGTA to a final concentration of 5 mM. Under the low calcium conditions, bacteria were incubated an additional 1.5 hours at 37°C shaking at 225 rpm. Secreted proteins were separated from intact bacteria by centrifugation at 16000 x g, 10 minutes and
aspiration of the supernatant. For PEGylation experiments, PEG maleimide was added to a final concentration of 5 mM, and samples were incubated 30 minutes at 37°C before being quenched by addition of 10 mM DTT. PEG maleimide (Sigma Aldrich, 63187) was prepared as a 150 mM stock solution in DPBS (Cytiva) and stored at -20°C. Proteins were precipitated through addition of TCA to a final concentration of 10% v/v and analyzed by western blotting.

2.3.4 HeLa Cell Cytotoxicity Assay

HeLa cells were maintained in DMEM (Cytiva) supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO₂. For rounding experiments, HeLa cells were seeded into a 6-well plate at 1.2x10⁶ cells per well. Cultures of PAKΔPopDAΔExsEΔExoSTY expressing the PopD cysteine variants were grown overnight in LB supplemented with 300 ug/mL carbenicillin. After ~15 hrs of incubation, PAK cultures were diluted to OD₆₀₀ = 0.1 and grown to mid-log phase (OD₆₀₀ = 0.8-1.0). HeLa cultures were washed twice with warm DPBS and replenished with warm DMEM (no FBS). PAK were added to HeLa cell monolayers to an MOI of 10, and cultures were observed over 2 hrs for signs of cell rounding. Concentrations of bacteria in culture were calculated based on the assumption that an OD₆₀₀ of 1 = 2.0x10⁶ cells/mL.

2.3.5 Isolation of Translocators Associated with HeLa Cell Membranes.

Cultures of PAKΔPopDAΔExsEΔExoSTY expressing PopD cysteine variant constructs were grown overnight in LB supplemented with 300 ug/mL carbenicillin, shaking at 225 rpm in a 37°C incubator. HeLa cells were passaged into T25 flasks (2.4 x 10⁶ cells per
flask) and incubated overnight at 37°C. After approx. 15 hrs of incubation, PAK cultures were
diluted to an OD600 = 0.1 into antibiotic-free media and grown for 2.5 hours to reach mid-logarithmic phase. HeLa cells were washed twice with 1 mL of 37°C DPBS and then replenished with 2 mL of 37°C DMEM media without FBS. PAK were added to HeLa monolayers to an MOI of 30 and incubated 1 hr at 37°C, 5% CO2. Media was removed and cells were washed twice with pre-warmed DPBS, then incubated with 2 μM rPFO in DPBS for 30 minutes shaking at room temperature. The membrane fraction was separated from the cytosol by centrifugation for 10 minutes at 2000 x g, 4°C. Pellets were resuspended in 0.1% Triton X-100 for 30 min at 4°C, then centrifuged 18,000 x g, 15 min at 4°C to separate bacteria from the solubilized membrane fraction. Supernatant was chloroform/methanol precipitated and resuspended in 1X loading buffer (see Section 2.3.7) for Western blotting analysis.

2.3.6 PEGylation of Membrane Associated PopD

HeLa cells were infected with PAKΔPopDΔExsEΔExoSTY::PopD (cysteine variants) as described in the previous section (2.3.5). After infection and washes, cells were incubated in the presence or absence of 2 μM rPFO in reducing buffer (DPBS supplemented with 5 mM TCEP and 1 mM EDTA) for 30 minutes at room temperature. Concentrations of rPFO were quantified by measuring the A280 value (Beckman) and calculating the concentration using the Beer-Lambert law and a molar absorptivity coefficient of 74260 M⁻¹ cm⁻¹. Cells were scraped into microfuge tubes (PFO-treated samples) or left in the flask, and washed once with 1 mL of DPBS. Cells were incubated with 5 mM PEG maleimide in reducing buffer for 30 minutes at 37°C and then quenched by addition of
DTT to a final concentration of 10 mM. After quenching, intact HeLa cells were permeabilized with 2 μM rPFO to release the cytosolic fraction and scraped into a microfuge tube. Samples were pelleted at 2000 x g for 10 minutes at 4°C, then resuspended in 210 μL lysis buffer (DPBS supplemented with 0.1% Triton X-100) and incubated for 30 minutes shaking at 4°C. Samples were centrifuged at 18000 x g for 15 minutes at 4°C to pellet insoluble components. 200 μL of supernatant were collected and chloroform-methanol precipitated for western blot analysis.

