Interactions of a secreted Pseudomonas aeruginosa aminopeptidase with bacterial outer membrane molecules: characterization and implications for bacterial pathogenic phenotypes.

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Abstract

The protein *Pseudomonas aeruginosa* aminopeptidase (PaAP) is secreted by the opportunistic, Gram-negative bacterial pathogen *Pseudomonas aeruginosa*. PaAP expression appears to be upregulated in clinical isolates of *P. aeruginosa*, especially in strains found in the lungs of cystic fibrosis patients, likely indicating that it serves a role in the development of pathogenic phenotypes. However, the function(s) that PaAP serves in pathogenic contexts are as yet unknown, as are the mechanisms by which PaAP accomplishes them. This work seeks to identify molecules of the Gram negative outer membrane with which PaAP interacts in order to understand the role that PaAP plays in the establishment and maintenance of *P. aeruginosa* infections. Particular attention is given to determining whether PaAP interacts with lipopolysaccharide (LPS), which is the major constituent of the Gram negative outer membrane and a potent toxin and immune system agonist. To accomplish this goal, a range of biochemical analyses are used, particularly immunochemistry techniques. While results from these assays are preliminary, our findings suggest that PaAP does not interact with *P. aeruginosa* LPS, suggesting that proteins or non-LPS saccharides are the primary interaction partners of PaAP in the outer membrane.
Introduction

*P. aeruginosa* is a Gram-negative bacterium that is found ubiquitously in soil and water habitats (Gellatly and Hancock, 2013). However, it is also an opportunistic pathogen, and in particular, persistently colonizes the lungs of patients with cystic fibrosis (CF). CF is caused by mutations in the *CTFR* gene, which encodes a protein that functions as a chloride ion channel in epithelial cell membranes (Ratjen and Döring, 2003). The mutated CTFR protein is incapable of transmitting chloride ions across the cell membrane, producing a number of biophysical problems that cause the characteristic symptoms of CF (Ratjen and Döring, 2003). The mucus that lines the epithelia of CF lungs is unusually viscous, creating a unique microenvironment and severely limiting the ability of CF patients to expel mucus from their lungs by coughing (Matsui et al., 1998; Ratjen and Döring, 2003). The inability to effectively clear pathogens once they have colonized the lungs makes CF lungs particularly vulnerable to chronic bacterial infections (Gellatly and Hancock, 2013; Ratjen and Döring, 2003; Worlitzsch et al., 2002). These infections are difficult to treat, and can persist for twenty years or more, causing lung damage from chronic bacterial infections to be the leading cause of morbidity and mortality in CF patients (Gellatly and Hancock, 2013; Marshall et al., 2015; Ratjen and Döring, 2003). Wholly apart from infecting the lungs of nearly 50% of CF patients, *P. aeruginosa* is also one of the most commonly encountered pathogens in hospitals (Hidron et al., 2008). In particular, *P. aeruginosa* frequently infects immunocompromised patients, and can establish infections in the eyes, skin, and essentially all other tissues (Hidron et al., 2008; Lyczak et al., 2000). As such, *P. aeruginosa* is highly clinically significant, and warrants further research commensurate with the risks that it poses.

A variety of factors contribute to the success of *P. aeruginosa* as an opportunistic pathogen. *P. aeruginosa* can survive in a wide range of environmental conditions and is metabolically
diverse, allowing it to infect virtually all human tissues (Green et al., 1974; Lyczak et al., 2000; Williams and Worsey, 1976). Furthermore, *P. aeruginosa* possesses several traits that make it highly resistant to antibiotics, including an outer membrane that is 10- to 100-fold less permeable to common antibiotics than similar Gram-negative species such as *E. coli*, and a highly effective efflux pump system that it uses to remove antibiotics from itself after they enter the cell (Hancock and Speert, 2000). Additionally, in much the same way as other multi-drug resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA), the widespread use of antibiotics to combat *P. aeruginosa* infections has only increased the species’ drug resistance by selecting for mutants that are resistant to additional classes of antibiotics (Hancock and Speert, 2000). Together, these factors limit the ways in which *P. aeruginosa* infections can be treated; thus, research into the mechanisms by which this species establishes and maintains colonization is needed to inform the development of new, successful therapeutics.

One important mechanism that contributes to the success of *P. aeruginosa* as a pathogen is its ability to form biofilms within hosts, especially in patients who are chronically infected, such as CF patients (Høiby et al., 2010). Biofilms are surface-associated bacterial communities that exist in a structured, bacterially-produced extracellular matrix that includes proteins, polysaccharides, DNA, outer membrane vesicles (which are discussed in greater detail below), and other trace components (Moreau-Marquis et al., 2008; Schooling and Beveridge, 2006). To enter this mode of growth, *P. aeruginosa* gene expression changes dramatically during biofilm development, such that over 50% of the *P. aeruginosa* proteome undergoes a six-fold or greater change in expression level between planktonic and biofilm growth (Sauer et al., 2002). Additional changes include increased production of exopolysaccharides, which are an important component of the biofilm extracellular matrix (Hentzer et al., 2001). Accordingly, biofilms differ greatly from
bacteria growing in planktonic form, and *P. aeruginosa* existing in biofilms therefore presents markedly different clinical challenges. Although components of the biofilm extracellular matrix activate both innate and adaptive immunity, this structure also prevents components of the immune system such as antibodies and leukocytes from accessing and killing the invading bacteria (Høiby et al., 2010). Additionally, *P. aeruginosa* biofilms are less susceptible to antibiotics than planktonic cultures both *in vivo* and *in vitro* (Park et al., 2014). Studies have shown that biofilms slow the diffusion of antibiotics to bacteria and that components of the biofilm matrix bind to certain antibiotics, suggesting that the physical properties of biofilms themselves may be responsible for this reduced sensitivity (Chiang et al., 2013; Jefferson et al., 2005). These findings highlight the clinical significance of biofilms and the need for further research into their formation, maintenance and molecular interactions.

Figure 1: SEM of *Pseudomonas aeruginosa* biofilms shows a structural morphology of densely-packed cells held within a bacterially-produced extracellular matrix. Adapted from Park et al., 2014.
Bacterially-derived outer membrane vesicles (OMVs) are also likely to be important in the ability of *P. aeruginosa* to cause inflammatory disease (Bauman and Kuehn, 2006; Manning and Kuehn, 2013). Gram-negative bacteria possess two cell membranes, an inner and outer membrane, which are separated by a region called the periplasm (Silhavy et al., 2010). These structures differ greatly from each other, and each contains a unique assortment of proteins, lipids, and other molecules that are relevant to their biological functions (Silhavy et al., 2010). OMVs are small structures that are formed when parts of the outer membrane bulge outward and “bleb” off the membrane, forming a detached, closed spherical structure containing periplasmic content encased by an outer-membrane layer (Fig. 2) (Kulp and Kuehn, 2010; Roier et al., 2016). OMV contents are variable between species, and within a given species their composition can change in response to different environmental conditions, such as oxidative stress or biofilm growth (Schwechheimer and Kuehn, 2015). This flexibility allows OMVs to serve numerous purposes, making them a dynamic tool for *P. aeruginosa* to interact with its environment (Manning and Kuehn, 2013). In CF patients, the environment that *P. aeruginosa* colonizes is the diseased CF lung. Accordingly, OMV functions in this context can include transporting virulence factors to host cells and protecting bacteria from outer-membrane targeting antimicrobials such as antibiotics (Manning and Kuehn, 2013). Interestingly, OMVs appear to play vital roles in the establishment and maintenance of *P. aeruginosa* biofilms (Kulp and Kuehn, 2010; Schooling and Beveridge, 2006). In *Helicobacter pylori*, a Gram-negative species that uses OMVs in similar ways as *P. aeruginosa*, exogenously added OMVs were found to induce the formation of biofilms, and biofilms containing OMVs were observed to be thicker and more robust than those lacking them (Yonezawa et al., 2009). Furthermore, the protein contents of OMVs isolated from *P. aeruginosa* biofilms and planktonic cells have been found to vary to an even greater degree than the proteomes of whole
cells, suggesting that OMV-associated proteins are important in establishing biofilm phenotypes (Park et al., 2014). Further supporting this notion is the finding that OMV-associated proteins constitute 30% of the protein diversity found in *P. aeruginosa* biofilm extracellular matrices (Toyofuku et al., 2012). Given the functional advantages conferred by OMVs, their flexible and diverse functionality, and their prominence in *P. aeruginosa* biofilms, it is likely that specific molecules in OMVs are involved in establishing the particular infection phenotypes found in CF patients.

