Aminopeptidase-Dependent Modulation of Bacterial Biofilms by *Pseudomonas aeruginosa* Outer Membrane Vesicles

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
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2019

ABSTRACT

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Abstract

Pseudomonas aeruginosa, known as one of the leading causes of morbidity and mortality in cystic fibrosis (CF) patients, secretes a variety of virulence-associated proteases. These enzymes have been shown to contribute significantly to *P. aeruginosa* pathogenesis and biofilm formation in the chronic colonization of CF patient lungs, as well as playing a role in infections of the cornea, burn wounds and chronic wounds. Our lab has previously characterized a secreted *P. aeruginosa* peptidase, PaAP, that is highly expressed in chronic CF isolates. This leucine aminopeptidase is also highly expressed during infection and in biofilms, and it associates with bacterial outer membrane vesicles (OMVs), structures known for their contribution to virulence mechanisms in a variety of Gram-negative species and one of the major components of the biofilm matrix. With this in mind, we hypothesized that PaAP may play a role in *P. aeruginosa* biofilm formation. Using a lung epithelial cell/bacterial biofilm coculture model, we show that PaAP deletion in a clinical *P. aeruginosa* background alters biofilm microcolony composition to increase cellular density, while decreasing matrix polysaccharide content and resistance to the antibiotic colistin. We recreate this phenotype using a pellicle biofilm model, in which bacteria are grown statically at the culture air-liquid interface, demonstrating that these phenotypes are not dependent on the coculture host cell substrate. We additionally show that OMVs from PaAP expressing strains, but not PaAP alone or in combination with

PaAP deletion strain-derived OMVs, could complement this phenotype. Finally, we found that OMVs from PaAP-expressing strains cause protease-mediated biofilm detachment, leading to changes in matrix and colony composition. OMVs mediated the detachment of biofilms formed by both non-self *P. aeruginosa* strains and *Klebsiella pneumoniae*, another respiratory pathogen, showing that this process may also be relevant in polymicrobial communities and acts on non-*P. aeruginosa* derived substrates. Our findings represent novel roles for OMVs and the PaAP aminopeptidase in the modulation of bacterial biofilm architecture.

Dedication

To my wonderful family and friends.

Without their love and support this never would have been possible.

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Acknowledgements

I am very appreciative of all the people who helped to make this dissertation possible. First, I would like to thank my advisor, Meta Kuehn, for her many hours of brainstorming, revising, and teaching. Her efforts have helped me to realize my own potential and develop as a scientist. I am also immeasurably grateful to my lab-mates, old and new, for their scientific inspiration, camaraderie, and never-ending emotional support. I would additionally like to thank my committee members, David Tobin, Dennis Ko, Mari Shinohara, and Soman Abraham, for their advice, support, and encouragement throughout the entire course of this project.

I would also like to express my immeasurable gratitude for amazing family and friends. My parents, Robert and Nina, and siblings, Brian, Kevin and Kate, have always supported my dreams and remind me what I am capable of every day. While they may not have understood what I was talking about, they somehow always found the right thing to say to make me laugh even during the hardest times. I am very appreciative of the wonderful friends I made during my time in this program, as well. Shannon and Dora, especially, always knew how to lift me up when I needed it most and reminded me to celebrate all my accomplishments. I am so grateful for you all.

1. Introduction

Bacteria interact with their environments through the secretion of cellular products into the extracellular milieu, and these microbial factors actively influence bacterial survival. For pathogenic species, secretion also provides a mechanism by which bacteria influence interactions with their host. This is of particular importance to biofilm microbial communities which rely on secreted material for protection from environmental factors, host immune systems, and antibiotic challenge. Factors secreted by biofilm-forming bacteria contribute to the structural integrity of the stationary colonies and serve functional roles in regulating such properties as antibiotic resistance, nutrient acquisition, and toxicity against host tissues. The secretion of outer membrane vesicles by Gram negative bacterial species has been documented for decades, and yet the functions of these particles and their impact on bacterial pathogenesis remain incompletely understood. In this work, we attempt to shed light on role of bacterial vesicles in biofilm formation, specifically through the study of a vesicle-bound aminopeptidase in the model organism *P. aeruginosa*.

1.1 Outer membrane vesicles

Outer membrane vesicles (OMVs) are extracellular spherical, membranous structures, 20-200 nm in diameter, that are formed and detached from the cell envelope of Gram-negative bacteria (Kuehn and Kesty 2005). OMVs contain distinct membrane protein, lipid, and soluble periplasmic content, and provide a mechanism by which

bacterial cargo and membrane lipids can be released from the parent cell (Orench-Rivera and Kuehn 2016). Vesicles have been implicated in bacterial virulence, biofilm formation, horizontal gene transfer, communication, stress responses, resistance to phage and antibiotics, and other functions critical to bacterial survival (Ellis and Kuehn 2010). Additionally, they have been found to be produced under a wide variety of bacterial growth conditions, including in both planktonic and biofilm cultures, in fresh and salt water, and inside eukaryotic cells as well as within mammalian tissues (Schwechheimer and Kuehn 2015). Understanding how these bacterial particles interact with their environments to promote bacterial survival and disease phenotypes is a topic of ongoing research in the field of host-microbe interactions.

1.1.1 Prevalence of OMVs

Vesicles were first described over 50 years ago as membranous blebs seen associated with bacterial cells in election microscopy (EM) images. Since then, vesicles, their contents, and the bacteria producing them have been studied extensively, and every Gram-negative bacterium studied to date has been shown to produce OMVs. Some of the diverse species in which they have been examined include *Escherichia coli* (Hoekstra et al. 1976), *Neisseria* spp. (Devoe and Gilchrist 1973), *Bacteroides* spp. (Grenier and Mayrand 1987), *Vibrio* spp. (Chatterjee and Das 1967), *Campylobacter jejuni* (Logan and Trust 1982), *Pseudomonas aeruginosa* (Kadurugamuwa and Beveridge 1995), and *Actinobacillus actinomycetemcomitans* (Nowotny et al. 1982).

While some of the first studies on bacterial vesicles concluded that they were byproducts of cell lysis or induced by nutrient limitation, more detailed analysis has found enrichment of OM content, periplasmic content, and specific lipid species in these particles, suggesting that they are secreted purposefully by the producing bacteria as a means to interact with their environment (McBroom et al. 2006). OMV production rates can vary substantially between producing species, but they often represent a major portion of bacterial cellular material. Vesicles from laboratory *E. coli* and *P. aeruginosa* cultures account for an estimated ~1% of all OM content present (Bauman and Kuehn 2006; Gankema et al. 1980; Wensink and Witholt 1981), while *Neisseria meningitidis* vesicles represent 8 to 12% of labeled protein and LPS in late log-phase cultures (Devoe and Gilchrist 1973). The substantial amount of cellular material, along with selective enrichment and exclusion of specific protein and lipid content into OMVs, further supports the conclusion that vesiculation is an active and purposeful mechanism by which bacteria secrete specific molecules into the environment.

Growth and nutrient conditions, including growth media, solid vs. liquid culture conditions, and media composition, can affect both levels of vesicle production and vesicle content (Orench-Rivera and Kuehn 2016). *Pseudomonas putida* grown in various laboratory media have been shown to display up to 3-fold differences in levels of vesicle production (Choi et al. 2014). Additionally, vesicle production by *Mycobacterium tuberculosis* cells depended on the levels of available iron in the media, suggesting that iron-limiting

conditions promote vesicle production by these cells (Prados-Rosales et al. 2014). Because iron is essential for bacterial survival and iron acquisition is a major hurdle in bacterial survival within a host, this suggests that vesicles may serve a function in iron acquisition. Similarly, under anoxic conditions, *Pseudomonas aeruginosa* biofilms were found to produce 6-fold greater levels of vesicles as compared to those under normal aerobic growth conditions (Toyofuku et al. 2014). Several studies have additionally found that bacteria show enhanced vesiculation after treatment with certain antibiotics, which may be related to the induction of stress responses (Orench-Rivera and Kuehn 2016). Finally, multiple studies have shown that vesiculation can be induced by exposure to host tissues or components, further emphasizing the potentially important roles for OMVs in host-pathogen interactions (Ellis and Kuehn 2010).

1.1.2 Outer membrane vesicle formation and composition

Vesicles are formed from regions of the cell envelope, and therefore the composition of the vesicle lipid bilayer mirrors that of the bacterial outer membrane (OM) (**Figure 1**). This includes membrane-associated lipoproteins as well as high concentrations of lipopolysaccharide (LPS) found in the outer leaflet of the OM. Vesicle cargo generally reflects bacterial periplasmic content, though cytosolic and inner membrane (IM) proteins have also been reported in vesicle isolations (Ellis and Kuehn 2010; Toyofuku et al. 2014). To date, the mechanism behind vesicle biogenesis has not been fully elucidated; however, several mechanisms have been proposed, and evidence gained from studies of bacterial

vesicles support a model of directed secretion. The Gram-negative cell envelope is composed of an outer membrane with an exterior leaflet formed of lipopolysaccharide (LPS), a traditional inner cytoplasmic membrane composed of phospholipids, and an intervening periplasm that promotes protein folding and contains a net-like layer of peptidoglycan (PG) (Schwechheimer and Kuehn 2015). Crosslinking between the OM and the PG help to maintain bacterial rigidity and stability, but also pose a barrier to OMV formation. While it has yet to be proven conclusively, studies examining bacterial mutants with decreased crosslinks were found to have hypervesiculation phenotypes, and constructs with increased crosslinks have hypovesiculation phenotypes, supporting the hypothesis that envelope crosslinking may be involved in regulating vesicle production (Schwechheimer and Kuehn 2013; Pathirana and Kaparakis-Liaskos 2016).

