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In Vitro Characterization of the *Pseudomonas aeruginosa* Cytotoxin ExoU

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ABSTRACT

In Vitro Characterization of the *Pseudomonas aeruginosa* Cytotoxin ExoU

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The *Pseudomonas aeruginosa* type III secretion system delivers effector proteins directly into target cells, allowing the bacterium to modulate host cell functions. ExoU is the most cytotoxic of the known effector proteins and has been associated with more severe infections in humans. Previous studies have shown that ExoU is a patatin-like A_2 phospholipase requiring the cellular host factors phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) and ubiquitin for its activation *in vitro*. We demonstrated that $PI(4,5)P_2$ also induces oligomerization of ExoU and that this $PI(4,5)P_2$-mediated oligomerization does not require ubiquitin. Single amino acid substitutions in the C-terminal membrane localization domain of ExoU reduced both its activity as well as its ability to form higher-order complexes in transfected cells and *in vitro*. Combining inactive truncated ExoU proteins partially restored phospholipase activity and cytotoxicity, indicating that ExoU oligomerization may have functional significance. Our results indicate that $PI(4,5)P_2$ induces oligomerization of ExoU, which may be a mechanism by which this co-activator enhances the phospholipase activity of ExoU.

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CHAPTER ONE

INTRODUCTION

Background and Clinical Significance of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative bacterium which can be isolated from a variety of sources such as water, soil, and certain animal species (1-3). Genome sizes of individual *P. aeruginosa* strains range from 5.5 to 7.3 million base pairs (4, 5), contributing to the genetic diversity that may allow them to survive in different environmental niches (3, 6). Although numerous *Pseudomonas* species have been described, *P. aeruginosa* is the most relevant to human disease (7-10).

P. aeruginosa is considered an opportunistic pathogen, one that primarily affects immunocompromised patients (11-13), but in rare cases it can also cause "hot tub" folliculitis (14, 15) and contact lens-induced corneal infections (16, 17) in healthy individuals. *P. aeruginosa* is also responsible for a substantial proportion of hospital-acquired infections, including bloodstream, urinary tract, and lung infections (18). These infections are a result of medical interventions such as catheterization or mechanical ventilation, and contribute to morbidity and mortality in patients who already have severe illnesses (19-21).

Patients with cystic fibrosis, a congenital disorder affecting the lungs as well as the gastrointestinal and reproductive systems (22), are particularly susceptible to *Pseudomonas* infection (23). Many of these patients are colonized during childhood (24) and fail to eradicate the organism despite antibiotic therapy (25), leading to recurrent bouts of respiratory infections (26). While the specific molecular mechanisms that allow chronic colonization of *P. aeruginosa*

and conversion to an acute illness are still being characterized, there appears to be a host of bacterial factors that allow *P. aeruginosa* to better adapt to the mucus-filled cystic fibrosis lung environment (27, 28). Advanced cases of the disease often progresses to respiratory failure and require lung transplantation (29). Interestingly, many patients become recolonized with *P. aeruginosa* post-transplant (30, 31). As lung transplantation is not available or recommended for all patients (32), developing methods to prevent and eradicate *P. aeruginosa* infections are of utmost importance.

Antibiotic Resistance

The emergence of antibiotic resistance has been a concern across bacterial species, and *Pseudomonas aeruginosa* is no exception (33-36). Due to the high prevalence of *P. aeruginosa* strains resistant to commonly used antibiotics, such as fluoroquinolones, carbapenems, and extended-spectrum cephalosporins (36, 37), these infections have become increasingly difficult to treat. Clinicians have to resort to older antibiotics such as colistin that have serious side effect profiles (38, 39). As a result, the Infectious Diseases Society of America (IDSA) has declared multi-drug resistant *P. aeruginosa* to be part of the ESKAPE pathogens (40), with the Centers for Disease Control and Prevention (CDC) assigning it a threat level of "serious" and in need of new therapies (41).

P. aeruginosa has multiple mechanisms of antibiotic resistance. Intrinsic resistance towards penicillins and cephalosporins due to production of beta-lactamases have been reported since the 1970s (42). Treatment using newer antipseudomonal penicillins such as piperacillin has been more effective (43). However, overproduction of a mutated variant of the beta-

lactamase AmpC has caused a subset of *P. aeruginosa* strains to become resistant to these therapies as well (44). The acquisition of the OXA group of extended spectrum beta-lactamases and carbapenemases from other *Pseudomonas* species (45, 46) or members of the Enterobacteriaceae family (47, 48) has resulted in the emergence of multi-drug resistant (MDR) strains for which this entire class of antibiotics is rendered ineffective (49, 50).

Antibiotic resistance can arise from mutations in both genes that encode target proteins as well as those that regulate the expression of such proteins. As DNA gyrase and topoisomerase IV are targets for fluoroquinolones, mutations in the *gyrA*, *gyrB,* and *parC* genes are commonly found in resistant *P. aeruginosa* strains (51, 52). Additionally, the overexpression of multidrug efflux pumps MexEF-OprN and MexAB-OprM contribute to higher levels of fluoroquinolone resistance (53, 54). Drug efflux pumps also export aminoglycosides (55), carbapenems (56), and beta-lactamase inhibitors (57). Finally, loss of the porin OprD confers resistance against antibiotics that would normally enter the bacterium through this channel (58, 59).

The CDC recently reported that amongst hospital-acquired infections across the United States, 15.9% of *P. aeruginosa* isolates were multi-drug resistant (60), highlighting the need for alternative anti-infective agents. An alternate approach in drug development is the targeting of virulence factors (61).

Virulence Determinants

We define bacterial virulence genes as those encoding factors that promote disease (62). *Pseudomonas aeruginosa* harbors many such genes which contribute to its virulence to varying degrees. Bacterial virulence and host-pathogen interactions in *P. aeruginosa* have been studied

using different animal models of infection (63-65). Here I highlight a subset of both structural and secreted virulence factors produced by *P. aeruginosa* that have been characterized.

Structural virulence factors

Lipopolysaccharide (LPS) is found in most Gram-negative bacteria and is a major contributor to virulence (66, 67). LPS is part of the outer membrane and is composed of a core oligosaccharide, O-antigen, and a lipid A tail (68). Both Toll-like receptors 2 and 4 (TLR2 and TLR4) recognize LPS (69, 70), promoting recruitment and activation of neutrophils, clearance of bacteria, and survival in a mouse model of pneumonia (71). Recognition of LPS is mediated by a hyper-variable 82 amino acid portion of the TLR4 receptor; however, multiple forms of the pro-inflammatory Lipid A component are found in *P. aeruginosa* and can induce differential immune responses (70).

P. aeruginosa has a single polar flagellum which renders it capable of both swimming and swarming motility (72). The main flagella filament is composed of flagellin subunits; the Toll-like receptor 5 detects N-terminal amino acids of flagellin, inducing a proinflammatory IL-8 response (73). *P. aeruginosa* mutants defective in glycosylation or motility of flagella show significant attenuation of virulence as measured by LD_{50} (74). Counterintuitively, non-swimming bacteria are better able to evade immune cells, and this phenotype is independent from the flagellar structure itself (75). These findings highlight the complexity of host-pathogen interactions and their contribution to overall disease.

Type IV pili are another structural component of the bacterial surface important for motility of *P. aeruginosa*, specifically twitching and swarming motility (76, 77). Both flagella

and type IV pili are necessary for the formation of biofilms, immobile communities of bacteria that decrease susceptibility of *P. aeruginosa* to certain antibiotics (78, 79). Pili mediate adherence of the bacterium to epithelial cells (80). Recent studies have shown that upon contact with surfaces, pili can also serve as mechanosensors that increase cAMP and Vfr, a transcriptional regulator of multiple virulence genes (81, 82).

Secreted virulence factors

Five of the six major types of secretion systems in Gram-negative bacteria have been found in *P. aeruginosa* isolates (83). The majority of these secrete proteins into the extracellular milieu. Other secreted small molecules such as siderophores utilize efflux pumps to cross the bacterial membrane (84).

Siderophores are iron-scavanging compounds that are released then taken up again by the bacteria. *P. aeruginosa* produces several virulence-associated siderophores, including pyochelin and pyoverdine, the latter contributing to the bacterium's characteristic green color (85-87). The HasA/HasR hemophore system allows *P. aeruginosa* to further compete for available iron stores by acquiring hemoglobin released by lysed cells (88). During infection, *P. aeruginosa* induces transcription of genes involved in protein secretion system, inflammation, and the iron acquisition process, and it is thought that they coordinately contribute to virulence in an acute model of pneumonia in mice (89).

P. aeruginosa produces many proteases which promote disease. Of these, the elastase LasB is one of the earliest described virulence determinants. In a mouse burn model of infection, enhanced bacterial clearance and host survival were observed with elastase-deficient strains (90).

Elastase also increases permeability of the epithelial lung barrier and degrades surfactant D in lungs (91, 92). Along with the alkaline protease AprA, LasB degrades flagellin, thereby preventing detection by the immune system (93). Protease IV also evades the immune system by degrading surfactants and disrupting phagocytosis by alveolar macrophages (94).

Multiple toxins also contribute to the virulence of *P. aeruginosa*, including Exotoxin A, phospholipase C, and RhsT (95-97). Exotoxin A is an AB toxin which ADP-ribosylates elongation factor 2, inhibiting protein synthesis leading to host cell death (96). The hemolytic phospholipase C decreases lung function in a mouse model of *P. aeruginosa* infection (98) and suppresses oxidative burst in neutrophils *in vitro* (99). RhsT enters eukaryotic cells through an unknown mechanism and activates the inflammasome pathway (95).

These virulence determinants appear to group into the following general functions: nutrient acquisition, adherence and motility, immune cell evasion, and inflammation or cell death. Nutrients, adherence, and motility all would be expected to help bacterial survive and persist in the host organism. The effects of *P. aeruginosa* on the immune system, however, are more complex. *P. aeruginosa* has acquired multiple mechanisms of evading phagocytosis or detection by the immune system, but it also harbors many pro-inflammatory virulence factors. Manipulation of the host immune system in a way that benefits the bacteria likely requires balancing when and how much of each virulence determinant is expressed.

Virulence factors are not equally distributed amongst strains, so they likely contribute to the differing phenotypes observed across *P. aeruginosa* isolates. Furthermore, virulence factor production is not static, even in the same strain within the same host. Similarly, antibiotic

resistance profiles are highly variable across strains. To combat *P. aeruginosa* effectively, we may need to combine existing antibiotics with "anti-virulence factor" therapies.

Traditional antibiotics usually act by preventing replication or survival of bacteria, allowing for selection of antibiotic resistance in hosts as well as environmental reservoirs. On the other hand, anti-virulence therapies would only benefit the bacteria within an organism. By improving the overall fitness of *P. aeruginosa* specifically during infection, selective pressure and resistance towards new anti-virulence drugs may be delayed. Thus, virulence factors could serve as useful targets in the era of rising antibiotic resistance.

Figure 1 only depicts a small subset of virulence factors produced by *P. aeruginosa*, but the examples discussed show that a wide range of insults can be exerted on the host during infection.

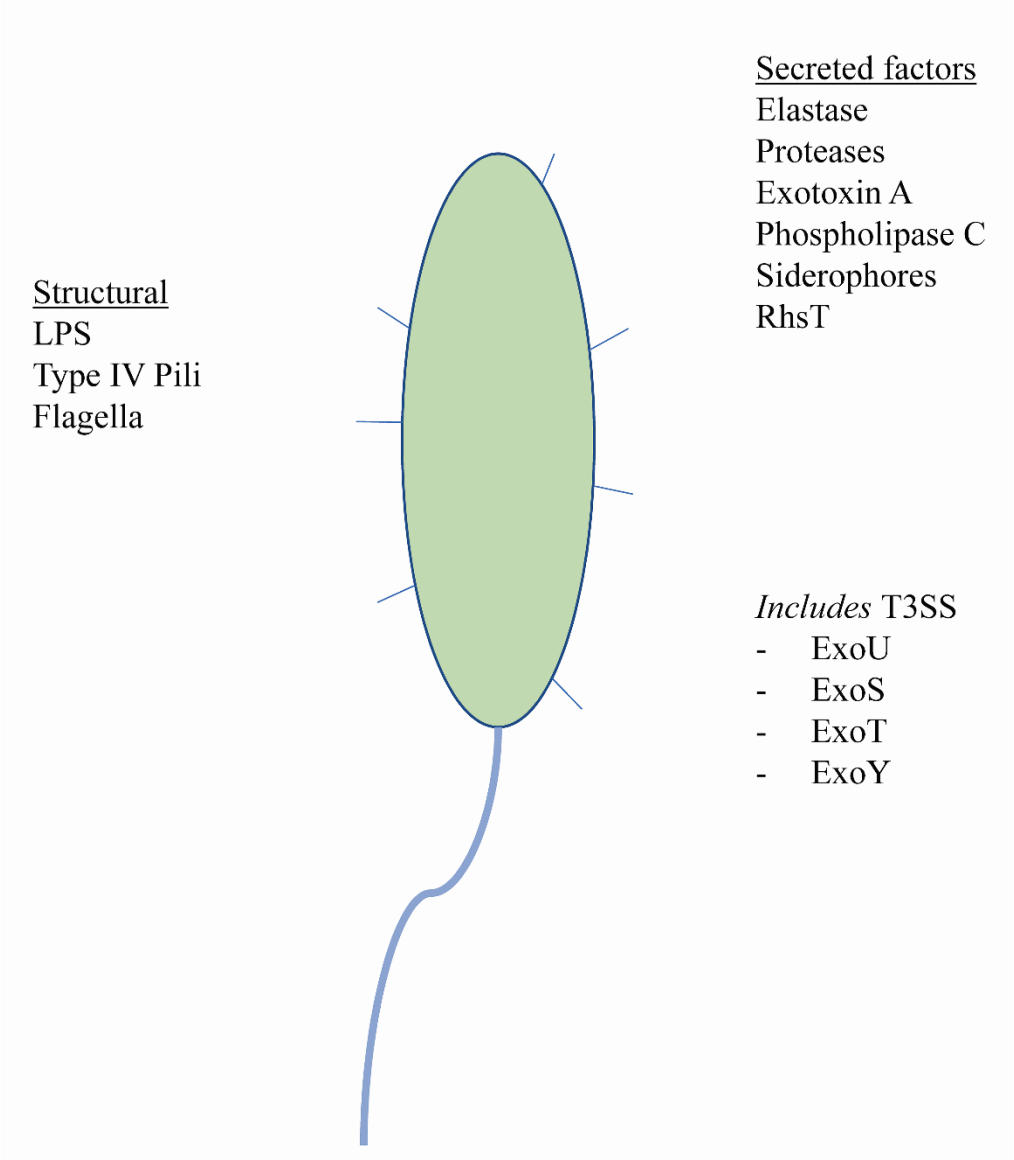


Figure 1: Virulence determinants of *Pseudomonas aeruginosa*.

A selection of *P. aeruginosa* virulence factors is depicted. They can be classified as either secreted factors or structural components of the bacterium. The focus of this work is on the Type 3 Secretion System (T3SS) and its effector proteins.

Type III Secretion System

Another important virulence determinant of *P. aeruginosa* is its type III secretion system. Type III secretion systems (T3SSs) are multi-protein complexes that form needle-like apparatuses on the surface of bacterial cells, allowing effector proteins to be delivered directly from the bacteria into the cytoplasm of the targeted cell. The T3SS is associated with worse outcomes in patients with bacteremia and ventilator-associated pneumonia (100, 101). Thus, the T3SS of *P. aeruginosa* and its effectors continue to be active targets of investigation in disease pathogenesis (102).

Structural Components

The T3SS is comprised of a multi-protein base, an extracellular needle apparatus, and a translocon (Figure 2). The base of the T3SS complex transverses the inner and outer membranes of the bacterium. PscC is the secretin, a protein that oligomerizes into a cylindrical channel on the outer membrane. A three-dimensional structure of the C-terminal domain of PscC generated using cryo-electron microscopy showed that it formed a ring-shaped dodecamer similar to YscC from *Yersinia enterocolitica* (103, 104). The structure and function of most proteins within the base have been inferred from T3SS homologues in other bacterial species (102, 105). PscN, the ATPase which provides energy for the secretion of effector proteins, has only been analyzed computationally for binding sites, whereas PscJ is predicted to form ring-like structures in the bacterial inner membrane based on the EscJ structure from *E. coil* (105, 106).

The needle apparatus of the *P. aeruginosa* T3SS is composed of PscF and is approximately 60-80 nm in length (107). As the hollow rod-like structure is only 7 nm wide

(107) with an inner diameter of 2.2 nm (108), effector proteins are thought to be unfolded as they pass through its lumen. The ruler protein PscP serves as a sensor of needle length, and its deletion results in the assembly of aberrantly long needle apparatuses (108).

The translocon is the portion of the T3SS that allows the effectors to cross through the eukaryotic plasma membrane. The translocators PopB and PopD assemble into hetero-oligomers that form the transmembrane pore (109). Electron microscopy showed that these oligomeric rings have an outer diameter of 8 nm and an inner lumen diameter of 4 nm (109). The translocator PcrV forms similar ring-like structures *in vitro* and is required for translocation of effector proteins through PopB/PopD pores inserted in the membrane (110, 111). PcrV is thought to control secretion by forming a needle tip and altering the conformation of the needle apparatus (112). Deletion of PcrV results in decreased lung injury and enhanced survival of rats in a lung model of infection (113). Purified PcrV induces TNF-α release from macrophages *in vitro* (114), suggesting that T3SS components can be detected by the host immune system independently of the secreted effector proteins. Given its roles in virulence, PcrV has become a popular target for vaccine development (115-117).

Other T3SS apparatus proteins have been implicated in triggering the immune system. The NLRC4 inflammasome has been reported to detect PrgJ from *Salmonella typhimurium*, leading to caspase-1 activation and cell death (118). Similarly, the *P. aeruginosa* T3SS needle protein PscF was shown to induce NLRC4-dependent caspase-1 cleavage in human U937 macrophages (118). Together, these studies demonstrate that the T3SS from *P. aeruginosa* is a complex virulence determinant, and future studies are needed to develop effective therapeutic agents against its regulators, components, or secreted effectors.

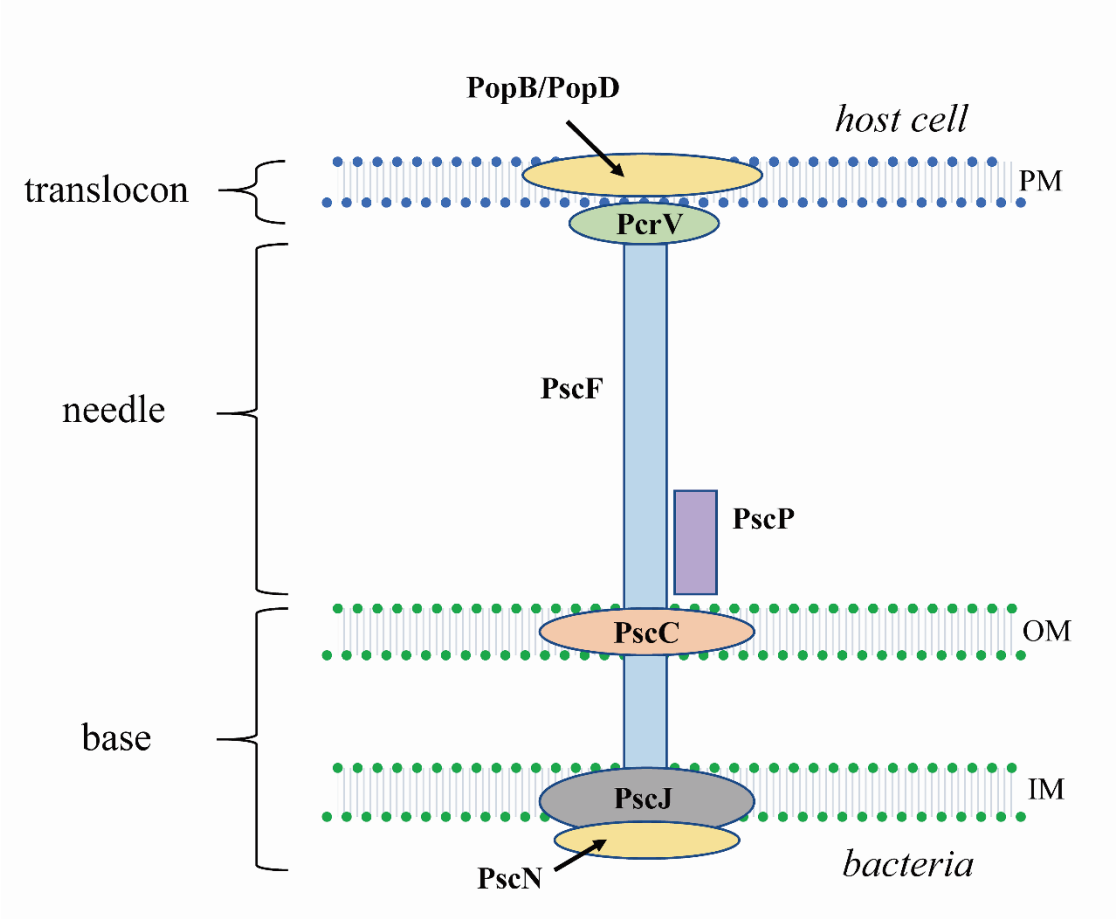


Figure 2: The type III secretion system apparatus.

The T3SS is a multi-protein complex consisting of a base, needle, and translocon. The base consists of the proteins that help secrete effector proteins across the inner membrane (IM) and outer membrane (OM) of the bacterial cell. The effectors transverse through the hollow needle without encountering the extracellular environment. The translocon allows proteins to cross the eukaryotic plasma membrane (PM) of targeted host cells.

Regulation of Secretion

The exact signal that triggers secretion of the *P. aeruginosa* T3SS during infection is not known, though it has been hypothesized that cell contact plays a role. *In vitro*, secretion is effectively stimulated by growing the bacteria under calcium-depleted conditions (119).

Secretion and transcription of effector proteins is tightly regulated by a signaling cascade involving ExsA, ExsC, ExsD, and ExsE (Figure 3). Under non-secreting conditions, ExsE and ExsC form a complex in the bacterial cytosol (120). ExsD binds to ExsA, the transcriptional activator of most T3SS genes, preventing ExsA from binding T3SS promoters (121).

When an external signal such as calcium depletion triggers the T3SS to turn on, ExsC enables efficient secretion of ExsE out of the bacterium (120). Secretion of ExsE results in free ExsC in the bacterial cytosol. ExsC is then able to bind ExsD and dissociate the ExsD:ExsA complex (121). The subsequently released ExsA is then free to bind DNA sequences upstream of the T3SS apparatus and effector genes to initiate their transcription (122, 123). This cascade results in an autofeedback loop as the operon containing the *exsA* gene also has an upstream ExsA-dependent promoter (124). Recently an additional promoter was discovered within the *exsCEBA* operon immediately upstream of *exsA* and found to be regulated by Vfr (virulence factor regulator), a global transcriptional regulator that controls expression of multiple virulence genes in *P. aeruginosa* (124).

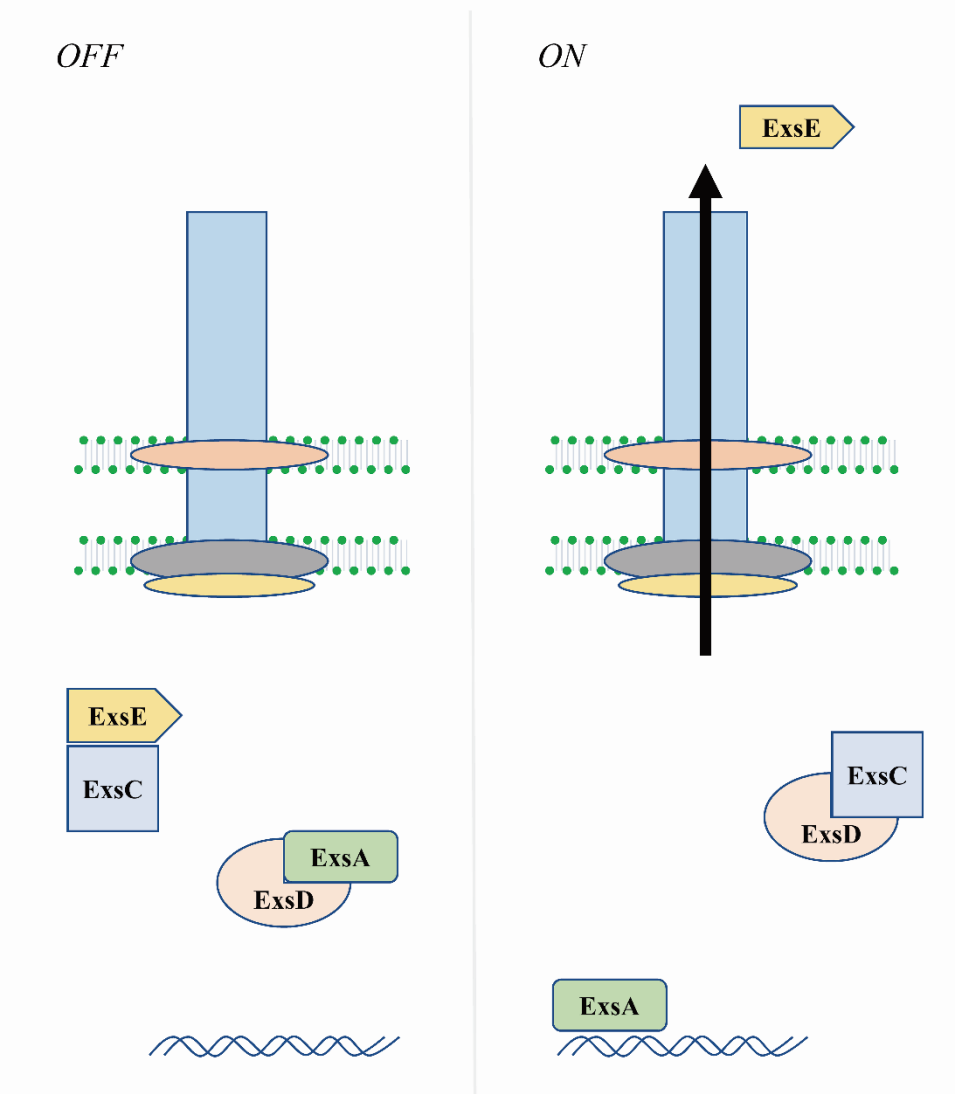


Figure 3: Regulation of T3SS secretion

T3SS secretion is regulated through a signaling cascade comprised of ExsA, ExsD, ExsC, and ExsE. When secretion is off (*left*), ExsE:ExsC and ExsD:ExsA complexes form in the bacterial cytosol. When secretion is triggered (*right*), ExsE is exported, ExsC sequesters ExsD, and ExsA is freed to transcriptionally activate T3SS-associated genes.

Effector Proteins

Pseudomonas aeruginosa is known to secrete six effector proteins: ExoS, ExoT, ExoU, ExoY, PemA, and PemB (102, 125). While ExoT and ExoY are found in 100% and 89% of *P. aeruginosa* strains in a study looking at environmental and clinical isolates from a variety of sources, the other effectors have a more variable distribution (126, 127). Interestingly, the *exoU* and *exoS* genes are rarely found in the same strains (126), though the significance of this observation is not known.

ExoS

ExoS is a bifunctional protein with ADP-ribosyltransferase (ADPRT) and GTPase-activating protein (GAP) activities (128). It is present in approximately 58-72% of *P. aeruginosa* isolates (102) and are particularly prevalent in isolates from chronic cystic fibrosis infections (129, 130). ExoS requires the cofactor SpcU, a member of the 14-3-3 family of proteins, for its secretion (131) and has a membrane localization domain for intracellular targeting after translocation into host cells (132, 133). Substitution of the residues responsible for either ADPRT or GAP enzymatic activities allowed for assessment of functions attributable to each domain (134).

The C-terminal ADPRT of ExoS acts upon a variety of substrates, including vimentin (135), immunoglobulin G (IgG) (136), as well as several Ras-related GTP-binding proteins (137-139). The ADPRT of ExoS inhibits DNA synthesis, disrupts the actin cytoskeleton, and induces cell rounding, leading to significant cell lysis within five hours after infection (134, 140). ExoS also ADP-ribosylates the ERM proteins (ezrin, radixin, and moesin), which are involved in modulating the cytoskeleton during cell motility and phagocytosis (141). The N-terminal GAP

of ExoS similarly affects cytoskeletal organization, except through inactivation of RhoA, Rac1, and Cdc42 GTPases (142). ExoS inactivation of GTPases alters their localization, shifting them from the membranes to the cytoplasm (143). The GAP activity of ExoS generally has lesser effects on cell rounding, epithelial barrier permeability, and phagocytosis compared to the ADPRT of ExoS (134, 144, 145). However, one unique GAP function is the upregulation of wild-type CFTR (cystic fibrosis transmembrane conductance regulator) levels on the surface of cells (146), though the significance of this effect on disease is unclear.

In addition to disrupting signal transduction and cytoskeletal pathways, ExoS induces T lymphocyte proliferation and pro-inflammatory cytokine and chemokine expression *in vitro* (147, 148). Similarly, effects of ExoS on the immune system are observed in animal models of infection. ExoS promotes bacterial survival in neutrophils and contributes to corneal opacification during *P. aeruginosa* eye infections (149). During pneumonia, ExoS has been shown to block phagocytosis, intoxicate type I pneumocytes, and allow dissemination of bacteria into the bloodstream of infected mice (150).

ExoT

ExoT is a 53-kD protein which shares 75% amino acid identity with ExoS, and was originally thought to be a variant of ExoS (151, 152). ExoT also has ADPRT and GAP activities (152, 153) and shares the chaperone SpcS for type III secretion (154). As with ExoS, the GAP domain of ExoT targets RhoA, Rac1, and Cdc42, and causes cell rounding when expressed in Chinese Hamster Ovary (CHO) cells (153, 155). ExoT ADP-ribosylates Crk-I and Crk-II, proteins which are involved in cell adhesion and phagocytosis (156). The ADPRT functions also contribute to

cell rounding, and both the GAP and ADPRT portions of ExoT induce cytotoxicity in yeast (157).

ExoT has primarily been studied in the context of epithelial cell intoxication and cytoskeletal disruption *in vitro*. ExoT-secreting strains of *P. aeruginosa* are internalized less by macrophages and corneal epithelial cells (155, 158). ExoT was found to inhibit wound healing when A549 lung epithelial cell monolayers were subjected to mechanical injury and infection (159). The GAP activity of ExoT appears to be more important for its anti-internalization function, whereas the ADRPT activity accounts for much of its effects on wound healing (157).