The identification of biomolecules that produce OMV phenotypes and the mechanisms by which these phenotypes emerge is a major research focus of the Kuehn lab, and previous research into *P. aeruginosa* OMVs at the Kuehn lab serves as the basis for this project. By comparing the

![Figure 2: Schematic of OMV production and PaAP secretion. The bacterial membrane is composed of an inner membrane and outer membrane, separated by an aqueous region called the periplasm. OMVs form when parts of the outer membrane become less tightly linked to the periplasm, causing a region of membrane to bulge outward and “bleb” off, forming an enclosed sphere. PaAP is produced in the cytoplasm and secreted via the Type II secretion system, crossing each layer of the bacterial membrane. Adapted from Schwechheimer and Kuehn, 2015.](image-url)
contents of OMVs produced by pathogenic and non-pathogenic strains of *P. aeruginosa*, components were identified which may contribute to the roles of OMVs in pathogenesis. In a paper published in 2006, Bauman and Kuehn compared the protein composition of OMVs isolated from different strains of *P. aeruginosa* and found that a *P. aeruginosa* aminopeptidase (PaAP) was significantly enriched in strains of *P. aeruginosa* that had been isolated from CF patients and other clinical settings when compared to laboratory or environmental strains (Bauman and Kuehn, 2006). PaAP is a zinc-dependent, secreted aminopeptidase that cleaves N-terminal leucine residues from substrate proteins (Cahan et al., 2001). Subsequent quantitative analysis by Bauman and Kuehn suggested that PaAP was at least 65-fold enriched in OMVs from clinical isolates when compared to OMVs from a commonly used laboratory strain, suggesting a functionality in clinical *P. aeruginosa* phenotypes (Bauman and Kuehn, 2006). Several additional experimental findings have supported the notion that PaAP serves a role in pathogenic contexts. In their same 2006 study, Bauman and Kuehn found that human lung epithelial cells that were exposed to *P. aeruginosa* vesicles secreted interleukin-8 (IL-8) at significantly greater concentrations than untreated cells (Bauman and Kuehn, 2006). IL-8 attracts and activates neutrophils *in vivo*, thereby leading to the inflammation of nearby host cells (Bauman and Kuehn, 2006; Kharazmi et al., 1986). Inflammation is a characteristic symptom of chronically infected CF lungs and is a primary contributor to lung epithelial cell damage and loss of lung function (Kharazmi et al., 1986). The ability of *P. aeruginosa* OMVs to elicit IL-8 secretion in lung epithelia therefore suggests that OMVs may share responsibility for the extensive lung damage that occurs in CF patients. Furthermore, following the discovery that *P. aeruginosa* OMVs associate with and are internalized by human lung epithelial cells, it was found that OMVs enriched in PaAP associated at significantly greater rates than OMVs with less or no PaAP (Bauman and Kuehn, 2009). This
finding may indicate that PaAP-enriched OMVs can more efficiently transport virulence factors and other vesicle contents to host cells, thereby allowing *P. aeruginosa* to more effectively interact with its host upon infection. Additional studies have shown that PaAP expression increases 103-fold in the presence of human lung epithelial cells (Chugani and Greenberg, 2007). Because bacterial gene expression is tied to environmental cues, this finding offers further support to the idea that PaAP plays a mechanistic role in *P. aeruginosa* infection phenotypes. Finally, PaAP has been found to be among the most abundant proteins in the *P. aeruginosa* biofilm extracellular matrix and isolated biofilm OMVs (Toyofuku et al., 2012). Because the transition of *P. aeruginosa* to a biofilm mode of growth is characteristic of chronic infections in CF patients, this finding suggests that PaAP may serve a particular function in establishing chronic infection phenotypes (Pritt et al., 2007).

Given the significance of biofilms in chronic CF lung infections, and the apparent links between PaAP, biofilms, OMVs and lung epithelial cells, it appears likely that PaAP plays a role in the establishment of chronic infections by *P. aeruginosa*. However, the mechanistic function that PaAP plays remains unknown and requires further research. This work seeks to address this question by identifying PaAP interaction partners in order to build a more robust model of the mechanisms and functions of PaAP in *P. aeruginosa* pathogenesis. Preliminary results from the Kuehn lab have suggested that PaAP binds differentially to vesicles expressing different forms of lipopolysaccharide (LPS), which is the major constituent lipid of the outer leaflet of the bacterial outer membrane (Fig. 3). Therefore, particular attention is given to identifying and characterizing the putative interaction between PaAP and LPS. Using various biochemical and molecular biological techniques to determine the localization of PaAP and LPS under various conditions, the interactions between PaAP and a number of variants of LPS are characterized. Employed
techniques include immunochemistry assays such as Far Western blotting and co-immunoprecipitation, and biochemical assays such as co-localization staining and electrophoretic mobility shift assays. With these analyses, we hope to determine whether PaAP binds specifically to LPS, and, if it does, to what chemical moieties it binds. From these data, we hope to build a

Figure 3: Binding and co-sedimentation assay reveals differential binding of PaAP to OMVs produced by *P. aeruginosa* LPS mutants. OMVs isolated from various mutant strains of *P. aeruginosa* producing various isoforms of LPS were treated with PaAP or Bovine Serum Albumin (BSA) as a control, sedimented by centrifugation, separated by PAGE and visualized by Western blotting against PaAP with α-2939 primary antibody. PaAP+ samples are boxed in red, and PaAP is indicated with asterisks. PaAP is present only in the supernatants of strains AK957 and AK1401, indicating that it did not bind to the sedimented OMVs. Conversely, PaAP is present in both supernatants and pellets of strains AK44 and AK1012, indicating that it bound to and sedimented with the OMVs produced by these strains. MW ladder indicated in kDa. Blots generated by Dan Rodriguez. Used with permission from Dr. Meta Kuehn.
more complete picture of the role that PaAP plays in OMV functionality and bolster our understanding of how OMVs contribute to bacterial pathogenesis. Together, a better understanding of the mechanisms by which \textit{P. aeruginosa} establishes infections will allow future insight into potential therapies.

**Materials and methods**

**Cell lines and strains**

The clinical \textit{P. aeruginosa} isolate strain S470 was used as the primary subject of this research study, and was used in the isolation of membrane molecules and native PaAP (Duke University Hospital). Strain S470APKO5 (S470-ΔPaAP) was used to purify membrane components (Bauman and Kuehn, 2009). \textit{E. coli} strain BL21-pRlap expressing transgenic PaAPΔ53K was used as an expression platform for synthesis of PaAP.

**PaAP purification from \textit{E. coli} inclusion bodies**

BL21-pRlap was grown overnight at 37°C with agitation in Superbroth containing chloramphenicol (25 µg/mL). The culture was diluted to OD$_{600}$=0.1 in fresh media and grown to OD$_{600}$=0.5, at which point 1mM IPTG was added to the culture to induce PaAP expression. The culture was incubated as above for 2 hours, and cells were collected by centrifugation in a SH-3000 (BK) rotor at 3500rpm for 15 minutes at room temperature. Cells were frozen at -80°C, then thawed and resuspended in lysis buffer (for this and all other buffers see Table 1). Cells were disrupted in a M-110L Microfluidizer® Materials Processor (Microfluidics). Recovered PaAP inclusion bodies were then washed 3 times each with inclusion body wash buffers a, b, then c for a total of 9 washes. Pellets were resuspended in 5 mL of solubilization buffer and insoluble material was removed with a final centrifugation. The supernatant was diluted in refolding solution
to a final concentration of 50 µg/mL, and PaAP was refolded in a 10°C water bath for 48 hours with agitation. Refolded PaAP was dialyzed against cold dialysis buffer overnight.

PaAP activity was assayed using PaAP activity assay described by Sarvonsky (Sarvonsky et al., 2009). 20-200 pmol protein and 120 nmol L-leucine p-nitroanilide (Leu-NA-HCl) were diluted into 200 µL AP buffer, allowed to incubate at room temperature for 0-30 minutes, and activity was measured by determining OD405. Dialyzed PaAP was concentrated by centrifugation using 10 kDa-pore centrifuge filters and stored at -20°C at a final concentration of 250 µg/mL.

Membrane fractionation.

*P. aeruginosa* outer membranes were isolated using a selective detergent treatment protocol adapted from Thein et al. (Thein et al., 2010). LB broth containing carbenicillin (100 µg/mL) was seeded with stationary-phase S470 culture and incubated overnight at 37°C with agitation. Cells were pelleted by centrifugation, and each pellet was then resuspended in 1mL of resuspension buffer. Lysozyme (1mg) was added to each sample, and the solution was incubated for 5 minutes at room temperature. 4 mL of deionized water was added to each sample, and samples were mixed thoroughly and incubated for half an hour at room temperature to allow spheroplast formation. 6 mL of OM lysis buffer was added to each sample, followed minimal powdered DNase, and samples were mixed until translucent, homogeneously viscous, and no longer cloudy. Samples were then combined and centrifuged in a Beckman TLA-100.3 rotor at 41,000 rpm at 10°C for one hour. The supernatant was discarded, and pellets were washed in OM lysis buffer and spun down as above. The supernatant was discarded, pellets were resuspended in dH2O to wash, split into 500 µL aliquots (each corresponding to 25 mL of the original culture) and spun down at 12,000 rpm in a tabletop centrifuge for 5 minutes at room temperature. Pellets were washed again with dH2O,
followed by OM lysis buffer, and finally an additional 4 washes with dH$_2$O. Supernatants were removed, and pellets were stored at -20°C for later use.

_Far Western blots._

A549 human lung epithelial cells were grown to confluence in 10 cm plates, washed, and harvested in lysis buffer. Cells were washed again in lysis buffer and resuspended in SDS-PAGE loading buffer. 5 mL cultures S470-ΔPaAP cells were grown overnight from freezer stocks, pelleted, and resuspended in SDS-PAGE loading buffer. Samples were run on a 12% Mini-PROTEAN® TGX Stain-Free™ gel (Bio-Rad Catalog #4568045) and blotted onto a nitrocellulose membrane. Membranes were blocked with Odyssey blocking buffer for 1 hour at room temperature with rotation, and PaAP was added to a final concentration of 2.5 µg/mL. Blots were incubated overnight at 4°C with rotation, then washed 3 times with TBST to remove unbound PaAP. PaAP was then visualized by Western blotting using α-2939 rabbit polyclonal antibody and Odyssey goat anti-rabbit fluorescent secondary antibody and imaged using an Odyssey imaging apparatus.