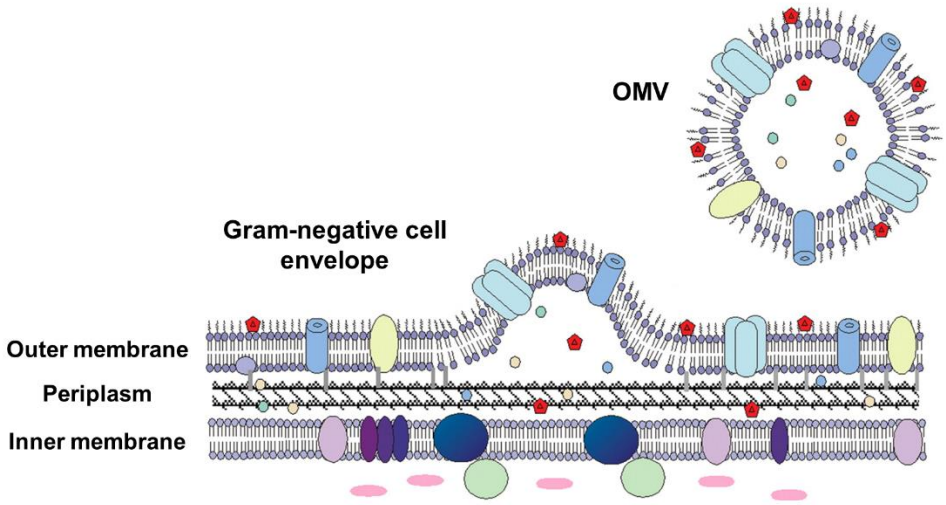

Figure 1: OMVs are formed form bacterial OM and periplasmic content
This model shows the production of a Gram-negative outer membrane vesicle containing outer membrane and periplasmic components of the Gram-negative cell envelope. The OM is a bilayer membrane with an inner leaflet of phospholipids an outer leaflet containing lipopolysaccharide (LPS). Consequently, OMVs contain OM lipids and membrane proteins, as well as soluble content from the periplasm. Proteins and molecules secreted by the cell into the extracellular environment may also be externally bound to the surface of OMVs. This figure was adapted from a previous publication (Kuehn and Kesty 2005).

Vesicle formation may also be caused or impacted by lipid species present in the OM. Multiple studies have noted that vesicles are often enriched in specific lipid species, as membrane content in OMVs is not identical to that of the OM of the producing cells (Bonnington and Kuehn 2014; Schwechheimer and Kuehn 2015). Certain lipids may impact OM flexibility, providing an area of the OM more susceptible to blebbing and subsequent vesiculation. Unsaturated and branched chain fatty acids were found to be enriched in *Pseudomonas syringae* OMVs, while *P. aeruginosa* vesicles contain longer, more

saturated fatty acids (Kulkarni, Swamy, and Jagannadham 2014; Tashiro et al. 2011). These results also indicate that lipid enrichment in OMVs and the OM conditions that create susceptible regions for vesiculation may vary significantly between species. Different forms of LPS may also impact vesiculation. For instance, in *P. aeruginosa* a charged form of this polysaccharide is preferentially packaged into OMVs (Tashiro et al. 2011). Temperature changes may additionally contribute to vesicle formation, likely as a result of increased membrane fluidity (Schwechheimer and Kuehn 2015).

The accumulation of lipid-binding, envelope, and/or misfolded proteins in regions of low PG crosslinking or specific OM lipid composition may also contribute to vesiculation, as they impact both membrane fluidity and curvature . In *P. aeruginosa*, it has been found that the quorum sensing signaling molecule PQS can bind to OM LPS and induce increased membrane curvature, promoting OMV formation. In fact, exogenous addition of PQS in cell cultures was sufficient to stimulate vesiculation (Mashburn and Whiteley 2005). Notably, PQS also induces membrane blebbing of eukaryotic cells, which do not have LPS (Schertzer and Whiteley 2012), and mutants that do not express PQS are still capable of vesiculation (MacDonald and Kuehn 2013), indicating that this molecule is not solely responsible for vesicle formation. While more study will be required to fully determine the mechanisms responsible for OMV production in Gram negative bacteria, it is likely that lipid composition, protein localization and binding, modulation of cross-links, and environment-dependent characteristics all play a role in this process.

1.1.3 Vesicle interactions with host cells and modulation of immune responses

While the full impact of vesicles on bacterial biology is still a subject of ongoing research, they are appreciated as important mediators of bacterial pathogenesis. In addition to the wide breadth of *in vitro* data supporting this, vesicles from diverse species have been observed *in vivo* in host tissues and fluids, sometimes far from the sites of original infection. Vesiculating *Helicobacter pylori* has been detected in gastric biopsies (Fiocca et al. 1999), and *N. meningitidis* vesicles have been seen in both the blood and cerebrospinal fluid from meningitis patients (Stephens et al. 1982; Brandtzaeg et al. 1992). Additionally, *B. burgdorferi* vesicles have been detected in the bladder, liver, heart, spleen, and brain tissues of infected mice, indicating the systemic dissemination of OMVs (Dorward, Schwan, and Garon 1991). Recently, Jang et al also showed that intraperitoneal OMV administration in mice led to the spread of vesicles throughout the body, as they were detected in the liver, lungs, spleen, and kidney within 3 hours of administration (Jang et al. 2015).

In vitro studies have additionally noted significant increases in bacterial vesiculation in response to host tissues, suggesting that signals from the host may be involved in the regulation of OMV production. For instance, *B. burgdorferi* bacteria were found to produce significant levels of OMVs after incubation with human skin (Beermann et al. 2000). Several studies also report that pathogenic bacteria may produce as much as 25-fold more OMVs than their non-pathogenic counterparts (Lai, Listgarten, and

Hammond 1981), further suggesting that host factors can influence OMV production. Based on the increasingly obvious involvement of OMVs in bacterial pathogenesis, a substantial amount of research in the field has been dedicated to understanding how these particles interact with host cells and their producing bacteria to mediate host-microbe interactions.

Studies have repeatedly shown that OMVs can associate with and be internalized by host cells. This is relatively unsurprising, as vesicles are derived from the OM of their producing bacteria and therefore display many of the same surface-associated virulence determinants and adhesins. Due to their small size, however, OMVs may be able to infiltrate host tissues inaccessible to whole bacterial cells. Vesicle binding to host cells has been documented for diverse bacterial species, including *E. coli*, *Shigella flexneri*, *Actinbacillus actinomycetemcomitans*, *B. burgdorferi*, *H. pylori*, and *P. aeruginosa* (Ellis and Kuehn 2010). In some species, this is mediated by bacterial toxins. Heat labile toxin (LT) produced by enterotoxigenic *E. coli* (ETEC) is enriched in OMVs and binds to both LPS and glycolipid receptors on host epithelial cells, facilitating vesicle-cell association (Horstman and Kuehn 2002). The *P. aeruginosa* aminopeptidase PaAP was also found to mediate OMV associations with host cells (Bauman and Kuehn 2009). In some cases, OMV-host cell interactions can interfere with normal interactions between the bacteria and these cells. *B. burgdorferi* vesicles, for example, inhibit the association of whole *Borrelia* cells with human endothelial cells, likely as a result of both the vesicles and bacteria using

the same cell-surface receptor systems, leading to competitive binding (Shoberg and Thomas 1993).

In many studies, bacterial OMVs have also been shown to be internalized by host cells. In the case of ETEC OMVs, LT binds the ganglioside GM_1 on the surface of host cells, allowing for vesicle internalization via lipid-raft mediated endocytosis (Horstman and Kuehn 2002). In *E. coli* K1, vesicle uptake is mediated by the outer membrane protein (OMP) OmpA, which interacts with the surface receptor Ecgp on brain microvascular endothelial cells (N. V. Prasadarao 2002; N. Prasadarao et al. 1996). Host cell factors can additionally impact OMV internalization, though these are less well-characterized. Several studies have additionally suggested that certain OMVs may fuse with the host cell membrane, though this process is poorly characterized and not well understood. Intracellular *Salmonella* was found to release LPS that intercalated into host cell membranes, potentially via OMV-mediated trafficking (Portillo, Stein, and Finlay 1997). *A. actinomycetemcomitans* OMVs also rapidly transferred a lipid-tracking dye to host cell membranes (Demuth et al. 2003). More recently, membrane material from *Legionella pneumophila* OMVs was shown to incorporate into eukaryotic membrane systems by both Förster resonance energy transfer (FRET) and infrared (IR) spectroscopy (Jäger et al. 2015). While these results are intriguing, more rigorous study will be needed to determine whether these data actually indicate fusion of bacterial OMV membrane content with host plasma membranes.