There have been many studies investigating the importance of ExoT *in vivo*. An ExoT-deficient strain was less able to reach the liver relative to its parental strain in a murine pneumonia model (160). ExoT also promoted dissemination of *P. aeruginosa* to the spleen; however, virulence (as determined by LD50) was not increased in a mouse model of pneumonia (161). As with ExoS, the ADPRT activity of ExoT is important for bacterial survival, neutrophil apoptosis, and corneal opacification in *P. aeruginosa* keratitis in mice (149).

Due to its ability to target proteins involving the cell division and signal transduction pathways, ExoT is being investigated as a potential therapeutic against cancer (162). The ADPRT and GAP activities of ExoT block cytokinesis, the last step of cell division, at different stages. Inhibition of cytokinesis by its ADPRT activity occurs through disruption of Crk and syntaxin-2 localization to the midbody (163). ExoT also induces apoptosis through both of its functional domains. The ADPRT interferes with integrin-mediated pro-survival signaling through ADP-ribosylation of Crk-I and disruption of focal adhesions required for cell attachment (164). In contrast, the GAP of ExoT induces apoptosis through the intrinsic (mitochondria-

mediated) pathway (165). ExoT could be a promising candidate as aberrant growth and survival are features common to most cancers.

ExoY

ExoY is a 42-kD adenylate cyclase with homology to CyaA of *Bordatella pertussis* and EF (edema factor) from *Bacillus anthracis* (166). Like other T3SS effectors, ExoY requires a eukaryotic cofactor for its enzymatic activity (166), which was recently identified as filamentous actin (F-actin) (167). ExoY localizes to the host cell cytoplasm, induces intercellular gaps, and causes cell rounding *in vitro* (168, 169). In a mouse model of pneumonia, ExoY-secreting *P. aeruginosa* induced pulmonary edema and hemorrhage and impaired re-formation of the endothelial barrier, but no difference in survival compared to a mutant strain (170). Other studies have questioned the role that ExoY plays in virulence, as deletion of *exoY* only resulted in minor decreases in cytotoxicity and bacterial numbers recovered from the lungs of infected mice (171, 172).

More recently, however, ExoY was shown to mediate hyperphosphorylation of Tau, a microtubule-associated protein, and render it insoluble (173). Infection with ExoY-producing *P. aeruginosa* strains led to mislocalization of Tau and decreased assembly of microtubules (174). ExoY is also able to bind F-actin and prevent disassembly of actin filaments (167). New studies have found that ExoY dampens the immune response by suppressing proinflammatory cytokine production and inflammasome activation (175, 176). These findings suggest that while ExoY may not be the most potent virulence factor on its own, its effects can still contribute to *P. aeruginosa* disease pathogenesis.

PemA/PemB

PemA and PemB were recently shown to be translocated into host cells through the T3SS. PemA and PemB (*Pseudomonas* effectors discovered by machine learning) were found using a bioinformatic approach based on comparison of sequenced open reading frames (ORFs) to those of other known T3SS effectors from related species (125). However, these effectors were not cytotoxic towards HeLa cells or *S. cerevisiae* (125). Although PemA and PemB are not likely to be virulence factors, they are worth recognition, as their discovery suggests there is a possibility that more T3SS effectors are still unidentified in *P. aeruginosa*.

The Effector Protein ExoU

Discovery

ExoU was identified by two research groups as a novel effector protein contributing to virulence of the *P. aeruginosa* PA103 strain in mice (177, 178). Hauser and colleagues discovered the 74 kD protein in culture supernatants when comparing a non-cytotoxic transposon mutant to its parental strain. PepA (later renamed ExoU) contained a five-amino acid peptide sequence identical to those found at the N-terminus of the known T3SS effectors ExoS and ExoT, and they demonstrated secretion of PepA was dependent on a functional T3SS apparatus (177). Frank and colleagues discovered ExoU when investigating why deletion of *exoT* from PA103 did not abrogate its cytotoxic effects during infection. They noted that the G+C content of *exoU* was 59%, less than the flanking sequences or overall genome (65% and 67%, respectively) and suggested that acquisition of *exoU* may have occurred through a transposable

element (178). Both groups reported that ExoU production was associated with *in vitro* cytotoxicity and lung injury or mortality in infected mice (177, 178).

The region downstream of *exoU* contains an open reading frame that encodes for SpcU, a 15 kD protein that serves as a chaperone to ExoU (179). Chaperones have been described for other T3SS effector proteins in *P. aeruginosa* as well as from other bacterial species; while they are required for translocation of the effector, the chaperone itself remains in the bacterium (154, 180, 181).

Multiple groups have noted that ExoU is a variable trait and that clinical and environmental strains harboring *exoU* typically do not have *exoS* (182-184). Since both ExoS and ExoU have significant contributions to the virulence of *P. aeruginosa*, it is possible that redundancy of the end effects of these toxins caused the loss of either gene. Alternatively, ExoS+ and ExoU+ strains could have evolved independently and phylogenetically different from each other. The *exoU* gene has been found within distinct genomic islands which vary depending on the specific *P. aeruginosa* strain (185). The chromosomal site that harbors these genomic islands is considered a "hot spot" for recombination and is flanked by highly conserved sequences (182). Given that very few *P. aeruginosa* strain have both *exoU* and *exoS* (or have neither), *exoU* was likely acquired by horizontal gene transfer independently from the rest of the T3SS-encoding genes.

Clinical Significance

ExoU has been associated with increased morbidity and mortality in patients with ventilator-associated pneumonia (101) and bacteremia (186). A study analyzing a diverse

collection of 328 *P. aeruginosa* clinical and environmental isolates found that 23% of those strains contained the *exoU* gene (187). Another epidemiologic study found that approximately half of the isolates obtained from eye and ear infections carried *exoU* (184) and that ExoU+ isolates are more prevalent in contact lens-associated keratitis compared to non-contact lens-associated infections (188). ExoU-secreting isolates from patients with hospital-acquired pneumonia were also more virulent than non-secreting strains when tested in mice (127), suggesting mice may be a useful model organism for studying acute pneumonia.

Interestingly, one study reported that fluoroquinolone resistance was associated with carriage of *exoU* in their collection of clinical isolates, where ExoU+ strains were more likely to harbor *gyrA* mutations and increased efflux pump phenotypes (189). Since then, multiple groups have found significant associations between *exoU* presence with resistance against fluoroquinolones in strains isolated from a variety of sources, including urinary tract infections, otitis media, corneal ulcers, and respiratory infections (190-193). However, *exoU*-containing isolates were found to have either negative or no correlation with multidrug resistance in the most recent studies (186, 194).

Virulence factor associations with antibiotic resistance can come about either due to co-acquisition of the genes due to proximity of the alleles (e.g. on a single plasmid) or through independent acquisition events. The latter scenario may involve products encoded by one gene benefiting the other. Agnello and colleagues investigated why *exoU*, but not *exoS*, is associated with fluoroquinolone resistance by introducing *parC* mutations into three strains with each genotype (195). They discovered that mutation of *parC* in the *exoU* background conferred a fitness advantage over its parental strain during co-culture, whereas a *parC* mutation in the *exoS*

strains resulted in fitness defects (195). Additionally, strains with both *exoU* and mutated *parC* alleles had an increased mutation frequency, whereas the reverse was observed in their *exoS* counterparts (195). Fluoroquinolone resistance conferring a fitness advantage to ExoU+ strains is an intriguing hypothesis, and further investigation may inform both treatment and drug development approaches.

Domains and Structure

ExoU is a 687-amino acid modular protein comprised of three domains corresponding to three major functions: chaperone-binding, phospholipase activity, and membrane localization (Figure 4). The boundaries of these domains and their associated functions were initially determined by alignment to known proteins and mutagenesis of the different regions of ExoU.

Amino acid residues 3-123 mediate binding of ExoU to its chaperone SpcU, which is necessary for efficient translocation by the T3SS (179). A large internal portion of the protein is homologous to patatin-like phospholipases and contains a catalytic dyad consisting of S142 and D344 (196-198), so this region we term the phospholipase (PLA_2) domain. Finally, the C-terminal region (residues 550 to 687) was found to be necessary for targeting ExoU to the plasma membrane of host cells and was thus named the membrane localization domain (MLD) (199, 200). Truncation of either the N- or C-terminal ends of ExoU renders it noncytotoxic (201), while transposon-based linker-insertion mutagenesis of ExoU also confirmed that multiple regions of the protein were required for function (198, 202).

Prior to the crystallization of ExoU, structural characterization of the protein was limited. Circular dichroism showed that ExoU was an alpha-helix rich protein (203). The conformation

of ExoU was examined by double electron-electron resonance (DEER) and electron paramagnetic resonance (EPR) spectroscopy with ExoU variants containing nitroxide spin-labeled cysteine substitutions (204, 205). These studies showed that the distance between spin labels at ExoU residue 137 (in the PLA_2 domain) and residue 643 (in the MLD) ranged from 22 to 38 angstroms (204), suggesting ExoU by itself is a flexible protein that adopts multiple conformations.

Nearly fifteen years after its discovery, ExoU was crystallized complexed to its chaperone SpcU by two independent research groups (206, 207). The crystal structures confirmed that ExoU had multiple domains. While the amino acid residues boundaries drawn for each domain differed slightly between their two models, both the PLA_2 domain and MLD roughly corresponded to those proposed previously (199). However, one major difference was observed with binding to SpcU. A series of amino acid residues between the PLA2 and MLD of ExoU formed contacts with SpcU. Thus, we have updated our model to reflect that the chaperone binding domain consists of two parts, one at the N-terminus and one internal peptide within ExoU (Figure 4). Approximately 25% of ExoU remained disordered, consistent with the previous finding that ExoU is able to adopt different conformations (206, 207). This flexibility may explain why previous attempts to crystallize ExoU in the absence of a stabilizing chaperone were unsuccessful.

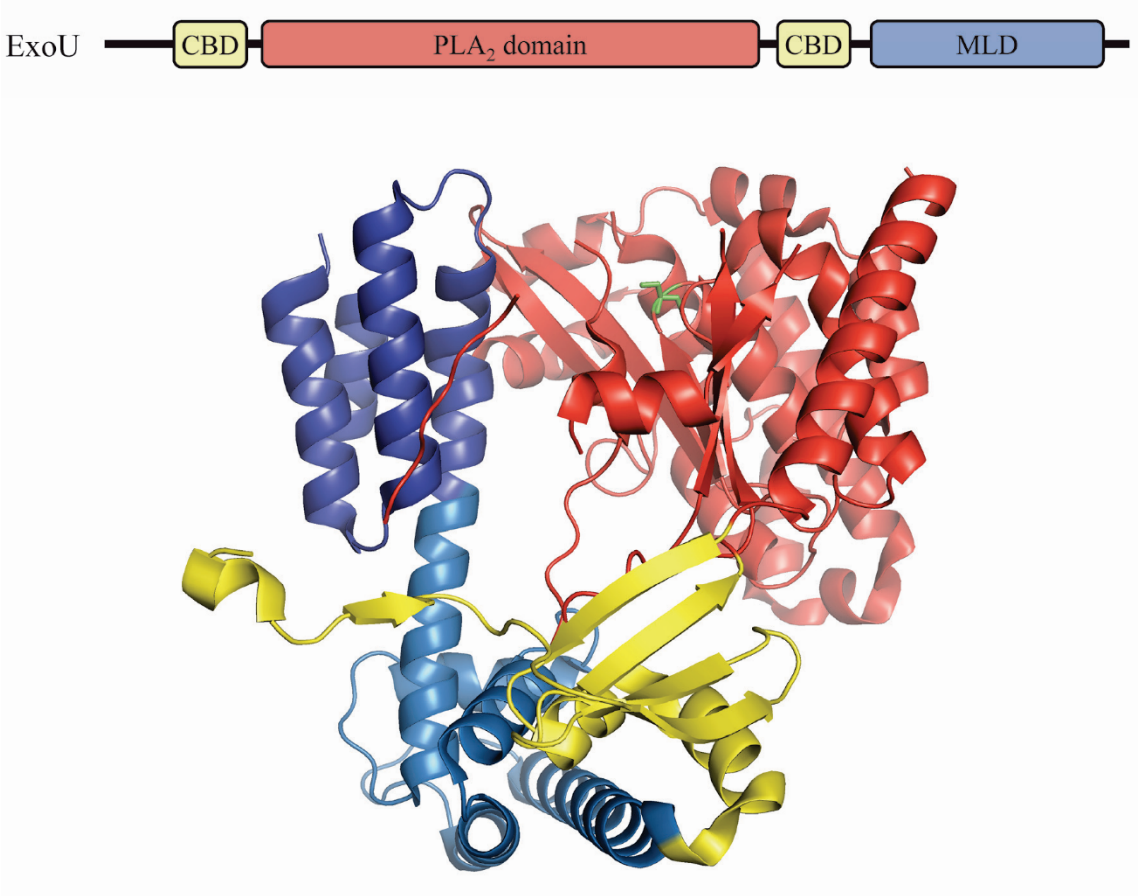


Figure 4: Functional domains and structure of ExoU

ExoU is composed of three functional domains. The chaperone binding domain (CBD, in yellow) is composed of two parts – the N-terminus and an internal region. The phospholipase domain (PLA2, in red) shares homology to the patatin-like phospholipases. The membrane localization domain (MLD in blue) mediates targeting of ExoU to the eukaryotic plasma membrane. The catalytic residue serine-142 is resolved in this structure and shown in green. Image was created using PyMOL. PDB ID: 3TU3

Localization

The crystal structure also revealed that ExoU contains a four-helical bundle within the C-terminal half of the MLD (206). Four-helical bundles are conserved motifs that mediate membrane binding in a variety of proteins, including toxins from other bacterial species (208, 209). It was previously known that either deletion of the MLD or substitution of specific amino acids in this domain disrupted ExoU localization, changing its distribution from mainly membrane-associated to cytoplasmic (196, 199, 200). Unsurprisingly, many of these amino acids are within the four-helical bundle.

Tyson and colleagues demonstrated that ExoU binds phosphatidyl-4,5-bisphosphate ($PI(4,5)P_2$), a plasma membrane phosphoinositide, with high affinity *in vitro* (Kd ~110 nM) and that substitution of arginine-661 decreased this interaction (210). This arginine residue is found in the L3 loop of the MLD four-helical bundle and is conserved in ExoU homologues from *P. fluorescens* and *Photorhabdus asymbiotica* (206, 210). This interaction was observed during expression in HeLa cells also, where wildtype ExoU localized to the plasma membrane, but the ExoU-R661L variant did not. Disruption of $PI(4,5)P_2$ distribution in yeast also led to mislocalization of ExoU, confirming that $PI(4,5)P_2$ is the lipid mediator involved (210). Together, these results show that R661 localizes ExoU to the inner leaflet of the host cell plasma membrane through interactions with $PI(4,5)P_2$.

$PI(4,5)P_2$) accounts for only 1% of the total phospholipid content in mammalian plasma membranes (211) but serves as an important factor for localizing endogenous proteins to the plasma membrane of mammalian cells and for regulating these proteins (212-214). It appears that ExoU has appropriated this localization system for its own benefit.

Ubiquitination

In mammalian cells, ExoU was discovered to be di-ubiquitinated at lysine-178 (215). Ubiquitination, the covalent addition of ubiquitin molecules onto lysine residues of target proteins, is a common protein modification mediated by a combination of E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases (216). The best studied form is Lys-48 linked poly-ubiquitination, where chains of ubiquitin molecules are added to proteins destined for the proteosome and subsequent degradation (217). This process is essential for maintaining desired levels of any particular protein, as well as the recycling of misfolded or damaged proteins (217). Mono-ubiquitination is important in the localization and trafficking of proteins, endocytosis, histone modification, and DNA damage repair (218). The roles of di-ubiquitination are less clear, but this rare modification has been implicated in the regulation of protein interactions and endosomal localization (219, 220).

Mutational analyses showed that the amino acid at positions 679-683 were required for di-ubiquitination of ExoU, localization to the plasma membrane, cytotoxicity, and phospholipase activity of ExoU (215). Substitution of tryptophan-681 was sufficient to abrogate ubiquitination and localization of ExoU, but not cytotoxicity (215). *In vitro* phospholipase activity of purified ExoU-W681A did decrease to 20% of wild-type ExoU; however, recombinant ExoU purified from *E. coli* is not ubiquitinated as it is in mammalian cells. Furthermore, K178R (a variant that is mutated at the ubiquitination site) only exhibited slightly less phospholipase activity and no difference in cytotoxicity (215). Even though the phospholipase activity defects observed with K178R and W681A did not translate to cytotoxicity decreases in transfected HeLa cells, but it is still possible that di-ubiquitination of ExoU could affect virulence of *P. aeruginosa* in the

context of infection. Nonetheless, these results called into question whether di-ubiquitination was important for ExoU function *in vivo*.

However, recent work shows that di-ubiquitination may have subtle effects on ExoU inside the cell (207). Gendrin and colleagues observed that non-ubiquitinated ExoU (carrying the K178R substitution) localized to the plasma membrane as previously reported (215), but ubiquitinated ExoU was targeted to both the plasma membrane and endosomes (207). It is still unknown whether this translates to a virulence defect during infection, but future investigations into which E3 ubiquitin ligases are responsible for di-ubiquitinating ExoU may shed light on possible functions of this modification.

ExoU Function

Virulence effects in vivo

ExoU is a major virulence determinant in many models of infection. Experiments using acute pneumonia mouse models showed that higher colony-forming units (CFUs) were recovered from the lungs of mice infected with ExoU-producing strains compared to an isogenic mutant, and mice infected with those ExoU+ strains succumbed to death more quickly and at much lower inoculums (161, 221). ExoU also contributes to corneal disease severity in a scarification model of infection (222). *P. aeruginosa* has also been studied in co-culture with *Acanthamoeba castellanii* and *Dictyostelium discoideum*, where ExoU expression and translocation is induced and contributes to necrotic cell death of these amoeba (223-225).

Howell and colleagues observed that *exoU* expression is induced early in the course of acute pneumonia and that delaying it improved survival of infected mice (226). This was

correlated with both an increase of the amount of ExoU present in the lungs of infected mice, as well as increased bacterial burden. These results suggest the effects of ExoU on the host is rapid and its presence has a major virulence effect.

Much of the virulence observed with ExoU *in vivo* is thought to be a result of its effects on the immune system. ExoU is proinflammatory, eliciting IL-8 production through activation of the mitogen-activated protein kinase/Jun N-terminal kinase (MAPK/JNK) pathway (227, 228). Consistent with the function of IL-8 is a chemoattractant, a greater influx of neutrophils was observed in the lungs of mice infected with ExoU-secreting bacteria (228, 229). However, one would hypothesize that more neutrophils would enable the host organism to better phagocytose and kill bacteria. Yet, all the studies mentioned previously showed the opposite phenotype, where ExoU production was associated with both neutrophil recruitment and higher bacterial burden.

Diaz and colleagues started to address this question by examining the immune response to ExoU-producing strains of *P. aeruginosa* both *in vitro* and *in vivo* (230, 231). Using a fluorescence resonance energy transfer (FRET)-based assay, they were able to observe and quantify ExoU translocation into recruited immune cells. While they detected a catalytically inactive ExoU variant in 40% of recovered cells, they could not detect any cells injected with wild-type ExoU (230). One explanation for this finding could be that ExoU kills cells too rapidly after translocation and only the remaining un-intoxicated immune cells could be isolated from the lung tissues. Infection of primary human neutrophils *in vitro* showed that ExoU induces loss of membrane integrity and host cell lysis (231), supporting the hypothesis that

killing of phagocytic cells *in vivo* is what allows ExoU-producing bacteria to cause more severe disease.

The majority of the cells injected with ExoU during murine early pneumonia are neutrophils and macrophages, rather than lung epithelial cells (230). Interestingly, when mice are co-infected with an ExoU-producing strain and either *P. aeruginosa* lacking *exoU* or nonpathogenic *E. coli*, the latter bacteria were able to persist in the lung (231). Together, these results show that the increased virulence of ExoU-producing *P. aeruginosa* results primarily from the disruption of the innate immune system.

Phospholipase Activity

ExoU causes rapid lysis of many types of eukaryotic cells. ExoU is extremely cytotoxic when expressed in yeast and causes their vacuole membranes to fragment (197, 232). ExoU-secreting strains cause cell rounding and membrane damage in CHO cells (168) and cell death within 1-3 hours of infecting J774 macrophages, A549 bronchial epithelial cells, and primary human neutrophils (127, 233, 234).

ExoU was discovered to be a phospholipase A_2 (PLA_2) enzyme when two general $cPLA_2$ and $iPLA_2$ phospholipase inhibitors, methyl arachidonyl fluorophosphonate (MAFP) and haloenol lactone suicide substrate (HELSS), were found to inhibit cytotoxicity of ExoU towards yeast and mammalian cells (196, 197). A $sPLA_2$ inhibitor was unable to prevent cell lysis (196). ExoU was predicted to be a patatin-like PLA_2 based on amino acid alignment, and substitutions in the predicted catalytic residues serine-142 or aspartate-344 prevented ExoU from killing CHO

cells (197). Subsequent studies showed that purified recombinant ExoU hydrolyzed lipids in the presence of eukaryotic cell extracts (197, 203).

PLA_2 enzymes differ from other phospholipases in where they cleave the lipid substrate (235) (Figure 5). Cleavage of phospholipids by ExoU releases free fatty acids, including arachidonic acid, which generate pro-inflammatory eicosanoids and also induce oxidative stress (229, 235, 236). Incubation with antioxidants increased cell viability after infection with ExoU+ *P. aeruginosa* (237), suggesting low amounts of ExoU may have non-lytic effects that contribute to its virulence.

Indeed, a small number of studies have suggested ExoU phospholipase activity can have effects on platelet activation through arachidonic acid and eicosanoid production. After 24 hours of infection, increased white blood cell counts and platelet concentrations are observed in a mouse pneumonia model (238). Thromboxane A2 (TXA2), an eicosanoid produced from arachidonic acid that induces platelet aggregation, as well as platelet microparticles levels in the plasma were increased in mice infected with an ExoU+ strain compared to an isogenic deletion mutant (238). Thrombi and fibrin deposits were also detected in the lungs. These results suggest that in addition to immunosuppression resulting from direct PLA_2-mediated phagocytic cell killing, ExoU may indirectly promote a procoagulatory state that also contributes towards *P. aeruginosa* virulence during pneumonia and sepsis (238, 239).

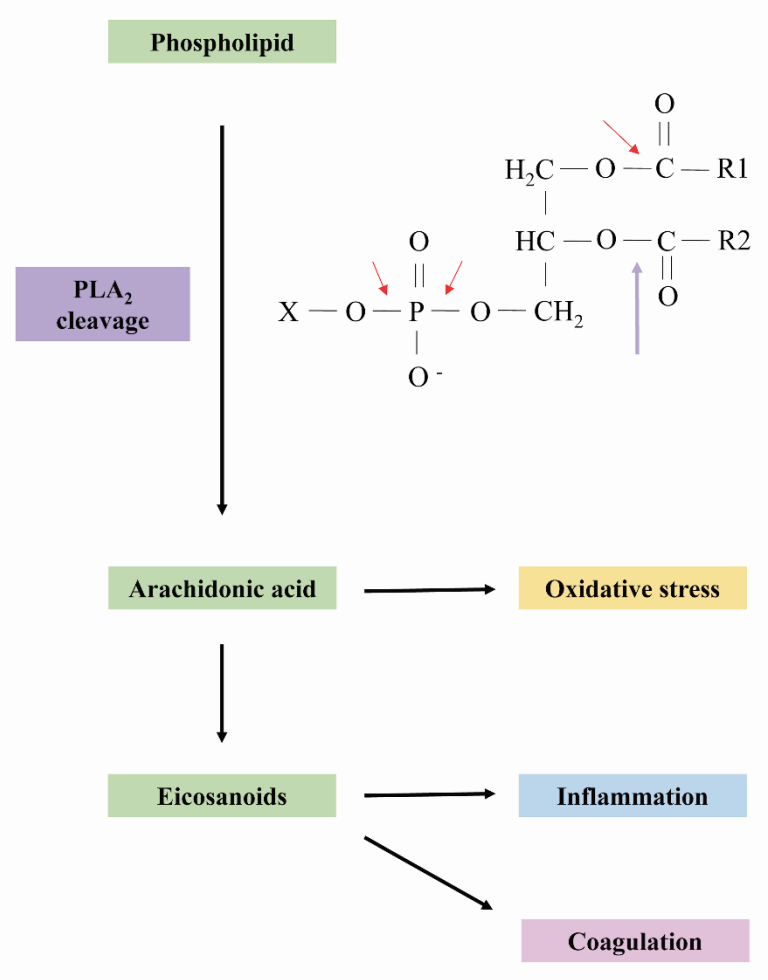


Figure 5: Phospholipase A_2 activity

Phospholipases differ in which part of the phospholipid substrate it cleaves. PLA_2 cleaves at the sn-2 location and releases arachidonic acid. Arachidonic acid can generate oxidative stress through non-enzymatic means, or be converted to eicosanoids by cyclo-oxygenases, which then cause inflammation and pro-coagulatory states. Purple arrow indicates PLA_2 cleavage site. Red arrows represent bonds cleaved by the other phospholipases PLA_1, PLD, and PLC. X represents the phospholipid head group, and R1 and R2 refer to the variable-length fatty acid chains.

Cofactors of ExoU

Previous work has shown that ExoU requires eukaryotic cell cofactors for activation, which ensures that its toxic phospholipase activity is manifested only following injection into host cells. Sato and colleagues were the first to discover a protein that activated ExoU *in vitro* (240). The protein was identified as superoxide dismutase 1 (SOD1) by mass spectrometry on activating fractions of cellular lysates. They demonstrated SOD1 of yeast or bovine origin were sufficient for phospholipid hydrolysis by ExoU; however, known inhibitors of the enzymatic function of SOD1 did not affect its ability to activate ExoU (240).

A follow-up study suggested that the main protein responsible for activating ExoU was not SOD1, but rather ubiquitin or ubiquitinated proteins (241), which are found in mammalian cells and as contamination in commercially available SOD1. Ubiquitin is thought to be so widespread because it plays an important role in maintaining protein homeostasis through the proteasome pathway, where damaged proteins are ubiquitinated and targeted for degradation (242). However, ubiquitin is not present in bacteria. It is therefore an attractive eukaryotic host cell activator for a T3SS effector protein, but ubiquitin by itself only weakly activates ExoU (243).

Several research groups had shown that a cofactor for ExoU resided in the insoluble/particulate fraction from fractionated HeLa cell extracts (215, 243). However, it was only recently that $PI(4,5)P_2$ was identified as this activating component (243). While $PI(4,5)P_2$ alone caused little *in vitro* activation of ExoU, it dramatically enhanced ExoU phospholipase activity in the presence of ubiquitin (243). Thus ExoU has a complex mechanism of action that involves at least two host cell factors.

$PI(4,5)P_2$ is an essential phospholipid found predominantly on the inner leaflet of eukaryotic plasma membranes (244, 245). It is often enriched in microdomains within these membranes and has a highly negatively charged headgroup at physiological pH, thus capable of mediating strong electrostatic interactions with metal cations, basic peptides, and proteins (244). $PI(4,5)P_2$ binds numerous cytoskeletal proteins (e.g. gelsolin, vinculin, profilin) and is involved in the regulation of many cellular processes dependent on cytoskeletal dynamics or membrane trafficking, including motility, phagocytosis, and cell adhesion (246). We can now include enhancing ExoU activity as another function of $PI(4,5)P_2$, but the mechanisms behind this activation are still being characterized.

Mechanisms of action

The process by which ExoU causes cell lysis after localization to the plasma membrane is poorly defined. It may directly cleave a broad spectrum of lipids in the plasma membrane to disrupt membrane integrity (197), or more specifically target $PI(4,5)P_2$ and its associated functions (247). PI(4,5)P2 negatively regulates multiple caspases (248), so it is possible that ExoU binding disrupts its native function and indirectly induces apoptosis. However, ExoU-induced cytotoxicity occurs within 2-3 hours (168), suggesting a mechanism independent of $PI(4,5)P_2$-mediated signaling is responsible.

Additional details regarding the molecular aspects of the mechanism of action of ExoU are beginning to emerge. Addition of di-ubiquitin and liposomes containing $PI(4,5)P_2$ resulted in significant conformational changes within the four-helical bundle of ExoU, with some portions exhibiting increased mobility and others showing less mobility (205). These results suggested a

model whereby contact with lipid membranes containing $PI(4,5)P_2$ caused unfolding of the four-helical bundle and insertion of ExoU into the lipid membrane for enhanced access to its substrate. Whether these conformational changes associated with $PI(4,5)P_2$ binding could have additional effects on ExoU remains unclear.

In this regard, it is interesting that Frank and colleagues previously reported evidence of intragenic complementation in ExoU (201). When transfected individually into CHO cells, constructs expressing truncated variants of ExoU caused only background levels of cytotoxicity. However, when co-transfected, certain pairs of these constructs resulted in partial reconstitution of ExoU cytotoxicity. These observations suggested that individual ExoU molecules were functionally interacting with each other, but the mechanism by which this occurs is unknown.

CHAPTER TWO

RESULTS

Due to its cytotoxicity towards multiple types of immune and epithelial cells (168, 177, 178, 233) and its relevance to human disease, the mechanisms of ExoU activity are of particular interest. In this chapter, I describe the discovery of $PI(4,5)P_2$-induced ExoU oligomerization. Oligomerization of ExoU and ExoU variants was characterized both in cells and *in vitro*. $PI(4,5)P_2$ mediated intragenic complementation *in vitro*, indicating oligomerization of ExoU is relevant in eukaryotic cells. Together, these results describe a new role for $PI(4,5)P_2$ in the organization and function of ExoU.

ExoU forms stable complexes in mammalian cells.

The MLD targets ExoU to the inner leaflet of the plasma membrane following injection into mammalian cells. Examination of transfected and infected cells using confocal microscopy has demonstrated that this membrane localization occurs in a punctate pattern (196, 200). One possible explanation for this pattern is that ExoU molecules interact with each other upon contact with the plasma membrane.

To study this phenomenon, we examined HeLa cells transfected with constructs expressing differentially tagged variants of ExoU. We used ExoU harboring a catalytic substitution (S142A) for these experiments (196, 197), as enzymatically active ExoU rapidly lyses cells, which precludes further examination. Constructs expressing ExoU-S142A tagged with either an N-terminal Flag epitope (ExoU-S142A-Flag) or N-terminal GFP (ExoU-S142A-

GFP) were transfected into HeLa cells, and immunoprecipitation experiments were then performed. Note that a proportion of the ExoU molecules expressed within eukaryotic cells are di-ubiquitinated, resulting in the presence of a second, more slowly migrating ExoU band on SDS-polyacrylamide gels as seen in Figure 6 (215). We observed that ExoU-S142A-GFP co-immunoprecipitated with ExoU-S142A-Flag, indicating that ExoU self-associates and forms complexes within HeLa cells.