PaAP co-sedimentation

1. _OM co-sedimentation._ S470-ΔPaAP membrane extract pellets were resuspended in 150 µL PBS + 1% bovine serum albumin (BSA), and each sample was then split into two 75 µL samples. Whole-cell samples were obtained from cultures seeded with frozen glycerol stocks of S470-ΔPaAP grown overnight at 37°C with rotation in LB broth containing carbenicillin. Aliquots of overnight culture were pelleted by centrifugation and resuspended in 150 µL PBS + 1% BSA. Additionally, a mock trial to be used as a negative control was made by adding 150 µL of PBS + 1% BSA to a sterile Eppendorf tube. 500 ng of PaAP from vesicle-free supernatant was added to treated samples, while nothing was
added to untreated control samples. Samples were incubated for 1 hour at 37°C to allow
binding of PaAP, then centrifuged at 20,000 rpm in a table-top centrifuge for 7.5 minutes
at room temperature. Pellets were washed 3 times in PBS, and following the final wash
were resuspended in SDS-PAGE loading buffer and frozen at -20°C until later use.

Frozen samples were thawed by boiling and treated by pulse sonication before being boiled
again for 5 minutes. Samples were then loaded into a 12% Mini-PROTEAN® TGX Stain-
Free™ gel (Bio-Rad Catalog #4568045). The gel was run at 100V for 40 minutes and then
120V for 1 hour, and gel contents were transferred to a PVDF membrane (GE Healthcare,
Product Code RPN303F). The blot was then visualized by Western blotting using rabbit α-
2939 polyclonal antibody and Odyssey goat α-rabbit fluorescent secondary antibody, both
in Odyssey blocking buffer, and imaged using an Odyssey imaging apparatus.

2. *LPS co-sedimentation.* Two samples of 100 µg of S470 LPS were diluted in PBS + 1%
BSA, and one mock trial was made by the addition of PBS + 1% BSA to a sterile Eppendorf
tube. The samples were blocked at 37°C for one hour in a water bath, and 500 µg of PaAP
from vesicle free supernatant was added to one of the tubes containing LPS and to the
mock, while 10 µL of PBS + 1% BSA was added to the LPS control sample. All samples
were incubated at 37°C for 1 hour, then centrifuged at 42,000 rpm in a Beckman TLA-
100.3 rotor for 2 hours at 10°C. Pellets were not visible, so the supernatant was carefully
removed and stored for later analysis, and 150 µL of dH₂O was added to the tube as if
resuspending “invisible” pellets to wash the samples. Samples were spun down again, and
washes were repeated twice for a total of 3 washes. After the final wash, samples were resuspended in SDS-PAGE loading buffer and frozen at -20°C until later use.

LPS ± PaAP supernatant aliquots were thawed at room temperature and diluted into SDS-PAGE loading buffer. LPS ± PaAP pellet and supernatant samples were treated by boiling, and each sample was pulse sonicated three times with multiple quick pulses before being boiled again. Samples were run on 15% acrylamide SDS-polyacrylamide gels at 50V for half an hour before voltage was increased to 80V. Samples were run until loading dye reached the bottom of each gel.

Gels were trimmed, and one gel was stained following instructions for Silver Stain Plus™ (BioRad, Catalog # 161-0449). The contents of the remaining gel were transferred to nitrocellulose membrane for Western blotting. PaAP on the membrane was then visualized using Western blotting as in the OM co-sedimentation analysis.

Protein-lipid overlay assays.

Various LPS and other biologically relevant samples were spotted directly onto nitrocellulose membranes for Far Western blot analysis. PaAP from vesicle-free S470 supernatant was diluted in PBS to a final concentration of 25 µg/mL. S470-ΔPaAP crude membrane extracts were thawed and resuspended in 1 mL PBS + 1% SDS. LPS samples from the following sources were diluted to a final concentration of 500 µg/mL in spotting buffer or dH2O: S470, PAO1, Salmonella typhimurium (wild type), S. typhimurium (truncated mutant Re), E. coli (truncated mutant Rd), and V. cholerae (wild type). E. coli total membrane extract was also diluted in spotting buffer or dH2O to a final concentration of 500 µg/mL. Samples were spotted directly onto each of two
nitrocellulose membranes by application with capillary spotting pipets; 3 1 µL applications of each sample were spotted per sample.

Membranes were left to dry at room temperature in the dark for one hour, then blocked in fat blot buffer + 3% milk for one hour at room temperature with rotation. Blocking buffer was replaced by fat blot buffer + 3% milk + 0.1% Tween-20. PaAP from vesicle-free supernatant was added to one blot to allow binding to spotted samples (final concentration 50 ng/mL), while the other was left as an untreated control. Blots were incubated overnight at 4°C with shaking, and then washed with fat blot buffer + 0.1% Tween 3 times for 5 minutes each at room temperature with agitation. Following washes, the blots were subjected to Western blotting using rabbit α-2939 polyclonal primary antibody and fluorescent Odyssey goat α-rabbit secondary antibody, both in fat blot buffer + 3% milk.

Additional trials were performed as above using S470 LPS (1 µg/mL) as a probe instead of PaAP. Blots were spotted, blocked, and probed as previously. Blots were then visualized one of two ways:

1) blots were treated with rabbit anti-\textit{P. aeruginosa} antibody ab68538 (Abcam) and visualized as above, or

2) blots were treated with mouse anti-\textit{E. coli} LPS antibodies and visualized using a fluorescent Odyssey goat α-mouse secondary antibody.

All blots were imaged on an Odyssey imaging apparatus, and patterns of fluorescence were analyzed to determine where the PaAP or LPS probes were localized on the blots.

\textit{Co-immunoprecipitation}.

Dynabeads® Protein A for Immunoprecipitation (ThermoFisher Scientific Catalog #10001D) were resuspended by gentle vortexing, pelleted with a handheld magnet, and resuspended in PBST
containing 50 µg/mL α-2939 polyclonal antibody. Beads were incubated with rotation for 10 minutes then washed once with PBST and twice with conjugation buffer. Beads were then resuspended in conjugation buffer containing 5mM BS3 crosslinking reagent (ThermoFisher Scientific Catalog #21580). Crosslinking proceeded with incubation as above for 30 minutes, then was quenched by the addition of 1M Tris HCl (pH 7.5) at 1/20th of the sample volume. Quenching proceeded with incubation for 15 minutes, and beads were then pelleted and washed 3 times with PBST. +PaAP samples were resuspended in PaAP diluted to 50 µg/mL in PBST, while –PaAP controls were resuspended in PBST. Samples were incubated for 30 minutes to allow PaAP binding, pelleted, and washed 3 times with PBST. +LPS samples were resuspended in LPS diluted to 10 µg/mL in PBST, while –LPS controls were resuspended in PBST. Samples were incubated for 1 hour to allow LPS binding, and beads were then pelleted and thoroughly washed with PBST, incubating as above for 10 minutes, for a total of 3 washes. Samples were magnetically pelleted, resuspended in 2X SDS-PAGE loading buffer, and incubated at 55°C for 10 minutes to elute antibody-bound molecules. Supernatants were collected and stored at -20°C for later analysis.

Samples were then thawed in a 95°C heating block and run on polyacrylamide gels (either 15% polyacrylamide with SDS or 4–20% Mini-PROTEAN® TGX™ Precast Protein Gel [Bio-Rad Catalog #4561094]). Gels were visualized by silver staining following the instructions for Silver Stain Plus™ or by dual-stain colocalization followed by silver staining. Dual-stained gels were first stained using Pro-Q® Emerald 300 Glycoprotein Gel and Blot Stain Kit (ThermoFisher Scientific Catalog #P21857) per the company’s instructions to visualize saccharides (i.e. LPS). Band locations were determined by photographing gels visualized by UV302 transillumination. Gels were then stained using SYPRO® Ruby Protein Gel Stain (ThermoFisher Scientific Catalog #S12000) per the company’s instructions to visualize proteins (i.e. PaAP), and band locations were
again determined by photographing gels visualized by UV\textsubscript{302} transillumination. The positions of saccharide bands were then compared to determine whether co-colocalization of PaAP and LPS had occurred. The position of the LPS control was noted in silver stained gels, and other lanes were compared to the LPS control to determine whether LPS was present.

*Electrophoretic mobility shift assay.*

Two 25 µg and one 10 µg samples of PaAP were diluted into 100 µL of dialysis buffer. 25 µg of S470 LPS was added to one of the 25 µg and the 10 µg PaAP samples, while the remaining 25 µg sample was left untreated as a control. 25 µg of LPS was also diluted into 100 µL of dialysis buffer as an additional control. Each sample was split into two separate aliquots, and all samples were incubated at 25°C or 37°C for half an hour to allow binding of PaAP to LPS. Samples were then added to native PAGE loading buffer, loaded into a non-denaturing 4–20% Mini-PROTEAN\textsuperscript{®} TGX™ Precast Protein Gel (Bio-Rad Catalog #4561094), and run at 80V for approximately 150 minutes using native running buffer.

Gels were then visualized either by Ruby staining or Western blotting. Ruby stained gels were stained using SYPRO\textsuperscript{®} Ruby Protein Gel Stain (ThermoFisher Scientific Catalog #S12000) per the company’s instructions and visualized on a UV\textsubscript{302} transilluminator. Gels and nitrocellulose membranes to be Western blotted were soaked in native transfer buffer for 10 minutes at room temperature prior to transfer. The transfer was carried out at 15V for 30min. Membranes were then treated by Western blotting with α2939 rabbit polyclonal antibody and Odyssey goat α-rabbit 2° and visualized on an Odyssey imaging apparatus.

To test whether PaAP behaved as expected under native PAGE conditions, an additional gel was run using two PaAP samples: native PaAP in native PAGE loading buffer and denatured PaAP in
SDS-PAGE loading buffer as a reference. Samples were loaded, run, and visualized as described above.