Due to their observed interactions with host cells and packaging of bacterial products, OMVs are considered "long range virulence factors" that can both deliver virulence determinants to host cells and protect these molecules from degradation in the extracellular environment (Ellis and Kuehn 2010). Indeed, OMVs from many pathogenic species have been shown to contain virulence-associated molecules, and often these factors are more resistant to degradative treatments such as proteases. Cytotoxic activity has often been observed from OMVs. For example, vesicles produced by *E. coli* O157:H7 cells were found to package Shiga toxin, a well-known cytotoxin and mediator of *E. coli* pathogenesis (Kolling and Matthews 1999). Some *E. coli* strains may also package cytolysin A, another bacterial toxin. Leukotoxin, which kills human polymorphonuclear leukocytes and monocytes, was found enriched in *A. actinomycetemcomitans* OMVs, resulting in cell lysis (Kato, Kowashi, and Demuth 2002).

In addition to packaging of potent bacterial toxins, vesicle derivation from the bacterial OM means they also contain a number of non-specific microbe-associated molecular patterns (MAMPs), molecules known to induce significant immune responses in host tissues. Unsurprisingly, therefore, OMVs have been repeatedly shown to stimulate immune responses in a variety of *in vitro* and *in vivo* infection models. When administered to mice intranasally, *N. meningitidis* vesicles elicited both a mucosal and systemic bactericidal immune response (Saunders et al. 1999). LPS likely serves as a natural adjuvant in this type of reaction, whereas surface antigens may stimulate more specific

immune responses. In fact, multiple studies have noted a potent TLR4 response to OMVs, likely due to Toll-like receptor sensing of OMV-associated LPS (Kaparakis-Liaskos and Ferrero 2015). OMVs can also induce significant cytokine responses in tissue culture cells. *H. pylori*, *P. aeruginosa*, *L. pneumophila*, and *Klebsiella pneumoniae* vesicles all stimulate potent CXCL8 responses to host epithelial cells, and the induction of several other cytokines was noted in the case of *Legionella* (Ismail, Hampton, and Keenan 2003; Bauman and Kuehn 2006; Galka et al. 2008; Lee et al. 2012). *H. pylori* OMVs also induced pattern recognition receptor (PRR) signaling in gastric epithelial cells, as determined by the induction of MAPK and AP-1 signaling (Allison et al. 2009). Additionally, OMVs from *H. pylori*, *N. gonorrhoeae*, and *P. aeruginosa* stimulated host epithelial cells to produce antimicrobial human β defensins (Kaparakis et al. 2010), while *Haemophilus influenzae* vesicles induced the production of the antimicrobial peptide LL-37 (Sharpe, Kuehn, and Mason 2011). OMVs have also been shown to affect host immune cells, resulting in the production of various cytokines by macrophages and neutrophils, as well as inducing dendritic cell maturation (Kaparakis-Liaskos and Ferrero 2015).

Based on these activities, as well as their non-proliferative nature and packaging of natural adjuvants, OMVs are now being exploited for use in vaccines. Vesicles, either produced naturally by target bacterial pathogens or created using detergent treatment of membranes have been used for the creation of vaccines against *Neisseria meningitidis*, *Mycobacterium tuberculosis*, and various enteric pathogens (Acevedo et al. 2014). Several

N. meningitidis vaccines have been successfully used in countries around the world for decades (Holst et al. 2014). Additionally, OMVs from *N. meningitidis*, based on their successful clinical use, are being considered as adjuvants in vaccines against other antigens, including herpes virus type 2 glycoprotein (Campo et al. 2010) and various allergens (Norheim et al. 2012).

1.1.4 Other functions of OMVs

Vesicles have other diverse effects on bacterial biology which may impact bacterial growth and survival, including mediation of resistance to antibiotics and phage, influences on bacterial interspecies interactions, and modulation of biofilm formation. This section briefly outlines the role vesicle play in these activities.

1.1.4.1 Resistance to antibiotics, phage, and stress

Vesiculation has been proposed as a mechanism by which bacteria increase survivability in response to a variety of known stressors. Antibiotic treatment, for example, can significantly increase vesiculation due to induction of the SOS stress response (Maredia et al. 2012). Increased vesicle production was also found to increase the resistance of *E. coli* cells to antibiotics that target the bacterial OM, including colistin and polymyxin B (Manning and Kuehn 2011). In this same study, exogenously added OMVs conferred resistance to these antibiotics in vesicle-depleted bacterial cultures, suggesting that OMVs can interact with stressor molecules, reducing bactericidal effects on the target cells. These results are supported by another study in which

Stenotrophomonas maltophilia treated with the β-lactam antibiotic imipenem was found to produce vesicles containing β-lactamase, which subsequently protected the bacteria against challenge with this antibiotic (Devos et al. 2016, 2015). It was further shown that vesicles can exhibit protective effects during bacterial attacks by phage (Manning and Kuehn 2011). The *E. coli*-specific T4 bacteriophage formed complexes with *E. coli* OMVs, and formation of these complexes reduced the ability of the phage to kill bacteria. Therefore, OMVs were found to act as "decoys" to prevent bacteriophage attack against whole bacterial cells.

In addition to their potentially protective role against external stressors, vesicles have been shown to help alleviate internal stress in bacteria, such as from the over-accumulation of misfolded proteins. This was exemplified in the analysis of a *degP* deletion mutant strain of *E. coli*. DegP is a chaperone-protease that normally degrades and removes misfolded proteins from the cell. In the absence of this chaperone, the periplasmic density increased and vesicles were found to contain these misfolded substrates, supporting a mechanism in which vesicles act as receptacles for cellular "garbage" in the absence of proper disposal functions (Schwechheimer and Kuehn 2015, 2013). When combined with a hypovesiculating mutant, the *degP* mutation was toxic and inhibited growth of the *E. coli* cells, suggesting that OMV-mediated removal of toxic, misfolded protein is a significant bacterial stress response pathway.

1.1.4.2 Interspecies interactions

Vesiculation is a mechanism by which bacteria release complex groups of molecules into the extracellular environment, and vesicles can therefore serve as a means by which producing cells interact, both symbiotically or antagonistically, with other bacteria in their environment. In some cases, vesicle cargo can serve as "public goods" utilized by cohabitating strains. For example, packaging of β-lactamases in *Staphylococcus aureus* membrane vesicles exogenously conferred ampicillin resistance to the susceptible *E. coli* DH5α strain (Ismail, Hampton, and Keenan 2003). *Bacteroides ovatus* OMVs have been shown to package inulin-degrading enzymes, which can support the growth of species unable to utilize this resource (Rakoff-Nahoum, Coyne, and Comstock 2014). On the other hand, vesicles can also deliver toxic compounds to competing bacteria, enabling the producer strain to scavenge nutrients from the resulting cell debris (Evans et al. 2012).

Finally, OMVs have been found to package chromosomal, plasmid, and viral DNA and RNA, which can be transmitted to other strains, and multiple studies have implicated these particles in the process of horizontal gene transfer (HGT) (Domingues and Nielsen 2017). This genetic material can be either exposed on the vesicle surface or contained in the lumen (Renelli et al. 2004; Fulsundar et al. 2014), and packaging into the vesicle lumen additionally protects nucleic acids from degradation (Dorward, Garon, and Judd 1989). Transmission of OMV-associated genetic material has been documented for such diverse receiving species as *N. gonorrhoeae* (Dorward, Garon, and Judd 1989), *E. coli* (Yaron et al.

2000), *Acinetobacter baylyi* (Fulsundar et al. 2014), and *Thermus thermophilus* (Blesa and Berenguer 2015), and OMV-mediated HGT has found to be heritable to subsequent generations of bacterial cells (Domingues and Nielsen 2017).

1.1.4.3 Biofilm formation

Recent studies on the composition and architecture of biofilms demonstrate that OMVs play a critical role in these bacterial communities. Not only have vesicles been found integrated into the biofilm extracellular matrix, cells in biofilms also produce greater levels of OMVs compared to planktonically grown counterparts. Additionally, bacterial cargo can contain biofilm adhesion and dispersion factors that aid in the development of a robust microcolony structure. This topic is reviewed more extensively in the Section 1.3.