Purified rExoU is monomeric *in vitro.*

We next determined whether ExoU complexes occurred *in vitro*. Recombinant ExoU (rExoU) with a C-terminal 6xHN tag was purified and subjected to size-exclusion chromatography followed by multi-angle light scattering (SEC-MALS), a sensitive method for separating and analyzing oligomeric protein species in solution (249). rExoU eluted as a single peak at approximately 75 kD, consistent with the molar mass of this 687-amino acid protein (Figure 7). We were not surprised by this result, as ExoU was found to be a monomer when crystallized (206, 207). However, the structures of ExoU that have been resolved were of ExoU in complex with its chaperone SpcU, a state that is only present in the bacteria and presumably inactive. rExoU has phospholipase activity but is not ubiquitinated (200), so it is possible that it cannot form complexes without this post-translational modification. Nonetheless, this result indicates that rExoU by itself does not form stable complexes *in vitro* and suggests that additional factors may be necessary to facilitate ExoU complex formation.

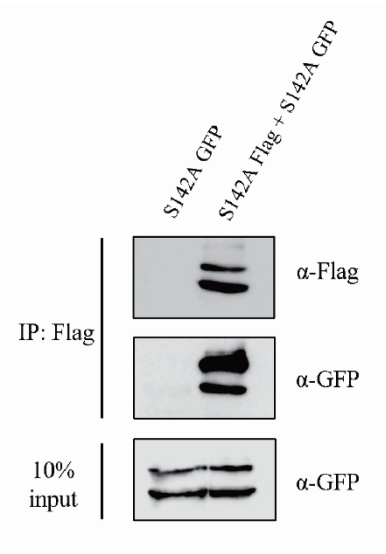


Figure 6: ExoU forms complexes in transfected HeLa cells.

Constructs expressing ExoU tagged with either Flag or GFP epitopes and containing the S142A catalytic substitution were transfected into HeLa cells. Lysates were immunoprecipitated with Flag antibody, and eluates probed with Flag or GFP antibodies. Expression of GFP-tagged ExoU-S142A was confirmed by examining 10% of total clarified lysates. Some ExoU molecules are di-ubiquitinated following injection into eukaryotic cells, resulting in the presence of a second, more slowly migrating ExoU band. α-Flag, antibody against the Flag tag; α-GFP, antibody against the GFP tag.

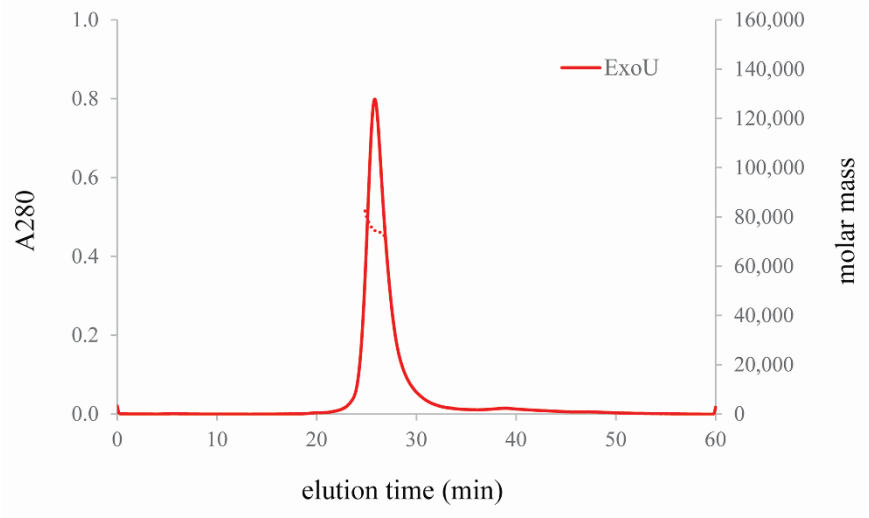


Figure 7: Purified recombinant ExoU is a monomer *in vitro*.

SEC-MALS was performed on rExoU alone. Light scattering measurements indicated that the resulting peak corresponded to proteins with a molecular mass of approximately 75 kD. At least two independent samples were analyzed and a representative elution profile shown. Normalized absorbance at 280 nm (A280, solid line) and molar mass (g/mol, dotted line) are graphed.

PI(4,5)P_2 but not ubiquitin is required for ExoU complex formation.

Since ExoU requires ubiquitin for phospholipase activity, we next examined whether the addition of ubiquitin was sufficient to cause ExoU complex formation *in vitro*. Mono-ubiquitin was mixed with rExoU at a 1:1 ratio and examined by SEC-MALS. rExoU and ubiquitin eluted at separate times, and no peak corresponding to ExoU complexes was observed (Figure 8A). No complexes were seen even when ExoU was incubated with a four or ten-fold molar excess of ubiquitin (Appendix I). Similarly, the addition of monomeric ubiquitin to rExoU did not change the migration of rExoU following electrophoresis through non-denaturing blue-native (BN) polyacrylamide gels (Figure 8B). These results indicate that any association between purified rExoU and ubiquitin under the conditions of these assays is weak or transient and that ubiquitin does not induce ExoU complex formation.

ExoU binds to PI(4,5)P_2 to localize to host cell plasma membranes, and this binding enhances ExoU phospholipase activity in the presence of ubiquitin. We therefore examined whether PI(4,5)P_2 facilitated ExoU complex formation. rExoU was incubated with PI(4,5)P_2 at a 1:1 molar ratio. PI(4,5)P_2 induced formation of an ExoU species with a molecular weight of approximately 450 kD, substantially higher than was observed with ExoU alone (Figure 9). Increasing or decreasing the PI(4,5)P_2 concentration relative to ExoU resulted in corresponding changes in the magnitude of the high-molecular weight peak (Appendix II), indicating this phenomenon is dose-dependent. The presence of a distinct, narrow peak suggests that relatively stable complexes of ExoU can form *in vitro* in the presence of PI(4,5)P_2. For brevity, we will refer to these complexes as "oligomers," although it is also possible that they are less ordered aggregates of ExoU protein.

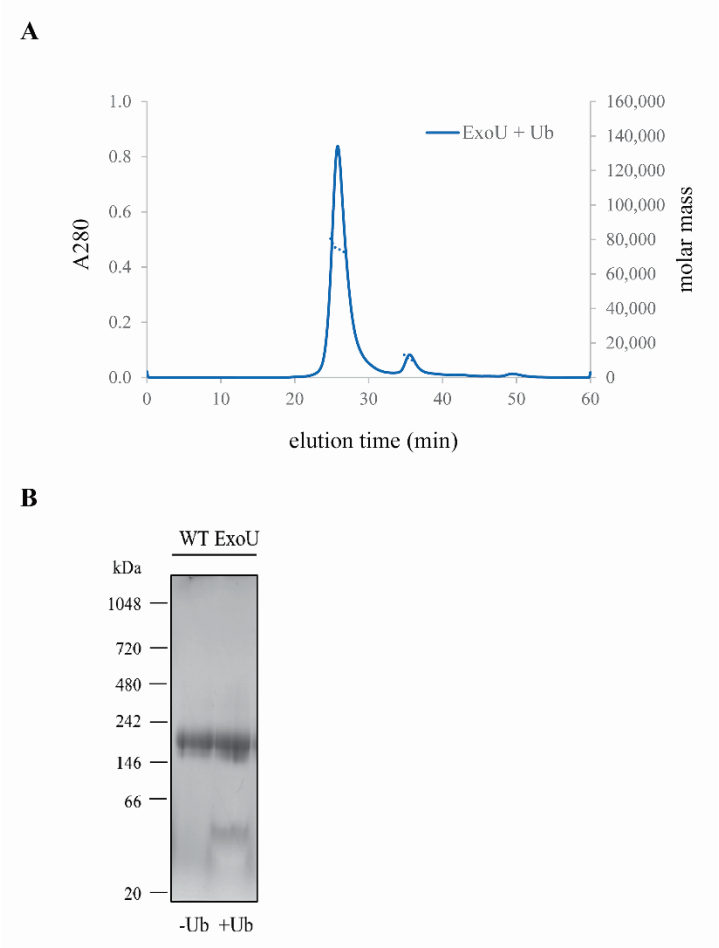


Figure 8: Oligomerization of ExoU is ubiquitin-independent.

(A) An equimolar mixture of rExoU and ubiquitin (Ub) was assessed by SEC-MALS for induction of oligomerization. The graph represents elution profiles of each sample, with normalized absorbance (solid line) and molar mass (dotted line). (B) Samples of rExoU with or without addition of ubiquitin were examined by non-denaturing blue-native gel electrophoresis. Gels were further washed and stained with Coomassie Blue to better visualize separated bands. kDa values refer to the molecular weight of the proteins present in the marker, and only reflect relative migration, not accurate masses of the bands.

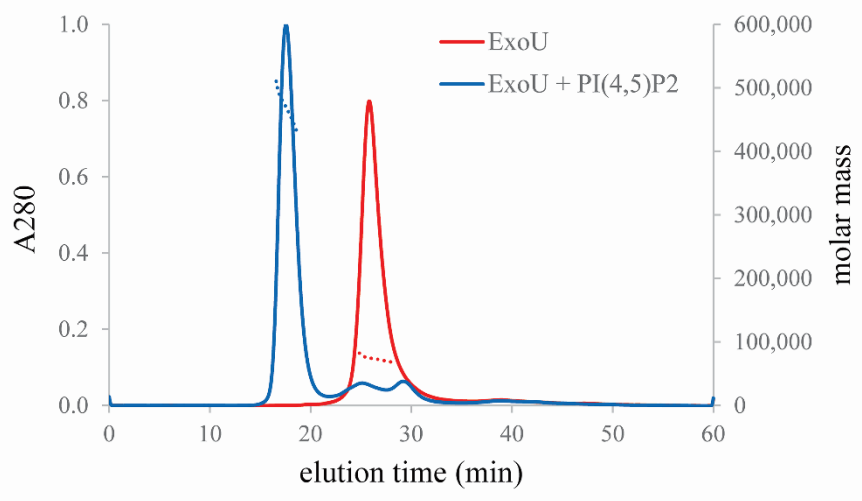


Figure 9: PI(4,5)P_2 induces oligomerization of ExoU

SEC-MALS was performed on samples containing rExoU with or without PI(4,5)P_2. Samples were mixed at a 1:1 ratio of lipid to protein. Light scattering measurements indicated a molecular mass of approximately 450 kD for proteins in the earlier peak. At least two independent samples were analyzed and a representative elution profile is shown. Normalized absorbance at 280 nm (A280, solid lines) and molar mass (g/mol, dotted lines) are graphed.

To further examine these oligomers as well as complexes formed between ExoU and its two cofactors, we used bis-sulfosuccinimidyl suberate (BS3) to irreversibly crosslink mixed samples of rExoU, monomeric ubiquitin, and PI(4,5)P_2. Since BS3 is an amine-reactive crosslinker, it affects proteins but not lipids such as PI(4,5)P_2. Crosslinked samples were run on denaturing SDS-PAGE gels. In the absence of BS3, no higher-order bands were observed (Figure10). Interestingly, addition of BS3 to rExoU alone caused the appearance of a second band that migrated at approximately 140 kDa. It is unclear what this band represents. Previous studies have shown that ExoU is a flexible protein that can adopt multiple conformations in the absence of cofactors (204, 205), and the BS3 crosslinking may have stabilized an otherwise transient conformation of ExoU that migrates more slowly in gels. There is precedence for this scenario, as a membrane-permeable analog of BS3 created intramolecular crosslinks that resulted in monomeric Tau proteins separating into two distinct bands on SDS-polyacrylamide gels (250). On the other hand, the recently crystallized ExoU homolog from *Pseudomonas fluorescens* was found to have a dimeric structure (210), so it is possible that *P. aeruginosa* ExoU also transiently dimerizes, and crosslinking captured this weak interaction.

Crosslinked samples containing both ExoU and PI(4,5)P_2 formed multiple higher-ordered species (Figure 10). The presence of ubiquitin did not alter these banding patterns, and no diminution of the ubiquitin band was observed to suggest that ubiquitin was being incorporated into the more slowly migrating rExoU bands, indicating that ubiquitin does not play a major factor in ExoU oligomerization *in vitro* (Figure 10). Taken together, these results further support a role for PI(4,5)P_2, but not monomeric ubiquitin, in the oligomerization of ExoU.

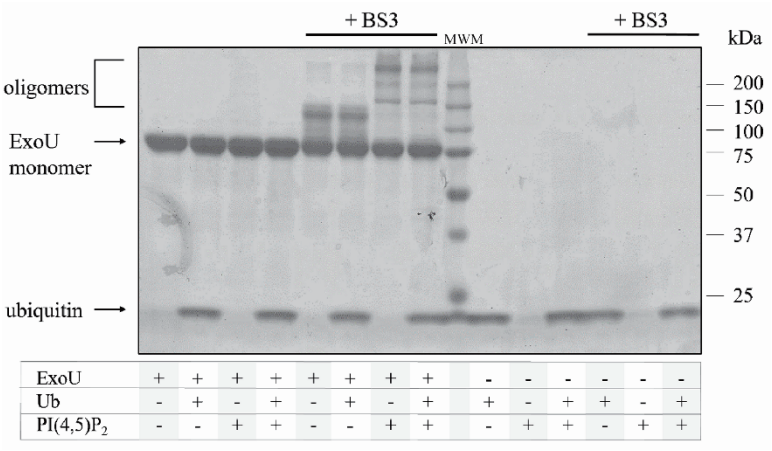


Figure 10: BS3 crosslinking captures oligomeric species induced by PI(4,5)P_2

Samples containing all of the possible combinations of rExoU and its cofactors Ub and PI(4,5)P_2 were incubated with the crosslinker BS3. Equal volumes of sample were loaded on SDS-polyacrylamide gels, and bands were visualized by Coomassie Blue staining. MWM, molecular weight marker.

Oligomerization of ExoU only occurs in the presence of specific phospholipids.

As mentioned, ExoU is targeted to the inner leaflet of the host cell plasma membrane, where it interacts with many different phospholipids. We therefore investigated whether ExoU oligomerization is a phenomenon that occurs only in the presence of $PI(4,5)P_2$ or whether it can be triggered by other plasma membrane lipids. One possibility was that phospholipids capable of activating the phospholipase activity of ExoU were also capable of inducing ExoU complex formation. In this regard, previous work had shown that $PI(4,5)P_2$ strongly co-activated ExoU in the presence of ubiquitin, phosphatidylinositol 4-phosphate (PI(4)P) had a minor effect, and most of the other examined phospholipids had no effect (243). We examined the ability of different phospholipids to cause ExoU oligomerization using rExoU in SEC-MALS experiments.

PI(4)P caused a similar elution pattern as $PI(4,5)P_2$ (Figure 11), although a smaller proportion of rExoU was in the oligomeric form. When rExoU was incubated with ten-fold excess PI(4)P, oligomerization was enhanced, suggesting that ExoU has a lower affinity for PI(4)P than $PI(4,5)P_2$. Decreased oligomerization would be consistent with previous observations that PI(4)P activates ExoU to a much lesser degree than $PI(4,5)P_2$ (243).

Next we tested phosphatidylcholine (PC), a neutral phospholipid that did not activate ExoU in the presence of ubiquitin (243). Unlike the previous experiments with $PI(4,5)P_2$ and PI(4)P, we found that ExoU pre-incubated with PC remained in a monomeric state (Figure 12A). Ten-fold excess of PC resulted in aggregated protein in the void volume, but no ExoU oligomers were observed.

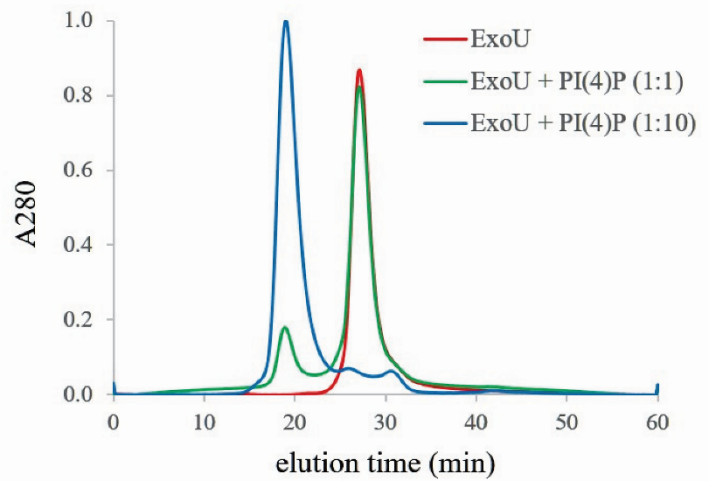


Figure 11: PI(4)P induces oligomerization of ExoU less effectively than PI(4,5)P_2.

rExoU was incubated with PI(4)P, which only activates ExoU slightly as compared to PI(4,5)P_2. Samples were prepared at the indicated protein to lipid ratios and analyzed by SEC-MALS. SEC-MALS was performed on at least two independent samples; a representative elution profile is shown.

We were curious whether the inability of PC to induce oligomerization of ExoU was due to its charge. At physiological pH, the headgroups of PI(4)P and PI(4,5)P_2 would have net charges of -2 and -4, respectively, whereas the headgroup of PC would be neutral. To address this question, we chose to test phosphatidylinositol 3,5-bisphosphate [PI(3,5)P_2], a second phosphoinositide with the same number of phosphate groups as PI(4,5)P_2. However, PI(3,5)P2 does not activate ExoU *in vitro* (243). Using SEC-MALS, we determined that like PC, PI(3,5)P_2 was incapable of inducing ExoU oligomerization at any of the concentrations tested (Figure 12B).

These protein-lipid samples were also crosslinked with BS3 to stabilize intermolecular ExoU interactions, then run on both blue native and SDS polyacrylamide gels. As expected, samples containing PI(4)P but not those containing PC or PI(3,5)P_2 showed a pattern of more slowly migrating bands on the SDS-PAGE gel similar to those of PI(4,5)P_2 (Figure 13). However, as with the SEC-MALS findings, a larger proportion of ExoU in the PI(4)P-containing samples remained monomeric. Similar results were seen on the BN-PAGE gel, where PI(4)P was less effective at inducing ExoU oligomerization than PI(4,5)P_2. Together, these data indicate that only certain phospholipids have the ability to induce oligomerization of ExoU and that phospholipids capable of activating ExoU also cause ExoU oligomerization.

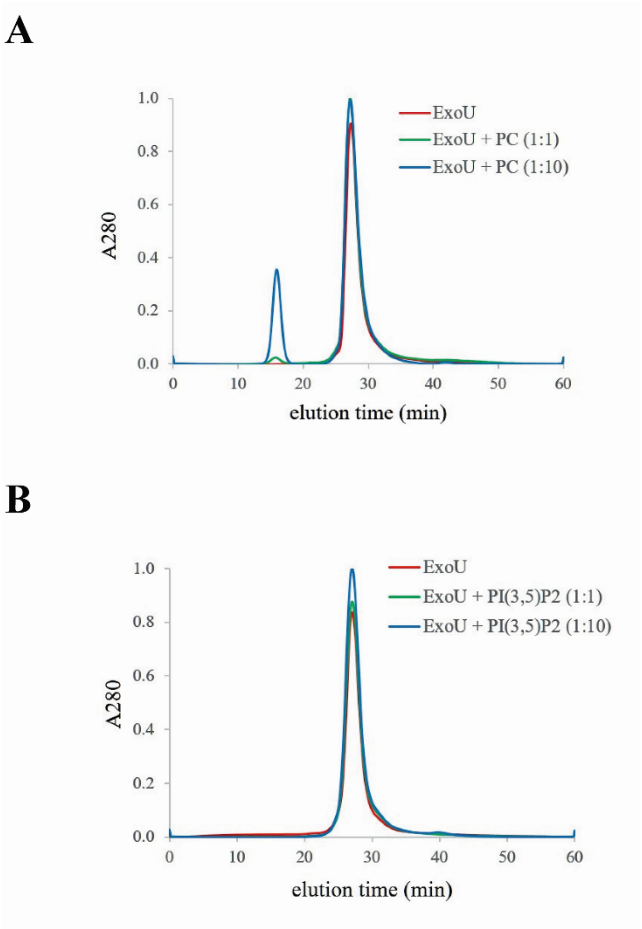


Figure 12: PC and PI(3,5)P_2 do not induce oligomerization of ExoU.

rExoU was incubated with either equal concentrations of or ten-fold excess of the indicated phospholipids and analyzed by SEC-MALS. Elution profiles containing normalized absorbance at 280 nm (A280) are graphed. (A) The elution pattern of rExoU incubated with PC, a representative neutral lipid that does not activate ExoU. (B) The elution profile of rExoU incubated with PI(3,5)P_2, which is a polar but non-activating phosphoinositide. SEC-MALS was performed on at least two independent samples; representative results are shown.

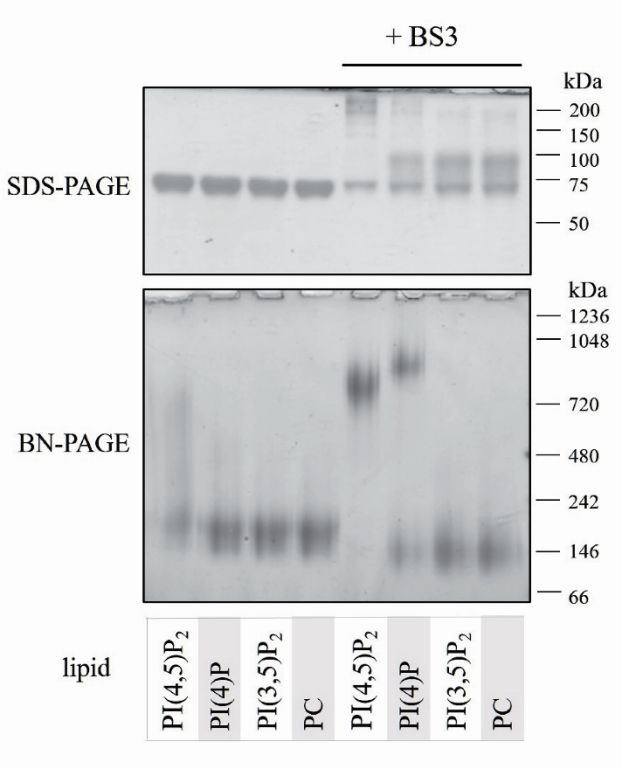


Figure 13: Crosslinking shows oligomer-inducing capacity of phospholipids differ.

Samples containing rExoU and lipids PI(4,5)P_2, PI(4)P, PI(3,5)P_2, and PC were examined for complex formation through crosslinking. After incubation with the crosslinker BS3, equal volumes of samples were loaded and run on SDS and BN polyacrylamide gels. Bands were visualized by Coomassie Blue staining.

Single amino acid substitutions in the linker region of the PLA_2 domain do not disrupt oligomerization of ExoU.

We next examined which regions or amino acid residues of ExoU were necessary for the formation of oligomers. We first focused on the PLA_2 domain of ExoU, structurally defined as amino acids residues 102 through 471 (206). The C-terminal portion of this domain, which we termed the "linker region" (amino acid residues 358-471), does not share homology to patatin-like phospholipases and has no attributed function. Furthermore, the crystal structure of ExoU shows that this region is not located on the same face of the protein predicted to interact with the plasma membrane (206), making it a possible surface for intermolecular ExoU-ExoU interactions. Therefore, we selected this linker region for further investigation.

As part of a study to identify residues required for ExoU cytotoxicity, error-prone PCR-based mutagenesis on the linker region had been performed to obtain a collection of constructs expressing ExoU variants containing single amino acid substitutions (251). This collection of constructs had been screened for substitutions that rendered ExoU partially or fully non-cytotoxic following transfection into HeLa cells. The three substitutions that caused the largest decreases in cytotoxicity were A384G, V462F, and V423F (251). Since it was conceivable that these ExoU variants were less cytotoxic because of defects in oligomerization, we investigated them further. A384G is located in disordered part of the ExoU structure, but appears to be located near the catalytic site and would be predicted to face the membrane following ExoU binding to $PI(4,5)P_2$. It may therefore play a more direct role in catalysis. V423 and V462, however, are located in β-strand 10 and α-helix 15, which are remote from the catalytic site and are predicted to be exposed on the side of membrane-docked ExoU monomer, where they would

be available to interact with other ExoU monomers (206). We therefore focused on these two valine residues.

We first verified that expression of ExoU-V423F and ExoU-V462F in HeLa cells resulted in less cytotoxicity. Respective decreases of 71% and 42% in cell lysis relative to wild-type ExoU were observed, as measured by lactate dehydrogenase release (Figure 14A). We purified these ExoU variants and used them to perform *in vitro* phospholipase assays to confirm that they had attenuated phospholipase activity. We observed that when supplemented with cofactors, recombinant ExoU-V423F and ExoU-V462F exhibited very little activity compared to wild-type rExoU, even after 4 hr of incubation with substrate (Figure 14B). These results show that an intact linker region is necessary for full ExoU cytotoxicity and phospholipase activity *in vitro*, even though this region is far removed from the active site of the PLA_2 domain.

We next tested whether V423F and V462F substitutions disrupted the ability of ExoU to oligomerize. First, we generated Flag- and GFP-tagged variants of ExoU-V423F and ExoU-V462F that also contained the S142A substitution to render them completely non-cytotoxic for co-immunoprecipitation experiments. These experiments showed that both ExoU-S142A/V423F-Flag and ExoU-S142A/V462F-Flag were capable of interacting with their respective GFP-tagged variants (Figure 15A). Interestingly, only a single ExoU band was observed with ExoU-V423F and ExoU-V462F, indicating that these amino acid substitutions prevented the di-ubiquitination of ExoU that normally occurs within eukaryotic cells. Since interactions between the Flag- and GFP-tagged variants could still be detected in the absence of this post-translational modification, we can conclude ubiquitination is not required for complex

formation and that our *in vitro* experiments should not be affected by our use of non-ubiquitinated rExoU.

When we performed SEC-MALS using purified proteins, both rExoU-V423F and rExoU-V462F eluted in single peaks, matching our results from the wild-type rExoU experiments. Pre-incubation of these linker region variants with $PI(4,5)P_2$ also resulted in a phenotype similar to that of wild-type rExoU, where a portion of the monomeric protein shifted into an oligomeric state (Figure 15B). Given that the valine-to-phenylalanine substitutions in these proteins would be predicted to cause a significant change in the secondary structure of the linker region, but had no detectable effect on ExoU complex formation, we conclude that these portions of the linker region of the PLA_2 domain are not necessary for ExoU oligomerization.

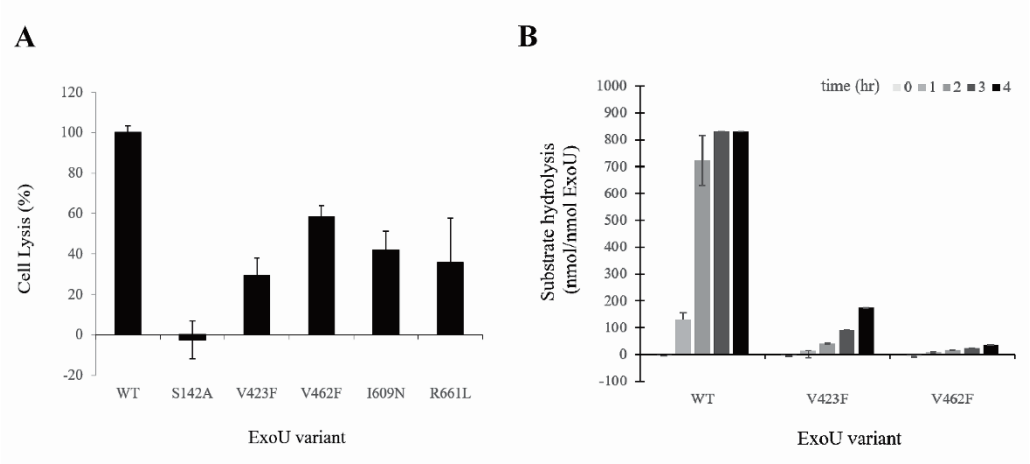


Figure 14: Amino acid substitutions in the PLA_2 linker region disrupt ExoU function

(A) ExoU variants with substitutions in the PLA_2 domain or MLD were tested for cytotoxicity following expression in HeLa cells by measurement of LDH release. Cell lysis was normalized to LDH released from cells transfected with wild-type (WT) ExoU. (B) Substrate hydrolysis of ExoU variants was analyzed relative to WT ExoU after the indicated number of hours. Each sample was supplemented with ubiquitin and $PI(4,5)P_2$ to allow for induction of phospholipase activity. For both (A) and (B), experiments were performed in triplicate, results represent means, and error bars represent standard deviations. All samples were significantly decreased compared to wild-type ($p<0.05$).

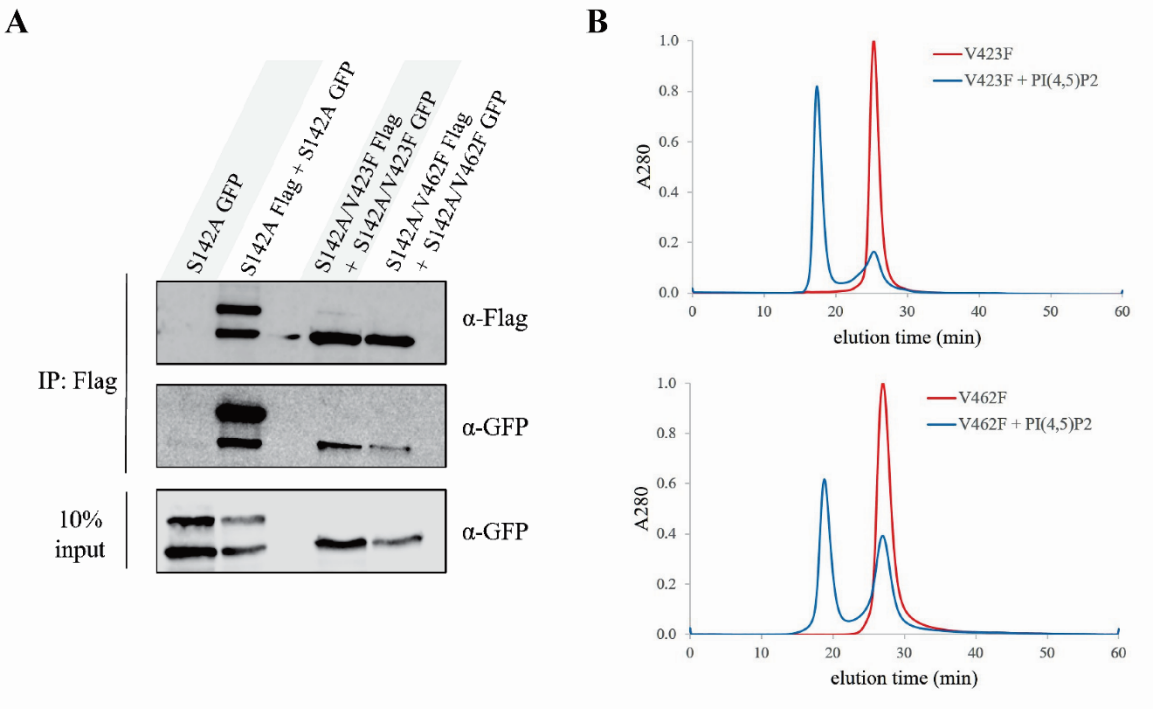


Figure 15: Selected mutations in the PLA$_2$ domain do not disrupt oligomerization.