**Table 1: Buffers and Reagents**

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis buffer</td>
<td>18 mL 50 mM Tris, 20 mM EDTA, pH 8.0 [TE], 0.8 mL lysozyme [5mg/mL in dH₂O], 2.0 mL 5M NaCl, 2.0 mL Triton X-100 [25%v/v solution in dH₂O]</td>
</tr>
<tr>
<td>Inclusion body wash buffers</td>
<td>a) 50 mM Tris, 20 mM EDTA, pH 8.0 + 1% Triton X-100</td>
</tr>
<tr>
<td></td>
<td>b) 50 mM Tris, 20 mM EDTA, pH 8.0</td>
</tr>
<tr>
<td></td>
<td>c) 50 mM Tris, pH 8.0</td>
</tr>
<tr>
<td>Solubilization buffer</td>
<td>6 M guanidine HCl, 50 mM Tris, pH 8.0</td>
</tr>
<tr>
<td>Refolding solution</td>
<td>0.5 M arginine, 50 mM Tris pH 8.0, 1 mM CaCl₂, 50 µM ZnCl₂</td>
</tr>
<tr>
<td>AP Buffer</td>
<td>50 mM Tris HCl, pH 8.0, 1 mM CaCl₂, 50 µM ZnCl₂</td>
</tr>
<tr>
<td>Dialysis buffer</td>
<td>20 mM Tris HCl, pH 8.0, 50 mM NaCl, 1 mM CaCl₂, 50 µM ZnCl₂</td>
</tr>
<tr>
<td>Resuspension buffer</td>
<td>0.2 M Tris HCl pH 8.0, 1 M sucrose, 1 mM EDTA</td>
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<tr>
<td>OM lysis buffer</td>
<td>50 mM Tris HCl pH 8.2, 2% w/v Triton X-100, 10 mM MgCl₂</td>
</tr>
<tr>
<td>Spotting buffer</td>
<td>250:500:200:2 of Chloroform:Methanol:50 mM HCl:Ponceau S</td>
</tr>
<tr>
<td>Fat blot buffer</td>
<td>50 mM Tris HCl, pH 7.5, 150 mM NaCl</td>
</tr>
<tr>
<td>SDS-PAGE loading buffer</td>
<td>0.5 M Tris-HCl, pH 6.8, 10% glycerol, 1% w/v SDS, 0.29% β-mercaptoethanol, 0.01% v/v of 2% bromophenol blue</td>
</tr>
<tr>
<td>TBS</td>
<td>50 mM Tris HCl, 0.15 M NaCl, pH 7.4</td>
</tr>
<tr>
<td>TBST</td>
<td>50 mM Tris HCl, 0.15 M NaCl, pH 7.4 + 0.1% Tween-20</td>
</tr>
<tr>
<td>SDS-PAGE running buffer</td>
<td>14.4 g/L glycine, 3 g/L Tris HCl, 1 g/L SDS, pH ~8.4</td>
</tr>
<tr>
<td>SDS-PAGE transfer buffer</td>
<td>0.2 M glycine, 25 mM Tris HCl, 2% w/v SDS, 20% methanol</td>
</tr>
<tr>
<td>Odyssey blocking buffer</td>
<td>TBS + 3% w/v milk + 0.2% v/v NaN</td>
</tr>
<tr>
<td>Native PAGE loading buffer</td>
<td>0.2 M Tris HCl, pH 8.6, 20% glycerol, 0.005% bromophenol blue</td>
</tr>
<tr>
<td>Native PAGE running buffer</td>
<td>0.24M Tris, 1.92M glycine</td>
</tr>
<tr>
<td>Native PAGE transfer buffer</td>
<td>10X: 0.12M Tris, 4.8M glycine. 1X: 1:10 of 10X in dH₂O</td>
</tr>
<tr>
<td>Conjugation buffer</td>
<td>100 mM sodium phosphate, 150 mM NaCl</td>
</tr>
<tr>
<td>PBST</td>
<td>PBS + 0.1% Tween-20</td>
</tr>
<tr>
<td>Protein molecular weight standards (Western blots)</td>
<td>Precision Plus Protein™ Dual Color Standards (Bio-Rad catalog #1610374)</td>
</tr>
<tr>
<td>Protein molecular weight standards (Gel stains)</td>
<td>Precision Plus Protein™ Unstained Protein Standards (Bio-Rad catalog #1610363)</td>
</tr>
</tbody>
</table>
Figure 4: outline of major assays used in this study, and candidates for PaAP binding used in each. Not pictured are the isolation of PaAP from *E. coli* inclusion bodies or from vesicle-free supernatants.
Results

Far Western blotting reveals multiple interaction PaAP interaction partners in both mammalian and bacterial cells

Far Western blotting assays were used to determine whether PaAP could bind specific components in bacterial and mammalian cells. Similarly to standard Western blots, Far Western blots begin by separating samples by SDS-PAGE and transferring them to a nitrocellulose membrane. However, rather than visualizing samples at this stage, a native-state “probe” protein (in this case PaAP) is applied to the blot, where it binds to “targets” (PaAP interaction partners) on the membrane. The probe protein is then subsequently visualized, and each band on the blot represents a distinct target for the probe (Wu et al., 2007).

In this study, the contents of whole-bacterial lysates of *P. aeruginosa* strain S470ΔPaAP and mammalian lung epithelial A549 cell lysates were used as target samples, and PaAP was used as a probe. PaAP binding was then detected with α-2939 polyclonal anti-PaAP primary antibody followed by Odyssey goat secondary antibody (Fig. 5). Untreated PaAP was used as a positive control for antibody activity, and to demonstrate where PaAP localized on the gel relative to non-
PaAP species to which it bound (lane 2). The broad smear is visible extending below the expected position of PaAP (56 kDa) represents multiple degradation products of various molecular weights, rather than distinct entities with which the antibody reacted. The multiple bands that are observed for the lysate sample of strain S470-ΔPaAP (lane 3), which does not itself produce PaAP, therefore represent non-PaAP components in the sample to which PaAP bound. This suggests that PaAP can bind at least four distinct molecular weight components in bacteria. Additionally, a faint but distinct band was visible in the sample containing a lysate of mammalian lung epithelial cells (lane 4). This band indicates that PaAP may bind a low molecular weight component of the mammalian cell.

**PaAP co-sediments with bacterial membrane components**

Native co-sedimentation assays were used to reveal PaAP binding partners in a context that better replicates physiological conditions. In this assay, *P. aeruginosa* outer membrane components were isolated using the selective detergent treatment protocol described by Thein, in which nonionic, nondenaturing detergents are used to isolate membrane species and separate them from other cellular components (Johnson, 2013; Thein et al., 2010). Fractionated membranes and nondenatured whole-cell samples were treated with PaAP in nondenaturing conditions or left untreated as a control, incubated to allow PaAP to bind interaction partners. Centrifugation was used to separate less-soluble cellular components, (including membrane molecules) from soluble components (such as cytosolic proteins in whole-cell samples and unbound PaAP), and after extensive washing to remove unbound material, PaAP that co-sedimented with these components was then visualized by anti-PaAP Western blotting (Fig 6). A fluorescent band at 56 kDa would indicate that PaAP had bound to a species in the membrane or whole cell samples, while fluorescent bands at other molecular weights would indicate either degradation products of PaAP.
that bound to species, or background binding by the antibody to non-specific components in the samples. The latter two possibilities could be distinguished based on whether the bands were present in both the incubations with and without PaAP, or not. The presence of a discrete band at 56 kDa in the membrane +PaAP sample (lane 3) and the absence of this band in the –PaAP sample (lane 4) shows that exogenously added PaAP co-sedimented with bacterial membranes. In addition to this band, both membrane and whole cell samples incubated with and without PaAP (lanes 3 through 6) have a single band at approximately 20kDa, with a streak proceeding from the band to the end of the gel, indicating this band is not PaAP-dependent and may be due to non-specific reactivity of the antibody with a component in the bacterial membrane. Both PaAP-treated and untreated whole cell samples possessed identical fluorescence patterns (lanes 5 and 6), suggesting these were anti-PaAP cross reactive species and not indicative of PaAP binding.

**Figure 6: PaAP co-sediments with fractionated P. aeruginosa membranes.** Fractionated membrane and whole-cell samples were treated with PaAP or left untreated, incubated to allow binding, and pelleted by centrifugation. PaAP was then visualized Western blotting. PaAP in the control lane is marked with a red asterisk, and all other bands that represent antibody binding are marked with white asterisks. Lanes: 1. Protein molecular weight standards with molecular weights in kDa, 2. untreated PaAP, 3. fractionated membrane + PaAP, 4. fractionated membrane – PaAP, 5. whole cell + PaAP, 6. whole cell – PaAP, 7. mock (+ PaAP).
Direct co-sedimentation of PaAP with LPS was also assessed (Fig. 7). This assay was more specific than previous co-sedimentation attempts because it could have shown that PaAP bound to LPS directly, whereas whole cell or OM co-sedimentation did not identify the molecules to which PaAP bound. In this protocol, PaAP and LPS were co-incubated to allow binding (LPS incubated without PaAP was used as a control), washed repeatedly to remove unbound material from LPS, then samples containing either sedimented LPS or unbound material in the first supernatant were loaded on SDS-PAGE, transferred to nitrocellulose, and the membrane subjected to anti-PaAP Western blotting. Unfortunately, in these samples, PaAP was only visible in the PaAP-loaded positive control lane, but was not visible in any of the pellets or the supernatant samples of the co-sedimentation incubations. The fact that there was no PaAP detected even in the supernatant samples when PaAP was added (lanes 4, 8), suggested that the amount of PaAP used in these assays was insufficient to detect.