1.2 Model organism: Pseudomonas aeruginosa

Pseudomonas aeruginosa is a Gram-negative bacterium and prominent opportunistic pathogen. Due to its high adaptability this organism can survive under a variety of diverse conditions, including not only on host tissues but also in soil and water reservoirs, as well as on hospital surfaces, contributing to infection initiation and spread. When found in environmental reservoirs or in host tissues during infection, *P. aeruginosa* often forms bacterial communities known as biofilms. Additionally, the production of outer membrane vesicles by *P. aeruginosa* under a variety of growth conditions has been confirmed by multiple studies (Schooling and Beveridge 2006; Bauman and Kuehn 2006;

Toyofuku et al. 2014). In this dissertation, *P. aeruginosa* is used as a model organism for studying the role of OMVs in bacterial biofilm development, and the following sections provide information on the biology and pathogenesis of this bacterium.

1.2.1 *P. aeruginosa* infections

P. aeruginosa is an opportunistic pathogen and, as such, can cause disease in a variety of immunocompromised patients. Its virulence is enhanced both by its high level of innate antibiotic resistance and its ability to adapt to changing environments, which allows the bacterium to survive under a wide range of conditions. While some *P. aeruginosa* infections are community-acquired, most of these cases involve highly immunocompromised patients, such as the elderly or those suffering from cystic fibrosis (CF). More often, however, severe *P. aeruginosa* infections are nosocomial, and infect those undergoing treatment for other conditions or the implantation of a foreign medical device, such as ventilators or catheters. Based on the high level of hospital-acquired, antibiotic resistant *P. aeruginosa* infections, the Centers for Disease Control (CDC) has classified this bacterium an ESKAPE pathogen and the World Health Organization has determined it to be a serious risk to public health (Skariyachan et al. 2018).

Following host colonization, *P. aeruginosa* establishes acute infections, which, under certain conditions, can progress to chronic disease. Most acute infections, as noted above, are nosocomial and associated with implanted devices and/or an immunocompromised host. For example, *P. aeruginosa* is the second most common cause

of ventilator-associated pneumonia (VAP), which occurs when ventilator insertion damages host epithelial surfaces, providing a reservoir for bacterial colonization (Gellatly and Hancock 2013). The plastic surface of the ventilator tube can also serve as a substrate for bacterial colonization, which can both cause and exacerbate these lung infections. Similarly, the plastic surface of catheter tubes may be contaminated with *P. aeruginosa* or serve as a colonization surface for bacterial biofilms, causing antibiotic-resistant urinary tract infections. Additionally, contact lenses may harbor *P. aeruginosa*, leading to significant corneal infection and damage. This bacterium is also known to cause severe infections in burn wound patients (Moradali, Ghods, and Rehm 2017).

While the danger of nosocomial acute *P. aeruginosa* infections is well documented as a significant public health hazard, the bacterium is perhaps most notorious for its contribution to chronic infections. In such cases, the immunocompromised status of the host prevents bacterial clearance, creating an environment in which the bacteria can persist against host immune responses and antibiotic interventions over long periods of time. In the case of CF, patients have a mutation in the CFTR ion transport channel, which causes dehydration of airway surface liquid (ASL), creating a dense, mucous-rich lung environment which prevents proper bacterial clearance. After colonization, the host mounts a strong immune response, mainly mediated by the recruitment of neutrophils to the lungs. Along with bacterial cytotoxicity, this immune activation causes significant damage to lung tissues, further exacerbating disease phenotypes. Due to high antibiotic

resistance in *P. aeruginosa* strains, these infections often cannot be adequately treated and may persist in the same patient for over several decades (Gellatly and Hancock 2013). During this time, the bacteria will adapt to the host, amplifying antibiotic-resistant small colony variants, downregulating traditional virulence factors to aid in immune evasion, and establishing expression of genes to promote mucoid phenotypes to better protect growing colonies. The damage caused both by the bacteria and host responses makes *P. aeruginosa* infection one of the major sources of morbidity and mortality in cases of CF. This type of long-term, destructive infection is also often seen in cases of chronic wounds, in which the bacteria and host responses prevent proper wound healing.

1.2.2 *P. aeruginosa* as a model organism for biofilm formation

While disease progression and bacterial biology can differ greatly between chronic and acute *P. aeruginosa* infections, recent research suggests that even short-term infections can include some degree of biofilm formation, which is a defining characteristic of *P. aeruginosa* pathogenesis and a source of antibiotic resistance. Due to high instance of biofilm formation during both infection and under laboratory culture conditions, *P. aeruginosa* is considered a model organism for the study of biofilm biology. The process of biofilm formation is detailed more extensively in the following section.

1.2.3 *P. aeruginosa* quorum sensing

Many bacteria, including *P. aeruginosa*, regulate cooperative behaviors via chemical signaling known as quorum sensing (QS). This density-dependent regulation

allows for the bacteria to sense the relative density of self-bacteria in the surrounding environment and tune their production of target genes accordingly. By engaging in this type of cell-to-cell communication, *P. aeruginosa* cells can conserve resources by synthesizing products such as virulence factors only when sufficient bacteria are present to create the desired outcome. Additionally, this prevents premature sensing of virulence factor production by the host immune system during infeciton, which aids in preventing clearance during early stages of colonization .

P. aeruginosa encodes two homoserine lactone (HSL)-based QS systems: Las and Rhl. Each of these include a transcriptional regulator (LasR or RhlR), an acylhomoserine lactone (AHL) signal (3-oxo-C12-HSL and C4-HSL), and an AHL synthase (LasI and RhlI) (Williams and Cámara 2009). The bacterium also produces a third signal, 2-heptyl-3-hydroxy-4(1H)-quinolone, known as *Pseudomonas* quinolone signal (PQS). These systems are regulated in a hierarchical structure, wherein LasR regulates RhlR and PQS positively, and RhlR regulates PQS negatively (Kariminik, Baseri-Salehi, and Kheirkhah 2017). Several separate studies have shown that at least 300 genes, and up to 11% of the *P. aeruginosa* genome, are subject to AHL-based regulation (Whiteley, Lee, and Greenberg 1999; Schuster et al. 2003; Wagner et al. 2003).

It is well-established in the field that QS control influences bacterial virulence and can have significant effects on biofilm formation (de Kievit 2009). Specifically, deletion of the Las system prevents biofilm maturation, and Rhl is involved in transcription of genes

required for synthesis of the polysaccharide Pel. QS additionally controls production of rhamnolipids, which are important for the maintenance of nutrient channels in mature biofilms and impacts swarming motility which influences biofilm composition and morphology. The expression of multiple virulence determinants, including alkaline protease, elastase, rhamnolipids, phospholipase B, exotoxin A, and pyocyanin, are also controlled by this hierarchy of QS signaling. Additionally, several of the secretion systems expressed by *P. aeruginosa*, including OMV-biogenesis, are controlled by QS systems. This adds an additional layer of QS control to bacterial virulence, as many of the aforementioned virulence factors are secreted by these systems (Turkina and Vikström 2019). QS molecules can also impact the host immune system, interfering with mucociliary clearance, epithelial tight junctions, NF-κB and MAPK signaling, and immune cell functions (Kariminik, Baseri-Salehi, and Kheirkhah 2017; de Kievit 2009; Turkina and Vikström 2019).

Due to the innate involvement of QS systems in *P. aeruginosa* biofilm formation and bacterial virulence, compounds that disrupt QS signaling are being considered as antibacterial strategies, especially in cases of antibiotic-resistant, biofilm-based chronic infections (Defoirdt 2018). This includes molecules found by high-throughput screens to inhibit QS signaling, compounds such as flavonoids which block AHL receptors, and antibodies which can sequester signal molecules and induce a host immune response.

1.2.4 *P. aeruginosa* virulence

P. aeruginosa releases a wide variety of virulence determinants into the extracellular environment. These bacterial products determine bacterial pathogenesis and mediate host responses to the bacterium. Interestingly, a relatively large portion of these factors display proteolytic activity. In fact, nearly 3% of *P. aeruginosa* genes are predicted proteases, and several of these are prominent sources of virulence produced by *P. aeruginosa*. In this section, we discuss proteolytic virulence factors, including elastases A and B, alkaline protease, and protease IV, as well as non-proteolytic molecules that can contribute to *P. aeruginosa* pathogenesis.

1.2.4.1 P. aeruginosa proteases

Four major endoproteases secreted by the *P. aeruginosa* type 2 secretion system (T2SS) have been studied extensively and shown to contribute substantially to *P. aeruginosa* virulence in a number of different infection models: elastase B, elastase A, alkaline protease, and protease IV (Hoge et al. 2010). Elastase B (LasB), a member of the M4 thermolyin peptidase family, is perhaps the most well-characterized virulence determinant produced by this organism and confers significant pathogenic potential to the bacterium. Importantly, it has significant elastolytic activity (Tulnier et al. 1989). Degradation of elastin increases collagen in the lung, and this activity is partially responsible for lung fibrosis observed in CF patients (Voynow, Fischer, and Zheng 2008). It has also been found to degrade IgG (Bainbridge and Fick 1989) and IgA (Heck et al.