(A) Flag- and GFP-tagged ExoU variants containing substitutions in the linker region of the PLA$_2$ domain (V423F and V462F) were examined for the ability to interact within transfected cells by co-immunoprecipitation. The presence of the proteins in eluates and input was determined through immunoblotting with the indicated antibodies. The upper bands, which represent di-ubiquitinated ExoU, were absent in the lanes containing the V423F or V462F variant proteins. α-Flag, antibodies against the Flag tag; α-GFP, antibodies against the GFP tag. (B) Linker region variants ExoU-V423F and ExoU-V462F were purified and samples prepared with or without PI(4,5)P$_2$. Samples were subjected to SEC-MALS to determine the ability of ExoU variants to oligomerize.

MLD substitutions alter ExoU oligomerization.

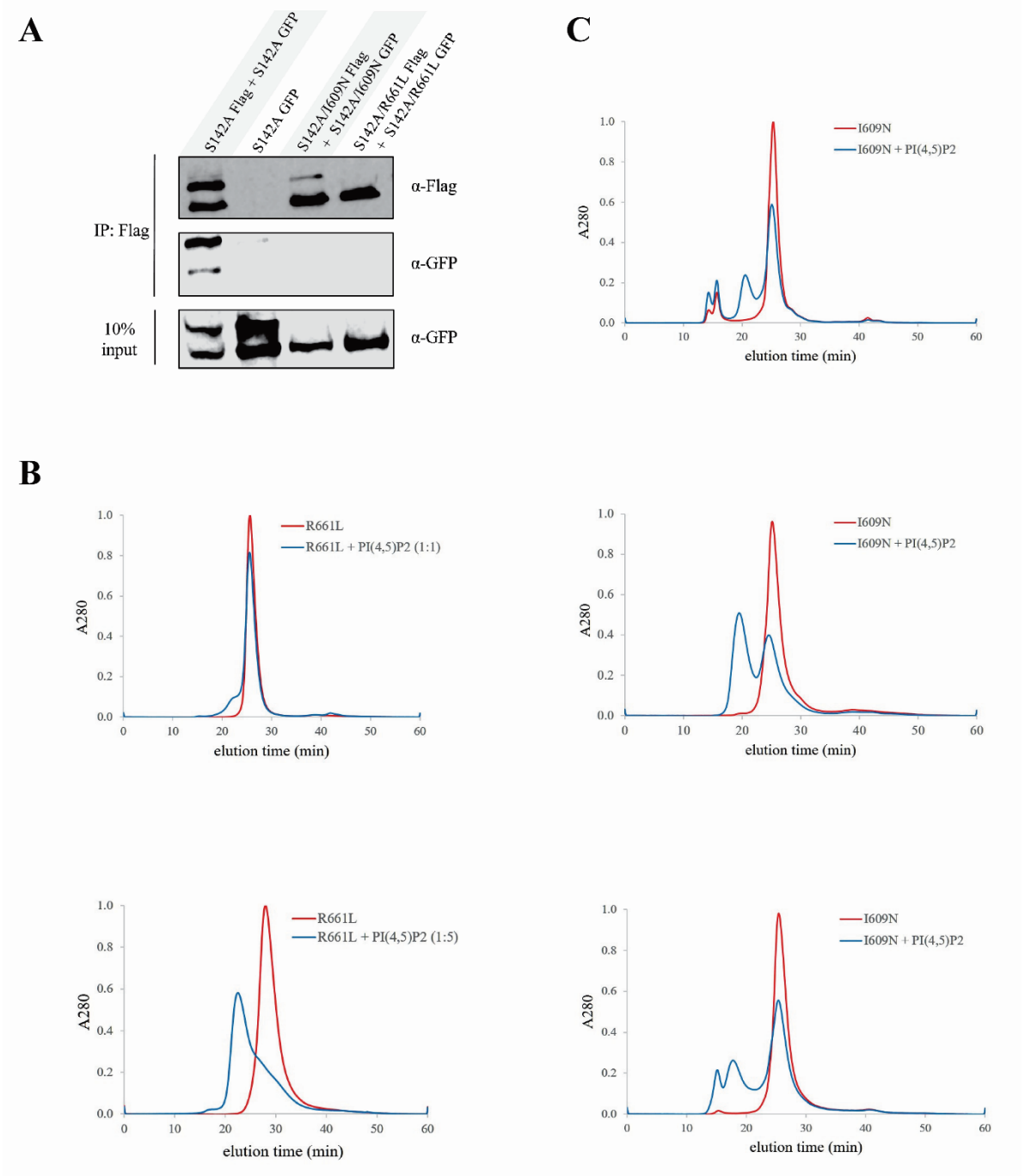
Previous work had shown that amino acid substitutions in the MLD of ExoU decrease cytotoxicity towards mammalian cells, disrupt localization to the plasma membrane, and reduce bacterial virulence in mice (200). Two ExoU variants in this domain, ExoU-I609N and ExoU-R661L, show opposing phenotypes with regard to activation by the cofactors ubiquitin and $PI(4,5)P_2$. ExoU-I609N is relatively insensitive to activation by ubiquitin, but in excess ubiquitin conditions still becomes synergistically activated by $PI(4,5)P_2$. In contrast, ExoU-R661L is activated by ubiquitin but does not respond to the co-activator $PI(4,5)P_2$ (243). As previously demonstrated (200), both of these MLD variants exhibited a significant decrease in cell lysis compared to wild-type ExoU when expressed via transfection in HeLa cells (Figure 14A). For these reasons, we decided to examine whether these amino acid residues played a role in oligomerization.

Co-immunoprecipitation experiments using Flag- or GFP-tagged ExoU MLD variants were performed. Both ExoU-S142A/I609N-Flag and ExoU-S142A/R661L-Flag were unable to pull down their corresponding GFP-tagged versions (Figure 16A). Interestingly, the more slowly migrating band was faint or absent with the I609N and R661L variants, indicating that like V423F and V462F, these amino acid substitutions affected the di-ubiquitination of ExoU. Nonetheless, these experiments show that in a cellular context, neither ExoU-I609N nor ExoU-R661L are able to form stable complexes.

To test whether these proteins can form oligomers *in vitro*, purified recombinant ExoU-I609N and ExoU-R661L were incubated with $PI(4,5)P_2$ and then subjected to SEC-MALS (Figure 16B and 16C). Both MLD variants individually eluted with calculated masses

corresponding to monomers; however, pre-incubation with PI(4,5)P_2 yielded different results. In the presence of equimolar amounts of PI(4,5)P_2, the majority of ExoU-R661L remained in monomeric form, with a very small proportion eluting in a slightly earlier peak (Figure 16B). The protein present in the peak had a calculated mass of approximately 115 kD, less than that of an ExoU dimer, so it is unclear whether it is an unfolded or misfolded monomer, or if it is a mixture of unstable dimers dissociating back into monomers. The proportion of protein in this earlier peak increased when the protein to lipid ratio was increased to 1:5 (Figure 16B). However, unlike the oligomeric peak observed with wild-type ExoU (Figure 9), these oligomers eluted at a later time point in a sloping, broad peak with a calculated mass ranging from 150 kD to 300 kD. These results suggest that the R661L substitution causes defects in ExoU oligomerization, although the presence of excess PI(4,5)P_2 may allow ExoU-R661L to form smaller complexes *in vitro*.

Curiously, ExoU-I609N appeared to exist in multiple states when incubated with equimolar amounts of PI(4,5)P_2 (Figure 16C). A variable proportion of the protein remained as monomers, while the rest eluted as higher-order oligomers. These oligomers eluted at different times across the three experiments with variable calculated molar masses. This contrasts with wild-type ExoU, which consistently formed oligomers with a mass of around 450 kD (Figure 9). Unlike all of the previous ExoU variants tested, ExoU-I609N also had a tendency to form large aggregates, as indicated by the peaks detected around 15 min after sample injection (corresponding to the void volume of the column). This suggests that in the presence of PI(4,5)P_2, ExoU-I609N partially unfolds, resulting in a less globular conformation which aggregates and that these changes may also block earlier steps in the oligomer-forming process.


A
S142A Flag + S142A GFP
S142A Flag
S142A/I609N Flag + S142A/I609N GFP
S142A/R661L Flag + S142A/R661L GFP
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α-Flag
α-GFP
10% input
α-GFP
B
R661L
R661L + PI(4,5)P2 (1:1)
A280
elution time (min)
R661L
R661L + PI(4,5)P2 (1:5)
C
I609N
I609N + PI(4,5)P2
I609N
I609N + PI(4,5)P2
I609N
I609N + PI(4,5)P2

Figure 16: Substitutions in the MLD of ExoU affect oligomerization.

(A) ExoU containing catalytic (S142A) and MLD (I609N or R661L) substitutions were used to determine whether ExoU complexes are disrupted. Co-immunoprecipitation experiments show that Flag-tagged ExoU variants did not pull down GFP-tagged variants co-expressed in HeLa cells. The upper bands, which represent di-ubiquitinated ExoU, were faint or absent in the lanes containing the I609N or R661L variant proteins. α-Flag, antibodies against the Flag tag; α-GFP, antibodies against the GFP tag. (B) Purified recombinant ExoU-R661L was analyzed for oligomerization in the presence or absence of $PI(4,5)P_2$ using SEC-MALS. Samples containing a 1:1 (top) or 1:5 (bottom) ratio of protein to lipid were tested. (C) SEC-MALS was performed on ExoU-I609N samples with or without equimoles of $PI(4,5)P_2$. Three separate sets of experiments are shown. Each row represents a pair of experiments performed on the same day. ExoU-I609N plus $PI(4,5)P_2$ demonstrated variable elution profiles, which was not observed with the other ExoU variants.

Although it is clear that the I609N substitution alters oligomerization, additional experiments will be necessary to elucidate how and why these differing oligomeric states are formed.

One explanation for the preceding results is that the MLD variants of ExoU form unstable oligomers or monomers during the course of immunoprecipitation or SEC-MALS. To examine this possibility, we performed BS3 crosslinking experiments on samples containing ExoU-I609N or ExoU-R661L. We reasoned that crosslinking might stabilize weak or transient interactions that would not otherwise be observable by co-immunoprecipitation or SEC-MALS.

WT ExoU and the two MLD variants were incubated with $PI(4,5)P_2$ at a 1:1 protein to lipid ratio and crosslinked with BS3. Under these conditions, SDS polyacrylamide gels showed that ExoU-I609N formed a banding pattern indistinguishable from that of wild-type ExoU (Figure 17A). Slightly less prominent oligomeric bands were observed with crosslinked ExoU-R661L plus $PI(4,5)P_2$ as compared to WT ExoU. When the same samples were run through non-denaturing BN-PAGE, a similar trend was observed. The oligomeric band was most prominent in the WT ExoU-containing sample, followed by ExoU-I609N, and barely detectable with ExoU-R661L. We then repeated these experiments using a 1:5 protein to lipid ratio. This time, no reduction of higher-order oligomer bands was observed on SDS-PAGE gels with either ExoU MLD variant (Figure 17B). Under non-denaturing conditions, excess $PI(4,5)P_2$ appeared to significantly increase the amount of oligomeric ExoU-I609N and ExoU-R661L, but as seen with the SEC-MALS experiments, the distribution or range in size of these complexes may not completely mimic WT oligomers (Figure 17B). These results suggest that ExoU-I609N and ExoU-R661L form unstable oligomers in the presence of $PI(4,5)P_2$.

Taken together, our co-immunoprecipitation, SEC-MALS, and crosslinking experiments show that an intact MLD is necessary for both cytotoxicity and normal PI(4,5)P_2-mediated oligomerization of ExoU.

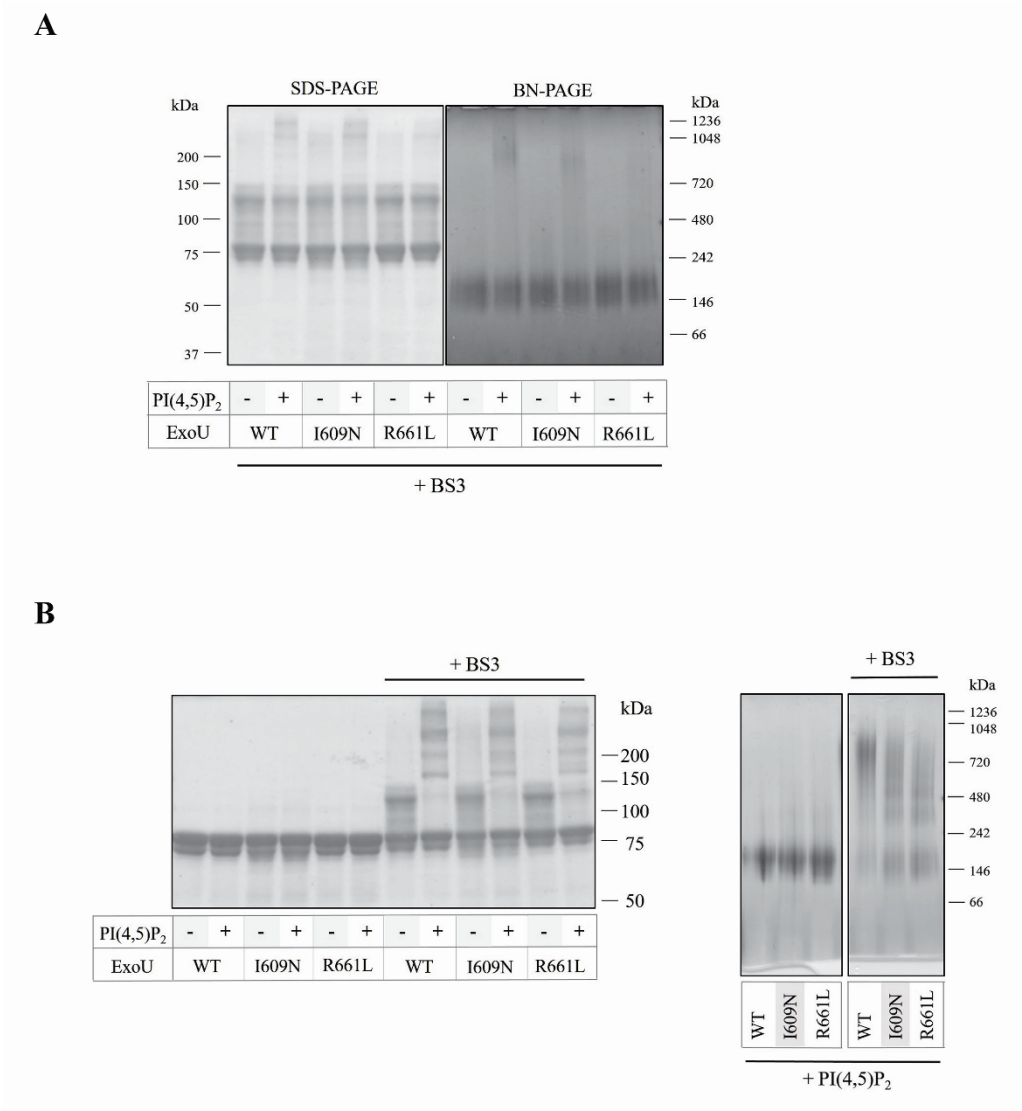


Figure 17: BS3 crosslinking captures unstable oligomers formed by MLD variants

ExoU variants with I609N or R661L substitutions were analyzed by BS3 crosslinking for detection of oligomers induced by PI(4,5)P2. (A) Equimolar mixtures of ExoU variants and lipid were incubated with the crosslinker BS3, then run on SDS and BN polyacrylamide gels. (B) Proteins were mixed with PI(4,5)P2 at a 1:5 molar ratio and crosslinked. Equal volumes of samples were loaded and separated through SDS-PAGE (*left)* or BN-PAGE (*right)*. Bands were visualized by Coomassie Blue staining. kDa values for BN-PAGE refer to the molecular weight of the proteins present in the marker, and only reflect relative migration, not accurate masses of the bands.

Truncated ExoU proteins exhibit intragenic complementation.

Finck-Barbançon and colleagues showed that specific non-cytotoxic ExoU truncation variants could functionally complement each other and partially restore cell lysis following expression by co-transfection (201). Since this phenomenon, referred to as intragenic complementation, often implies protein multimerization (252-255), we attempted to verify this hypothesis.

Truncated variants of the 687-amino-acid ExoU protein were generated. ExoU 100-687 lacks the N-terminal 99 amino acids, and ExoU 1-550 lacks the C-terminal 137 amino acids (Figure 18A). When constructs expressing these truncated proteins were transfected individually into HeLa cells, both induce cytotoxicity only slightly above background levels (Figure 18B). However, co-transfection of constructs expressing ExoU 1-550 and ExoU 100-687 resulted in 69% cell lysis relative to wild-type ExoU, indicating that intragenic complementation occurred with these ExoU truncations. Introducing the S142A catalytic substitution into the truncation variants reduced cytotoxicity to background levels (Figure 18B), showing that cell lysis required the phospholipase activity of ExoU and was not occurring by some other mechanism. These results confirm that ExoU undergoes intragenic complementation following expression in HeLa cells.

To determine whether intragenic complementation of ExoU also occurred *in vitro*, the same truncated ExoU proteins were purified recombinantly and tested for phospholipase activity. As with wild-type ExoU, ExoU 1-550 and ExoU 100-687 individually or in combination were inactive when ubiquitin and PI(4,5)P2 were absent (Figure 18C). When ubiquitin was added, wild-type ExoU caused substrate hydrolysis but the truncated variants, individually or in

combination, did not. PI(4,5)P2 alone did not activate wild-type ExoU, the individual truncation variants, or the combination of truncation variants, as no substrate hydrolysis was observed even after 24 hrs (Appendix III). In concordance with previous studies (243), $PI(4,5)P_2$ dramatically enhanced the phospholipase activity of wild-type ExoU in the presence of ubiquitin (Figure 18C). However, the individual truncated proteins remained inactive despite incubation with both cofactors. Finally, when ExoU 1-550 and ExoU 100-687 were together incubated with both ubiquitin and $PI(4,5)P_2$, phospholipase activity was restored (Figure 18C). Although substrate hydrolysis by the combined truncation variants consistently lagged behind WT ExoU, they were always able to achieve maximal phospholipase activity after longer incubation with both co-factors and the substrate being cleaved. Interestingly, phospholipase activity resulting from intragenic complementation of the truncation variants was higher than that of WT ExoU incubated with ubiquitin alone (Figure 18C). Regardless, these results are consistent with those from the HeLa cell cytotoxicity experiments, and show that the two known ExoU cofactors are sufficient for mediating intragenic complementation of ExoU phospholipase activity *in vitro*.

Finally, we wished to examine whether ExoU 1-550 and ExoU 100-687 oligomerize, as this would be a likely mechanism by which intragenic complementation occurs. Co-immunoprecipitation experiments were performed following transfection of HeLa cells with constructs expressing the same two ExoU truncation variants containing catalytic mutations to prevent cell lysis. The more slowly migrating band was absent from the samples containing the truncated proteins, indicating that the truncations affected the di-ubiquitination of ExoU. A faint band was reproducibly observed when ExoU S142A/1-550-Flag was used to pull down ExoU-S142A/100-687-GFP (Figure 19). To further demonstrate this interaction, the Flag- and GFP-

epitope tags were swapped and the experiment was repeated. Again, a faint band was reproducibly observed (Figure 19). The faintness of this band may be the result of lower affinity interactions between truncated ExoU molecules relative to wild-type ExoU molecules. Consistent with this explanation is that truncated ExoU variants also exhibited only partial restoration of cytotoxicity and phospholipase activity. It may be possible that the di-ubiquitinated population of ExoU forms more stable complexes and thus result in higher cytotoxicity in cells, but *in vitro* all of the rExoU proteins are non-ubiquitinated so the difference between WT ExoU and the phospholipase activity resulting from intragenic complementation is somewhat less prominent (Figure 18B and 18C).

In summary, these results show that ExoU 1-550 and ExoU 100-687 form complexes that exhibit intragenic complementation of both cytotoxicity and phospholipase activity *in vitro*. Thus, oligomerization may have functional consequences and impact ExoU activity.

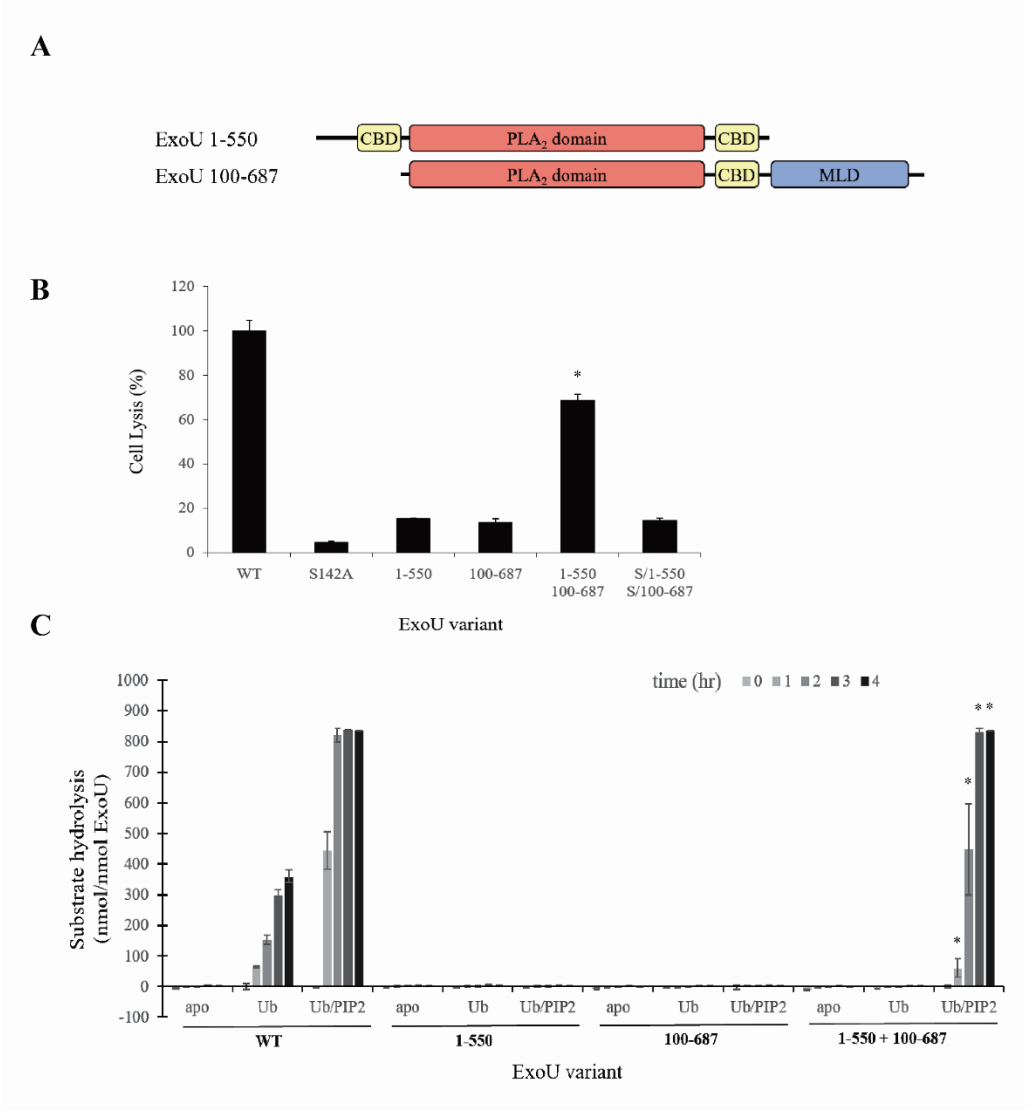


Figure 18: ExoU truncations exhibit intragenic complementation.

(A) Schematic depiction of the ExoU truncation variants. The PLA_2 domain is shown in red, the MLD is shown in blue, and the chaperone-binding domain is shown in yellow (CBD). (B) Cytotoxicity of the truncation variants ExoU 1-550 and ExoU 100-687 was quantified by LDH release following expression by transfection into HeLa cells. Cell lysis was normalized to LDH released from cells transfected with WT ExoU. "S" denotes S142A catalytic substitution. (C) Phospholipase activity of each truncated ExoU variant alone (apo), with ubiquitin (Ub), or supplemented with both ubiquitin and $PI(4,5)P_2$ (Ub/PIP2) was measured. Hydrolysis of a synthetic phospholipid substrate was calculated after the indicated hours of incubation. * indicates a significant increase ($p < 0.05$) in cytotoxicity or substrate hydrolysis as compared to either ExoU truncation variant alone. Values represent means from three samples and error bars represent standard deviations.

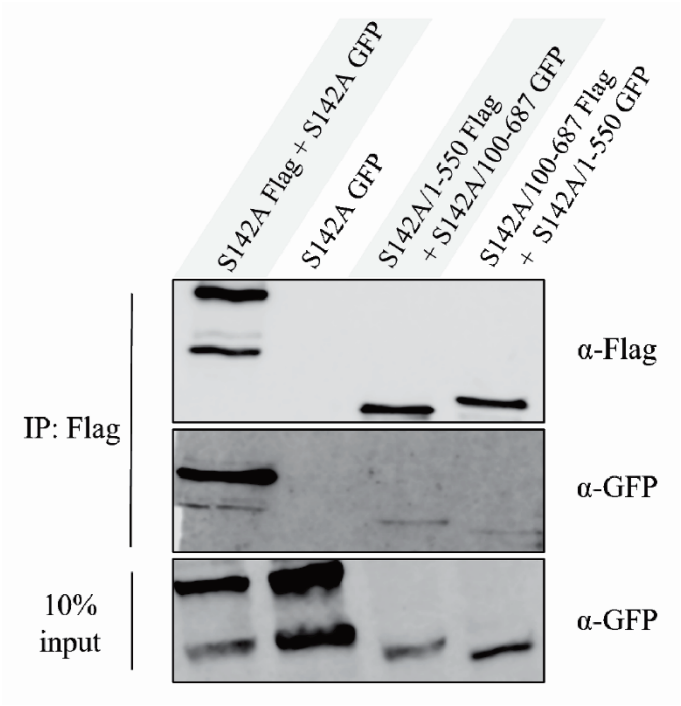


Figure 19: ExoU truncation variants oligomerize.

Co-immunoprecipitation experiments were performed to examine interactions between truncated ExoU variants. The upper bands, which represent di-ubiquitinated ExoU, were absent in the lanes containing the truncated variant proteins. α-Flag, antibodies against the Flag tag; α-GFP, antibodies against the GFP tag. "S" denotes S142A catalytic substitution.

CHAPTER THREE

DISCUSSION

ExoU is a major virulence determinant of *P. aeruginosa* and plays an important role in the progression of infections caused by this bacterium. To efficiently lyse host cells, ExoU must localize to the host cell plasma membrane and in the process interact with two different cofactors. We have identified another aspect to the complex mechanism of action of this phospholipase.

Summary of Findings

Upon interaction with $PI(4,5)P_2$ and to a lesser extent PI(4)P, ExoU undergoes oligomerization. Co-immunoprecipitation and SEC-MALS experiments with ExoU variants harboring single amino acid substitutions indicated that although α-helix 15 and β-strand 10 of the linker region of the PLA_2 domain did not directly mediate oligomer formation, the MLD was involved. Some of the same MLD residues critical for membrane localization also affected the oligomerization of ExoU. Finally, we examined the functional implications of ExoU oligomerization. We confirmed previous reports that truncated ExoU proteins exhibit intragenic complementation in transfected cells. Intragenic complementation occurred *in vitro* and required both cofactors, ubiquitin and $PI(4,5)P_2$. Lastly, these truncated ExoU proteins formed complexes, suggesting a mechanism by which the restoration of ExoU function occurred. Taken together, our results indicate that ExoU undergoes oligomerization during intoxication of host cells and suggest that this oligomerization may play a role in ExoU activity.

Oligomerization of phospholipases

Formation of oligomers is not unique to ExoU but is widespread among phospholipases. Crystallization studies have shown oligomerization to occur with both bacterial and eukaryotic phospholipases, including several PLA_2 enzymes found in snake venom (256-258), PldA of *Yersinia pseudotuberculosis* (259), human pancreatic phospholipase A_2 (260), and calcium-independent cytosolic phospholipase A_2 (261). PldA is a phospholipase A_1 that forms a homodimer which requires Ca^{2+} ions for both catalytic activity and dimerization, and the crystal structure showed those ions coordinated with residues from both monomer subunits (259). The oligomers formed by phospholipases from the snake venom of different species vary in size, forming dimers, trimers, or hexamers (256, 257, 262). Interestingly, the hexamer reported for the PLA_2 from *Ophiophagus hannah* consisted of three homodimers (256). While calcium ions can mediate oligomerization of some venom PLA_2 enzymes, carbohydrates have also been shown to act as an inducing ligand (262, 263).

Our SEC-MALS results show that when ExoU is incubated with $PI(4,5)P_2$ at a 1:1 ratio, it consistently forms oligomers approximately 450 kD in size, corresponding to the molecular mass of six ExoU molecules. However, the exact arrangement and conformation of individual subunits within these complexes remains unknown.

Lipid-mediated oligomerization

Our results suggest that localization to the host cell plasma membrane, and in particular to $PI(4,5)P_2$ in the inner leaflet of the plasma membrane, causes ExoU oligomerization. This prediction is consistent with other proteins that undergo multimerization upon contact with

membranes (264, 265). Similar to ExoU, the Ebola virus matrix protein VP40 binds $PI(4,5)P_2$, which triggers oligomerization at the plasma membrane (266). Phosphatidylinositol-specific phospholipase C (PI-PLC) of *Staphylococcus aureus* interacts with phosphatidylcholine in membranes, which causes dimerization (267). In contrast, Gag protein of HIV forms oligomers in the cytosol, and these oligomers bind to $PI(4,5)P_2$ at the plasma membrane, which induces further assembly (268). ExoU is therefore another example of a microbial phospholipase that uses host cell membrane contact as a signal to undergo multimerization.