2.3.7 Western Blotting

After precipitation, protein pellets were resuspended in an appropriate volume of 1X SDS sample buffer (50 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 5 mM EDTA, 16% 2-mercaptoethanol and 0.02% bromophenol blue) and heat shocked at 95°C for 5 min. Samples were run on a 12.5% acrylamide gel and subsequently transferred onto a PVDF membrane at 90V for 90 minutes at 4°C. Membrane was blocked in TBST with 5% w/v milk for 1 hr, room temperature. After three 15 min washes with 10 mL of TBST, membranes were incubated in primary antibody overnight at 4°C. Indicated primary antibodies were prepared in TBST supplemented with 0.25 g BSA (Fisher, Fraction V) and 0.02% sodium azide at the following concentrations: anti-PopD 1:1000 (Thermo Fisher, G3284, Day 35) and anti-phosphoGSK 1:1000 (Cell Signaling, 9336S). Membrane was washed with TBST and then incubated for 1 hour in a solution of 2.5% w/v milk and 1:20,000 HRP-conjugated anti-rabbit IgG (Cell Signaling, 7074P2) in TBST. Membranes were washed and incubated 5 minutes in a solution of ECL detection reagent (Amersham) for imaging.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Purpose</th>
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<tbody>
<tr>
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<td>Cysteine Mutagenesis</td>
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<tr>
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<td>NruIR</td>
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<td>PopD_cys_mut_check_F</td>
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<td>Sanger sequencing</td>
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Table 1: Primers used in this study.
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<td>PEGylation and T3SS secretion experiments</td>
</tr>
<tr>
<td>PAK ΔPopDExsEExoSTY::pUCP18_PopD_GSK40_A84C</td>
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Table 2: Bacterial strains used in this study.
2.4 Discussion

To gain insight into the structure and function of the *P. aeruginosa* translocon pore, we employed assays to map out the topology of the translocon protein PopD upon membrane insertion *in vivo*. We used a GSK assay to show that the N-terminus and a loop domain of PopD are exposed to the cytosolic face of the host cell membrane. To determine the locations of regions not reported by the GSK assay, we next worked to develop a site-specific, single-cysteine labeling assay using PEG maleimide. Four single cysteine variants of PopD were generated: PopD^A43C, PopD^A84C, PopD^A212C, and PopD^A292C. The PopD^A43C cysteine was located in the N-terminal region of PopD, which was previously shown to be exposed to the host cell cytosol. PopD^A292C was located in the C-terminus of the protein, which is believed to be accessible to the extracellular space (Armentrout and Rietsch 2016). The PopD^A84C and PopD^A212C cysteines were located in putative extracellular or intracellular loop domains, respectively. All of the variants were shown to associate with HeLa cell membranes and form functional translocon pores. All secreted PopD cysteine variants isolated from culture media were PEGylated *in vitro*, confirming the presence of a free cysteine residue.

Due to the membrane impermeability of PEG maleimide, we predicted that PopD^A43C and PopD^A212C would be labeled only after permeabilization of the host membrane with rPFO, while PopD^A84C and PopD^A292C would be labeled regardless of membrane permeabilization. We show that the PopD^A212C cysteine is PEGylated only after permeabilization of the host plasma membrane, suggesting a location in a cytosolic loop domain. This indicates that the failure of GSK208 to get phosphorylated was likely
because of hindered accessibility to Akt kinases. However, PopD^{A212C} was labeled with low efficiency, and the PopD^{A43C}, PopD^{A84C}, and PopD^{A292C} variants were not effectively PEGylated in vivo, even under membrane-permeabilizing conditions.

To determine why PEGylation was not very effective in native membranes, I next explored the possibility that steric hindrance could be affecting the PEGylation efficiency. By treating membrane associated translocons with 2% SDS prior to PEGylation, PopD^{A212C} was labeled with greater efficiency than what was seen after membrane permeabilization with rPFO alone. SDS solubilization also allowed for successful labeling of the PopD^{A43C} variant in native membranes, which was not PEGylated to detectable levels after permeabilization with rPFO. Since labeling efficiency is improved upon SDS solubilization, it seems likely that steric hindrance is playing a role in shielding cysteines from PEG maleimide.

This could indicate that the substituted cysteines are located in transmembrane domains. While this may be a possibility for the PopD^{A84C} and PopD^{A292C} variants, it doesn’t seem likely for PopD^{A43C} due to the suggested cytosolic exposure of the N-terminus. Steric hindrance could also be due to a close association between PopD and cytoskeletal filaments of the host cell, as was demonstrated for the IpaC translocator of S. flexneri (Russo et al 2019). Additionally, cysteine residues could be located close to an interface with PopB or within other domains of secondary structure that make them inaccessible for PEGylation. While PopD^{A292C} was predicted to be accessible to the extracellular
space, the C-terminus was also shown to interact with the PcrV needle tip (Armentrout et al 2016) and this residue may be inaccessible as a result.