1990), as well as various components of the complement system (Schultz and Miller 1974). Notably, IgG degradation products also prevent bacterial phagocytosis by neutrophils, likely due to the blocking of surface receptors on the immune cells (Bainbridge and Fick 1989). LasB can additionally degrade surfactant proteins (Mariencheck et al. 2003), contribute to corneal infections via degradation of the stromal proteoglycan extracellular matrix (Kessler, Kennah, and Brown 1977), and increase lung epithelium permeability (Azghani, Miller, and Peterson 2000).

Elastase A (LasA), also known as stapholysin, displays less elastolyic activity compared to LasB, but can also affect epithelial cell tight junctions, contributing to bacterial invasion of host tissues (Fleiszig, Zaidi, and Pier 1995). Importantly, this enzyme also causes lysis of *Staphylococcus aureus* cells, which confers a competitive advantage to *P. aeruginosa* during the early stages of lung colonization (Smith, Blackman, and Foster 2000). Protease IV (PIV) cleaves the clotting factor fibrinogen and may contribute significantly to hemorrhage seen during *P. aeruginosa* infection (Elliott and Cohen 1986). It also has been found to degrade plasminogen, IgG, and several complement components (Engel et al. 1998), as well as contribute significantly to corneal keratitis. Finally, alkaline protease (AprA), also known as aeruginolysin, degrades laminin (Heck, Morihara, and Abrahamson 1986), an important part of basal lamina, and through this activity may potentially contribute to bacterial invasion and hemorrhagic tissue necrosis. It has additionally been found, along with LasB, to inactivate γ-interferon and TNF-α (Horvat

and Parmely 1988; Parmely et al. 1990). AprA can also rapidly degrade the pro-inflammatory cytokine IL-6 (Matheson, Potempa, and Travis 2006).

1.2.4.2 Other *P. aeruginosa* virulence factors

Like other pathogenic bacteria, many of the cell products made by *P. aeruginosa* contribute to the bacterium's pathogenicity or elicit host immune responses. This includes motility factors, non-proteolytic secreted proteins, lectins, and phenazines, as well as bacterial products which contribute to biofilm formation (Al-Wrafy et al. 2017).

While *P. aeruginosa* is well known for its ability to form stationary biofilm colonies, the bacterium is, in fact, motile. It possesses one polar flagellum that mediates the majority of the bacterium's motility, as well as multiple type IV pili. The flagellar appendage contributes not only to bacterial chemotaxis but can also initiate an inflammatory response from the host and mediate surface interactions with epithelial cells via binding with asialylated glycolipid (Lyczak, Cannon, and Pier 2000). *P. aeruginosa* pili, on the other hand, mediate the bacterium's adherence to host cell membranes and other surfaces, and this activity is critical to the initial stages of biofilm formation. Pili can additionally mediate bacterial twitching motility and avoidance of the host immune system (Driscoll, Brody, and Kollef 2007).

Like other bacterial pathogens, *P. aeruginosa* produces a type 3 secretion system (T3SS) that can deliver toxic molecules to the extracellular environment and host cells. It is frequently associated with acute infections and causes increased mortality in infected

patients (Gellatly and Hancock 2013). Interestingly, throughout the course of chronic infection, *P. aeruginosa* strains usually lose the ability to express this system, likely as a way for the bacterium to evade host immune responses to infection and prevent bacterial clearance. The *P. aeruginosa* T3SS has four effector proteins which are expressed variably in different strains: ExoY, ExoS, ExoT, and ExoU. ExoS, ExoT and ExoU are all known to cause host cell dysfunction and death, while ExoY causes disruption of the actin cytoskeleton and inhibition of bacterial uptake by host cells (Hauser 2009). To date, the full role of each of these effectors in pathogenesis remains unclear, though it is thought that they allow the bacterium to exploit breaches in the epithelial layer, leading to delays in wound healing, and they may promote cell injury that causes the symptoms of bacterial pneumonia (Gellatly and Hancock 2013).

Other virulence factors expressed by *P. aeruginosa* include lipopolysaccharide, several lectins, pyocyanin, and rhamnolipids. As with other Gram-negative pathogens, LPS from *P. aeruginosa* is an important MAMP and can cause significant immune activation as well as endotoxic shock in the host (Pier 2007). Two lectins, LecA and LecB, are also present in the bacterial outer membrane and contribute to bacterial adhesion to host cells (Chemani et al. 2009). Pyocyanin in a bacterial phenazine that aids in bacterial iron acquisition and can have pro-inflammatory effects, causing additional damage to host tissues. Interestingly, this molecule is pigmented and gives *P. aeruginosa* cultures their characteristic green appearance (Lau et al. 2004). Rhamnolipids are bacterial

surfactant molecules that aid in biofilm formation and swarming motility. They have additionally been found to disrupt host epithelial layers and prevent mucociliary clearance. These molecules have also been shown to have anti-microbial effects against non-self bacteria, thereby aiding in competition during colonization (Read et al. 1992).

1.2.5 *P. aeruginosa* OMVs

Vesicles produced by *P. aeruginosa* have been described relatively extensively in the literature, but their biological functions and biogenesis mechanisms are still incompletely understood. In this section, we provide an overview of what is currently known about these OMVs. We outline mechanisms of *P. aeruginosa* OMV biogenesis, including a bilayer couple model involving intercalation of PQS into the OM, as well as OMV composition, known biological functions, and involvement in host-microbe interactions.

1.2.5.1 *P. aeruginosa* OMV biogenesis

PQS, a *P. aeruginosa* QS molecule, has been found to be a major component of *P. aeruginosa* OMVs and an inducer of vesicle biogenesis. In fact, Mashburn et al found that 86% of the PQS produced by *P. aeruginosa* is packaged into OMVs (Mashburn and Whiteley 2005). Additionally, several studies have shown that deletion of PQS reduces OMV formation, and that addition of exogenous PQS is sufficient to restore vesiculation in these strains. This occurs without the need for the PQS receptor, indicating that PQS-stimulated vesicle production was not due to QS signaling, but potentially a physical

response to the QS molecule. Using a red blood cell model, Schertzer et al demonstrated that PQS can insert into a lipid membrane nonspecifically and induce membrane curvature. This finding prompted the researchers to propose a bilayer-couple model of OMV biogenesis in *P. aeruginosa*. In this model, PQS binds to LPS in the outer leaflet of the OM, due in part to the molecule's hydrophobic nature, inducing asymmetric expansion of the outer leaflet. This asymmetry forces the membrane to buckle, and the curvature increases until the OM buds from the surface of the bacterial cell, creating a vesicle (Schertzer and Whiteley 2012). It has since been shown that PQS insertion into the membranes of other bacteria can also lead to vesiculation (Horspool and Schertzer 2018).

While PQS-mediated OMV biogenesis appears to be a major mechanism for vesicle production in planktonic *P. aeruginosa*, additional studies have noted that vesiculation occurs in strains deficient in PQS (MacDonald and Kuehn 2013). Therefore there must be multiple routes for *P. aeruginosa* OMV biogenesis, and further study will be needed to determine the mechanism and conditions that influence this. While cell lysis is not the typical mode of vesicle production, current research suggests that a certain subset of biofilm vesicles are created from cell lysis products. This produces a subpopulation of OMVs with different sizes and antimicrobial properties as compared to PQS-induced vesicles (Cooke et al. 2019). This is unsurprising, considering the relatively high level of bacterial lysis seen in biofilms, which contributes to the accumulation of extracellular DNA in the biofilm matrix. However, this likely does not explain OMV formation in the

absence of PQS in planktonic cultures, and more study will be required to define these biogenesis mechanisms.

1.2.5.2 Composition of *P. aeruginosa* OMVs

Like vesicles from all other Gram-negative species, *P. aeruginosa* OMVs are formed form the OM and periplasmic content of producer cells, and generally reflect their content. The membrane of *P. aeruginosa* OMVs, however, has been found to preferentially enriched in B-band LPS (Kadurugamuwa and Beveridge 1995). As expected, these membranes have also been found to contain multiple outer membrane proteins (OMPs) that are also normally present in whole cell OMs, though no significant enrichment for any of these proteins has yet been noted in OMVs (Toyofuku et al. 2012; Choi et al. 2014).

Several specific virulence factors have been identified in OMV preparations, including LasA, phospholipase C, hemolysin, and alkaline phosphatase. Additionally, protease activity has been detected in these samples (Kadurugamuwa and Beveridge 1995). Interestingly, *P. aeruginosa* vesicles were also found to harbor anti-microbial activity against bacteria of different species (Kadurugamuwa and Beveridge 1996). More recent studies have used proteomics to study the protein content of planktonic and biofilm-derived OMVs, and these studies have reported vesicle packaging of proteins involved in antibiotic resistance, proteolysis, and host-microbe interactions (Choi et al. 2014; Toyofuku et al. 2012). It has also been noted that growth conditions, stress conditions, and mode of growth (i.e. biofilm vs. planktonic) can alter the composition and size of

OMVs (Cooke et al. 2019; MacDonald and Kuehn 2013; Kadurugamuwa and Beveridge 1996).