$PI(4,5)P_2$ may trigger conformational changes in an ExoU monomer that expose surfaces capable of forming molecular interactions with other ExoU monomers. As recently suggested (205), the α-helices of the four-helical bundle might unfold to facilitate inter-monomer interactions between α-helices that replace intra-monomer interactions. Alternatively, MLD binding to $PI(4,5)P_2$ may simply serve to colocalize a number of ExoU molecules to the same patch of membrane. $PI(4,5)P_2$ molecules have been observed to cluster together in the inner leaflet of the plasma membrane (269), and binding of multiple ExoU molecules to these $PI(4,5)P_2$-enriched patches of membranes could by itself cause the formation of tightly packed ExoU complexes. A similar process has been described for the phosphatidylinositol-specific phospholipase C (PI-PLC) of *Listeria monocytogenes.* This PI-PLC binds to anionic phospholipids; when anionic phospholipids are concentrated, PI-PLC aggregates form and were readily detected by OD_{350}. When the anionic phospholipids are diluted with neutral lipids prior to incubation with PI-PLC, these aggregates are not observed (270). It remains formally possible that the early ExoU peak observed in the SEC-MALS experiments represents multiple ExoU monomers bound to $PI(4,5)P_2$ micelles, but with limited ExoU: ExoU intermolecular

interactions. However, the absence of a visible precipitate or aggregate in the void of the column and the intragenic complementation results (showing that a ExoU truncation variant lacking the MLD could be complemented) argue against this interpretation.

Functional effects of oligomerization

Another important question is whether PI(4,5)P_2-mediated ExoU oligomerization has functional consequences. In other words, does oligomerization enhance the overall phospholipase activity of ExoU? Two lines of reasoning suggest that this is the case. First, ExoU undergoes intragenic complementation, which in many proteins has been associated with catalytic cooperativity. We confirmed previous results (201) by showing that ExoU 1-550 and ExoU 100-687 caused little cytotoxicity when individually expressed in HeLa cells but that cell lysis was restored to nearly two-thirds of wild-type ExoU levels when constructs expressing these variants were co-transfected into HeLa cells. We observed that the phospholipase activities of these individual truncated ExoU proteins were partially restored *in vitro* following co-incubation with PI(4,5)P_2 and ubiquitin, demonstrating that these two factors are sufficient for ExoU intragenic complementation.

A detailed structural explanation for intragenic complementation has been determined for several proteins, such as β-galactosidase (271) and alkaline phosphatase (252) of *Escherichia coli.* In these cases, the proteins have been shown to dimerize or oligomerize, and the formation of these complexes induces cooperative interactions between the monomers that enhance activity. Our finding that ExoU truncation variants co-immunoprecipitated when co-expressed in HeLa cells suggests that a similar mechanism may occur with ExoU.

Second, many examples of functional activation by oligomerization exist in both bacterial and eukaryotic phospholipases. Multimerization of *Staphylococcus aureus* PI-PLC and *E. coli* outer membrane phospholipase A is associated with enhanced activity (267, 272). In mammalian cells, group VIa calcium-independent PLA_2 ($iPLA_2$) forms an oligomeric complex of 270-350 kD (273). Splice variants of this phospholipase bound the full-length protein and negatively regulated its activity during the late G_1 and S phases of the cell cycle (274, 275). The authors speculated that this reduction in activity was due to the disruption of active oligomers by the inactive truncated forms of $iPLA_2$.

Interestingly, protein complex formation can also act to inhibit phospholipase activity. PlaB, a phospholipase A made by *Legionella pneumophila*, forms dimers and tetramers of low or no activity, but upon dissociation into monomers this enzyme becomes highly active (276). Many snake venom phospholipases oligomerize in such a way that the active sites are turned inwards or buried inside the oligomer (257, 277). It is thought that this arrangement prevents or reduces the ability of substrates to engage the active site, thus rendering the phospholipase inactive. This could be a protective mechanism for the host, as it needs to store the venom without suffering the negative effects of the phospholipases itself.

Our results suggest that $PI(4,5)P_2$-mediated oligomerization likely contributes to the enhancement of ExoU activity. However, more studies are needed to determine how $PI(4,5)P_2$ binding causes these quaternary structural changes, why these structural conformations increase phospholipase activity, why ubiquitin is required for $PI(4,5)P_2$ activation of ExoU, and in what order these events occur inside the cell.

Model of ExoU activity

Our results add to the overall mechanism by which ExoU intoxicates host cells (Figure 20). Within the *P. aeruginosa* bacterium, the N-terminus of ExoU is recognized by the SpcU chaperone and the type III secretion apparatus to facilitate secretion and injection. Once in the host cell cytosol, ExoU coopts a host cell trafficking system by using $PI(4,5)P_2$ as a marker for the inner leaflet of the plasma membrane. The MLD of ExoU binds to $PI(4,5)P_2$ to juxtapose ExoU against the membrane. These interactions cause conformational changes in ExoU that facilitate oligomerization. PI(4)P is also capable of inducing ExoU oligomerization *in vitro*; however, it is unclear whether PI(4)P contributes to this process inside cells. In the presence of ubiquitin (or ubiquitinated proteins), $PI(4,5)P_2$ enhances the phospholipase activity of ExoU. This in turn leads to cleavage of plasma membrane phospholipids and eventually cell lysis. Additional studies will be necessary to understand the functional role played by oligomerization in these processes.

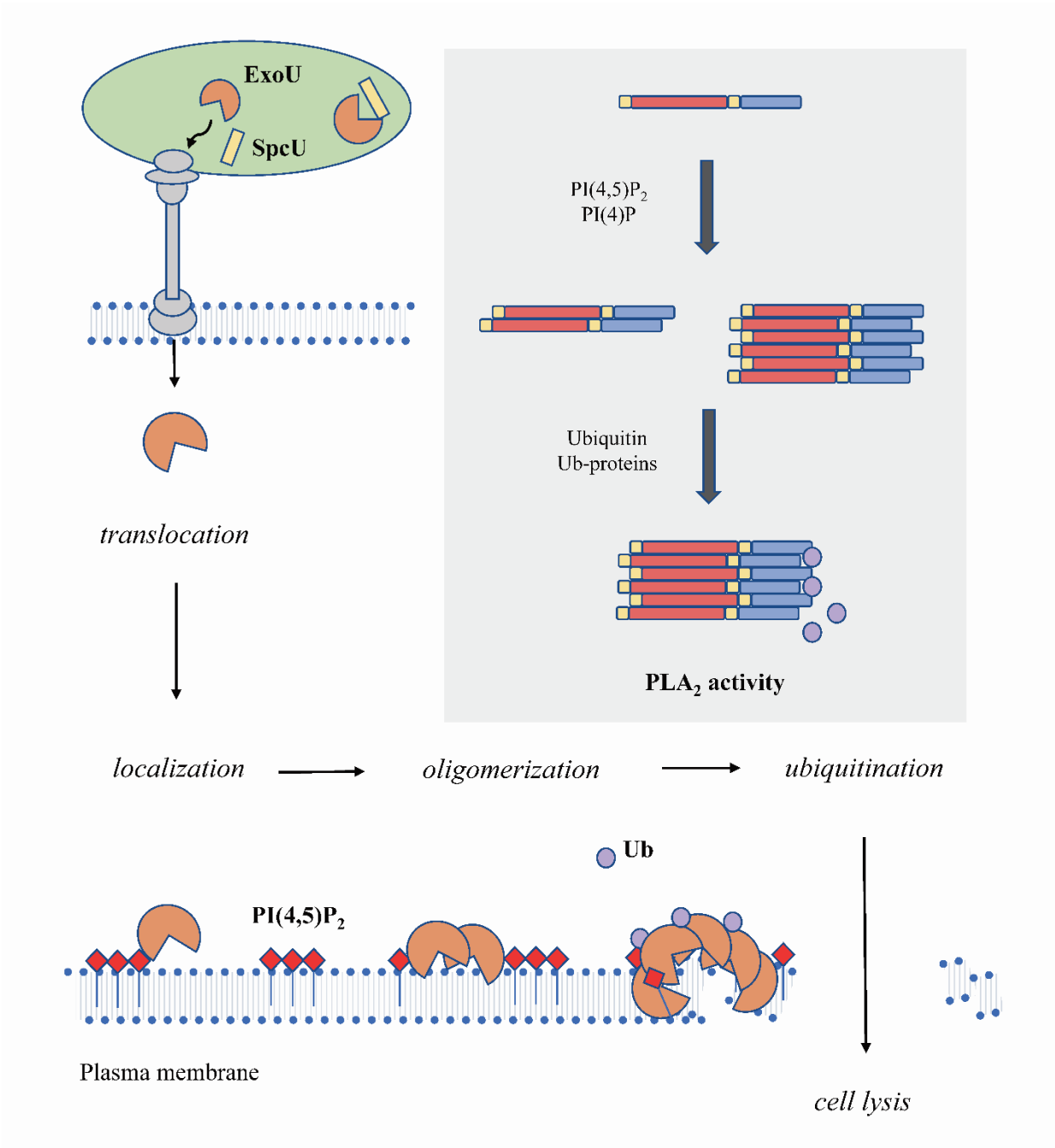

ExoU
SpcU
translocation
PI(4,5)P2
PI(4)P
Ubiquitin
Ub-proteins
PLA2 activity
localization
oligomerization
ubiquitination
Ub
PI(4,5)P2
Plasma membrane
cell lysis

Figure 20: A new model of ExoU activity

We propose this new model of ExoU activity based on the results from *in vitro* experiments. ExoU is injected into the host cell through the T3SS. Following translocation, ExoU binds $PI(4,5)P_2$ at the plasma membrane, where it then becomes oligomerized. Oligomerization may occur in multiple steps, forming dimers before assembling into higher-order oligomers. ExoU is then ubiquitinated, and this oligomeric form of ExoU causes rapid cell lysis. Both $PI(4,5)P_2$ and PI(4)P are capable of inducing ExoU oligomerization *in vitro* (gray box). However, the phospholipase A_2 (PLA_2) activity of ExoU requires addition of ubiquitin (Ub) or ubiquitinated proteins (Ub-proteins).

Future directions

A major question that is still unanswered is what portions of ExoU are necessary for $PI(4,5)P_2$-mediated oligomerization to occur. Our search for these amino acid residues had mixed results. Based upon the assumption that ExoU oligomerization was an important step in ExoU phospholipase activation, we focused on ExoU amino acid substitution variants that showed defects in cytotoxicity and phospholipase activity. Substitution of select residues in the linker region of the phospholipase domain resulted in decreased cytotoxicity towards HeLa cells but did not abrogate $PI(4,5)P_2$-mediated oligomerization. These findings indicate that this portion of the phospholipase domain plays an important role in the enzymatic activity of ExoU but that this role is distinct from oligomerization.

In contrast, substitutions of residues I609 or R661 in the MLD did result in a defect in oligomerization. R661 extends in a disordered loop between α-helix 24 and α-helix 25 of the MLD four-helical bundle, which plays a critical role in the interaction between ExoU and $PI(4,5)P_2$. It was previously postulated that the positive charge of the side chain of the arginine at residue 661 interacted with the negatively charged phosphate group of $PI(4,5)P_2$ to facilitate the interaction between these two molecules (210). Consistent with this hypothesis is that ExoU-R661L did not exhibit enhanced activation in the presence of $PI(4,5)P_2$ (243). It is therefore likely that the R661L substitution disrupts oligomerization by preventing ExoU from recognizing or interacting with $PI(4,5)P_2$ effectively. This is supported by the observation that high concentrations of $PI(4,5)P_2$ were able to induce oligomerization of ExoU-R661L. While SEC-MALS showed that ExoU-R661L oligomers formed by excess $PI(4,5)P_2$ were smaller than those formed by wild-type ExoU, the higher-order species captured by BS3 crosslinking were not

significantly different. This suggests that the slowly-migrating bands on SDS-PAGE gels could represent transient oligomers or an intermediate stage of oligomerization that both WT and ExoU-R661L undergo, but only WT ExoU can form the ~450 kD oligomer required for optimal activity.

$PI(4,5)P_2$ can serve as a substrate for ExoU, meaning it must be able to bind the catalytic pocket within the phospholipase domain of ExoU. We do not know which residues in this region are required, nor if this is a high affinity lipid-protein interaction. However, it does leave open the possibility that $PI(4,5)P_2$-mediated oligomerization could be induced through two distinct binding events. Our results with ExoU-R661L are still consistent with this hypothesis. Mutation of one residue may only reduce oligomerization partially, but if the other PI(4,5)P2-binding sites are also mutated, it is possible that oligomerization of ExoU-R661L would be completely abrogated.

In contrast, ExoU-I609N was still activated by $PI(4,5)P_2$ but less effectively than wild-type ExoU (243). It was therefore unclear what impact this substitution would have on ExoU oligomerization. We observed variability in SEC-MALS elution profiles of ExoU-I609N across experiments. In each case, ExoU-I609N eluted as a monomer, but after incubation with $PI(4,5)P_2$, much of the protein eluted as a range of peaks. The elution time of each peak was consistent with protein complexes smaller than those in the oligomer peak observed with wild-type ExoU.

There are several possible explanations for these peaks. First, unlike the wild-type protein, ExoU-I609N may become unstable or disordered when incubated with $PI(4,5)P_2$. Although circular dichroism experiments of ExoU-I609N (in the absence of $PI(4,5)P_2$) revealed

no gross differences in secondary structure compared to wild-type ExoU (200), the range of oligomer peaks could reflect different degrees of protein unfolding in the presence of $PI(4,5)P_2$. Second, I609 is within α-helix 22 of the four-helical bundle of the MLD (206), so it is possible that this helix serves as a contact point between adjacent ExoU molecules. Mutation of I609 may disrupt a subset of interactions within a large ExoU complex, and the importance of this disruption may vary from complex to complex, resulting in the heterogeneity of oligomers in the elution profiles we observed. Finally, ExoU molecules may normally undergo a step-wise progression from monomers to dimers to larger oligomers. ExoU-I609N could slightly change the conformation of ExoU, altering the spacing, rotation, or exposure of amino acid side chains at surfaces required for progression of oligomerization, halting this process at different steps in different complexes. Regardless of the mechanism, these results indicate that the specific residues within the four-helical bundle of the MLD play critical roles in the ability of ExoU to form oligomers.

In the future, we should not only identify which amino acid residues are necessary for ExoU oligomerization, but also determine where the interface between ExoU molecules within the oligomer are. These residues may not be the same. This is particularly important if multiple amino acid residues bind $PI(4,5)P_2$ and are independently capable of inducing ExoU oligomerization. Identifying which portions of ExoU interact with adjacent molecules within the oligomer will allow construction of ExoU variants that maintain the ability to bind $PI(4,5)P_2$ but are incapable of oligomerization. This would separate the two different functions of $PI(4,5)P_2$ and could in turn inform the role of oligomerization in phospholipase activity. Finally, the

elucidation of ExoU: ExoU interfaces will allow for more targeted design of drugs that directly interfere with ExoU oligomerization.

Given the rise in antibiotic resistance, the need for alternative treatments is undisputed. One option is to develop drugs targeting virulence factors, but so far there has only been limited success in developing new therapeutic drugs targeting ExoU. An obvious approach is to inhibit the phospholipase activity of ExoU. While MAFP and HELSS were effective at inhibiting ExoU activity (196, 197), they also inhibit mammalian phospholipases and would not be viable options. A library screen of small molecule compounds identified a candidate, which was named pseudolipasin A, that could inhibit phospholipase activity of ExoU similarly to MAFP but did not affect endogenous $cPLA_2$ activity in bone marrow-derived macrophages (278). Another research group identified arylsulfonamides as another class of molecules that can inhibit ExoU cytotoxicity in yeast and mammalian cell lines (279). However, no studies testing these compounds in animal models of infection have been published thus far.

Other options are to target downstream effects of ExoU phospholipase activity, such as signaling pathways induced by arachidonic acid (AA) release. There is some evidence that COX-2 inhibitors can improve survival of mice infected with ExoU-producing strains (280), presumably by decreasing production of prostaglandins and eicosanoids from AA and reducing the overall inflammatory response induced by ExoU. However, as with the phospholipase inhibitors, drugs that target the host cell proteins, particularly those involving the immune system, would likely have more significant adverse side effects. Thus, we believe targeting the toxin itself is the better approach. By aiming to disrupt oligomerization of ExoU, we can

diminish ExoU function indirectly, avoiding the issue of enzymatic activities that are conserved across the prokaryotic and eukaryotic domains.

In conclusion, this dissertation has described work shedding new insight on the mechanisms of ExoU function. We discovered that in addition to localization and co-activation of ExoU, $PI(4,5)P_2$ is capable of inducing oligomerization of ExoU. ExoU is a virulence factor produced by *P. aeruginosa* that contributes to more severe disease in patients who are hospitalized or have underlying chronic illnesses. Though many questions still remain, this initial characterization of $PI(4,5)P_2$-mediated oligomerization provides another feature of ExoU that can be exploited in the race to develop better therapies against this important pathogen.

CHAPTER FOUR

MATERIALS AND METHODS

Bacterial strains and plasmids

Chemically competent *E. coli* strains Top10 (Invitrogen, Carlsbad, CA) and XL10 Gold (Stratagene, La Jolla, CA) were used in the cloning of mammalian expression vectors expressing ExoU variants. Chemically competent *E. coli* BL21 bacteria (DE3, Invitrogen) were used for bacterial expression and purification of ExoU protein. Unless otherwise stated, all bacteria were grown in Luria-Bertani (LB) broth with 100 μg/mL ampicillin. *exoU* alleles were sub-cloned into pcDNA3.1NT-GFP (Invitrogen), pEcoli-Cterm 6×HN (Clontech, Mountain View, CA), or p3×Flag-CMV-7.1 (Sigma-Aldrich, St. Louis, MO). Specific strain genotypes and plasmid characteristics are listed in Table 1.

Cloning of ExoU expression constructs

All cloning procedures used are based on standard protocols for traditional restriction enzyme-based cloning. To generate pcDNA3.1 NT-GFP constructs for expression of ExoU truncation variants in mammalian cells, *exoU* alleles were amplified with primers containing AgeI and KpnI sites using high-fidelity DNA polymerases Pfu Ultra (Agilent Technologies, Santa Clara, CA) or Phusion (New England BioLabs, Ipswich, MA), following the manufacturers' instructions. PCR products were separated by electrophoresis through 1% agarose gels and desired bands were excised and purified using the QIAquick Gel Extraction kit (Qiagen, Valencia, CA). pcDNA3.1 NT-GFP and the PCR products both were digested with

AgeI and KpnI enzymes for two hours at 37˚C, then the QIAquick PCR Purification kit (Qiagen) was used to remove contaminating nucleotides and enzymes. The resulting fragments were mixed at a vector to insert molar ratio of 1:3, for a combined total of 100-200 ng DNA per 10 uL reaction, and ligated using T4 DNA ligase (Invitrogen) for 20 mins at room temperature. Transformation into Top10 *E. coli* was performed by gently adding 2 uL of the ligation reaction directly to the bacteria, then incubating the mixture for 30 min on ice, 30-45 seconds at 42˚C, then 2 min on ice. Cells were allowed to recover for 1 hr in 250 uL SOC media (provided with competent cells from Invitrogen) at 37˚C. 50-150 uL was plated on LB plates supplemented with ampicillin for selection of transformants. Colonies after overnight incubation were screened for presence of the *exoU* gene using the AmpliTaq Gold (Thermo Fisher) or AccuStart (VWR) PCR master mixes using manufacturer-provided protocols.

To generate p3xFlag CMV-7.1 ExoU constructs, *exoU* alleles carrying the indicated mutations were PCR amplified from their corresponding pcDNA3.1 NT-GFP plasmids with primers harboring NotI and KpnI sites. Both vector and PCR insert were digested with NotI and KpnI, and otherwise the same protocol for pcDNA3.1 NT-GFP cloning used.

Protein purification constructs were generated by subcloning *exoU* alleles from the corresponding mammalian vectors into pEcoli-Cterm-6xHN using the same approach, except with primers containing HindIII and NotI sites and digesting with the respective restriction enzymes. Primers used for generating expression plasmids are listed in Table 2. All constructs were sequence verified by the Northwestern Sequencing Core Facility.

Protein purification

Recombinant 6×HN-tagged ExoU protein was purified using an optimized version of a protocol previously described (243). BL21 (DE3) bacteria harboring the desired ExoU purification constructs were grown in 10 mL of LB media overnight at 37˚C, then sub-cultured into 1 L of TPM (20 g tryptone, 15 g yeast extract, 8 g NaCl, 2 g Na_2HPO_4, and 1 g KH_2PO_4, per liter) supplemented with ampicillin (100 μg/mL). The subculture was grown to an OD_{600} of 0.6-0.8 at 30˚C. Subsequently, the bacteria were induced with isopropyl beta-D-thiogalactopyranoside (0.25 mM) incubated at 16˚C. After overnight induction, cells were centrifuged at 6340 x *g* for 15-20 minutes, then resuspended in 100 mL of pre-chilled lysis/loading buffer: 10 mM Tris (pH 8.3), 500 mM NaCl, and 5 mM beta-mercaptoethanol containing cOmplete Mini EDTA-free Protease Inhibitor Tablets (Roche Applied Science, Indianapolis, IN). Bacteria were lysed by sonication for 10 mins (10-second pulses with 10-second pauses after each pulse) on ice at an amplitude of 60% using a Q500 Sonicator equipped with a ½-inch probe (Qsonica, Newton, CT). Lysates were then centrifuged at 17600 x *g* to remove insoluble debris.

Proteins was purified from the cleared lysates using an Akta protein purification system with a 5 mL HisTrap FF nickel column connected to a HiPrep 26/60 Superdex 200 gel filtration column (GE Healthcare Life Sciences, Marlborough, MA). Proteins were eluted from the HisTrap nickel column using an elution buffer containing 10 mM Tris (pH 8.3), 500 mM NaCl, and 500 mM imidazole. The gel filtration buffer used was 10 mM Tris (pH 8.3), 500 mM NaCl, and 5 mM beta-mercaptoethanol. All steps were performed at 4˚C. Eluted fractions containing ExoU were pooled and concentrated using Vivaspin centrifugal concentrators (GE Healthcare)

per manufacturer instructions. Purified ExoU was run on 10% SDS-polyacrylamide gels and stained with SimplyBlue SafeStain (Invitrogen) to determine purity. Aliquots of protein were flash frozen and stored at -80˚C for future use.

Cell culture and transfections

HeLa cells (ATCC) were grown in Minimum Essential Medium (Corning, Corning, NY) supplemented with 10% heat-inactivated fetal bovine serum (Gemini Bio Products, West Sacramento, CA or Gibco, Gaithersburg, MD) and incubated at 37˚C with 5% CO_2. Cells were passaged every 2-3 days to maintain approximately 60-90% confluency.

HeLa cells were transfected with the indicated plasmids using X-tremeGENE 9 (Roche) at a 6:1 ratio following the manufacturer's instructions. For lactate dehydrogenase release assays, HeLa cells were seeded at 5×10^4 cells/well in 24-well plates and allowed to grow overnight pre-transfection. For each well, a transfection mixture containing 1.5 μL of transfection reagent, 25 μL of serum free Minimal Essential Medium (Corning), and 250 ng of each plasmid was prepared and incubated at room temperature for 30-45 min. Mixtures were then added dropwise to cells that were newly replenished with fresh media. For co-immunoprecipitation experiments, HeLa cells were seeded at approximately 2×10^6 cells per 100 mm dish one day prior to transfection. For each dish, 36 μL of transfection reagent was added to 600 μL of serum free medium, then incubated with 6 μg of each plasmid before adding dropwise to cells.

Co-immunoprecipitation and Western blotting

Co-immunoprecipitation was performed 48-72 hr after transfection of HeLa cells. Cells were detached from the plates by mechanical scraping or trypsinization and washed in pre-chilled phosphate buffered saline (PBS) three times. Cells were then resuspended in 500 uL of the following lysis buffer: 50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 supplemented with a cOmplete Mini Protease Inhibitor Tablet (Roche). After 30 min of rotation at 4˚C, cells were further lysed by mechanical shearing through a 27-gauge needle. Lysates were cleared by centrifugation at 18000 x *g* for 15 mins. Immunoprecipitation was performed using ANTI-FLAG M2 affinity resin (Sigma) as recommended in instruction manual. Briefly, affinity resin was rinsed with IP wash buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl) three times then mixed with the cleared cell lysate, rotating at 4˚C. After two hours of incubation, resin was washed five times with IP wash buffer to remove unbound proteins. Bound complexes were then eluted by incubating the resin with 150 uL of a 3X FLAG peptide stock solution (150 ng/uL in IP wash buffer, Sigma) for 1 hr, rotating at 4˚C.

A total of 50 μL of cell lysates (10% v/v of input) and 50 μL of eluates were run on 10% SDS-polyacrylamide gels for 1-1.5 hr at 100 V. Proteins were then transferred to nitrocellulose membranes for 1 hr at 250 mA using the semi-dry Trans-Blot system (Bio-Rad, Hercules, CA). Membranes were blocked for at least 1 hr with 5% powdered milk in PBS (blocking buffer). Western blotting was performed using a rabbit anti-Flag antibody (Sigma, 1:2500) and rabbit anti-GFP (Invitrogen, 1:1250) or Living Colors JL-8 mouse antibody (Clontech, 1:1250) for detection of GFP. Primary antibodies were diluted in blocking buffer containing 0.1% Tween-20 and incubated with nitrocellulose membranes for 2-24 hours, then washed three times with PBS-

T (0.1% Tween-20 in PBS). Detection of primary antibodies was achieved using secondary antibodies IRdye 680LT goat anti-rabbit IgG (LI-COR, Lincoln, NE) and IRdye 800CW goat anti-mouse (LI-COR) that were diluted 1:4000 in blocking buffer containing 0.1% Tween-20. After a 1 hr incubation with secondary antibodies, membranes were washed three times with PBS-T. Images of the blots were acquired through the 700 nm and 800 nm channels (2 minutes each) using the ImageStudio 3.0 software with the LI-COR Odyssey Fc imaging system.

Lipid Preparation

The phospholipids PI(4)P, PI(3,5)P_2, PI(4,5)P_2 ,and PC (Avanti Polar Lipids, Alabaster, AL) were dissolved in chloroform, and aliquots were taken from these stocks and dried under a steady N2 stream in glass tubes. The lipid film was then dried further under vacuum for 15 min to ensure complete evaporation of chloroform. Lipids were resuspended in the same buffers that were used for dialysis of ExoU proteins in each experiment. Each lipid suspension was vortexed repeatedly at room temperature, then sonicated at 20% amplitude with 10-second pulses followed by a 50-second pause after each pulse. Suspensions were sonicated for five pulses each or until the solution was clear using the Q500 sonicator with a 1/16” microtip probe (QSonica).

Size-exclusion chromatography/multi-angle light scattering (SEC-MALS)

PI(4)P, PI(3,5)P_2, PI(4,5)P_2 ,and PC (Avanti Polar Lipids) were individually reconstituted in a 10 mM Tris (pH 7.5), 100 mM NaCl buffer as described above . Purified rExoU or its variants were dialyzed in 1 L of the same buffer for at least 1 hr prior to preparation of samples.

ExoU proteins were mixed with either lipids or bovine monoubiquitin (Sigma) at a 1:1 molar ratio unless otherwise indicated in the graph. 250-300 μL of each sample was prepared.

SEC-MALS was performed on each sample after overnight incubation on ice. At least 400 μg of each sample was injected and run at a flow rate of 0.5 mL/min on a Superdex 200 10/300 GL column (GE Healthcare) for size exclusion coupled with a Wyatt DAWN HELEOS II multi-angle light scattering detector and Wyatt T-rEx differential refractive index detector (Wyatt Technology Corp, Santa Barbara, CA). Columns were pre-equilibrated overnight in 10 mM Tris (pH 7.5) and 100 mM NaCl, and a dn/dc of 0.194 was determined in this buffer. Bovine serum albumin (Sigma-Aldrich) was used for calibration.

Data was collected for 60 min following sample injection using the ChemStation software package (Agilent). SEC-MALS was performed on at least two independent samples for each condition; however, only experimental runs performed on the same day were analyzed and presented together. All calculations and data analysis was performed using the standard default settings included in the ASTRA software (version 5.3.4.19, Wyatt).

Blue-native gel electrophoresis

For the experiment determining whether ubiquitin formed complexes with ExoU, samples containing either purified ExoU alone or with bovine mono-ubiquitin (Sigma) were prepared using equal volumes of individual 20 μM protein stocks in 20 mM HEPES (pH 7.5) and 100 mM KCl. Samples were incubated overnight on ice prior to electrophoresis.

For all BN-PAGE experiments, equal volumes of each sample were examined using the Invitrogen BN-PAGE system. NativePAGE Novex gels (4-16% Bis-Tris) were run at a constant

voltage (150 V) at room temperature for 2 hr using pre-chilled buffers prepared per manufacturer instructions. The running buffer consisted of 50 mM BisTris and 50 mM Tricine, pH 6.8. Light blue cathode buffer (running buffer containing 0.002% G-250) was chosen for these experiments as these were non-detergent samples. Gels were subsequently stained with SimplyBlue SafeStain (Invitrogen) and washed overnight in dH_2O.

Crosslinking

Purified recombinant ExoU, ExoU variants, and bovine mono-ubiquitin (Sigma) were dialyzed in 20 mM HEPES, 100 mM KCl, pH 7.5 for 1-2 hr at 4˚C. $PI(4,5)P_2$, PI(4)P, $PI(3,5)P_2$, and PC (Avanti Polar Lipids) were reconstituted in the same buffer, and samples were prepared by mixing 20 μM of rExoU proteins with each of the ligands at a molar ratio of 1:5 unless otherwise indicated. After overnight incubation, samples were crosslinked with bis(sulfosuccinimidyl)suberate (BS3, Thermo Scientific, Rockford, IL) at a final crosslinker concentration of 250 mM for 2 hr on ice, then quenched with 1M Tris, pH 7.5 for 15 min at room temperature. Samples were electrophoresed through SDS or BN polyacrylamide gels and stained with SimplyBlue SafeStain (Invitrogen) for visualization of proteins.