*α*-2939 polyclonal antibody and other primary antibodies react nonspecifically to lipids in PLO blot assay

To visualize PaAP interactions with LPS or other lipids, multiple Far Western protein-lipid overlay (PLO) blots were conducted, using a variety of spotting conditions and antibodies. In these assays,
if the PaAP binds to the sample spotted on a membrane, then that spot will fluoresce when incubated with a fluorescently-tagged antibody raised the α-2939 anti-PaAP antibody. If the spots fluoresce only in blots treated with the probe molecule, but not in blots that are not treated, then the signal indicates that the probe molecule is specifically binding to the spotted content. The lipids spotted in the PLO blots conducted in this study did show fluorescence, but the spots were fluorescent in blots both PaAP-treated and untreated conditions indicating the anti-PaAP antibody bound the lipids independent of PaAP.

In the first blots conducted in this manner (Fig. 8), PaAP, S470 LPS, S470-ΔPaAP outer membranes, E. coli total membrane extract, and V. cholerae LPS all fluoresce, regardless of whether they were treated with the PaAP probe (Fig. 8B). Similar results were found across multiple trials, including trials using new samples and reagents, and in trials using dH2O instead of fat blot spotting buffer to apply samples to the membrane.

Additional attempts to visualize PaAP interacting with LPS were made using LPS as the probe molecule, and PaAP spotted onto the membrane. These assays were conducted in the same manner as those described previously, with PaAP and the LPS spotted onto a membrane then incubated
with LPS to allow binding. Blots were then visualized with either anti-\textit{E. coli} LPS antibody (Fig. 9) or anti-\textit{Pseudomonas} antibody ab68538 (Fig. 10). In addition to PaAP and LPS spots, the same lipid and membrane samples that were used in the blot shown in Fig. 8 were also included in these blots. The goal of including these additional samples was not to determine whether the LPS probe bound to the spotted lipid/membrane samples, as this was not relevant to the questions posed in this study, but rather to determine whether the primary detecting antibodies being used in these trials also cross-reacted with these samples.

The LPS-probed blots produced different patterns of fluorescence from those observed in trials using PaAP as the probe and the \textit{α}-2939 antibody for detection. However, similarly to these initial trials, all blots probed with LPS and visualized with anti-LPS antibodies produced the same patterns of fluorescence, regardless of whether they were treated with the LPS probe. In particular, the \textit{E. coli} LPS antibody (Fig. 9) detected neither S470 LPS nor PaAP, while ab68535 (Fig. 10) detected both molecules, making each antibody unusable in the PLO assays. Additionally, each of the anti-LPS antibodies cross-reacted with a number of other molecules, further adding to their unsuitability for further use in this work.
Therefore, while all +probe blots showed some degree of fluorescence, this was insufficient to determine whether interaction had occurred because the −probe controls showed the same behavior. Due to differences in fluorescence brought about by stochasticity and slight variation of experimental conditions, the quantification of spot brightness between blots was not feasible. Therefore, quantitatively comparing spot brightness between blots either treated or not treated with probe was an invalid way to determine whether the probe molecule was interacting with any spotted samples. It is therefore impossible to determine whether PaAP bound to any of the spotted samples with these results.

Elecrophoretic mobility shift was not observed in S470 LPS co-incubated with PaAP.

In the electrophoretic mobility shift assay (EMSA), proteins are subjected to electrophoresis using buffers and a gel that lack denaturing agents, such as SDS. Under these conditions, PaAP should retain its active conformation and its ability to bind other molecules through non-covalent interactions that rely on secondary or tertiary protein structures. In native PAGE, LPS-bound PaAP would differ from unbound PaAP in its mass, charge, and other biochemical characteristics, causing bound and unbound PaAP to migrate different distances on the gel. In preparation for the mobility shift assay, various concentrations of PaAP were co-incubated with S470 LPS in PaAP
dialysis buffer, in which PaAP is stable and correctly folded as determined by PaAP activity assays conducted at multiple points during long-term periods of storage. Samples were incubated at room temperature or 37°C to allow the PaAP to bind LPS under conditions that mimic either the general environment (i.e. room temperature) or the human body (37°C). Samples were then separated by native PAGE as described above, and Ruby staining was used to visualize proteins in the gel (Fig. 11). Surprisingly, gels showed a strong and similar laddered pattern in all lanes containing LPS, including the LPS-only control, suggesting that the Ruby stain had visualized LPS; conversely, no signal was visible in the PaAP-only control lane.

![Figure 10: EMSA fails to show presence of binding of PaAP to S470 LPS. LPS and PaAP were co-incubated at 37°C and 25°C and subjected to non-denaturing PAGE. Samples were detected by Ruby staining. Lanes: 1 and 6: Protein molecular weight standards with masses indicated in kDa, 2 and 7: PaAP (25µg), 3 and 8: LPS, 4 and 9: PaAP (25 µg) + LPS, 5 + 10: PaAP (10 µg) + LPS. Samples 1-5 were incubated at 37°C, while samples 6-10 were incubated at 25°C. All LPS lanes contained 25 µg S470 LPS.](image-url)
The lack of any bands in the PaAP control lane suggested that PaAP had not migrated through the gel as expected, and may instead have accumulated in the loading well. To determine whether this was the case, an additional native PAGE was run in the same manner as the EMSA, but only SDS-treated PaAP and native PaAP samples were used. After the run, the contents of the gel were transferred to a nitrocellulose membrane and visualized by Western blotting with α-2939 polyclonal rabbit antibody and Odyssey goat anti-rabbit fluorescent secondary antibody (Fig.12). SDS-treated PaAP is clearly visible as a distinct band at approximately 60 kDa, which is slightly greater than the predicted mass of 53 kDa. In the native PaAP lane, however, there was little-to-no evidence of protein entering the gel, although there is a smudge and relatively intense fluorescent species at the top of the gel, near the well.

*LPS does not co-immunoprecipitate with PaAP*

In the co-immunoprecipitation assay used in this study, antibodies coupled to magnetic beads are used to specifically pull a target antigen out of solution, as well as any molecules to which the antigen is bound. After washing to remove nonspecifically bound contents, the interacting
molecules can be separated by SDS-PAGE and visualized. This assay therefore operates under similar principle as co-sedimentation by allowing native-state molecules to interact in solution; however, in co-immunoprecipitation, the specificity of the capturing antibody for the target antigen determines which components are pelleted, rather than differential solubility as in co-immunoprecipitation. Due to the strength and specificity of antigen-antibody interactions, and the ease with which magnetic beads can be separated from the supernatant, this assay therefore allows for greater specificity than co-sedimentation while also using fewer reagents.

For this co-immunoprecipitation assay, the polyclonal PaAP-reactive α-2939 antibody was used as the capturing antibody, and PaAP was used as the bait antigen. Beads coated in antibody-anchored PaAP were then treated with LPS, beads were washed thoroughly to remove any unbound or non-specifically bound material, and antibody-bound material was eluted from the beads using 2X SDS-PAGE loading buffer before being separated by SDS-PAGE and visualized by silver staining, or Ruby and Emerald stains (Fig. 13, and data not shown). In the silver-stained gels (Fig. 13A), both immunoprecipitation reactions that contained PaAP, with and without LPS, showed characteristic bands at 53 and 22 kDa, which represent the fully processed active form of PaAP and an abundant degradation fragment of PaAP, respectively. The fact that PaAP was present in these incubations regardless of LPS was expected because anti-PaAP antibody was used in immunocapturing. The immunoprecipitation sample that contained LPS but lacked PaAP (lane 5) showed bands at approximately 50 and 25 kDa. Smudges at these locations also might be present in the immunoprecipitation samples that contained PaAP, with (lane 4) or without (lane 6) LPS. Antibodies are large, heavy proteins with a molecular weight of approximately 150 kDa, and are composed of two linked polypeptides termed the H and L chain (Janeway et al., 2001). The H chain weighs approximately 50 kDa, and the L chain approximately 25 kDa (Janeway et al., 2001).
The bands in lane 5 therefore likely represent the H and L chains of the capturing antibody. The untreated LPS control lane (i.e. directly added, not immunoprecipitated LPS) shows a distinct silver-stained band at approximately 12.5 kDa, which is within the normal range for the molecular weight of LPS. However, this lane is not present in the PaAP+LPS immunoprecipitation sample (lane 5), indicating that LPS did not co-immunoprecipitate with PaAP. In the Ruby stained gel, (Fig. 13B), all the same bands are visible as in the silver stained gel with the exception of the band in the LPS control lane. This is expected since Ruby stain does not typically detect LPS. Emerald staining (used to visualize LPS) of this gel did not produce any visible bands, and thus the image of the gel is not pictured. It is not clear why lane 3 (LPS-only control) did not yield a visible band upon Emerald staining.

Figure 12: LPS does not co-immunoprecipitate with PaAP. α-2939 polyclonal antibody was crosslinked to magnetic beads and used to immunoprecipitated PaAP and molecules bound by PaAP. Beads were treated with 12.5 µg PaAP, followed by 5 µg S470 LPS. Samples were separated by SDS-PAGE and visualized using silver and Ruby staining. PaAP bands indicated with asterisks, and bands at 50 kDa and 25 kDa are marked with arrows. A 12.5 kDa band in the LPS-only control lane is marked with two asterisks. A) Gel following silver staining. B) The same gel following ruby staining. Lanes: 1. Protein molecular weight standards with masses indicated in kDa, 2. untreated PaAP control, 3. untreated LPS control, 4. PaAP + LPS co-IP, 5. LPS-only co-IP, 6. PaAP-only co-IP.