1.2.5.3 *P. aeruginosa* OMV functions and interaction with host cells

As mentioned above, the packaging of known virulence determinants into *P. aeruginosa* OMVs has been noted by multiple studies (Kadurugamuwa and Beveridge 1997; Toyofuku et al. 2012). It is therefore unsurprising that these vesicles also display immunostimulatory activity against host cells. In a tissue culture macrophage model, vesicles were shown to elicit MIP-2, TNF-α, IL-1β, and IL-6 responses from the host cells. These responses were significantly increased as compared to treatment with corresponding levels of purified LPS, and the results indicated the involvement of both vesicle membrane material and protein cargo in immune stimulation (Ellis, Leiman, and Kuehn 2010). *P. aeruginosa* OMVs have also been found to associate with and be internalized by lung epithelial cells. Upon association, they produced a potent IL-8 response from the host cells, which was significantly higher when the OMVs were isolated from certain clinical *P. aeruginosa* isolates (Bauman and Kuehn 2009).

P. aeruginosa OMVs have additionally been observed as major components of biofilm matrices. More in-depth discussion of the relationship between vesicles and biofilms is found in the following section.

1.3 Biofilm formation

While most bacteria are studied *in vitro* as planktonic (free-swimming) communities, recent studies suggest that up to 85% of bacterial species have the ability to form biofilms (Jamal et al. 2018). Biofilms are stationary, multicellular bacterial communities surrounded by a dense extracellular matrix composed of extrapolymeric substance (EPS). The physical and molecular properties of biofilm bacteria provide strong resistance to a variety of bacterial stressors, including antibiotic treatment, environmental extremes, and host immune attack (Flemming et al. 2016).

P. aeruginosa forms biofilms under both infectious and non-infectious conditions and is considered a model organism for the study of biofilm development. Biofilms are more common and well-studied in cases of chronic *P. aeruginosa* infection, such as CF and chronic wound infections, but this process has also been documented in cases of acute infection (Schaber et al. 2007). Formation of biofilm colonies, known as microcolonies, during infection creates a physical barrier to bacterial clearance, and prevents antibiotic-mediated bacterial killing. Accordingly, the discovery of novel anti-biofilm therapies and research into biofilm mechanisms that may help inform these therapies are priorities in both clinical and basic science research.

1.3.1 Stages of biofilm maturation

In its planktonic state, *P. aeruginosa* expresses both flagella and pili, which allow it to move freely throughout its environment. Biofilm formation can be induced in these

motile bacteria by a number of stimuli, including location of a suitable surface, environmental stress conditions, sensing of host tissues, and predation. Once this process is induced and the bacteria locate a suitable surface for colonization, the cells terminate expression of motility apparatuses and begin the formation of multicellular microcolonies (Gellatly and Hancock 2013). An overview of this process is shown in **Figure 2**.

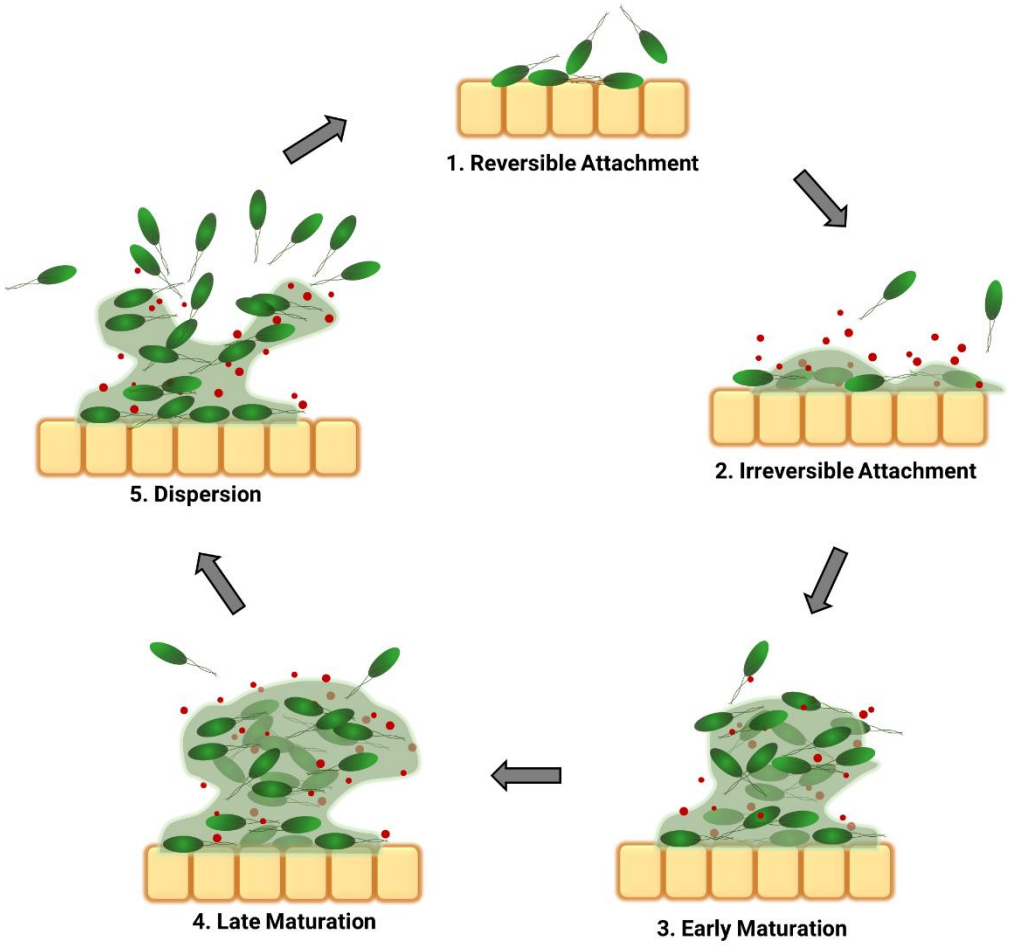

Figure 2: The stages of biofilm formation

Biofilm formation begins with an initial interaction of the bacterial cells with either a host cell or abiotic substrate. This stage is called reversible attachment (**1**) because the cells may leave the developing structure if the environment is unsuitable. As the process continues, they secrete EPS which causes irreversible attachment (**2**) to the substratum. The colony then undergoes several stages of maturation (**3**,**4)**, during which the colony develops more complex structures. Finally, as the colony experiences nutrient limitation or other cues indicating the need for a change in environment, cells will disperse (**5**) from the colony structure and find new substrates to colonize, beginning the process again.

After the bacteria initially attach to the host or environmental substrate, they enter a second stage of irreversible attachment, in which they associate more firmly with the substrate. The microcolony then grows both through cell division and recruitment of planktonic cells to the colony structure. Throughout the process, the biofilm bacteria secrete EPS, including polysaccharides, proteins, eDNA, and OMVs, to create a dense extracellular matrix. As the colony grows, the matrix surrounds the cells providing a physical barrier to protect against environmental extremes, dehydration and nutrient limitation, and host immune attack or predation. During colony maturation, depending on growth conditions and nutrient availability, it can take on a mushroom-shaped structure and channels may form through the structure to aid in water and nutrient delivery. If nutritional needs overwhelm the colony, or the bacteria receive other environmental cues that are interpreted as a need to move, the colony can disperse (Mann and Wozniak 2012). Single motile cells may release from the colony structure to find a new, potentially more habitable, environmental niche, or the entire colony may dissemble, releasing a number of active cells all at once through a process known as seeding dispersal (Purevdorj-Gage, Costerton, and Stoodley 2005).

1.3.2 The biofilm matrix

The biofilm matrix serves an invaluable function in the protection of biofilm microcolonies, and its function is directly related to its component parts. The major components of the *P. aeruginosa* biofilm matrix are polysaccharides, proteins, eDNA, and

OMVs. Three main polysaccharides have been described in the *P. aeruginosa* biofilm extracellular matrix: Psl, Pel, and alginate. Psl is important to initial bacterial attachment and also forms a web-like skeleton with eDNA, providing structural support to the matrix. Pel has also been shown as critical to matrix structural integrity and pellicle development, and either Psl or Pel are required for microcolony development (de Kievit 2009). Alginate production is not necessary for *P. aeruginosa* biofilm formation, but it does lead to the formation of mucoid colonies, which, as the name suggests, form a dense, mucous-like matrix. Mucoidy and alginate, as well as their respective contributions to pathogenesis, have both been studied extensively, as they are often observed in *P. aeruginosa* isolates from chronically-infected CF patients (Rivera and Nicotra 1982).

eDNA has been found to be an important component of the matrices of early biofilms and less important to colony structure as the biofilm matures, as was shown by DNase treatments of flow cell PAO1 biofilms (Whitchurch et al. 2002). In both of these stages of biofilm maturation, however, eDNA helps to define the structure of biofilm colonies and can influence the formation of mushroom-shaped microcolonies (de Kievit 2009). eDNA is released through programmed cell lysis of specific biofilm cells, and at least one pathway involved in this process has been shown to be under quorum sensing control (Allesen-Holm et al. 2006). Clinically, DNase is used to help disrupt *P. aeruginosa* lung infections and allow for better penetration of antibiotics to biofilm-associated cells

(Smith et al. 2017). eDNA can also interact with OMVs found in the biofilm matrix (Schooling, Hubley, and Beveridge 2009).