Lactate dehydrogenase release assays

Lactate dehydrogenase (LDH) release was measured using the CytoTox96 Non-radioactive Cytotoxicity Assay Kit (Promega, Madison, WI) following manufacturer's instructions. Approximately 20-24 hr after transfection with the indicated plasmids, 100 μL of supernatant was removed from each well and centrifuged in a 96-well V-bottom plate at 250 x *g*

for five minutes to pellet any detached cells. 50 μL of cleared supernatant was then added to 50 μL of substrate and incubated for 30 min in the dark at room temperature. After addition of 50 μL of Stop solution, A490 was measured. All conditions were performed in triplicate. After subtracting values from negative controls (untransfected cells), results were reported as a cell lysis normalized to LDH release from cells transfected with wild-type ExoU (WT set as 100%).

Phospholipase activity assays

Phospholipase activity of purified ExoU proteins was assessed using a commercial $cPLA_2$ assay kit (Cayman Chemical, Ann Arbor, MI) based on the manufacturer's instructions and protocols described previously (210, 243). Samples containing ExoU proteins, with or without ubiquitin and/or $PI(4,5)P_2$, were prepared at equimolar ratios and incubated overnight on ice. The final reaction mixture for each sample contained 200 μL of a 1.5 mM stock substrate (arachidonoyl thio-phosphatidylcholine), 10 μL of 25 mM DTNB (5,5'-dithio-*bis* (2-nitrobenzoic acid), and 20 μL of the sample being tested.

Absorbance (A_{405}) was measured after addition of substrate at the times indicated. Background absorbance (measured from wells containing the substrate and DTNB only) was subtracted from all values obtained. Substrate hydrolysis (in units of nmol per nmol of ExoU) was calculated using the equation $A_{405}/10.00 \times 0.230$ mL/nmol of where 10.00 is the pathlength-adjusted extinction coefficient of DTNB and 0.230 mL is the reaction volume. The final reactions contained 100 pmol of each ExoU variant per well. Each experimental condition was tested in triplicate.

Statistics

For LDH release assays, a Student's *t* test was used to make two-sample comparisons between each ExoU variant and wild type ExoU, or between co-expression of truncated variants and each single truncation transfection. For phospholipase assays, Student's *t* tests were used to make two-sample comparisons between each ExoU variant and wild-type ExoU, or between co-incubated truncated variants and each truncation alone at the same time point. A p value < 0.05 was considered significant.

Table 1 Bacterial strains and plasmids used in this study

Bacterial Strains		
Name	**Description**	**Reference**
XL10 Gold	Tet^r Δ(*mcr*A)183 Δ(*mcr*CB-*hsd*SMR-*mrr*)173 *end*A1 *sup*E44 *thi-1 rec*A1 *gyr*A96 *rel*A1 *lac* Hte [F′ *pro*AB *lac*I^q ZΔM15 Tn10 (Tet^r) Amy Cam^r]	Stratagene
Top10	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen
BL21 (DE3)	F-*ompT hsdS*$_B$ (r$_B$-m$_B$-) *gal dcm* (DE3)	Invitrogen
Plasmids		
Name	**Description**	**Reference**
pcDNA3.1 NT-GFP	for expression in mammalian cells; N-terminal GFP tag	Invitrogen
p3xFLAG-CMV-7.1	for expression in mammalian cells; N-terminal 3xFlag tag	Sigma-Aldrich
pEcoli-Cterm 6xHN	for purification from *E. coli*; C-terminal 6xHN tag	Clontech
pcDNA3.1 NT-GFP ExoU	for expression of wild-type ExoU-GFP	(198)
pcDNA3.1 NT-GFP S142A	for expression of ExoU-GFP with S142A substitution	(198)
pcDNA3.1 NT-GFP V423F	for expression of ExoU-GFP with V423F substitution	(251)
pcDNA3.1 NT-GFP V462F	for expression of ExoU-GFP with V462F substitution	(251)
pcDNA3.1 NT-GFP I609N	for expression of ExoU-GFP with I609N substitution	(200)
pcDNA3.1 NT-GFP R661L	for expression of ExoU-GFP with R661L substitution	(200)
pcDNA3.1 NT-GFP S142A/V423F	for expression of ExoU-GFP with S142A and V423F substitutions	(251)

pcDNA3.1 NT-GFP S142A/V462F	for expression of ExoU-GFP with S142A and V462F substitutions	(251)
pcDNA3.1 NT-GFP S142A/I609N	for expression of ExoU-GFP with S142A and I609N substitutions	(200)
pcDNA3.1 NT-GFP S142A/R661L	for expression of ExoU-GFP with S142A and R661L substitutions	(200)
pcDNA3.1 NT-GFP S142A/1-550	for expression of ExoU 1-550 GFP with S142A substitution	This study
pcDNA3.1 NT-GFP S142A/100-687	for expression of ExoU 100-687 GFP with S142A substitution	This study
p3xFLAG-CMV-7.1 ExoU	for expression of wild-type ExoU-FLAG	(251)
p3xFLAG-CMV-7.1 S142A	for expression of ExoU-FLAG with S142A substitution	(251)
p3xFLAG-CMV-7.1 S142A/V423F	for expression of ExoU-FLAG with S142A and V423F substitutions	This study
p3xFLAG-CMV-7.1 S142A/V462F	for expression of ExoU-FLAG with S142A and V462F substitutions	This study
p3xFLAG-CMV-7.1 S142A/I609N	for expression of ExoU-FLAG with S142A and I609N substitutions	This study
p3xFLAG-CMV-7.1 S142A/R661L	for expression of ExoU-FLAG with S142A and R661L substitutions	This study
p3xFLAG-CMV-7.1 1-550	for expression of ExoU 1-550 FLAG	*
p3xFLAG-CMV-7.1 100-687	for expression of ExoU 100-687 FLAG	(251)
p3xFLAG-CMV-7.1 S142A/1-550	for expression of ExoU 1-550 FLAG with S142A substitution	This study
p3xFLAG-CMV-7.1 S142A/100-687	for expression of ExoU 100-687 FLAG with S142A substitution	This study
pEcoli-Cterm 6xHN ExoU	for purification of ExoU	(200)
pEcoli-Cterm 6xHN S142A	for purification of ExoU with S142A substitution	(200)
pEcoli-Cterm 6xHN V423F	for purification of ExoU with V423F substitution	(251)
pEcoli-Cterm 6xHN V462F	for purification of ExoU with V462F substitution	(251)

pEcoli-Cterm 6xHN I609N	for purification of ExoU with I609N substitution	(200)
pEcoli-Cterm 6xHN R661L	for purification of ExoU with R661L substitution	(200)
pEcoli-Cterm 6xHN 1-550	for purification of ExoU 1-550	**
pEcoli-Cterm 6xHN 100-687	for purification of ExoU 100-687	This study

* Unpublished, generated by Jeffrey Veesenmeyer
** Unpublished, generated by Gregory Tyson

Table 2 Primers used in this study

For generation of pcDNA3.1 NT-GFP and p3XFLAG-CMV7.1 constructs	
Name	**Primer Sequence**
5' AgeI ExoU	5'-AAAACCGGTACATATCCAATCGTTGG-3'
5' AgeI ExoU 100	5'-AAAACCGGTACGGCCACCATTGACCAGTCTG-3'
3' KpnI ExoU 550	5'-AAAGGTACCTCACGCATCGAGTCGC-3'
3' KpnI ExoU 687	5'-AAAGGTACCTCATGTGAACTCCTTATTCC-3'
5' NotI ExoU	5'-AAAGCGGCCGCAATGCATATCCAATCGTTGGGG-3'
5' NotI ExoU 100	5'-AAAGCGGCCGCAATGCGGCCACCATTGACCAGT-3'
For generation of pEcoli-Cterm 6xHN constructs	
Name	**Primer Sequence**
5' HindIII ExoU 100	5'-AAAAAGCTTATGCGGCCACCATTGACCAGTCTG-3'
3' NotI ExoU 687	5'-AAAGCGGCCGCCTGTGAACTCCTTATTCCG-3'

References

1. **Kidd TJ, Ritchie SR, Ramsay KA, Grimwood K, Bell SC, Rainey PB.** 2012. *Pseudomonas aeruginosa* exhibits frequent recombination, but only a limited association between genotype and ecological setting. PLoS One **7:**e44199.

2. **Scaccabarozzi L, Leoni L, Ballarini A, Barberio A, Locatelli C, Casula A, Bronzo V, Pisoni G, Jousson O, Morandi S, Rapetti L, Garcia-Fernandez A, Moroni P.** 2015. *Pseudomonas aeruginosa* in Dairy Goats: Genotypic and Phenotypic Comparison of Intramammary and Environmental Isolates. PLoS One **10:**e0142973.

3. **Martins VV, Pitondo-Silva A, Manco Lde M, Falcao JP, Freitas Sdos S, da Silveira WD, Stehling EG.** 2014. Pathogenic potential and genetic diversity of environmental and clinical isolates of *Pseudomonas aeruginosa*. APMIS **122:**92-100.

4. **Ozer EA, Allen JP, Hauser AR.** 2012. Draft genome sequence of the *Pseudomonas aeruginosa* bloodstream isolate PABL056. J Bacteriol **194:**5999.

5. **Klockgether J, Cramer N, Wiehlmann L, Davenport CF, Tummler B.** 2011. *Pseudomonas aeruginosa* Genomic Structure and Diversity. Front Microbiol **2:**150.

6. **Ozer EA, Allen JP, Hauser AR.** 2014. Characterization of the core and accessory genomes of *Pseudomonas aeruginosa* using bioinformatic tools Spine and AGEnt. BMC Genomics **15:**737.

7. **Jun SR, Wassenaar TM, Nookaew I, Hauser L, Wanchai V, Land M, Timm CM, Lu TY, Schadt CW, Doktycz MJ, Pelletier DA, Ussery DW.** 2015. Diversity of *Pseudomonas* Genomes, Including Populus-Associated Isolates, as Revealed by Comparative Genome Analysis. Appl Environ Microbiol **82:**375-383.

8. **Hameed A, Shahina M, Lin SY, Liu YC, Young CC.** 2014. *Pseudomonas hussainii* sp. nov., isolated from droppings of a seashore bird, and emended descriptions of *Pseudomonas pohangensis*, *Pseudomonas benzenivorans* and *Pseudomonas segetis*. Int J Syst Evol Microbiol **64:**2330-2337.

9. **Scales BS, Dickson RP, LiPuma JJ, Huffnagle GB.** 2014. Microbiology, genomics, and clinical significance of the *Pseudomonas fluorescens* species complex, an unappreciated colonizer of humans. Clin Microbiol Rev **27:**927-948.

10. **Remold SK, Purdy-Gibson ME, France MT, Hundley TC.** 2015. *Pseudomonas putida* and *Pseudomonas fluorescens* Species Group Recovery from Human Homes Varies Seasonally and by Environment. PLoS One **10:**e0127704.

11. **Migiyama Y, Yanagihara K, Kaku N, Harada Y, Yamada K, Nagaoka K, Morinaga Y, Akamatsu N, Matsuda J, Izumikawa K, Kohrogi H, Kohno S.** 2016. *Pseudomonas aeruginosa* Bacteremia among Immunocompetent and Immunocompromised Patients: Relation to Initial Antibiotic Therapy and Survival. Japanese Journal of Infectious Diseases **69:**91-96.

12. **Sousa D, Justo I, Dominguez A, Manzur A, Izquierdo C, Ruiz L, Nebot M, Bayas JM, Celorrio JM, Varona W, Llinares P, Miguez E, Sanchez E, Carratala J.** 2013. Community-acquired pneumonia in immunocompromised older patients: incidence, causative organisms and outcome. Clin Microbiol Infect **19:**187-192.

13. **Metersky ML, Frei CR, Mortensen EM.** 2016. Predictors of *Pseudomonas* and methicillin-resistant *Staphylococcus aureus* in hospitalized patients with healthcare-associated pneumonia. Respirology **21:**157-163.

14. **Segna KG, Koch LH, Williams JV.** 2011. "Hot tub" Folliculitis from a nonchlorinated children's pool. Pediatr Dermatol **28:**590-591.

15. **Molina-Leyva A, Ruiz-Ruigomez M.** 2013. *Pseudomonas* folliculitis in Arabian baths. Dermatol Online J **19:**18959.

16. **Sharma R, Jhanji V, Satpathy G, Sharma N, Khokhar S, Agarwal T.** 2013. Coinfection with *Acanthamoeba* and *Pseudomonas* in contact lens-associated keratitis. Optom Vis Sci **90:**e53-55.

17. **Batta P, Goldstein MH.** 2010. Severe pseudomonal keratitis in an infrequent daily disposable contact lens wearer. Eye Contact Lens **36:**181-182.

18. **Rosenthal VD, Al-Abdely HM, El-Kholy AA, AlKhawaja SA, Leblebicioglu H, Mehta Y, Rai V, Hung NV, Kanj SS, Salama MF, Salgado-Yepez E, Elahi N, Morfin Otero R, Apisarnthanarak A, De Carvalho BM, Ider BE, Fisher D, Buenaflor MC, Petrov MM, Quesada-Mora AM, Zand F, Gurskis V, Anguseva T, Ikram A, Aguilar de Moros D, Duszynska W, Mejia N, Horhat FG, Belskiy V, Mioljevic V, Di Silvestre G, Furova K, Ramos-Ortiz GY, Gamar Elanbya MO, Satari HI, Gupta U, Dendane T, Raka L, Guanche-Garcell H, Hu B, Padgett D, Jayatilleke K, Ben Jaballah N, Apostolopoulou E, Prudencio Leon WE, Sepulveda-Chavez A, Telechea HM, Trotter A, Alvarez-Moreno C, Kushner-Davalos L.** 2016. International Nosocomial Infection Control Consortium report, data summary of 50 countries for 2010-2015: Device-associated module. Am J Infect Control doi:10.1016/j.ajic.2016.08.007.

19. **Tumbarello M, De Pascale G, Trecarichi EM, Spanu T, Antonicelli F, Maviglia R, Pennisi MA, Bello G, Antonelli M.** 2013. Clinical outcomes of *Pseudomonas aeruginosa* pneumonia in intensive care unit patients. Intensive Care Med **39:**682-692.

20. **Pena C, Gomez-Zorrilla S, Oriol I, Tubau F, Dominguez MA, Pujol M, Ariza J.** 2013. Impact of multidrug resistance on *Pseudomonas aeruginosa* ventilator-associated pneumonia outcome: predictors of early and crude mortality. Eur J Clin Microbiol Infect Dis **32:**413-420.

21. **Melzer M, Welch C.** 2013. Outcomes in UK patients with hospital-acquired bacteraemia and the risk of catheter-associated urinary tract infections. Postgrad Med J **89:**329-334.

22. **Ehre C, Ridley C, Thornton DJ.** 2014. Cystic fibrosis: an inherited disease affecting mucin-producing organs. Int J Biochem Cell Biol **52:**136-145.

23. **Grassme H, Becker KA, Zhang Y, Gulbins E.** 2010. CFTR-dependent susceptibility of the cystic fibrosis-host to *Pseudomonas aeruginosa*. Int J Med Microbiol **300:**578-583.

24. **Kidd TJ, Ramsay KA, Vidmar S, Carlin JB, Bell SC, Wainwright CE, Grimwood K, Investigators AS.** 2015. *Pseudomonas aeruginosa* genotypes acquired by children with cystic fibrosis by age 5-years. J Cyst Fibros **14:**361-369.

25. **Mayer-Hamblett N, Rosenfeld M, Gibson RL, Ramsey BW, Kulasekara HD, Retsch-Bogart GZ, Morgan W, Wolter DJ, Pope CE, Houston LS, Kulasekara BR, Khan U, Burns JL, Miller SI, Hoffman LR.** 2014. *Pseudomonas aeruginosa* in vitro phenotypes distinguish cystic fibrosis infection stages and outcomes. Am J Respir Crit Care Med **190:**289-297.

26. **Yum HK, Park IN, Shin BM, Choi SJ.** 2014. Recurrent *Pseudomonas aeruginosa* Infection in Chronic Lung Diseases: Relapse or Reinfection? Tuberc Respir Dis (Seoul) **77:**172-177.

27. **Paulsson M, Singh B, Al-Jubair T, Su YC, Hoiby N, Riesbeck K.** 2015. Identification of outer membrane Porin D as a vitronectin-binding factor in cystic fibrosis clinical isolates of *Pseudomonas aeruginosa*. J Cyst Fibros **14:**600-607.

28. **McCaslin CA, Petrusca DN, Poirier C, Serban KA, Anderson GG, Petrache I.** 2015. Impact of alginate-producing *Pseudomonas aeruginosa* on alveolar macrophage apoptotic cell clearance. J Cyst Fibros **14:**70-77.

29. **Samano MN, Pego-Fernandes PM, Fonseca Ribeiro AK, Turaca K, Abdalla LG, Fernandes LM, Correia AT, Jatene FB.** 2013. Lung transplantation in patients with cystic fibrosis. Transplant Proc **45:**1137-1141.

30. **Walter S, Gudowius P, Bosshammer J, Romling U, Weissbrodt H, Schurmann W, von der Hardt H, Tummler B.** 1997. Epidemiology of chronic *Pseudomonas aeruginosa* infections in the airways of lung transplant recipients with cystic fibrosis. Thorax **52:**318-321.

31. **Syed SA, Whelan FJ, Waddell B, Rabin HR, Parkins MD, Surette MG.** 2016. Reemergence of Lower-Airway Microbiota in Lung Transplant Patients with Cystic Fibrosis. Ann Am Thorac Soc **13:**2132-2142.

32. **Smyth AR, Bell SC, Bojcin S, Bryon M, Duff A, Flume P, Kashirskaya N, Munck A, Ratjen F, Schwarzenberg SJ, Sermet-Gaudelus I, Southern KW, Taccetti G, Ullrich G, Wolfe S, European Cystic Fibrosis S.** 2014. European Cystic Fibrosis Society Standards of Care: Best Practice guidelines. J Cyst Fibros **13 Suppl 1:**S23-42.

33. **Datta S, Roy S, Chatterjee S, Saha A, Sen B, Pal T, Som T, Basu S.** 2014. A five-year experience of carbapenem resistance in Enterobacteriaceae causing neonatal septicaemia: predominance of NDM-1. PLoS One **9:**e112101.

34. **Cullen IM, Manecksha RP, McCullagh E, Ahmad S, O'Kelly F, Flynn R, McDermott TE, Murphy P, Grainger R, Fennell JP, Thornhill JA.** 2013. An 11-year analysis of the prevalent uropathogens and the changing pattern of *Escherichia coli* antibiotic resistance in 38,530 community urinary tract infections, Dublin 1999-2009. Ir J Med Sci **182:**81-89.

35. **Musicha P, Cornick JE, Bar-Zeev N, French N, Masesa C, Denis B, Kennedy N, Mallewa J, Gordon MA, Msefula CL, Heyderman RS, Everett DB, Feasey NA.** 2017. Trends in antimicrobial resistance in bloodstream infection isolates at a large urban hospital in Malawi (1998-2016): a surveillance study. Lancet Infect Dis doi:10.1016/S1473-3099(17)30394-8.

36. **Weiner LM, Webb AK, Limbago B, Dudeck MA, Patel J, Kallen AJ, Edwards JR, Sievert DM.** 2016. Antimicrobial-Resistant Pathogens Associated With Healthcare-Associated Infections: Summary of Data Reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2011-2014. Infect Control Hosp Epidemiol **37:**1288-1301.

37. **Edelsberg J, Weycker D, Barron R, Li X, Wu H, Oster G, Badre S, Langeberg WJ, Weber DJ.** 2014. Prevalence of antibiotic resistance in US hospitals. Diagn Microbiol Infect Dis **78:**255-262.

38. **Justo JA, Bosso JA.** 2015. Adverse reactions associated with systemic polymyxin therapy. Pharmacotherapy **35:**28-33.

39. **Oliveira MS, Prado GV, Costa SF, Grinbaum RS, Levin AS.** 2009. Polymyxin B and colistimethate are comparable as to efficacy and renal toxicity. Diagn Microbiol Infect Dis **65:**431-434.

40. **Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, Scheld M, Spellberg B, Bartlett J.** 2009. Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. Clin Infect Dis **48:**1-12.

41. **Centers for Disease Control and Prevention OoID.** 2013. Antibiotic resistance threats in the United States. http://www.cdc.gov/drugresistance/threat-report-2013.

42. **Garber N, Friedman J.** 1970. Beta-lactamase and the resistance of *Pseudomonas aeruginosa* to various penicillins and cephalosporins. J Gen Microbiol **64:**343-352.

43. **Lodise TP, Jr., Lomaestro B, Drusano GL.** 2007. Piperacillin-tazobactam for *Pseudomonas aeruginosa* infection: clinical implications of an extended-infusion dosing strategy. Clin Infect Dis **44:**357-363.

44. **Berrazeg M, Jeannot K, Ntsogo Enguene VY, Broutin I, Loeffert S, Fournier D, Plesiat P.** 2015. Mutations in beta-Lactamase AmpC Increase Resistance of *Pseudomonas aeruginosa* Isolates to Antipseudomonal Cephalosporins. Antimicrob Agents Chemother **59:**6248-6255.

45. **Poirel L, Girlich D, Naas T, Nordmann P.** 2001. OXA-28, an extended-spectrum variant of OXA-10 beta-lactamase from *Pseudomonas aeruginosa* and its plasmid- and integron-located gene. Antimicrob Agents Chemother **45:**447-453.

46. **Zhang R, Liu Z, Li J, Lei L, Yin W, Li M, Wu C, Walsh TR, Wang Y, Wang S, Wu Y.** 2017. Presence of VIM-Positive *Pseudomonas* Species in Chickens and Their Surrounding Environment. Antimicrob Agents Chemother **61**.

47. **Toleman MA, Rolston K, Jones RN, Walsh TR.** 2003. Molecular and biochemical characterization of OXA-45, an extended-spectrum class 2d' beta-lactamase in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother **47:**2859-2863.

48. **Dortet L, Flonta M, Boudehen YM, Creton E, Bernabeu S, Vogel A, Naas T.** 2015. Dissemination of carbapenemase-producing Enterobacteriaceae and *Pseudomonas aeruginosa* in Romania. Antimicrob Agents Chemother **59:**7100-7103.

49. **Hall LM, Livermore DM, Gur D, Akova M, Akalin HE.** 1993. OXA-11, an extended-spectrum variant of OXA-10 (PSE-2) beta-lactamase from *Pseudomonas aeruginosa*. Antimicrob Agents Chemother **37:**1637-1644.

50. **Sanschagrin F, Couture F, Levesque RC.** 1995. Primary structure of OXA-3 and phylogeny of oxacillin-hydrolyzing class D beta-lactamases. Antimicrob Agents Chemother **39:**887-893.

51. **Mouneimne H, Robert J, Jarlier V, Cambau E.** 1999. Type II topoisomerase mutations in ciprofloxacin-resistant strains of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother **43:**62-66.

52. **Akasaka T, Tanaka M, Yamaguchi A, Sato K.** 2001. Type II topoisomerase mutations in fluoroquinolone-resistant clinical strains of *Pseudomonas aeruginosa* isolated in 1998

and 1999: role of target enzyme in mechanism of fluoroquinolone resistance. Antimicrob Agents Chemother **45:**2263-2268.

53. **Pasca MR, Dalla Valle C, De Jesus Lopes Ribeiro AL, Buroni S, Papaleo MC, Bazzini S, Udine C, Incandela ML, Daffara S, Fani R, Riccardi G, Marone P.** 2012. Evaluation of fluoroquinolone resistance mechanisms in *Pseudomonas aeruginosa* multidrug resistance clinical isolates. Microb Drug Resist **18:**23-32.

54. **Llanes C, Kohler T, Patry I, Dehecq B, van Delden C, Plesiat P.** 2011. Role of the MexEF-OprN efflux system in low-level resistance of *Pseudomonas aeruginosa* to ciprofloxacin. Antimicrob Agents Chemother **55:**5676-5684.

55. **Hocquet D, Vogne C, El Garch F, Vejux A, Gotoh N, Lee A, Lomovskaya O, Plesiat P.** 2003. MexXY-OprM efflux pump is necessary for a adaptive resistance of *Pseudomonas aeruginosa* to aminoglycosides. Antimicrob Agents Chemother **47:**1371-1375.

56. **Ikonomidis A, Tsakris A, Kantzanou M, Spanakis N, Maniatis AN, Pournaras S.** 2008. Efflux system overexpression and decreased OprD contribute to the carbapenem heterogeneity in *Pseudomonas aeruginosa*. FEMS Microbiol Lett **279:**36-39.

57. **Li XZ, Zhang L, Srikumar R, Poole K.** 1998. Beta-lactamase inhibitors are substrates for the multidrug efflux pumps of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother **42:**399-403.

58. **Fukuoka T, Ohya S, Narita T, Katsuta M, Iijima M, Masuda N, Yasuda H, Trias J, Nikaido H.** 1993. Activity of the carbapenem panipenem and role of the OprD (D2) protein in its diffusion through the *Pseudomonas aeruginosa* outer membrane. Antimicrob Agents Chemother **37:**322-327.

59. **Huang H, Hancock RE.** 1993. Genetic definition of the substrate selectivity of outer membrane porin protein OprD of *Pseudomonas aeruginosa*. J Bacteriol **175:**7793-7800.

60. **Weiner LM, Fridkin SK, Aponte-Torres Z, Avery L, Coffin N, Dudeck MA, Edwards JR, Jernigan JA, Konnor R, Soe MM, Peterson K, Clifford McDonald L.** 2016. Vital Signs: Preventing Antibiotic-Resistant Infections in Hospitals - United States, 2014. Am J Transplant **16:**2224-2230.

61. **Hauser AR, Mecsas J, Moir DT.** 2016. Beyond Antibiotics: New Therapeutic Approaches for Bacterial Infections. Clin Infect Dis **63:**89-95.

62. **Wassenaar TM, Gaastra W.** 2001. Bacterial virulence: can we draw the line? FEMS Microbiol Lett **201:**1-7.

63. **Kukavica-Ibrulj I, Facchini M, Cigana C, Levesque RC, Bragonzi A.** 2014. Assessing *Pseudomonas aeruginosa* virulence and the host response using murine models of acute and chronic lung infection. Methods Mol Biol **1149:**757-771.

64. **O'Callaghan D, Vergunst A.** 2010. Non-mammalian animal models to study infectious disease: worms or fly fishing? Curr Opin Microbiol **13:**79-85.

65. **Steinert M, Leippe M, Roeder T.** 2003. Surrogate hosts: protozoa and invertebrates as models for studying pathogen-host interactions. Int J Med Microbiol **293:**321-332.

66. **Gupta RS.** 2011. Origin of diderm (Gram-negative) bacteria: antibiotic selection pressure rather than endosymbiosis likely led to the evolution of bacterial cells with two membranes. Antonie Van Leeuwenhoek **100:**171-182.

67. **Cryz SJ, Jr., Pitt TL, Furer E, Germanier R.** 1984. Role of lipopolysaccharide in virulence of *Pseudomonas aeruginosa*. Infect Immun **44:**508-513.

68. **Bystrova OV, Lindner B, Moll H, Kocharova NA, Knirel YA, Zahringer U, Pier GB.** 2004. Full structure of the lipopolysaccharide of *Pseudomonas aeruginosa* immunotype 5. Biochemistry (Mosc) **69:**170-175.

69. **Erridge C, Pridmore A, Eley A, Stewart J, Poxton IR.** 2004. Lipopolysaccharides of *Bacteroides fragilis*, *Chlamydia trachomatis* and *Pseudomonas aeruginosa* signal via toll-like receptor 2. J Med Microbiol **53:**735-740.

70. **Hajjar AM, Ernst RK, Tsai JH, Wilson CB, Miller SI.** 2002. Human Toll-like receptor 4 recognizes host-specific LPS modifications. Nat Immunol **3:**354-359.

71. **Ramphal R, Balloy V, Jyot J, Verma A, Si-Tahar M, Chignard M.** 2008. Control of *Pseudomonas aeruginosa* in the lung requires the recognition of either lipopolysaccharide or flagellin. J Immunol **181:**586-592.

72. **Toutain CM, Zegans ME, O'Toole GA.** 2005. Evidence for two flagellar stators and their role in the motility of *Pseudomonas aeruginosa*. J Bacteriol **187:**771-777.

73. **Verma A, Arora SK, Kuravi SK, Ramphal R.** 2005. Roles of specific amino acids in the N terminus of *Pseudomonas aeruginosa* flagellin and of flagellin glycosylation in the innate immune response. Infect Immun **73:**8237-8246.

74. **Arora SK, Neely AN, Blair B, Lory S, Ramphal R.** 2005. Role of motility and flagellin glycosylation in the pathogenesis of *Pseudomonas aeruginosa* burn wound infections. Infect Immun **73:**4395-4398.

75. **Lovewell RR, Collins RM, Acker JL, O'Toole GA, Wargo MJ, Berwin B.** 2011. Step-wise loss of bacterial flagellar torsion confers progressive phagocytic evasion. PLoS Pathog **7:**e1002253.

76. **Kohler T, Curty LK, Barja F, van Delden C, Pechere JC.** 2000. Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. J Bacteriol **182:**5990-5996.

77. **Bradley DE.** 1980. A function of *Pseudomonas aeruginosa* PAO polar pili: twitching motility. Can J Microbiol **26:**146-154.

78. **O'Toole GA, Kolter R.** 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol Microbiol **30:**295-304.

79. **Hengzhuang W, Hoiby N, Ciofu O.** 2014. Pharmacokinetics and pharmacodynamics of antibiotics in biofilm infections of *Pseudomonas aeruginosa in vitro* and *in vivo*. Methods Mol Biol **1147:**239-254.

80. **Woods DE, Straus DC, Johanson WG, Jr., Berry VK, Bass JA.** 1980. Role of pili in adherence of *Pseudomonas aeruginosa* to mammalian buccal epithelial cells. Infect Immun **29:**1146-1151.

81. **Inclan YF, Persat A, Greninger A, Von Dollen J, Johnson J, Krogan N, Gitai Z, Engel JN.** 2016. A scaffold protein connects type IV pili with the Chp chemosensory system to mediate activation of virulence signaling in *Pseudomonas aeruginosa*. Mol Microbiol **101:**590-605.

82. **Persat A, Inclan YF, Engel JN, Stone HA, Gitai Z.** 2015. Type IV pili mechanochemically regulate virulence factors in *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A **112:**7563-7568.

83. **Bleves S, Viarre V, Salacha R, Michel GP, Filloux A, Voulhoux R.** 2010. Protein secretion systems in *Pseudomonas aeruginosa*: A wealth of pathogenic weapons. Int J Med Microbiol **300:**534-543.

84. **Hannauer M, Yeterian E, Martin LW, Lamont IL, Schalk IJ.** 2010. An efflux pump is involved in secretion of newly synthesized siderophore by *Pseudomonas aeruginosa*. FEBS Lett **584:**4751-4755.

85. **Cobessi D, Celia H, Pattus F.** 2005. Crystal structure at high resolution of ferric-pyochelin and its membrane receptor FptA from *Pseudomonas aeruginosa*. J Mol Biol **352:**893-904.