The close interaction between individual PopD and PopB subunits in the translocon pore may also play a role in lowering labeling efficiency. While a few subunits per pore may be labeled successfully, recruitment of the large PEG maleimide polymers to the translocon will block further labeling by shielding free cysteines on the other subunits. SDS solubilization could counteract this by individually solubilizing the pore components and rendering more cysteines available for labeling. This could also explain the discrepancy in labeling efficiency between the PEGylation and GSK tag phosphorylation assays. As phosphate groups are small relative to PEG maleimide, phosphorylation of the pore complex won’t have the same effect in shielding other subunits from modification.
R THREE

CONCLUSION

3.1 Summary and Concluding Remarks
The PEGylation assay allowed successful identification of residue A212C as part of a PopD cytosolic loop domain, but the conditions must be further optimized in order to study the topologies of the other generated cysteine variants. Solubilization of PopD variant translocons with SDS prior to PEGylation allowed for successful labeling, which suggests that the cysteine residues are blocked from being accessed by PEG maleimide upon membrane insertion. Future troubleshooting should focus on identifying the source of this steric hindrance and making modifications to the assay that maximize labeling while still maintaining the native topology of PopD. Based on my results and the troubleshooting I have done so far, I outline below several directions which could be worth exploring for further optimization of this assay.

3.2 Future Directions
If steric hindrance from cytoskeletal filaments is precluding labeling, employing a different cell type as a model for infection may be a good solution. Erythrocyte ghosts are a good candidate, provided contact between the membrane and PcrV needle tip is sufficient for activation of the T3SS. By releasing the red blood cell cytosolic components prior to infection and leaving just the membrane, we may be able to get inserted translocons with minimal steric hindrance. While the absence of cytoskeletal
filaments might prevent the conformational change which allows for needle tip attachment to the translocon, it shouldn’t prevent insertion of the translocon itself into the membrane (Russo et al 2019). However, use of erythrocyte ghosts will prevent study of translocon functionality through the cytotoxicity assay. Additionally, GSK tag phosphorylation can’t be studied using this model due to removal of the Akt enzymes.

Testing smaller molecular weight PEGylation reagents may also be a viable method of avoiding interference due to steric hindrance. In addition to the PEG-5000 maleimide that we employed for this assay, a range of different molecular weight PEG reagents are available down to a minimum of 750 Da. The smaller size of these reagents may allow access to cysteine residues which were shielded from the bulkier PEG-5000 maleimide. As long as there is a significant enough molecular weight increase between unlabeled and labeled protein to visualize on a western blot, decreasing the size of the PEG maleimide used could improve labeling efficiency.

Additionally, optimization of the buffers and pH used for labeling could help further improve the efficiency. Although the PEGylation reaction is only specific for thiols between pH 6.5-7.5, raising the pH value may also improve the efficiency of labeling by shifting the equilibrium of thiol protonation/deprotonation in favor of thiolate (S-) formation. Because thiolates are better nucleophiles than the protonated thiol (SH) form, they may be more reactive with PEG maleimide. However, we will need to include the appropriate controls to ensure non-specific binding to amines does not occur at the higher pH. We also need to consider the hydrolysis of PEG maleimide to the unreactive
maleamic acid form, which occurs at greater rates as the pH of the buffer is raised (Hermanson 2013).

Pre-treatment of HeLa cells with the cysteine-blocking reagent iodoacetamide prior to infection may improve labeling efficiency by blocking the cysteines of native eukaryotic proteins from interacting with PEG maleimide. By subsequently quenching the iodoacetamide and infecting the HeLa cells with the PopD cysteine variants, we can increase the chance that the translocons will get labeled. Although there will be some turnover of the native HeLa proteins, this could still dramatically cut down the amount of off-target cysteines that may get labeled. Scaling up the concentration of PEG maleimide from 5 mM to 10-20 mM may also help improve labeling, as will a longer incubation with the labeling reagent. When considering longer incubations, however, we have to consider the tradeoff between maintaining the viability of attached HeLa cells and optimizing the labeling efficiency.

After the PEGylation assay has been further optimized, more PopD variants may be generated with cysteines substituted at the sites S101C, A108C, S115C, A172C, and A242C. The pUCP18_PopD^{S101C} plasmid has already been successfully generated, but needs to be transformed into strains of PAK and further characterized as was done with the other mutants. Primers have already been designed to generate the PopD^{A108C}, PopD^{S115C}, PopD^{A172C}, and PopD^{A242C} variants, and they have been tested to ensure functionality for the PCR reactions. PopD^{A172C} and PopD^{A242C} will report the cytosolic loop domain between H1 and A1, while PopD^{S101C}, PopD^{A108C}, and PopD^{S115C} will report
the extracellular H2-H1 loop domain. Ideally, at least three cysteine variants will be
generated within each putative loop domain in order to accurately report the topology.