While the polysaccharide and eDNA content of biofilm matrices have been studied extensively, the identity and functions of matrix proteins remain poorly understood. Three major proteins, however, have been characterized in *P. aeruginosa* biofilms: LecA, LecB, and CdrA. LecA and LecB are lectins (carbohydrate-binding proteins) with demonstrated sugar specificity. LecA is required for biofilm formation on polystyrene and stainless-steel surfaces, and its biofilm-modulating properties involve its galactose binding site. Interference with this site has also been shown to induce dispersion in mature biofilms (Diggle et al. 2006). LecB has been found to localize to the OM via interactions with the OMP OprF, and likely mediates biofilm formation through interactions with the galactose and mannose residues on Psl (Tielker et al. 2005; Funken et al. 2012). CdrA is a cell-associated protein that mediates intercellular interactions during biofilm formation and induces cellular auto-aggregation in *P. aeruginosa* cultures. This protein has also been shown to directly interact with the matrix polysaccharide Psl, which leads to protease-resistant intercellular interactions (Reichhardt et al. 2018). Recently, proteomics approaches have been used to help identify other proteins present in the extracellular matrix (Toyofuku et al. 2012). These studies have identified a variety of OMPs in the matrix which may be associated with OMVs, as well as several prominent virulence factors, including LasB and alkaline protease.

1.3.3 OMVs and biofilms

Outer membrane vesicles can be produced by both planktonic and biofilm-associated cells, and they have been shown to be a major component of biofilm matrix under a wide variety of growth conditions and in various models of biofilm formation from different Gram-negative species (Schooling and Beveridge 2006; Toyofuku et al. 2012; Wang, Chanda, and Zhong 2015). Schooling et al clearly demonstrated that OMVs are a major component of *P. aeruginosa* biofilms by imaging these structures directly using transmission electron microscopy (TEM), as well as isolating vesicles from biofilm-grown cells (Schooling and Beveridge 2006). Interestingly, biofilm bacteria have been found to produce significantly more OMVs than planktonically grown bacteria, and vesicle content can vary substantially based on the bacterial mode of growth. Exogenous addition of OMVs to low-vesiculating strains has also been found to enhance biofilm formation in *H. pylori* (Yonezawa et al. 2009). More recent work has demonstrated that vesicles may constitute up to 20% of the proteins identified in the *P. aeruginosa* biofilm matrix (Couto et al. 2015). These data suggest not only that OMVs are integral parts of biofilm biology, but also that the cells preferentially produce vesicles of different composition to aid in the process of biofilm formation and as a protective component of the biofilm matrix.

Recent research into biofilm OMVs has focused on identifying vesicle cargo as well as the roles that vesicle-associated molecules play in biofilm formation and integrity. Several studies have examined the matrix proteome and identified a number of proteins

that may be associated with biofilm-derived vesicles (Couto et al. 2015; Toyofuku et al. 2012). Proteins identified in these studies include outer membrane proteins (OMPs), flagella and pili-associated proteins, proteins involved in the transport of small molecules, and virulence factors, including adhesins and proteases. Research into the effect of these molecules on the biofilm matrix is ongoing, but several studies have noted a structural role for OMVs in biofilm formation. For instance, biofilm-derived OMVs can interact with extracellular DNA, another major component of the matrices of early biofilms, providing a potential role for the vesicles in the formation of a robust matrix architecture (Schooling, Hubley, and Beveridge 2009). The functional roles for OMV cargo in biofilms, however, remain poorly understood.

1.3.4 Models for studying biofilm formation

Biofilm formation occurs under a wide variety of both environmental and infectious conditions, and the models used for studying these communities *in vitro* reflect this diversity. While this offers many opportunities for the study of diverse biofilm-associated phenotypes, it also represents a major challenge in the field, as studies often cannot be compared. The most basic model for biofilm development, particularly in *P. aeruginosa*, uses a Crystal violet stain to examine the formation of biofilms at the air-liquid interface of static cultures. These biofilms, known as pellicles, can be grown in multi-well dishes, and this protocol allows for implementation of high-throughput studies (O'Toole 2011). Biofilm formation can also be seen in agar-plate colonies, and various studies,

including several examining matrix proteomes and vesicles, use this convenient method (Toyofuku et al. 2012; Schooling and Beveridge 2006). Certain biofilms are also known to form under varying levels of fluid flow, such as those formed on piping in water reservoirs or on the surface of catheters. Flow cell reactors are therefore used in some biofilm studies, and they induce the adherence of bacterial cells to the chosen substrate. Due to constant refreshing of culture media, these systems also allow for biofilms to be studied over longer periods of time and through greater degrees of maturation.

To model biofilm formation under infectious conditions, some researchers have developed models to study biofilms grown on host cell substrates. Usually, these models utilize epithelial cell lines, such as CFBE cells which harbor the CFTR cystic fibrosis mutation or other lung epithelial cell lines used to model respiratory infection (Moreau-Marquis et al. 2010; Chugani and Greenberg 2007). These models are thought to provide the bacteria with more infection-like condition, and the presence of the host cell can stimulate bacterial responses not seen on abiotic surfaces. Some research suggests, however, that bacterial cells may not come into direct contact with host cells during certain types of infection. This has prompted the development of artificial sputum media, which induces the formation of biofilm-like structures in suspension and may mimic some of the conditions the bacteria experience during CF lung infections (Sriramulu et al. 2005). Finally, certain models utilize different materials to mimic the substrate of interest, such as plastic tubing used for the study of catheter biofilms (Lebeaux et al. 2013). The wide

range of models used in biofilm research has posed significant problems in the field, especially when trying to compare the results between gene expression and proteomics experiments. The development and implementation of consistent models for biofilm research is therefore ongoing and represents an important question in the field of biofilm biology.

1.4 Bacterial aminopeptidases

1.4.1 Description and classification of aminopeptidases

Aminopeptidases are exopeptidases that selectively cleave a single amino acid residue from the N-terminus of substrate polypeptides. Typically, these enzymes are classified based on their substrate specificity, cellular localization, catalytic function, requirement for cofactors, and optimum pH. They are additionally divided into three main groups based on their sensitivity to certain inhibitors: metallo-aminopeptidases (which require coordination of divalent metallic cations), cysteine aminopeptidases, and serine aminopeptidases. Metallo-aminopeptidases are the most common of these, and compromise almost two-thirds of described bacterial aminopeptidases. In most cases, these enzymes require coordination of Zn^{+} ions for proper folding and activity. Cysteine and serine aminopeptidases, on the other hand, do not require ionic cofactors, but rather a reactive cysteine or serine residue (Gonzales and Robert-Baudouy 1996).

Aminopeptidases can additionally be classified as broad specificity or narrow, depending on whether they are able to cleave multiple different amino acids or only a

single amino acid residue, respectively. Most, though, are stereospecific and prefer L-form amino acids. Of the aminopeptidases identified in bacteria, 65% are cytoplasmic, 16% are found either in the Gram-negative periplasm or Gram-positive cell wall, and 16% are secreted into the extracellular environment (Gonzales and Robert-Baudouy 1996).

1.4.2 Traditional physiological roles of bacterial aminopeptidases

Based on their relatively straightforward enzymatic activity, most bacterial aminopeptidases are known to help in the degradation of proteins either as nutrient sources or to aid in protein turnover (Jarocki, Tacchi, and Djordjevic 2015). Like most organisms, bacteria require certain essential amino acids for growth. Aminopeptidases allow for the digestion of proteins or peptides to acquire these amino acids when they are not readily present in the environment. For example, *Lactococcus lactis*, a bacterium often used in the production of fermented milk products, encodes a complex system of proteases and peptidases to acquire its essential amino acids. After initial degradation of milk proteins by endoproteases, aminopeptidases scavenge the required amino acids from peptide fragments (Tan, Poolman, and Konings 1993). Aminopeptidases can also aid in protein turnover for their producing bacterial cells. During normal growth, bacteria establish an equilibrium between new protein synthesis and protein degradation, and aminopeptidase digestion allows for amino acids from degraded proteins to be re-used in new protein synthesis. This is particularly important for bacteria under nutrient-limiting conditions. Finally, bacterial aminopeptidases can aid in the maturation of endogenous

protein precursors that require processing before reaching their final, active forms (Gonzales and Robert-Baudouy 1996).

1.4.3 Bacterial protease immune-modulating and moonlighting functions

While traditional roles for bacterial proteases and aminopeptidases are relatively well-characterized, an emerging field of research has provided mounting evidence that many of these enzymes may serve additional functions in host immune regulation during infection, either through their enzymatic activity or secondary "moonlighting" functions. The proteases expressed by *P. aeruginosa* provide good examples of protease-mediated virulence activity, though many of these display promiscuous endoprotease activity, as opposed to the relatively specific activity of aminopeptidases (Hoge et al. 2010).