86. **Meyer JM, Neely A, Stintzi A, Georges C, Holder IA.** 1996. Pyoverdin is essential for virulence of *Pseudomonas aeruginosa*. Infect Immun **64:**518-523.

87. **Takase H, Nitanai H, Hoshino K, Otani T.** 2000. Impact of siderophore production on *Pseudomonas aeruginosa* infections in immunosuppressed mice. Infect Immun **68:**1834-1839.

88. **Minandri F, Imperi F, Frangipani E, Bonchi C, Visaggio D, Facchini M, Pasquali P, Bragonzi A, Visca P.** 2016. Role of Iron Uptake Systems in *Pseudomonas aeruginosa* Virulence and Airway Infection. Infect Immun **84:**2324-2335.

89. **Damron FH, Oglesby-Sherrouse AG, Wilks A, Barbier M.** 2016. Dual-seq transcriptomics reveals the battle for iron during *Pseudomonas aeruginosa* acute murine pneumonia. Sci Rep **6:**39172.

90. **Pavlovskis OR, Wretlind B.** 1979. Assessment of protease (elastase) as a *Pseudomonas aeruginosa* virulence factor in experimental mouse burn infection. Infect Immun **24:**181-187.

91. **Azghani AO, Connelly JC, Peterson BT, Gray LD, Collins ML, Johnson AR.** 1990. Effects of *Pseudomonas aeruginosa* elastase on alveolar epithelial permeability in guinea pigs. Infect Immun **58:**433-438.

92. **Alcorn JF, Wright JR.** 2004. Degradation of pulmonary surfactant protein D by *Pseudomonas aeruginosa* elastase abrogates innate immune function. J Biol Chem **279:**30871-30879.

93. **Casilag F, Lorenz A, Krueger J, Klawonn F, Weiss S, Haussler S.** 2015. The LasB Elastase of *Pseudomonas aeruginosa* Acts in Concert with Alkaline Protease AprA To Prevent Flagellin-Mediated Immune Recognition. Infect Immun **84:**162-171.

94. **Malloy JL, Veldhuizen RA, Thibodeaux BA, O'Callaghan RJ, Wright JR.** 2005. *Pseudomonas aeruginosa* protease IV degrades surfactant proteins and inhibits surfactant host defense and biophysical functions. Am J Physiol Lung Cell Mol Physiol **288:**L409-418.

95. **Kung VL, Khare S, Stehlik C, Bacon EM, Hughes AJ, Hauser AR.** 2012. An rhs gene of *Pseudomonas aeruginosa* encodes a virulence protein that activates the inflammasome. Proc Natl Acad Sci U S A **109:**1275-1280.

96. **Michalska M, Wolf P.** 2015. *Pseudomonas* Exotoxin A: optimized by evolution for effective killing. Front Microbiol **6:**963.

97. **Berka RM, Gray GL, Vasil ML.** 1981. Studies of phospholipase C (heat-labile hemolysin) in *Pseudomonas aeruginosa*. Infect Immun **34:**1071-1074.

98. **Wargo MJ, Gross MJ, Rajamani S, Allard JL, Lundblad LK, Allen GB, Vasil ML, Leclair LW, Hogan DA.** 2011. Hemolytic phospholipase C inhibition protects lung

function during *Pseudomonas aeruginosa* infection. Am J Respir Crit Care Med **184:**345-354.

99. **Terada LS, Johansen KA, Nowbar S, Vasil AI, Vasil ML.** 1999. *Pseudomonas aeruginosa* hemolytic phospholipase C suppresses neutrophil respiratory burst activity. Infect Immun **67:**2371-2376.

100. **El-Solh AA, Hattemer A, Hauser AR, Alhajhusain A, Vora H.** 2012. Clinical outcomes of type III *Pseudomonas aeruginosa* bacteremia. Crit Care Med **40:**1157-1163.

101. **Hauser AR, Cobb E, Bodi M, Mariscal D, Valles J, Engel JN, Rello J.** 2002. Type III protein secretion is associated with poor clinical outcomes in patients with ventilator-associated pneumonia caused by *Pseudomonas aeruginosa*. Crit Care Med **30:**521-528.

102. **Hauser AR.** 2009. The type III secretion system of *Pseudomonas aeruginosa*: infection by injection. Nat Rev Microbiol **7:**654-665.

103. **Kowal J, Chami M, Ringler P, Muller SA, Kudryashev M, Castano-Diez D, Amstutz M, Cornelis GR, Stahlberg H, Engel A.** 2013. Structure of the dodecameric *Yersinia enterocolitica* secretin YscC and its trypsin-resistant core. Structure **21:**2152-2161.

104. **Tosi T, Estrozi LF, Job V, Guilvout I, Pugsley AP, Schoehn G, Dessen A.** 2014. Structural similarity of secretins from type II and type III secretion systems. Structure **22:**1348-1355.

105. **Dash R, Hosen SM, Sultana T, Junaid M, Majumder M, Ishat IA, Uddin MM.** 2016. Computational Analysis and Binding Site Identification of Type III Secretion System ATPase from *Pseudomonas aeruginosa*. Interdiscip Sci **8:**403-411.

106. **Burns RE, McDaniel-Craig A, Sukhan A.** 2008. Site-directed mutagenesis of the *Pseudomonas aeruginosa* type III secretion system protein PscJ reveals an essential role for surface-localized residues in needle complex function. Microb Pathog **45:**225-230.

107. **Pastor A, Chabert J, Louwagie M, Garin J, Attree I.** 2005. PscF is a major component of the *Pseudomonas aeruginosa* type III secretion needle. FEMS Microbiol Lett **253:**95-101.

108. **Bergeron JR, Fernandez L, Wasney GA, Vuckovic M, Reffuveille F, Hancock RE, Strynadka NC.** 2016. The Structure of a Type 3 Secretion System (T3SS) Ruler Protein Suggests a Molecular Mechanism for Needle Length Sensing. J Biol Chem **291:**1676-1691.

109. **Schoehn G, Di Guilmi AM, Lemaire D, Attree I, Weissenhorn W, Dessen A.** 2003. Oligomerization of type III secretion proteins PopB and PopD precedes pore formation in *Pseudomonas*. EMBO J **22:**4957-4967.

110. **Gebus C, Faudry E, Bohn YS, Elsen S, Attree I.** 2008. Oligomerization of PcrV and LcrV, protective antigens of *Pseudomonas aeruginosa* and *Yersinia pestis*. J Biol Chem **283:**23940-23949.

111. **Goure J, Pastor A, Faudry E, Chabert J, Dessen A, Attree I.** 2004. The V antigen of *Pseudomonas aeruginosa* is required for assembly of the functional PopB/PopD translocation pore in host cell membranes. Infect Immun **72:**4741-4750.

112. **Lee PC, Stopford CM, Svenson AG, Rietsch A.** 2010. Control of effector export by the *Pseudomonas aeruginosa* type III secretion proteins PcrG and PcrV. Mol Microbiol **75:**924-941.

113. **Audia JP, Lindsey AS, Housley NA, Ochoa CR, Zhou C, Toba M, Oka M, Annamdevula NS, Fitzgerald MS, Frank DW, Alvarez DF.** 2013. In the absence of effector proteins, the *Pseudomonas aeruginosa* type three secretion system needle tip complex contributes to lung injury and systemic inflammatory responses. PLoS One **8:**e81792.

114. **Sing A, Roggenkamp A, Geiger AM, Heesemann J.** 2002. *Yersinia enterocolitica* evasion of the host innate immune response by V antigen-induced IL-10 production of macrophages is abrogated in IL-10-deficient mice. J Immunol **168:**1315-1321.

115. **Goure J, Broz P, Attree O, Cornelis GR, Attree I.** 2005. Protective anti-V antibodies inhibit *Pseudomonas* and *Yersinia* translocon assembly within host membranes. J Infect Dis **192:**218-225.

116. **Yang F, Gu J, Yang L, Gao C, Jing H, Wang Y, Zeng H, Zou Q, Lv F, Zhang J.** 2017. Protective Efficacy of the Trivalent *Pseudomonas aeruginosa* Vaccine Candidate PcrV-OprI-Hcp1 in Murine Pneumonia and Burn Models. Sci Rep **7:**3957.

117. **Thanabalasuriar A, Surewaard BG, Willson ME, Neupane AS, Stover CK, Warrener P, Wilson G, Keller AE, Sellman BR, DiGiandomenico A, Kubes P.** 2017. Bispecific antibody targets multiple *Pseudomonas aeruginosa* evasion mechanisms in the lung vasculature. J Clin Invest **127:**2249-2261.

118. **Zhao Y, Yang J, Shi J, Gong YN, Lu Q, Xu H, Liu L, Shao F.** 2011. The NLRC4 inflammasome receptors for bacterial flagellin and type III secretion apparatus. Nature **477:**596-600.

119. **Horsman SR, Moore RA, Lewenza S.** 2012. Calcium chelation by alginate activates the type III secretion system in mucoid *Pseudomonas aeruginosa* biofilms. PLoS One **7:**e46826.

120. **Urbanowski ML, Lykken GL, Yahr TL.** 2005. A secreted regulatory protein couples transcription to the secretory activity of the *Pseudomonas aeruginosa* type III secretion system. Proc Natl Acad Sci U S A **102:**9930-9935.

121. **Brutinel ED, Vakulskas CA, Yahr TL.** 2010. ExsD inhibits expression of the *Pseudomonas aeruginosa* type III secretion system by disrupting ExsA self-association and DNA binding activity. J Bacteriol **192:**1479-1486.

122. **Brutinel ED, Vakulskas CA, Brady KM, Yahr TL.** 2008. Characterization of ExsA and of ExsA-dependent promoters required for expression of the *Pseudomonas aeruginosa* type III secretion system. Mol Microbiol **68:**657-671.

123. **Vakulskas CA, Brady KM, Yahr TL.** 2009. Mechanism of transcriptional activation by *Pseudomonas aeruginosa* ExsA. J Bacteriol **191:**6654-6664.

124. **Marsden AE, Intile PJ, Schulmeyer KH, Simmons-Patterson ER, Urbanowski ML, Wolfgang MC, Yahr TL.** 2016. Vfr Directly Activates *exsA* Transcription To Regulate Expression of the *Pseudomonas aeruginosa* Type III Secretion System. J Bacteriol **198:**1442-1450.

125. **Burstein D, Satanower S, Simovitch M, Belnik Y, Zehavi M, Yerushalmi G, Ben-Aroya S, Pupko T, Banin E.** 2015. Novel type III effectors in *Pseudomonas aeruginosa*. MBio **6:**e00161.

126. **Feltman H, Schulert G, Khan S, Jain M, Peterson L, Hauser AR.** 2001. Prevalence of type III secretion genes in clinical and environmental isolates of *Pseudomonas aeruginosa*. Microbiology **147:**2659-2669.

127. **Schulert GS, Feltman H, Rabin SD, Martin CG, Battle SE, Rello J, Hauser AR.** 2003. Secretion of the toxin ExoU is a marker for highly virulent *Pseudomonas aeruginosa* isolates obtained from patients with hospital-acquired pneumonia. J Infect Dis **188:**1695-1706.

128. **Iglewski BH, Sadoff J, Bjorn MJ, Maxwell ES.** 1978. *Pseudomonas aeruginosa* exoenzyme S: an adenosine diphosphate ribosyltransferase distinct from toxin A. Proc Natl Acad Sci U S A **75:**3211-3215.

129. **Faraji F, Mahzounieh M, Ebrahimi A, Fallah F, Teymournejad O, Lajevardi B.** 2016. Molecular detection of virulence genes in *Pseudomonas aeruginosa* isolated from children with Cystic Fibrosis and burn wounds in Iran. Microb Pathog **99:**1-4.

130. **Hu H, Harmer C, Anuj S, Wainwright CE, Manos J, Cheney J, Harbour C, Zablotska I, Turnbull L, Whitchurch CB, Grimwood K, Rose B, investigators Fs.** 2013. Type 3 secretion system effector genotype and secretion phenotype of longitudinally collected *Pseudomonas aeruginosa* isolates from young children

diagnosed with cystic fibrosis following newborn screening. Clin Microbiol Infect **19:**266-272.

131. **Fu H, Coburn J, Collier RJ.** 1993. The eukaryotic host factor that activates exoenzyme S of *Pseudomonas aeruginosa* is a member of the 14-3-3 protein family. Proc Natl Acad Sci U S A **90:**2320-2324.

132. **Krall R, Zhang Y, Barbieri JT.** 2004. Intracellular membrane localization of *pseudomonas* ExoS and *Yersinia* YopE in mammalian cells. J Biol Chem **279:**2747-2753.

133. **Zhang Y, Barbieri JT.** 2005. A leucine-rich motif targets *Pseudomonas aeruginosa* ExoS within mammalian cells. Infect Immun **73:**7938-7945.

134. **Fraylick JE, La Rocque JR, Vincent TS, Olson JC.** 2001. Independent and coordinate effects of ADP-ribosyltransferase and GTPase-activating activities of exoenzyme S on HT-29 epithelial cell function. Infect Immun **69:**5318-5328.

135. **Coburn J, Dillon ST, Iglewski BH, Gill DM.** 1989. Exoenzyme S of *Pseudomonas aeruginosa* ADP-ribosylates the intermediate filament protein vimentin. Infect Immun **57:**996-998.

136. **Knight DA, Barbieri JT.** 1997. Ecto-ADP-ribosyltransferase activity of *Pseudomonas aeruginosa* exoenzyme S. Infect Immun **65:**3304-3309.

137. **Coburn J, Wyatt RT, Iglewski BH, Gill DM.** 1989. Several GTP-binding proteins, including p21c-H-ras, are preferred substrates of *Pseudomonas aeruginosa* exoenzyme S. J Biol Chem **264:**9004-9008.

138. **Coburn J, Gill DM.** 1991. ADP-ribosylation of p21ras and related proteins by *Pseudomonas aeruginosa* exoenzyme S. Infect Immun **59:**4259-4262.

139. **Pederson KJ, Vallis AJ, Aktories K, Frank DW, Barbieri JT.** 1999. The amino-terminal domain of *Pseudomonas aeruginosa* ExoS disrupts actin filaments via small-molecular-weight GTP-binding proteins. Mol Microbiol **32:**393-401.

140. **Frithz-Lindsten E, Du Y, Rosqvist R, Forsberg A.** 1997. Intracellular targeting of exoenzyme S of *Pseudomonas aeruginosa* via type III-dependent translocation induces phagocytosis resistance, cytotoxicity and disruption of actin microfilaments. Mol Microbiol **25:**1125-1139.

141. **Maresso AW, Baldwin MR, Barbieri JT.** 2004. Ezrin/radixin/moesin proteins are high affinity targets for ADP-ribosylation by *Pseudomonas aeruginosa* ExoS. J Biol Chem **279:**38402-38408.

142. **Henriksson ML, Sundin C, Jansson AL, Forsberg A, Palmer RH, Hallberg B.** 2002. Exoenzyme S shows selective ADP-ribosylation and GTPase-activating protein (GAP) activities towards small GTPases *in vivo*. Biochem J **367:**617-628.

143. **Krall R, Sun J, Pederson KJ, Barbieri JT.** 2002. In vivo rho GTPase-activating protein activity of *Pseudomonas aeruginosa* cytotoxin ExoS. Infect Immun **70:**360-367.

144. **Soong G, Parker D, Magargee M, Prince AS.** 2008. The type III toxins of *Pseudomonas aeruginosa* disrupt epithelial barrier function. J Bacteriol **190:**2814-2821.

145. **Mustafi S, Rivero N, Olson JC, Stahl PD, Barbieri MA.** 2013. Regulation of Rab5 function during phagocytosis of live *Pseudomonas aeruginosa* in macrophages. Infect Immun **81:**2426-2436.

146. **Tukaye DN, Kwon SH, Guggino WB.** 2013. The GAP portion of *Pseudomonas aeruginosa* type III secreted toxin ExoS upregulates total and surface levels of wild type CFTR. Cell Physiol Biochem **31:**153-165.

147. **Mody CH, Buser DE, Syme RM, Woods DE.** 1995. *Pseudomonas aeruginosa* exoenzyme S induces proliferation of human T lymphocytes. Infect Immun **63:**1800-1805.

148. **Epelman S, Bruno TF, Neely GG, Woods DE, Mody CH.** 2000. *Pseudomonas aeruginosa* exoenzyme S induces transcriptional expression of proinflammatory cytokines and chemokines. Infect Immun **68:**4811-4814.

149. **Sun Y, Karmakar M, Taylor PR, Rietsch A, Pearlman E.** 2012. ExoS and ExoT ADP ribosyltransferase activities mediate *Pseudomonas aeruginosa* keratitis by promoting neutrophil apoptosis and bacterial survival. J Immunol **188:**1884-1895.

150. **Rangel SM, Diaz MH, Knoten CA, Zhang A, Hauser AR.** 2015. The Role of ExoS in Dissemination of *Pseudomonas aeruginosa* during Pneumonia. PLoS Pathog **11:**e1004945.

151. **Kulich SM, Yahr TL, Mende-Mueller LM, Barbieri JT, Frank DW.** 1994. Cloning the structural gene for the 49-kDa form of exoenzyme S (exoS) from *Pseudomonas aeruginosa* strain 388. J Biol Chem **269:**10431-10437.

152. **Yahr TL, Barbieri JT, Frank DW.** 1996. Genetic relationship between the 53- and 49-kilodalton forms of exoenzyme S from *Pseudomonas aeruginosa*. J Bacteriol **178:**1412-1419.

153. **Krall R, Schmidt G, Aktories K, Barbieri JT.** 2000. *Pseudomonas aeruginosa* ExoT is a Rho GTPase-activating protein. Infect Immun **68:**6066-6068.

154. **Dey S, Datta S.** 2014. Interfacial residues of SpcS chaperone affects binding of effector toxin ExoT in *Pseudomonas aeruginosa*: novel insights from structural and computational studies. FEBS J **281:**1267-1280.

155. **Kazmierczak BI, Engel JN.** 2002. *Pseudomonas aeruginosa* ExoT acts *in vivo* as a GTPase-activating protein for RhoA, Rac1, and Cdc42. Infect Immun **70:**2198-2205.

156. **Sun J, Barbieri JT.** 2003. *Pseudomonas aeruginosa* ExoT ADP-ribosylates CT10 regulator of kinase (Crk) proteins. J Biol Chem **278:**32794-32800.

157. **Garrity-Ryan L, Shafikhani S, Balachandran P, Nguyen L, Oza J, Jakobsen T, Sargent J, Fang X, Cordwell S, Matthay MA, Engel JN.** 2004. The ADP ribosyltransferase domain of *Pseudomonas aeruginosa* ExoT contributes to its biological activities. Infect Immun **72:**546-558.

158. **Cowell BA, Chen DY, Frank DW, Vallis AJ, Fleiszig SM.** 2000. ExoT of cytotoxic *Pseudomonas aeruginosa* prevents uptake by corneal epithelial cells. Infect Immun **68:**403-406.

159. **Geiser TK, Kazmierczak BI, Garrity-Ryan LK, Matthay MA, Engel JN.** 2001. *Pseudomonas aeruginosa* ExoT inhibits in vitro lung epithelial wound repair. Cell Microbiol **3:**223-236.

160. **Garrity-Ryan L, Kazmierczak B, Kowal R, Comolli J, Hauser A, Engel JN.** 2000. The arginine finger domain of ExoT contributes to actin cytoskeleton disruption and inhibition of internalization of *Pseudomonas aeruginosa* by epithelial cells and macrophages. Infect Immun **68:**7100-7113.

161. **Shaver CM, Hauser AR.** 2004. Relative contributions of *Pseudomonas aeruginosa* ExoU, ExoS, and ExoT to virulence in the lung. Infect Immun **72:**6969-6977.

162. **Goldufsky J, Wood S, Hajihossainlou B, Rehman T, Majdobeh O, Kaufman HL, Ruby CE, Shafikhani SH.** 2015. *Pseudomonas aeruginosa* exotoxin T induces potent cytotoxicity against a variety of murine and human cancer cell lines. J Med Microbiol **64:**164-173.

163. **Shafikhani SH, Engel J.** 2006. *Pseudomonas aeruginosa* type III-secreted toxin ExoT inhibits host-cell division by targeting cytokinesis at multiple steps. Proc Natl Acad Sci U S A **103:**15605-15610.

164. **Wood S, Goldufsky J, Shafikhani SH.** 2015. *Pseudomonas aeruginosa* ExoT Induces Atypical Anoikis Apoptosis in Target Host Cells by Transforming Crk Adaptor Protein into a Cytotoxin. PLoS Pathog **11:**e1004934.

165. **Wood SJ, Goldufsky JW, Bello D, Masood S, Shafikhani SH.** 2015. *Pseudomonas aeruginosa* ExoT Induces Mitochondrial Apoptosis in Target Host Cells in a Manner That Depends on Its GTPase-activating Protein (GAP) Domain Activity. J Biol Chem **290:**29063-29073.

166. **Yahr TL, Vallis AJ, Hancock MK, Barbieri JT, Frank DW.** 1998. ExoY, an adenylate cyclase secreted by the *Pseudomonas aeruginosa* type III system. Proc Natl Acad Sci U S A **95:**13899-13904.

167. **Belyy A, Raoux-Barbot D, Saveanu C, Namane A, Ogryzko V, Worpenberg L, David V, Henriot V, Fellous S, Merrifield C, Assayag E, Ladant D, Renault L, Mechold U.** 2016. Actin activates *Pseudomonas aeruginosa* ExoY nucleotidyl cyclase toxin and ExoY-like effector domains from MARTX toxins. Nat Commun **7:**13582.

168. **Vallis AJ, Finck-Barbancon V, Yahr TL, Frank DW.** 1999. Biological effects of *Pseudomonas aeruginosa* type III-secreted proteins on CHO cells. Infect Immun **67:**2040-2044.

169. **Sayner SL, Frank DW, King J, Chen H, VandeWaa J, Stevens T.** 2004. Paradoxical cAMP-induced lung endothelial hyperpermeability revealed by *Pseudomonas aeruginosa* ExoY. Circ Res **95:**196-203.

170. **Stevens TC, Ochoa CD, Morrow KA, Robson MJ, Prasain N, Zhou C, Alvarez DF, Frank DW, Balczon R, Stevens T.** 2014. The *Pseudomonas aeruginosa* exoenzyme Y impairs endothelial cell proliferation and vascular repair following lung injury. Am J Physiol Lung Cell Mol Physiol **306:**L915-924.

171. **Lee VT, Smith RS, Tummler B, Lory S.** 2005. Activities of *Pseudomonas aeruginosa* effectors secreted by the Type III secretion system *in vitro* and during infection. Infect Immun **73:**1695-1705.

172. **Vance RE, Rietsch A, Mekalanos JJ.** 2005. Role of the type III secreted exoenzymes S, T, and Y in systemic spread of *Pseudomonas aeruginosa* PAO1 *in vivo*. Infect Immun **73:**1706-1713.

173. **Ochoa CD, Alexeyev M, Pastukh V, Balczon R, Stevens T.** 2012. *Pseudomonas aeruginosa* exotoxin Y is a promiscuous cyclase that increases endothelial tau phosphorylation and permeability. J Biol Chem **287:**25407-25418.

174. **Balczon R, Prasain N, Ochoa C, Prater J, Zhu B, Alexeyev M, Sayner S, Frank DW, Stevens T.** 2013. *Pseudomonas aeruginosa* exotoxin Y-mediated tau hyperphosphorylation impairs microtubule assembly in pulmonary microvascular endothelial cells. PLoS One **8:**e74343.

175. **He C, Zhou Y, Liu F, Liu H, Tan H, Jin S, Wu W, Ge B.** 2017. Bacterial Nucleotidyl Cyclase Inhibits the Host Innate Immune Response by Suppressing TAK1 Activation. Infect Immun **85**.

176. **Jeon J, Kim YJ, Shin H, Ha UH.** 2017. T3SS effector ExoY reduces inflammasome-related responses by suppressing bacterial motility and delaying activation of NF-kappaB and caspase-1. FEBS J doi:10.1111/febs.14199.

177. **Hauser AR, Kang PJ, Engel JN.** 1998. PepA, a secreted protein of *Pseudomonas aeruginosa*, is necessary for cytotoxicity and virulence. Mol Microbiol **27:**807-818.

178. **Finck-Barbancon V, Goranson J, Zhu L, Sawa T, Wiener-Kronish JP, Fleiszig SM, Wu C, Mende-Mueller L, Frank DW.** 1997. ExoU expression by *Pseudomonas aeruginosa* correlates with acute cytotoxicity and epithelial injury. Mol Microbiol **25:**547-557.

179. **Finck-Barbancon V, Yahr TL, Frank DW.** 1998. Identification and characterization of SpcU, a chaperone required for efficient secretion of the ExoU cytotoxin. J Bacteriol **180:**6224-6231.

180. **Brinkworth AJ, Malcolm DS, Pedrosa AT, Roguska K, Shahbazian S, Graham JE, Hayward RD, Carabeo RA.** 2011. *Chlamydia trachomatis* Slc1 is a type III secretion chaperone that enhances the translocation of its invasion effector substrate TARP. Mol Microbiol **82:**131-144.

181. **Scheibner F, Hartmann N, Hausner J, Lorenz C, Hoffmeister AK, Buettner D.** 2017. The type III secretion chaperone HpaB controls the translocation of effector and non-effector proteins from *Xanthomonas campestris pv. vesicatoria.* Mol Plant Microbe Interact doi:10.1094/MPMI-06-17-0138-R.

182. **Wolfgang MC, Kulasekara BR, Liang X, Boyd D, Wu K, Yang Q, Miyada CG, Lory S.** 2003. Conservation of genome content and virulence determinants among clinical and environmental isolates of *Pseudomonas aeruginosa.* Proc Natl Acad Sci U S A **100:**8484-8489.

183. **Roy-Burman A, Savel RH, Racine S, Swanson BL, Revadigar NS, Fujimoto J, Sawa T, Frank DW, Wiener-Kronish JP.** 2001. Type III protein secretion is associated with death in lower respiratory and systemic *Pseudomonas aeruginosa* infections. J Infect Dis **183:**1767-1774.

184. **Lomholt JA, Poulsen K, Kilian M.** 2001. Epidemic population structure of *Pseudomonas aeruginosa*: evidence for a clone that is pathogenic to the eye and that has a distinct combination of virulence factors. Infect Immun **69:**6284-6295.

185. **Kulasekara BR, Kulasekara HD, Wolfgang MC, Stevens L, Frank DW, Lory S.** 2006. Acquisition and evolution of the *exoU* locus in *Pseudomonas aeruginosa*. J Bacteriol **188:**4037-4050.

186. **Pena C, Cabot G, Gomez-Zorrilla S, Zamorano L, Ocampo-Sosa A, Murillas J, Almirante B, Pomar V, Aguilar M, Granados A, Calbo E, Rodriguez-Bano J, Rodriguez-Lopez F, Tubau F, Martinez-Martinez L, Oliver A, Spanish Network for Research in Infectious D.** 2015. Influence of virulence genotype and resistance profile in the mortality of *Pseudomonas aeruginosa* bloodstream infections. Clin Infect Dis **60:**539-548.

187. **Pirnay JP, Bilocq F, Pot B, Cornelis P, Zizi M, Van Eldere J, Deschaght P, Vaneechoutte M, Jennes S, Pitt T, De Vos D.** 2009. *Pseudomonas aeruginosa* population structure revisited. PLoS One **4:**e7740.

188. **Shen EP, Tsay RY, Chia JS, Wu S, Lee JW, Hu FR.** 2012. The role of type III secretion system and lens material on adhesion of *Pseudomonas aeruginosa* to contact lenses. Invest Ophthalmol Vis Sci **53:**6416-6426.

189. **Wong-Beringer A, Wiener-Kronish J, Lynch S, Flanagan J.** 2008. Comparison of type III secretion system virulence among fluoroquinolone-susceptible and -resistant clinical isolates of *Pseudomonas aeruginosa*. Clin Microbiol Infect **14:**330-336.

190. **Heidary Z, Bandani E, Eftekhary M, Jafari AA.** 2016. Virulence Genes Profile of Multidrug Resistant *Pseudomonas aeruginosa* Isolated from Iranian Children with UTIs. Acta Med Iran **54:**201-210.

191. **Park MH, Kim SY, Roh EY, Lee HS.** 2016. Difference of Type 3 secretion system (T3SS) effector gene genotypes (*exoU* and *exoS*) and its implication to antibiotics resistances in isolates of *Pseudomonas aeruginosa* from chronic otitis media. Auris Nasus Larynx doi:10.1016/j.anl.2016.07.005.

192. **Borkar DS, Acharya NR, Leong C, Lalitha P, Srinivasan M, Oldenburg CE, Cevallos V, Lietman TM, Evans DJ, Fleiszig SM.** 2014. Cytotoxic clinical isolates of *Pseudomonas aeruginosa* identified during the Steroids for Corneal Ulcers Trial show elevated resistance to fluoroquinolones. BMC Ophthalmol **14:**54.

193. **Sullivan E, Bensman J, Lou M, Agnello M, Shriner K, Wong-Beringer A.** 2014. Risk of developing pneumonia is enhanced by the combined traits of fluoroquinolone resistance and type III secretion virulence in respiratory isolates of *Pseudomonas aeruginosa*. Crit Care Med **42:**48-56.

194. **Aditi, Shariff M, Chhabra SK, Rahman MU.** 2017. Similar virulence properties of infection and colonization associated *Pseudomonas aeruginosa*. J Med Microbiol doi:10.1099/jmm.0.000569.

195. **Agnello M, Wong-Beringer A.** 2012. Differentiation in quinolone resistance by virulence genotype in *Pseudomonas aeruginosa*. PLoS One **7:**e42973.

196. **Phillips RM, Six DA, Dennis EA, Ghosh P.** 2003. *In vivo* phospholipase activity of the *Pseudomonas aeruginosa* cytotoxin ExoU and protection of mammalian cells with phospholipase A2 inhibitors. J Biol Chem **278:**41326-41332.

197. **Sato H, Frank DW, Hillard CJ, Feix JB, Pankhaniya RR, Moriyama K, Finck-Barbancon V, Buchaklian A, Lei M, Long RM, Wiener-Kronish J, Sawa T.** 2003. The mechanism of action of the *Pseudomonas aeruginosa*-encoded type III cytotoxin, ExoU. EMBO J **22:**2959-2969.

198. **Rabin SD, Hauser AR.** 2005. Functional regions of the *Pseudomonas aeruginosa* cytotoxin ExoU. Infect Immun **73:**573-582.