An additional co-IP trial was conducted to confirm the results of the first, then visualized after separating samples on a 4-20% gradient polyacrylamide gel (Fig. 14). In this gel, the PaAP control
lane showed characteristic bands at 53 and 22 kDa (lane 2), in addition to multiple higher molecular weight bands, likely impurities in this sample. Similar bands were observed in the PaAP-containing co-immunoprecipitation samples with (lane 4) and without (lane 6) LPS; these bands have apparent molecular weights of approximately 60 and 22kD, and these samples also have multiple higher-weight bands, similar to the PaAP-only control (lane 2). An uneven line at the leading edge of the samples in lanes 4, 5 and 6 demonstrates that the samples ran unevenly, and that therefore the ~60 kDa bands in lanes 4 and 6 actually represent PaAP. In contrast to the gel shown in Fig. 13, the LPS control lane in Fig. 14 (lane 3) has a strong, broad band from approximately 55-65 kDa, in addition to an additional broad band of much higher molecular weight. Unfortunately, the “diagnostic” LPS band at 12 kDa (see in Fig 13A) ran off the bottom of this gel. Nevertheless, as in Fig. 13, LPS bands (the 55-65 kDa and larger bands) were not present in the +PaAP/+LPS co-immunoprecipitation lane (lane 5), indicating that LPS did not co-precipitate with PaAP on the beads.

Figure 13: follow-up co-IP trial suggests that LPS does not co-immunoprecipitate with PaAP. PaAP and LPS were used in a co-immunoprecipitation assay as pictured in Fig. 13, using new samples, and visualized by silver staining. PaAP bands are marked with single asterisks, and prominent bands in the LPS-only control lane are marked with double asterisks.
Discussion

This study sought to confirm that PaAP interacts specifically with molecules of the *P. aeruginosa* outer membrane. Previous results at the Kuehn lab suggested that PaAP may bind to LPS, which is the major constituent of the Gram-negative outer membrane and a prominent toxin and immune agonist; therefore, particular attention was given to confirming whether this interaction does occur. By answering this question, we hope to build a more robust model of how PaAP contributes to the establishment of infections by *P. aeruginosa*. It is our hope that the creation of such a model can help direct future research into therapies to treat *P. aeruginosa* infections, especially in patients for whom these infections are difficult to treat, such as persons suffering from cystic fibrosis. The primary strategy used in this study was to co-incubate PaAP with various candidate interaction partners, in native or in some cases denaturing conditions, to allow binding to occur. Various treatments were then performed to separate samples based on characteristics such as binding affinity and mass, and immunochemistry was used to compare co-incubated samples to controls to determine whether any interaction had occurred.

*Far Western blots demonstrate that PaAP binds to both bacterial and mammalian partners*

Far Western blots were used to initially determine whether exogenously applied PaAP interacts with very complex mixtures containing all the components of bacterial and mammalian cells. The multiple bands present in Fig. 5 showed that such interactions do occur for both types of cells. The S470-ΔPaAP reaction showed that multiple interactions between PaAP and bacterial molecules occur, as 4 or more bands are evident this lane. However, given the fact that these proteins were collected from whole-cell samples, it is impossible to determine whether these bacterial bands are located in the outer membrane, and this result therefore does not directly answer the question posed in this study. Interestingly, a single band is also visible in the lane containing A549 cells, indicating
that PaAP also interacts with at least one component of mammalian cells. While the identities of these bands are currently unknown, an ongoing project at the Kuehn lab is to identify the molecules present in each band using mass spectrometry and comparing the results to the human and *P. aeruginosa* proteomes. If the localization and functions of the proteins that are identified by mass spectrometry are known, they could validate the results seen in my Far-Western assay directly. These results will significantly advance our model of PaAP functionality and directly answer whether PaAP binds to outer membrane proteins in *P. aeruginosa*.

However, this strategy is not without limitations. The SDS-PAGE step of Far Western blotting denatures sample proteins, which in some cases can cause them to lose the secondary and tertiary structures that define the active regions that are responsible for binding to other proteins (Wu et al., 2007). This can prevent proteins in the sample from being bound by PaAP as they would in their native state (Wu et al., 2007). Far Western blotting therefore might fail to identify all proteins to which PaAP binds in a given sample, making it necessary for other tests to be performed to identify additional PaAP binding partners. On the other hand, Far Western blotting does provide the molecular weight of any PaAP binding partners that are identified, and can therefore help confirm the results of mass spectrometry assays, making it altogether a technique worth using in future research.

*PaAP co-sedimented with fractionated membranes, but the assay is tenuous*

Unlike Far Western blotting, co-sedimentation allows PaAP to interact with candidate binding partners under non-denaturing conditions, which increases the likelihood of identifying interactions that occur in physiological contexts. However, after co-sedimentation is performed, both the exogenously applied PaAP and the tested sample are run together on an SDS-PAGE gel, such that it is impossible to determine whether multiple binding interactions occurred.
Furthermore, if samples are not absolutely pure, it is difficult to determine to which components in the sample PaAP bound.

Attempts to co-sediment PaAP with various components of *P. aeruginosa* (outer membranes, whole cell extracts, and isolated LPS) yielded mixed results. First, and most importantly, the results shown in Fig. 6 suggest that exogenously applied PaAP is able to bind to a component or components in *P. aeruginosa* outer membranes, because PaAP is only present in membrane samples that were treated with the protein (lane 3) and not in control samples (lane 4). Given that PaAP is relatively stable in PBS, and that the centrifugation performed to co-sediment PaAP and the membrane samples was done for short periods at low speeds that normally do not cause PaAP to pellet (see the mock sample in lane 7, which contained PaAP but no outer membrane), this result strongly suggests that PaAP binds to *P. aeruginosa* outer membranes. However, this result gives us less information than the Far Western discussed above, as they do not identify or even give the molecular weight of the component(s) of the membrane that interacted with PaAP. Furthermore, the purity of the outer membrane samples used in this assay is questionable. The authors who designed the outer membrane fractionation protocol used in this study contend that this protocol efficiently isolates Gram-negative outer membranes from other cellular components (Thein et al., 2010). However, the protocol did not employ more commonly used and widely accepted methods used in subcellular fractionation and outer-membrane isolation, such as density-gradient centrifugation. It is therefore possible that contaminants from other subcellular compartments, such as inner membranes or the cytoplasm, were present in the outer membrane samples. If so, molecules from these compartments may have bound to PaAP and produced the band visible in lane 3 of Fig. 6. However, in the original study by Thein et al., the purity of outer membranes isolated by the protocol used in this study was compared to outer
membranes isolated by other methods by mass spectrometry, and fractions isolated by this protocol were found to have fewer inner membrane contaminants than more complex methods and comparable levels of contamination by cytoplasmic proteins (Thein et al., 2010). Therefore, while we cannot be absolutely sure that outer membrane components caused the co-sedimentation of PaAP in this assay, the ease with which PaAP was pelleted and the purity of the outer membrane samples used in this assay suggest that PaAP does bind to molecules of the Gram-negative outer membrane.

While this part of Fig. 6 suggests that PaAP binds to *P. aeruginosa* outer membrane molecules, the results from the whole-cell samples in this assay are difficult to explain. The S470-ΔPaAP strain used to generate these samples has been engineered to not produce PaAP, and therefore no signal should be visible in the whole cell –PaAP lane (lane 6). However, this lane is identical to the +PaAP lane (lane 5), indicating that PaAP antibody-reactive bands are present, even in untreated samples. It is most likely that these bands represent factors non-specifically recognized by the polyclonal anti-PaAP α-2939 antibody used to detect PaAP in this assay. Such non-specific binding is not entirely unexpected, as the α-2939 antibody is a polyclonal serum, and thereby likely contains antibodies that will react against multiple PaAP epitopes, some of which may mimic other antigens to which the rabbits used to raise the antibody had been exposed (Hanly et al., 1995). More broadly speaking, these rabbits have likely been exposed to other Gram-negative bacteria or even *P. aeruginosa* prior to being used to generate the antibody, and therefore may possess other antibodies that react to antigens that are present in the whole-cell samples. These results show the utility of using more purified bacterial (or mammalian cell) fractions to assess the binding receptors for PaAP, as doing so can reduce the chances of cross-reactivity with off-target antigens. Furthermore, these results also illustrate one of the benefits of using monoclonal
antibodies in immunodetection, as these are far less likely to cross-react. One avenue for future research at the Kuehn lab is to perform antigen-specific antibody purification of the α-2939 prep to eliminate all antibodies that react to non-PaAP antigens; however, highly pure PaAP is needed to perform this assay, and the current system used to produce PaAP at the Kuehn lab yields only a partially-purified product.

However, regardless of the results of the whole cell assays, the fact remains that exogenously applied PaAP specifically bound to membrane components (lane 3), and this allows us to conclude that PaAP does bind a component of the *P. aeruginosa* membrane. Following this finding, further emphasis was placed on determining the specific component(s) of the outer membrane that interact with PaAP.