3.2.1 PEGylation may be used to study transmembrane PopD topology in the
presence of SDS through an AMS blocking assay.
While labeling in the presence of SDS alone doesn’t allow us to conclusively determine a
cysteine’s location, I believe topology can be still be studied if SDS is used in
conjunction with the blocking agent 4-acetamido-4′maleimidylstilbene-2,2′-disulfonic
acid (AMS) (Fig. 15A). AMS is a low molecular weight, membrane-impermeable
reagent which can irreversibly alkylate freely accessible cysteines to prevent them from
being labeled with PEG maleimide (Nagamori et al 2002, Cho et al 2007). Employing a
combined AMS blocking/PEG maleimide labeling assay will enable us to distinguish
residues located in intracellular or extracellular loops, transmembrane domains, or
interfaces with other proteins such as PcrV and PopB.

To study PopD topology using this reagent, we would first need to incubate intact HeLa
cells in the presence or absence of AMS after infection with PopD cysteine variants.
After quenching and sufficient washing to remove AMS, membranes can be solubilized
first with 0.1% Triton to remove bacteria, then with 2% SDS to disrupt the translocon.
Samples will then be incubated with PEG maleimide, and any unblocked cysteines
should be readily accessible for labeling. If a significant difference in PopD PEGylation
is seen between the AMS-blocked and unblocked samples, we can conclude that the
cysteine residue in question was extracellular (Fig. 15B). If there is no difference in
labeling, this could indicate the cysteine is located in a transmembrane domain, binding interface with another protein, or a cytosolic loop.

Blocking with AMS in the presence or absence of rPFO can allow us to determine if a residue is located in a cytosolic domain (Fig. 15C). Failure to PEGylate an SDS-solubilized PopD variant after AMS blocking under membrane-permeabilizing conditions indicates cytosolic exposure of the substituted cysteine. If there is no difference in PEGylation for samples treated with AMS in the presence or absence of membrane permeabilization, this could indicate the inserted cysteine is located in a transmembrane helix (Fig. 15D) or interacting with another protein.

Distinguishing residues that are located in protein-protein interfaces from those that are located in transmembrane domains could be done by treating infected, rPFO-permeabilized HeLa cells with AMS in the presence or absence of a protein denaturing agent such as urea or guanidinium chloride and observing the change in labeling efficiency (Fig. 15E). Denaturation of protein-protein interactions will expose any interacting cysteines to blocking by AMS and prevent subsequent PEGylation. If the protein interface is undisturbed, then the cysteines will not be blocked and will be readily accessible for PEGylation after SDS solubilization.

This assay should solve the problem of steric hindrance that I saw with the PEGylation assay, as the relatively small size of the AMS molecule will allow it to more easily access any cysteines located in the exposed loop domains or terminal regions of PopD.
To ensure this assay works, however, we first have to set up a control to ensure that the translocon pore doesn’t allow AMS to access the interior of the membrane in cells that haven’t been permeabilized with rPFO. While the translocon pore does have a diameter around 6.0 nm (Hauser 2009), it is also docked onto the PcrV needle tip which may block external molecules from entering through the pore. As a control we could identify a protein in the eukaryotic cytosol with a surface-accessible cysteine, such as thioredoxin. Intact, infected HeLa cells would be incubated in the presence or absence of AMS before solubilizing membranes and incubating with PEG maleimide to observe efficiency of thioredoxin labeling. If there is no difference in labeling efficiency between the AMS blocked and unblocked samples, this indicates that AMS cannot access the cytosol through the translocon.
A.

B. (added after membrane solubilization with SDS)

(added after membrane solubilization with SDS)

C. PopD (external cysteine) PopB

AMS PEGylation

AMS +
rPFO -

PEGylation ✓

AMS +
rPFO +

PEGylation ✓
Figure 15. Blocking of free cysteines with AMS will allow study of PopD transmembrane topology after SDS solubilization. (A) Structure of 4-acetamido-4’maleimidylstilbene-2,2’-disulfonic acid. (B) Incubation of intact host cells in the presence or absence of AMS allows identification of extracellular cysteines. (C) Incubation with AMS in the presence or absence of rPFO allows identification of cytosolic cysteines. (D) Transmembrane cysteines will be labeled with the same efficiency after AMS incubation, regardless of membrane permeabilization. (E) Incubation with AMS in the presence or absence of a denaturing agent allows identification of cysteines in interaction interfaces with other proteins.
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