199. **Rabin SD, Veesenmeyer JL, Bieging KT, Hauser AR.** 2006. A C-terminal domain targets the *Pseudomonas aeruginosa* cytotoxin ExoU to the plasma membrane of host cells. Infect Immun **74:**2552-2561.

200. **Veesenmeyer JL, Howell H, Halavaty AS, Ahrens S, Anderson WF, Hauser AR.** 2010. Role of the membrane localization domain of the *Pseudomonas aeruginosa* effector protein ExoU in cytotoxicity. Infect Immun **78:**3346-3357.

201. **Finck-Barbancon V, Frank DW.** 2001. Multiple domains are required for the toxic activity of *Pseudomonas aeruginosa* ExoU. J Bacteriol **183:**4330-4344.

202. **Schmalzer KM, Benson MA, Frank DW.** 2010. Activation of ExoU phospholipase activity requires specific C-terminal regions. J Bacteriol **192:**1801-1812.

203. **Sato H, Feix JB, Hillard CJ, Frank DW.** 2005. Characterization of phospholipase activity of the *Pseudomonas aeruginosa* type III cytotoxin, ExoU. J Bacteriol **187:**1192-1195.

204. **Benson MA, Komas SM, Schmalzer KM, Casey MS, Frank DW, Feix JB.** 2011. Induced conformational changes in the activation of the *Pseudomonas aeruginosa* type III toxin, ExoU. Biophys J **100:**1335-1343.

205. **Tessmer MH, Anderson DM, Buchaklian A, Frank DW, Feix JB.** 2017. Cooperative Substrate-Cofactor Interactions and Membrane Localization of the Bacterial Phospholipase A2 (PLA2) Enzyme, ExoU. J Biol Chem **292:**3411-3419.

206. **Halavaty AS, Borek D, Tyson GH, Veesenmeyer JL, Shuvalova L, Minasov G, Otwinowski Z, Hauser AR, Anderson WF.** 2012. Structure of the type III secretion effector protein ExoU in complex with its chaperone SpcU. PLoS One **7:**e49388.

207. **Gendrin C, Contreras-Martel C, Bouillot S, Elsen S, Lemaire D, Skoufias DA, Huber P, Attree I, Dessen A.** 2012. Structural basis of cytotoxicity mediated by the type III secretion toxin ExoU from *Pseudomonas aeruginosa*. PLoS Pathog **8:**e1002637.

208. **Geissler B, Tungekar R, Satchell KJ.** 2010. Identification of a conserved membrane localization domain within numerous large bacterial protein toxins. Proc Natl Acad Sci U S A **107:**5581-5586.

209. **Dondelinger Y, Declercq W, Montessuit S, Roelandt R, Goncalves A, Bruggeman I, Hulpiau P, Weber K, Sehon CA, Marquis RW, Bertin J, Gough PJ, Savvides S, Martinou JC, Bertrand MJ, Vandenabeele P.** 2014. MLKL compromises plasma membrane integrity by binding to phosphatidylinositol phosphates. Cell Rep **7:**971-981.

210. **Tyson GH, Halavaty AS, Kim H, Geissler B, Agard M, Satchell KJ, Cho W, Anderson WF, Hauser AR.** 2015. A novel phosphatidylinositol 4,5-bisphosphate binding domain mediates plasma membrane localization of ExoU and other patatin-like phospholipases. J Biol Chem **290:**2919-2937.

211. **Wenk MR, Lucast L, Di Paolo G, Romanelli AJ, Suchy SF, Nussbaum RL, Cline GW, Shulman GI, McMurray W, De Camilli P.** 2003. Phosphoinositide profiling in complex lipid mixtures using electrospray ionization mass spectrometry. Nat Biotechnol **21:**813-817.

212. **Janmey PA, Iida K, Yin HL, Stossel TP.** 1987. Polyphosphoinositide micelles and polyphosphoinositide-containing vesicles dissociate endogenous gelsolin-actin complexes and promote actin assembly from the fast-growing end of actin filaments blocked by gelsolin. J Biol Chem **262:**12228-12236.

213. **Skare P, Karlsson R.** 2002. Evidence for two interaction regions for phosphatidylinositol(4,5)-bisphosphate on mammalian profilin I. FEBS Lett **522:**119-124.

214. **Gilmore AP, Burridge K.** 1996. Regulation of vinculin binding to talin and actin by phosphatidyl-inositol-4-5-bisphosphate. Nature **381:**531-535.

215. **Stirling FR, Cuzick A, Kelly SM, Oxley D, Evans TJ.** 2006. Eukaryotic localization, activation and ubiquitinylation of a bacterial type III secreted toxin. Cell Microbiol **8:**1294-1309.

216. **Ebner P, Versteeg GA, Ikeda F.** 2017. Ubiquitin enzymes in the regulation of immune responses. Crit Rev Biochem Mol Biol **52:**425-460.

217. **Dikic I.** 2017. Proteasomal and Autophagic Degradation Systems. Annu Rev Biochem **86:**193-224.

218. **Dwane L, Gallagher WM, Ni Chonghaile T, O'Connor DP.** 2017. The Emerging Role of Non-traditional Ubiquitination in Oncogenic Pathways. J Biol Chem **292:**3543-3551.

219. **Nguyen LK, Kolch W, Kholodenko BN.** 2013. When ubiquitination meets phosphorylation: a systems biology perspective of EGFR/MAPK signalling. Cell Commun Signal **11:**52.

220. **Das-Bradoo S, Ricke RM, Bielinsky AK.** 2006. Interaction between PCNA and diubiquitinated Mcm10 is essential for cell growth in budding yeast. Mol Cell Biol **26:**4806-4817.

221. **Allewelt M, Coleman FT, Grout M, Priebe GP, Pier GB.** 2000. Acquisition of expression of the *Pseudomonas aeruginosa* ExoU cytotoxin leads to increased bacterial virulence in a murine model of acute pneumonia and systemic spread. Infect Immun **68:**3998-4004.

222. **Tam C, Lewis SE, Li WY, Lee E, Evans DJ, Fleiszig SM.** 2007. Mutation of the phospholipase catalytic domain of the *Pseudomonas aeruginosa* cytotoxin ExoU abolishes colonization promoting activity and reduces corneal disease severity. Exp Eye Res **85:**799-805.

223. **Pukatzki S, Kessin RH, Mekalanos JJ.** 2002. The human pathogen *Pseudomonas aeruginosa* utilizes conserved virulence pathways to infect the social amoeba *Dictyostelium discoideum*. Proc Natl Acad Sci U S A **99:**3159-3164.

224. **Abd H, Wretlind B, Saeed A, Idsund E, Hultenby K, Sandstrom G.** 2008. *Pseudomonas aeruginosa* utilises its type III secretion system to kill the free-living amoeba *Acanthamoeba castellanii*. J Eukaryot Microbiol **55:**235-243.

225. **Matz C, Moreno AM, Alhede M, Manefield M, Hauser AR, Givskov M, Kjelleberg S.** 2008. *Pseudomonas aeruginosa* uses type III secretion system to kill biofilm-associated amoebae. ISME J **2:**843-852.

226. **Howell HA, Logan LK, Hauser AR.** 2013. Type III secretion of ExoU is critical during early *Pseudomonas aeruginosa* pneumonia. MBio **4:**e00032-00013.

227. **Cuzick A, Stirling FR, Lindsay SL, Evans TJ.** 2006. The type III pseudomonal exotoxin U activates the c-Jun NH2-terminal kinase pathway and increases human epithelial interleukin-8 production. Infect Immun **74:**4104-4113.

228. **de Lima CD, Calegari-Silva TC, Pereira RM, Santos SA, Lopes UG, Plotkowski MC, Saliba AM.** 2012. ExoU activates NF-kappaB and increases IL-8/KC secretion during *Pseudomonas aeruginosa* infection. PLoS One **7:**e41772.

229. **Saliba AM, Nascimento DO, Silva MC, Assis MC, Gayer CR, Raymond B, Coelho MG, Marques EA, Touqui L, Albano RM, Lopes UG, Paiva DD, Bozza PT, Plotkowski MC.** 2005. Eicosanoid-mediated proinflammatory activity of *Pseudomonas aeruginosa* ExoU. Cell Microbiol **7:**1811-1822.

230. **Diaz MH, Hauser AR.** 2010. *Pseudomonas aeruginosa* cytotoxin ExoU is injected into phagocytic cells during acute pneumonia. Infect Immun **78:**1447-1456.

231. **Diaz MH, Shaver CM, King JD, Musunuri S, Kazzaz JA, Hauser AR.** 2008. *Pseudomonas aeruginosa* induces localized immunosuppression during pneumonia. Infect Immun **76:**4414-4421.

232. **Rabin SD, Hauser AR.** 2003. *Pseudomonas aeruginosa* ExoU, a toxin transported by the type III secretion system, kills *Saccharomyces cerevisiae*. Infect Immun **71:**4144-4150.

233. **Hauser AR, Engel JN.** 1999. *Pseudomonas aeruginosa* induces type-III-secretion-mediated apoptosis of macrophages and epithelial cells. Infect Immun **67:**5530-5537.

234. **Dacheux D, Toussaint B, Richard M, Brochier G, Croize J, Attree I.** 2000. *Pseudomonas aeruginosa* cystic fibrosis isolates induce rapid, type III secretion-dependent, but ExoU-independent, oncosis of macrophages and polymorphonuclear neutrophils. Infect Immun **68:**2916-2924.

235. **Birts CN, Barton CH, Wilton DC.** 2010. Catalytic and non-catalytic functions of human IIA phospholipase A2. Trends Biochem Sci **35:**28-35.

236. **Dennis EA, Norris PC.** 2015. Eicosanoid storm in infection and inflammation. Nat Rev Immunol **15:**511-523.

237. **Saliba AM, de Assis MC, Nishi R, Raymond B, Marques Ede A, Lopes UG, Touqui L, Plotkowski MC.** 2006. Implications of oxidative stress in the cytotoxicity of *Pseudomonas aeruginosa* ExoU. Microbes Infect **8:**450-459.

238. **Machado GB, de Assis MC, Leao R, Saliba AM, Silva MC, Suassuna JH, de Oliveira AV, Plotkowski MC.** 2010. ExoU-induced vascular hyperpermeability and platelet activation in the course of experimental *Pseudomonas aeruginosa* pneumosepsis. Shock **33:**315-321.

239. **Machado GB, de Oliveira AV, Saliba AM, de Lima CD, Suassuna JH, Plotkowski MC.** 2011. *Pseudomonas aeruginosa* toxin ExoU induces a PAF-dependent impairment of alveolar fibrin turnover secondary to enhanced activation of coagulation and increased expression of plasminogen activator inhibitor-1 in the course of mice pneumosepsis. Respir Res **12:**104.

240. **Sato H, Feix JB, Frank DW.** 2006. Identification of superoxide dismutase as a cofactor for the pseudomonas type III toxin, ExoU. Biochemistry **45:**10368-10375.

241. **Anderson DM, Schmalzer KM, Sato H, Casey M, Terhune SS, Haas AL, Feix JB, Frank DW.** 2011. Ubiquitin and ubiquitin-modified proteins activate the *Pseudomonas aeruginosa* T3SS cytotoxin, ExoU. Mol Microbiol **82:**1454-1467.

242. **Bett JS.** 2016. Proteostasis regulation by the ubiquitin system. Essays Biochem **60:**143-151.

243. **Tyson GH, Hauser AR.** 2013. Phosphatidylinositol 4,5-bisphosphate is a novel coactivator of the *Pseudomonas aeruginosa* cytotoxin ExoU. Infect Immun **81:**2873-2881.

244. **Brown DA.** 2015. PIP2Clustering: From model membranes to cells. Chem Phys Lipids **192:**33-40.

245. **Tan X, Thapa N, Choi S, Anderson RA.** 2015. Emerging roles of PtdIns(4,5)P2--beyond the plasma membrane. J Cell Sci **128:**4047-4056.

246. **Zhang L, Mao YS, Janmey PA, Yin HL.** 2012. Phosphatidylinositol 4, 5 bisphosphate and the actin cytoskeleton. Subcell Biochem **59:**177-215.

247. **Sato H, Frank DW.** 2014. Intoxication of host cells by the T3SS phospholipase ExoU: PI(4,5)P2-associated, cytoskeletal collapse and late phase membrane blebbing. PLoS One **9:**e103127.

248. **Mejillano M, Yamamoto M, Rozelle AL, Sun HQ, Wang X, Yin HL.** 2001. Regulation of apoptosis by phosphatidylinositol 4,5-bisphosphate inhibition of caspases, and caspase inactivation of phosphatidylinositol phosphate 5-kinases. J Biol Chem **276:**1865-1872.

249. **Folta-Stogniew E.** 2006. Oligomeric states of proteins determined by size-exclusion chromatography coupled with light scattering, absorbance, and refractive index detectors. Methods Mol Biol **328:**97-112.

250. **Paudel HK, Li W.** 1999. Heparin-induced conformational change in microtubule-associated protein Tau as detected by chemical cross-linking and phosphopeptide mapping. J Biol Chem **274:**8029-8038.

251. **Veesenmeyer JL.** 2010. Characterization of C-terminal Functional Domains of the *Pseudomonas aeruginosa* Cytotoxin ExoU.

252. **Hehir MJ, Murphy JE, Kantrowitz ER.** 2000. Characterization of heterodimeric alkaline phosphatases from *Escherichia coli*: an investigation of intragenic complementation. J Mol Biol **304:**645-656.

253. **Zhang Y, Billington CJ, Jr., Pan D, Neufeld TP.** 2006. Drosophila target of rapamycin kinase functions as a multimer. Genetics **172:**355-362.

254. **Turner MA, Simpson A, McInnes RR, Howell PL.** 1997. Human argininosuccinate lyase: a structural basis for intragenic complementation. Proc Natl Acad Sci U S A **94:**9063-9068.

255. **Gu Y, Wu J, Faucheu C, Lalanne JL, Diu A, Livingston DJ, Su MS.** 1995. Interleukin-1 beta converting enzyme requires oligomerization for activity of processed forms in vivo. EMBO J **14:**1923-1931.

256. **Zhang HL, Xu SJ, Wang QY, Song SY, Shu YY, Lin ZJ.** 2002. Structure of a cardiotoxic phospholipase A(2) from *Ophiophagus hannah* with the "pancreatic loop". J Struct Biol **138:**207-215.

257. **Gu L, Wang Z, Song S, Shu Y, Lin Z.** 2002. Crystal structures of an acidic phospholipase A(2) from the venom of *Naja Kaouthia*. Toxicon **40:**917-922.

258. **Jabeen T, Singh N, Singh RK, Jasti J, Sharma S, Kaur P, Srinivasan A, Singh TP.** 2006. Crystal structure of a heterodimer of phospholipase A2 from *Naja naja sagittifera* at 2.3 A resolution reveals the presence of a new PLA2-like protein with a novel cys 32-Cys 49 disulphide bridge with a bound sugar at the substrate-binding site. Proteins **62:**329-337.

259. **Bakholdina SI, Tischenko NM, Sidorin EV, Isaeva MP, Likhatskaya GN, Dmitrenok PS, Kim NY, Chernikov OV, Solov'eva TF.** 2016. Recombinant Phospholipase A1 of the Outer Membrane of Psychrotrophic *Yersinia pseudotuberculosis*: Expression, Purification, and Characterization. Biochemistry (Mosc) **81:**47-57.

260. **Xu W, Yi L, Feng Y, Chen L, Liu J.** 2009. Structural insight into the activation mechanism of human pancreatic prophospholipase A2. J Biol Chem **284:**16659-16666.

261. **Ackermann EJ, Kempner ES, Dennis EA.** 1994. Ca(2+)-independent cytosolic phospholipase A2 from macrophage-like P388D1 cells. Isolation and characterization. J Biol Chem **269:**9227-9233.

262. **Singh G, Gourinath S, Sarvanan K, Sharma S, Bhanumathi S, Betzel C, Yadav S, Srinivasan A, Singh TP.** 2005. Crystal structure of a carbohydrate induced homodimer of phospholipase A2 from *Bungarus caeruleus* at 2.1A resolution. J Struct Biol **149:**264-272.

263. **Sun MZ, Liu S, Yang F, Greenaway FT, Xu Y.** 2009. A novel phospholipase A2 from *Agkistrodon blomhoffii ussurensis* venom: purification, proteomic, functional and structural characterizations. Biochimie **91:**558-567.

264. **Kutluay SB, Bieniasz PD.** 2010. Analysis of the initiating events in HIV-1 particle assembly and genome packaging. PLoS Pathog **6:**e1001200.

265. **Dick RA, Barros M, Jin D, Losche M, Vogt VM.** 2015. Membrane Binding of the Rous Sarcoma Virus Gag Protein Is Cooperative and Dependent on the Spacer Peptide Assembly Domain. J Virol **90:**2473-2485.

266. **Johnson KA, Taghon GJ, Scott JL, Stahelin RV.** 2016. The Ebola Virus matrix protein, VP40, requires phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) for extensive oligomerization at the plasma membrane and viral egress. Sci Rep **6:**19125.

267. **Cheng J, Goldstein R, Stec B, Gershenson A, Roberts MF.** 2012. Competition between anion binding and dimerization modulates *Staphylococcus aureus* phosphatidylinositol-specific phospholipase C enzymatic activity. J Biol Chem **287:**40317-40327.

268. **Hendrix J, Baumgartel V, Schrimpf W, Ivanchenko S, Digman MA, Gratton E, Krausslich HG, Muller B, Lamb DC.** 2015. Live-cell observation of cytosolic HIV-1 assembly onset reveals RNA-interacting Gag oligomers. J Cell Biol **210:**629-646.

269. **Ji C, Zhang Y, Xu P, Xu T, Lou X.** 2015. Nanoscale Landscape of Phosphoinositides Revealed by Specific Pleckstrin Homology (PH) Domains Using Single-molecule Superresolution Imaging in the Plasma Membrane. J Biol Chem **290:**26978-26993.

270. **Chen W, Goldfine H, Ananthanarayanan B, Cho W, Roberts MF.** 2009. *Listeria monocytogenes* phosphatidylinositol-specific phospholipase C: Kinetic activation and homing in on different interfaces. Biochemistry **48:**3578-3592.

271. **Matthews BW.** 2005. The structure of *E. coli* beta-galactosidase. C R Biol **328:**549-556.

272. **Snijder HJ, Dijkstra BW.** 2000. Bacterial phospholipase A: structure and function of an integral membrane phospholipase. Biochim Biophys Acta **1488:**91-101.

273. **Tang J, Kriz RW, Wolfman N, Shaffer M, Seehra J, Jones SS.** 1997. A novel cytosolic calcium-independent phospholipase A2 contains eight ankyrin motifs. J Biol Chem **272:**8567-8575.

274. **Larsson PK, Claesson HE, Kennedy BP.** 1998. Multiple splice variants of the human calcium-independent phospholipase A2 and their effect on enzyme activity. J Biol Chem **273:**207-214.

275. **Manguikian AD, Barbour SE.** 2004. Cell cycle dependence of group VIA calcium-independent phospholipase A2 activity. J Biol Chem **279:**52881-52892.

276. **Kuhle K, Krausze J, Curth U, Rossle M, Heuner K, Lang C, Flieger A.** 2014. Oligomerization inhibits *Legionella pneumophila* PlaB phospholipase A activity. J Biol Chem **289:**18657-18666.

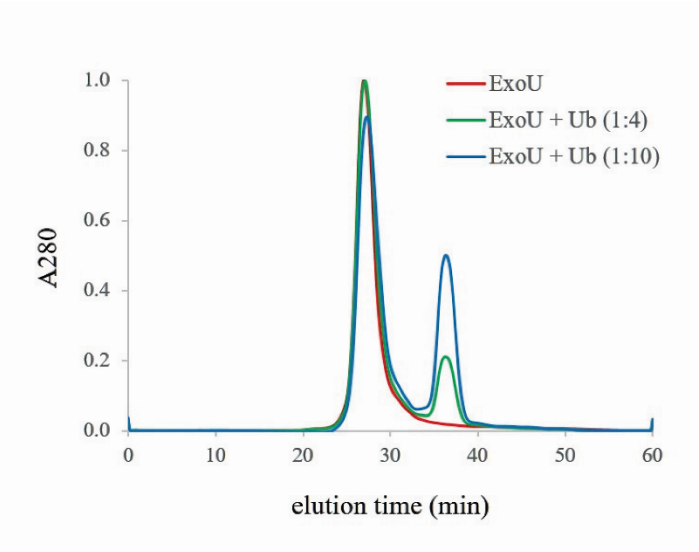
277. **Fremont DH, Anderson DH, Wilson IA, Dennis EA, Xuong NH.** 1993. Crystal structure of phospholipase A2 from Indian cobra reveals a trimeric association. Proc Natl Acad Sci U S A **90:**342-346.

278. **Lee VT, Pukatzki S, Sato H, Kikawada E, Kazimirova AA, Huang J, Li X, Arm JP, Frank DW, Lory S.** 2007. Pseudolipasin A is a specific inhibitor for phospholipase A2 activity of *Pseudomonas aeruginosa* cytotoxin ExoU. Infect Immun **75:**1089-1098.

279. **Kim D, Baek J, Song J, Byeon H, Min H, Min KH.** 2014. Identification of arylsulfonamides as ExoU inhibitors. Bioorg Med Chem Lett **24:**3823-3825.

280. **Sadikot RT, Zeng H, Azim AC, Joo M, Dey SK, Breyer RM, Peebles RS, Blackwell TS, Christman JW.** 2007. Bacterial clearance of *Pseudomonas aeruginosa* is enhanced by the inhibition of COX-2. Eur J Immunol **37:**1001-1009.

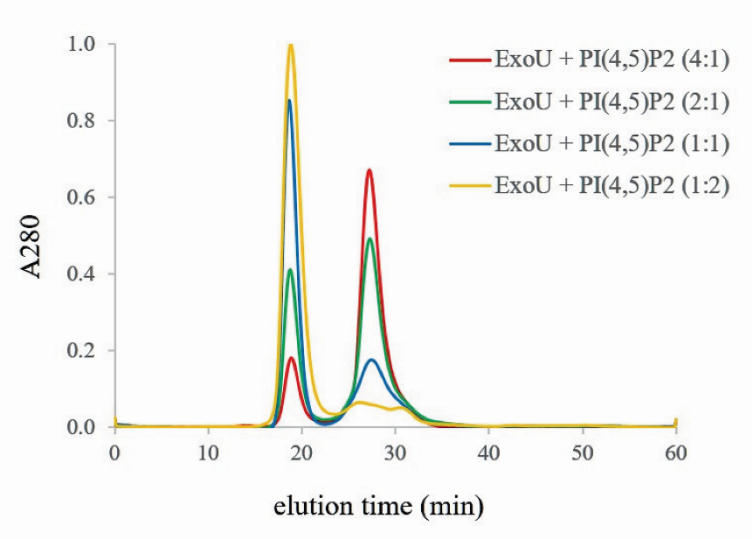
Appendix I



ExoU oligomerization is not observed with excess ubiquitin.

Samples containing differing ratios of ExoU to its cofactor ubiquitin (Ub) were examined for oligomerization. rExoU and Ub were mixed at molar ratios of 1:4 or 1:10 and assessed by SEC-MALS. The early peak corresponds to a molecular mass of approximately 75 kD (rExoU), and the later peak corresponds to a molecular mass of 8 kD (Ub). At least two independent samples were analyzed and a representative elution profile is shown. Normalized absorbance at 280 nm (A280) is graphed.

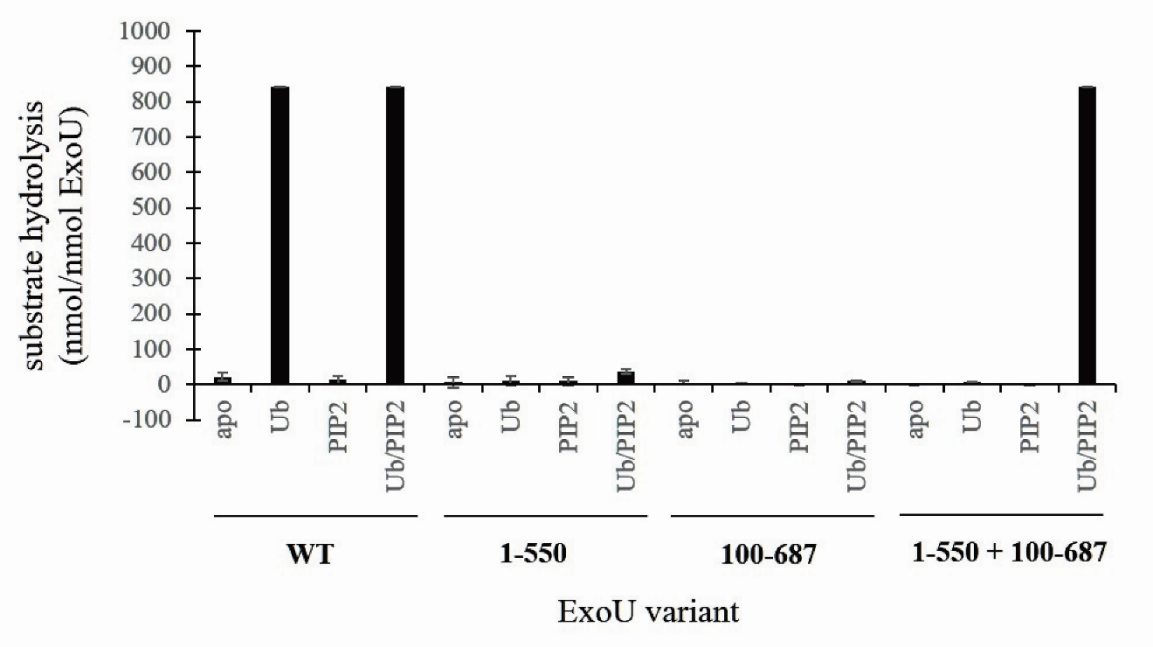
Appendix II



PI(4,5)P_2-mediated ExoU oligomerization is dose-dependent.

SEC-MALS was performed on samples containing the indicated ratios of rExoU to PI(4,5)P_2. At least two independent samples were analyzed and a representative elution profile is shown. Normalized absorbance at 280 nm (A280) is graphed

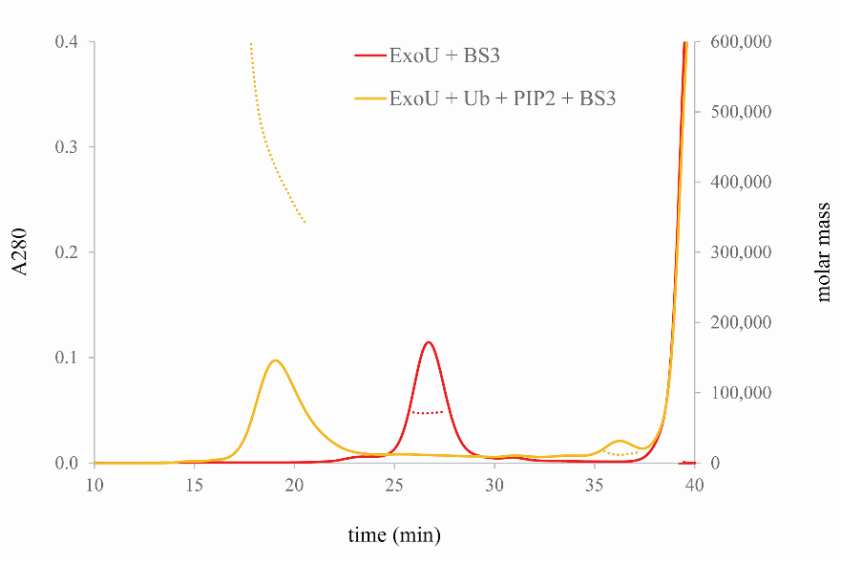
Appendix III



Ubiquitin or PI(4,5)P_2 alone are unable to mediate intragenic complementation.

Phospholipase activity of each truncated ExoU variant alone (apo), with ubiquitin (Ub),with PI(4,5)P_2 (PIP2), or supplemented with both ubiquitin and PI(4,5)P_2 (Ub/PIP2) was measured. Hydrolysis of a synthetic phospholipid substrate was calculated after 24 hours of incubation. Values represent means from three samples and error bars represent standard deviations.

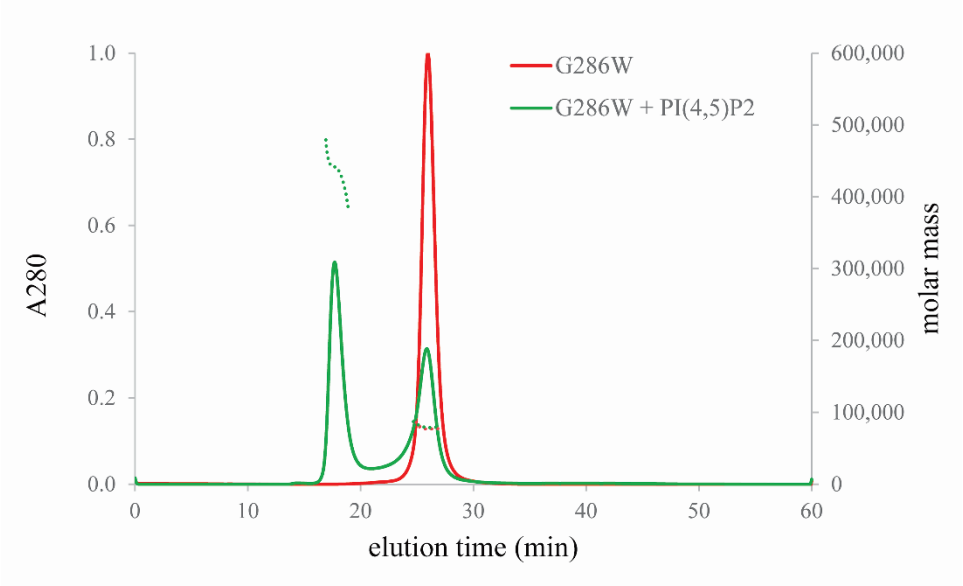
Appendix IV



SEC-MALS on BS3-crosslinked ExoU samples.

Samples containing rExoU alone or with equimolar ratios of its cofactors ubiquitin (Ub) and phosphatidylinositol 4,5-bisphosphate (PIP2) were crosslinked with BS3 then assessed by SEC-MALS. The elution profile from a preliminary experiment is shown. Normalized absorbance at 280 nm (A280, solid line) and molar mass (dotted line) are graphed.

Appendix V



The G286W substitution in the PLA$_2$ domain does not disrupt oligomerization.

Samples containing either purified rExoU-G286W alone or preincubated with an equimolar ratio of PI(4,5)P$_2$ were assessed by SEC-MALS. The elution profile from a preliminary experiment is shown. Normalized absorbance at 280 nm (A280, solid line) and molar mass (dotted line) are graphed.