The bacterial membrane is composed of lipid and protein determinants. We sought to investigate the interaction of PaAP with LPS, a principle component of the outer membrane of Gram-negative bacteria. Unfortunately, the LPS co-sedimentation assay did not provide any meaningful results. This assay theoretically works by exploiting the different solubilities of LPS and PaAP in aqueous saline solutions. LPS readily pellets when subjected to ultracentrifugation (~40,000rpm), while PaAP is less likely to pellet under these conditions. If PaAP-treated LPS were to test positive for PaAP by Western blotting, and a PaAP-only mock co-sedimentation trial test negative, then it would be possible to conclude that PaAP binds to LPS with high enough affinity to produce co-sedimentation. If PaAP does not bind to LPS, then PaAP should be present in the supernatant after LPS is pelleted. However, as shown in Fig. 7, PaAP was visible in neither the supernatant nor pellet fractions of pelleted LPS following co-sedimentation attempts. One likely explanation for this behavior is that PaAP did not co-sediment with LPS, and was instead present in the supernatant after LPS was pelleted. Subsequently, the already-dilute PaAP in the supernatant
was diluted even further when the sample was mixed with SDS PAGE loading buffer, so it is plausible that the concentration of PaAP in the supernatant was too low to be detected by Western blotting. Conversely, following pelleting and washing, LPS was resuspended in a smaller volume of SDS-PAGE loading buffer than the initial volume of the sample, so if co-sedimentation did occur PaAP would likely be present at detectable concentrations. Regardless, pelleted LPS was not visible to the naked eye following centrifugation, even when spin speeds and duration were increased. Based on prior experiments conducted by myself and others at the Kuehn lab, LPS likely still pelleted under these conditions; however, it is possible that the amount of LPS used in each co-sedimentation trial was too low to produce a visible pellet. In the interest of conserving reagents, it was decided that assays that made more efficient use of our limited stores of S470 LPS should be used instead of this co-sedimentation method, and further attempts at this assay were abandoned in favor of other techniques.

*Multiple antibody detection systems show off-target affinity in protein-lipid overlay blot analyses*

PLO blots were used to determine whether free PaAP or LPS could bind to each other when the other molecule was blotted onto the surface of a membrane. This assay is similar to a Western blot, except that it eliminates the PAGE and transfer steps and relies on the direct spotting of the target molecules onto a membrane (Dowler et al., 2002). The PLO blot technique therefore has the advantage of allowing sample molecules to be spotted in their native state without using solubilizing detergents such as SDS, thereby allowing for molecules to interact in conditions that more closely mimic physiological conditions. Furthermore, this technique eliminates the loss of sample due to inefficient transfer following gel electrophoresis; however, it also has certain disadvantages. First, molecules within samples are not separated by molecular weight prior to being probed with either PaAP or LPS. Therefore, if there are any contaminants in the sample,
they cannot be differentiated from the target molecule, and any nonspecific binding of contaminants to the probe molecule or the antibodies used to detect the probe cannot be differentiated from interactions between the probe and target molecules. An additional shortcoming of the PLO blot protocol is that it places large amounts of the target molecule in a single location. Therefore, while interactions between the target molecule and the detecting antibody may be weak or nonspecific, the high concentration of spotted target molecule might be detected, whereas lower concentrations more typical in standard Far Western blots would not produce a discrete signal.

These characteristics of the PLO blot assay may be responsible for the results that were obtained in this study. In every trial that was attempted, the antibody-based PaAP detection

![Diagram](image)

**Figure 14: diagram of possible antibody activity in PLO blots.** **A)** in this scenario, the probe molecule (PaAP, yellow Pac-Man) binds to the spotted target molecule (LPS, red squiggle). The antibody (green “Y”) detects both the PaAP bound to the LPS and the unbound LPS. **B)** PaAP is not bound to LPS, but the antibody still detects the spotted LPS, producing a fluorescent signal. While B represents what is occurring in the negative control, there is no way of knowing whether blots treated with PaAP behave like A or B.
method highlighted the same spots on all blots, regardless of whether or not they had been treated
with the PaAP probe molecule (Fig. 8). Because PaAP-treated and untreated blots displayed the
same fluorescence patterns, there is no way of knowing whether PaAP bound to LPS under the
conditions of the assay, as fluorescence produced by the antibody binding directly to the spotted
molecules (Fig. 15B) is indistinguishable from fluorescence produced by the antibody binding to
the probe molecule bound to the spotted molecule (Fig. 15A). This same problem was encountered
when LPS was used as the probe molecule and LPS-detecting antibodies were used as to visualize
interactions between the probe and spotted molecules (Figs. 9 and 10). In performing these blots,
we hoped to find an antibody that could bind specifically to LPS without binding to PaAP, such
that PaAP spots would only fluoresce when PaAP was bound by LPS. However, both antibodies
were unusable; in Fig. 9, the antibody detects neither PaAP nor S470 LPS, while in Fig. 10 the
antibody detects both molecules. In addition to these shortcomings, both LPS antibodies
demonstrated a high degree of cross-reactivity with other lipid/membrane samples, as visible in
spots 3 through 9 in Figs. 9 and 10. Because all probe-antibody combinations produced similar,
uninformative results, the PLO assay was not pursued further, and other tests that could better
differentiate between specific and non-specific antibody binding were pursued instead.

*LPS does not appear to co-immunoprecipitate with PaAP and PaAP does not appear to induce a
mobility shift of LPS*

Two assays that were expected to make better use of reagents and produce more meaningful results
were co-immunoprecipitation and electrophoretic mobility shift (EMSA). In co-
immunoprecipitation, samples containing untreated PaAP and LPS were separated by PAGE
alongside PaAP that had been bound to antibodies and co-incubated with LPS in non-denaturing
conditions to allow binding. Upon visualization, the untreated LPS control was compared to the
PaAP+LPS co-immunoprecipitation trial; if the both the characteristic bands from untreated PaAP and LPS were present in the co-immunoprecipitation trial, but absent in the PaAP-only and LPS-only co-immunoprecipitation control trials, then the assay would indicate that LPS had co-immunoprecipitated with PaAP and show that PaAP binds to LPS.

Taken by itself, the co-immunoprecipitation assay suggests that LPS does not co-immunoprecipitate with PaAP. Upon silver staining, the LPS control lane shows a single strong band at approximately 12.5 kDa (Fig. 13A). Single molecules of LPS can range in mass from approximately 10-20 kDa, depending on a variety of factors including the species and strain of bacteria producing the LPS and the isoform being expressed (Erridge et al., 2002). The band in the LPS control lane falls within this range, which would appear to suggest that the 12.5 kDa band is in fact S470 LPS as used in the co-IP assay. However, the 12.5 kDa band is not present in the PaAP+LPS co-immunoprecipitation sample, suggesting that LPS is incapable of binding PaAP under the conditions of the assay.

However, there are factors that need to be considered in interpreting this result. First, all samples used in the gel shown in Fig. 8 were treated with SDS, which binds to LPS and causes its molecular weight to increase to 50-100 kDa (Fomsgaard et al., 1990; Jann et al., 1975). Therefore, we should expect to see the LPS in the control lane to lie somewhere within this range, instead of at ~12.5 kDa. However, LPS behaved more normally in additional co-IP trials (Fig. 14). In the LPS control lane (lane 3), there is a strong, broad band from approximately 55-65 kDa, which better matches the predicted molecular weight of LPS in SDS-PAGE (Fomsgaard et al., 1990). While the broad appearance of the band may seem unusual, it is possible that the single broad band that is visible is actually several distinct bands in a laddering formation (which is typical for LPS) that were stained so heavily that they cannot be distinguished from each other (Fomsgaard et al.,
Nevertheless, the +PaAP/+LPS co-IP lane (lane 4) lacks a similar band, and instead has two narrow bands with apparent molecular weights of 60 and 22 kDa. The 60 kDa band appears to be somewhat heavier than the 53 kDa PaAP band in the PaAP control lane and falls within the range of molecular weights covered by the 55-65 kDa band in the LPS control lane, which might suggest that it is in fact LPS. However, the +PaAP/−LPS co-IP lane (lane 6) also has bands at ~60 and 22 kDa. Furthermore, the major degradation project of PaAP is approximately 22 kDa, which strongly suggests that PaAP is present in both the +PaAP/±LPS samples. Additionally, the leading edge of these samples appears to be slightly behind that of the untreated PaAP control, as indicated by the uneven line visible at the bottom right of Fig. 14; therefore, it is possible that the full contents of lanes 4 and 6 did not migrate as quickly as the contents of the PaAP control (lane 2), causing the apparent difference in mass. Overall, the strong similarity between 4 and 6, and the lack of the broad LPS band seen in lane 3 therefore suggest that PaAP is the major species present in the +PaAP/+LPS lane and that LPS is not present. This interpretation supports the conclusion that PaAP does not bind to LPS under the conditions of this assay.

As with co-immunoprecipitation, the EMSA used in this study attempted to promote the binding of native PaAP to LPS by co-incubating these samples in non-denaturing conditions. A further similarity between these assays is that the banding patterns of samples in the gel are used to determine whether binding occurs. The primary difference is that the EMSA theoretically allows proteins to retain their active conformations during electrophoresis, and allows intermolecular complexes to stay bound together. The physicochemical differences between these complexes cause the species to migrate at a different speed through the gel, and by visualizing and comparing co-incubated and untreated samples it is possible to determine whether binding has occurred.
However, the EMSA used in this study provided no such easy-to-interpret result. In Fig. 11, the LPS control lanes have a laddered banding pattern that is identical to those seen in all lanes which had been treated with PaAP. Given that the LPS samples that had been used to treat PaAP behaved identically to the LPS control, this result likely indicates that the co-incubated PaAP and LPS did not migrate together on the gel, as it is unlikely that LPS would migrate to the same locations when bound and unbound by PaAP. Furthermore, the lack of additional bands in either the PaAP control lane or the samples containing PaAP suggests that the observed banding patterns are caused by LPS alone. The most simplistic conclusion to be drawn from this result is that PaAP does not bind to LPS under the conditions of the electrophoretic mobility shift assay. If true, this result would be in agreement with the interpretation that PaAP does not bind to under the conditions of the co-immunoprecipitation assay. Taken together, these results suggest that PaAP does not bind to LPS.

However, additional idiosyncrasies in the EMSA call into question the validity of this conclusion. In non-denaturing PAGE, as employed in the EMSA, the amphipathic structure of LPS causes molecules to form aggregates that interact through their hydrophobic regions (Rodriguez and Hardy, 2015). The variable molecular weight of these aggregates causes LPS to appear as indistinct smears when visualized by imidazole-zinc staining (Rodriguez and Hardy, 2015); this behavior stands in stark contrast to the discrete bands that were observed in the EMSA, in which all lanes displayed discreet bands in a laddered pattern (Fig. 11). Rather, both the laddering behavior seen in Fig. 11 and the apparent molecular weight of the bands (between 50 and 150 kDa) are more typical of LPS in SDS-PAGE (Fomsgaard et al., 1990). The S470 LPS that was used in this and all other assays in this study was removed from a PBS-SDS buffer by repeated centrifugation and washing; therefore, one plausible reason for this behavior is that residual SDS
is present in the LPS stock solution. If true, this would have drastic implications for all results presented in this work. Even small amounts of Triton X-100, which is a milder detergent than SDS, can disrupt the high-affinity binding of LPS by CD14, which is a key component of the human immune response to LPS and has an extremely high affinity for LPS (Akashi et al., 2003). The presence of SDS in the LPS stock may similarly disrupt interactions between PaAP and LPS, leading to false negative results in the EMSA and co-IP experiments and potentially having great consequences for any conclusions drawn from this work. However, if there were sufficient SDS present in the LPS stock to produce a pattern typical of SDS-PAGE in LPS diluted into a non-denaturing PAGE buffer, it is surprising that the LPS control used in the SDS-PAGE of the co-IP assay did not consistently behave in the same fashion (Fig. 13A). Therefore, it is possible that some other mechanism caused this behavior in the LPS used in the EMSA.

Another anomalous aspect of the EMSA is the fact that LPS was visible at all following Ruby staining. Ruby stain binds to basic amino acids (lysine, histidine, arginine) and is also hypothesized to bind to the peptide backbone of proteins, and it is therefore traditionally used to specifically visualize proteins (Lopez et al., 2000). It is therefore surprising that this staining method visualized LPS, which is composed of lipid and saccharide groups. However, certain unique features of \textit{P. aeruginosa} LPS may be responsible for this result. Unlike the LPS produced by most species, \textit{P. aeruginosa} LPS frequently includes amino acids in its O-antigen, which in most cases is composed of various sugars (Erridge et al., 2002). It is possible that the Ruby stain molecules bound to amino acids in the LPS O-antigen, causing LPS to be visualized in the EMSA. Another unusual aspect of \textit{P. aeruginosa} LPS is that certain sugars in its O-antigen are modified to carry amide groups (Erridge et al., 2002; Wilkinson and Galbraith, 1975). The “peptide” bonds that characterize protein backbones are actually a series of amide bonds, and amide-substituted
sugars might be chemically similar enough to the peptide backbone of proteins to be bound by Ruby stain, causing or contributing to the staining of LPS in this assay. While either or both of these mechanisms may have contributed to the visibility of LPS on the EMSA gel, it is surprising that no LPS was visible in Ruby-stained co-IP gels (Fig. 13B). However, while SDS does not disrupt the interaction of Ruby stain with proteins, the effect of SDS on LPS in Ruby stained gels has not been characterized, and the presence of SDS in the co-IP gels may have had some effect that caused LPS to not be visualized.

In addition to these peculiarities, the lack of visible PaAP in the EMSA makes any conclusions drawn from this assay tenuous. The fact that PaAP cannot be seen in the control or mobility shift lanes is not surprising, due to a variety of factors intrinsic to native PAGE. In native PAGE, proteins are not as negatively charged as when they are treated with SDS, each molecule of which carries a negative charge (Arndt et al., 2012). Native proteins therefore generally do not experience as strong of a motive force when an electric field is applied to the gel, and many migrate far slower than their denatured counterparts, often causing them to have far greater apparent molecular weights than anticipated (Arndt et al., 2012). To counteract this problem, the pH in native PAGE may have to be fine-tuned to maximize the target protein’s charge and promote faster migration through the gel, while also ensuring that the protein does not denature under the new pH conditions (Arndt et al., 2012). Furthermore, native proteins retain their secondary and tertiary structures, causing them to be bulkier than SDS-treated proteins, which are denatured and therefore roughly linear (Arndt et al., 2012). This additional bulkiness means that some native proteins experience difficulties in migrating through the pores in a polyacrylamide gel, causing them to travel much shorter distances on the gel or even preventing them from entering the gel matrix entirely (Arndt et al., 2012). It is likely that these and possibly other factors prohibited PaAP from
entering the gel in the EMSA. The native PAGE shown in Fig. 12 supports this conclusion. In this blot, the band containing native PaAP has a distinct fluorescent smudge near the loading well, likely indicating the presence of PaAP that did not migrate well in the gel. To address this problem, in future trials the physical conditions of the assay will need to be optimized for the visualization of PaAP, possibly by altering the pH of the native sample and running buffers or by using a lower percentage of polyacrylamide in the gel. However, because neither LPS-treated nor untreated PaAP are visible in the gel it is impossible to determine whether PaAP bound to LPS using the existing results.

Summary and future directions

In summary, this work provided partial answers to the questions posed at the beginning of this study. Based on earlier results obtained at the Kuehn lab, it was hypothesized that PaAP would bind to molecules of the \textit{P. aeruginosa} outer membrane. This hypothesis was supported by the co-sedimentation of PaAP with isolated \textit{P. aeruginosa} outer membranes. The \textit{P. aeruginosa} outer membrane is composed mainly of proteins and LPS, both of which have many interactions with other molecules and could be suitable interaction partners for PaAP in a pathogenic context. Prior research at the Kuehn lab suggested that PaAP bound differentially to vesicles of \textit{P. aeruginosa} strains possessing various LPS mutations, leading us to hypothesis that PaAP binds to LPS. Altogether, the results of this study suggest that this interaction does not occur. This conclusion is best supported by the failure of PaAP to co-sediment or co-immunoprecipitate with \textit{P. aeruginosa} strain S470 LPS. However, these findings do not exhaustively prove that PaAP does not bind to LPS, as the conditions of these assays may have prevented such interaction from occurring.

There are many other possible reasons why PaAP may not have bound LPS in the assays used in this study, each of which could be pursued in further research. First, the binding of PaAP
to LPS may depend on the specific physiological context of LPS in the outer membrane, possibly due to interactions between PaAP, LPS, and/or other proteins. To test whether this is the case, outer membrane vesicles and purified outer membranes could be isolated from *P. aeruginosa* and subjected to protease treatment, then used in co-sedimentation or co-immunoprecipitation assays as in figures 3, 6, and 9. If samples treated in this way no longer bind to PaAP, these assays would indicate that PaAP binding is facilitated by proteins, either in part or in full, and not LPS alone. A second possible reason that PaAP did not bind to LPS in this study is that PaAP does not bind to the isoform of LPS produced by *P. aeruginosa* strain S470, but may still bind to other types of LPS. This possibility could be tested by repeating the experiments used in this study with other isoforms of *P. aeruginosa* LPS such as PA01 LPS, which was originally proposed as a part of this study but abandoned due to time constraints. However, the upregulation of PaAP in strain S470 and the pathogenic nature of this strain was one of the original reasons we decided to investigate the interaction between PaAP and LPS. If PaAP does not bind to the LPS produced by pathogenic strains, but does bind to the LPS produced by environmental strains, it is more difficult to make a case for the clinical significance of this interaction. Furthermore, prior research has shown that environmental *P. aeruginosa* strains produce less PaAP than clinical strains, so any interaction that does occur with LPS from environmental strains *in vitro* may not accurately represent what occurs under normal conditions.

Regardless of the reason why no interaction between LPS and PaAP was observed in this study, the difficulty of exhaustively proving that this interaction does not occur may indicate that future research should pursue other avenues. However, the clinical significance of PaAP has still not been established, and while it might not bind to LPS, the fact that it apparently interacts with OMVs and biofilms in pathogenic contexts suggests that PaAP plays a significant role in the establishment
and/or maintenance of *P. aeruginosa* infections. I suggest two major research projects for PaAP in the future. First, future projects should continue to investigate potential interactions between PaAP and other outer membrane molecules. One potential high-throughput method to identify PaAP binding partners is the continued use of mass spectrometry to identify PaAP interaction partners that are implicated by Far Western blotting. To overcome the limitations of using denatured samples, native PAGE could be used in the initial separation of samples, thereby allowing PaAP and the blotted proteins to interact under conditions that better recreate physiological contexts. Once interactions between PaAP and these other molecules are identified, future researchers can determine whether these interactions are clinically significant. If they are, scientists can work towards developing therapies to combat *P. aeruginosa* infections, which are difficult to treat with available antibiotics and pose a major threat to cystic fibrosis patients. The second major project that I suggest is a long-term study of PaAP expression patterns by *P. aeruginosa* in chronically infected CF patients. Such a study would help establish how PaAP contributes to *P. aeruginosa* infections by showing that expression is either maintained over the course of chronic infections or reduced after the transition from acute to chronic infection. Furthermore, if molecular studies help to identify therapies that target PaAP or other molecules with which PaAP interacts, doctors prescribing these treatments would need to account for how PaAP expression changes over time to ensure that the treatment is effective.

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