Aminopeptidases with virulence-modulating activity have been identified in a number of both bacterial and Protozoan species, and several of these studies suggest that the enzymes may be promising targets for inhibitor-based clinical therapies. In most cases, however, the full mechanism behind these virulence-related phenotypes has not been elucidated. For instance, the M29 family, cobalt-dependent aminopeptidase T from *Listeria monocytogenes* has been recently shown to affect bacterial growth and virulence during infection. This aminopeptidase localizes to the bacterial cytosol and was found to be required for invasion and survival inside human epithelial cells and murine macrophages. Deletion of the aminopeptidase resulted in nearly full attenuation of virulence in a mouse infection model (Cheng et al. 2015). Additionally, a *Mycobacterium tuberculosis* leucine

aminopeptidase (MtLAP) was found to be involved in bacterial growth and macrophage infection, and inhibition of aminopeptidase activity reduced both bacterial burden and lesions in the lungs of infected mice (Correa et al. 2017). In protists, knockout and inhibitor studies have shown a role for various aminopeptidases in the pathogenesis and virulence activity of *Acanthamoeba castellanii* (Huang et al. 2017), *Trichomonas vaginalis* (Puente-Rivera et al. 2017), and *Toxoplasma gondii* (Zheng, Jia, and Zheng 2015).

A limited number of residues within peptidases are required for enzymatic activity, and it therefore follows that other domains may be involved in non-proteolytic functions. For certain peptidases, these non-enzymatic activities, referred to as moonlighting functions, have direct impacts on host-microbe interactions. Many of these moonlighting functions have been shown to mediate bacterial adhesion to host cells or tissues (Jarocki, Tacchi, and Djordjevic 2015). A prime example of this is the C5a peptidase from *Streptococcus agalactiae*, which, through its enzymatic domain, degrades the C5a component of the complement system, a known neutrophil chemotaxin (Bohnsack et al. 1991). Additionally, its non-enzymatic domains confer important fibronectin adhesin activity. Deletion of the C5a peptidases reduces the bacterium's fibronectin binding abilities by over 50%, demonstrating the critical importance of this enzyme in mediating bacterial attachment to host substrates (Beckmann et al. 2002). Other moonlighting functions found in bacterial peptidases include plasminogen binding, which aids in fibrinolysis and subsequent bacterial escape from host defenses, as well as molecular Cpn

funcitons, which aid in the folding or unfolding of other macromolecules. Several bacterial enzymes have also been found to serve as cell-surface receptors (Jarocki, Tacchi, and Djordjevic 2015).

1.5 P. aeruginosa *leucine aminopeptidase (PaAP)*

The ***P****seudomonas* ***a****eruginosa* **a**mino**p**eptidase (PaAP) is a 56 kDa enzyme that preferentially cleaves N-terminal leucine amino acid residues (Cahan et al. 2001). The role of PaAP in *P. aeruginosa* biology has not been well-characterized; however, various studies examining diverse aspects of *P. aeruginosa* cellular processes have repeatedly suggested an important functional role for the aminopeptidase. This includes potential contributions to biofilm formation, infection, and bacterial survival. These studies are outlined in **Table 1** and detailed more extensively in the following sections.

Table 1: Previous studies characterizing PaAP and suggesting a role for the aminopeptidase in *P. aeruginosa* pathogenesis and biofilm formation

Authors	Publication Year	Major Findings
PaAP Identification		
Braun et al.	1998	Described PaAP accumulation in secretomes of *P. aeruginosa* strains under protease-limiting growth conditions
Cahan et al.	2001	Identified PaAP via leucine aminopeptidase activity in *P. aeruginosa* secreted material
Nouwens et al.	2003	Determined PaAP to be regulated by Las quorum sensing system
Michel et al.	2007	Characterized PaAP secretion via *P. aeruginosa* QS-regulated T2SS
Sarnovsky et al.	2009	Characterized PaAP activation and processing by other endogenous *P. aeruginosa* proteases
PaAP Involvement in Biofilm Formation and Pathogenesis		
Bauman et al.	2006	Characterized PaAP as a major component of *P. aeruginosa* OMVs. Determined PaAP to be more highly expressed in *P. aeruginosa* clinical isolates from chronically infected CF patients
Chugani et al.	2007	Described increased expression of PaAP in *P. aeruginosa* grown on host epithelial cells (as compared to TSB-grown bacteria)
Alvarez-Ortega et al.	2007	Described 21-fold increased PaAP expression under microaerobic conditions
Bauman et al.	2009	Determined PaAP mediates OMV internalization into lung epithelial cells
Toyofuku et al.	2012	Described PaAP as a major protein component of biofilm matrices Showed increased PaAP packaging into OMVs from biofilm-grown cells (compared to planktonic)

1.5.1 PaAP identification and classification

PaAP was first described by Braun et al, who noted accumulation of a ~58 kDa protein in *P. aeruginosa* secretomes under protease-limiting growth conditions (Braun et al. 1998). While they were unable to identify the protein, it was noted that it displayed significant identify with an aminopeptidase of *Streptomyces griseus.* Cahan et al further characterized this protein after they observed significant leucine aminopeptidase activity in the secreted material produced by *P. aeruginosa*. They traced the origin of this activity back to a single protein with 536 amino acid residues and found that the fully activated 56 kDa enzyme is encoded by the *P. aeruginosa* gene PA2939. PaAP displays homology to other known aminopeptidases, with 52% similarity to the SgAP aminopeptidase from *Streptomyces griseus*, 35-36% similarity to aminopeptidase Y from *Saccharomyces cerevisiae* and an aminopeptidase from *Bacillus subtilis*, and 29-32% similarity to VpAP, VcAP, and AcAP from *Vibrio proteolyticus*, *Vibrio cholerae*, and *Aeromonas caviae*, respectively. These enzymes additionally display many similar functional characteristics, including enzymatic activity, secretion as propetides, and binding of two zinc ions at the enzymatic active site. These enzymes are all members of the M28 family of Zn-dependent metalloproteases (Cahan et al. 2001).

In their characterization of PaAP, Cahan et al found that while the aminopeptidase was able to cleave N-terminal methionine and alanine at low levels, it displayed a substantial preference for N-terminal leucine amino acids. Additionally, the only

inhibitors active against the enzyme were dithiothreitol and Zn chelators, including tetraethylene pentaamine and 1,10 phenanthroline, which strongly suggested that, like the previously mentioned M28 metalloproteases, PaAP required zinc incorporation at its active site to display enzymatic activity (Cahan et al. 2001). This was later confirmed by Sarnovsky et al, who identified potential zinc-binding residues at the protein's Asp-308 and Asp-369 residues based on homology to SgAP (Sarnovsky et al. 2009). Based on its activity and coordination of zinc ions at its active site, PaAP was thus determined to also be a member of the M28 metalloprotease family. While many aminopeptidases are known to form oligomeric structures, VpAP and SgAP act as monomers of about 30 kDa (Cahan et al. 2001). While more research will be required to determine whether PaAP forms an oligomer, its close relationship to these other two aminopeptidases suggests that it may also function monomerically.

1.5.2 PaAP expression, secretion, and activation

Quorum sensing systems in *P. aeruginosa* contribute significantly to both bacterial virulence and biofilm formation, and analysis of QS mutants has shown that PaAP expression is mediated by the LasR QS regulator (Nouwens et al. 2003). As such, it is produced only when the bacteria reach a threshold density. Once it is expressed, it is secreted into the extracellular milieu by the *P. aeruginosa* Xcp Type 2 Secretion System (T2SS) (Braun et al. 1998; Michel, Durand, and Filloux 2007). T2SS form transmembrane complexes that span both the Gram-negative inner and outer membranes, and they are

used to secrete exoproteins bearing a Sec or Tat peptide secretion sequence (Cianciotto 2005). While T2SS are conserved over a wide range of bacterial species, the proteins they secrete are largely species-specific. *P. aeruginosa* encodes two different T2SS: the Hxc system, which is functional under phosphate limitation and mainly serves to secrete a specific alkaline phosphatase (Ball et al. 2002), and the more general Xcp system, through which PaAP is secreted.

In *P. aeruginosa*, most of the proteins identified to be secreted by T2SS are hydrolytic, and include the potent toxin elastase, exotoxin A and phospholipase C. This indicates that the system is intimately involved in virulence mechanisms for the bacteria. The Xcp system is known to be under the control of the both the Las and Rhl quorum sensing machinery (de Kievit 2009). Interestingly, Michel et al found that *P. aeruginosa* encodes genes for two separate, homologous Xcp systems (Michel, Durand, and Filloux 2007). While one is under QS control, the second they found to be produced constitutively. Of all the substrates examined in this study, PaAP was the only product found to be secreted solely by the QS-controlled machinery. This adds an additional layer of QS-mediated control to PaAP production, providing increased evidence that the aminopeptidase is expressed only under specific bacterial growth conditions and further suggesting that it may play a role in bacterial virulence.

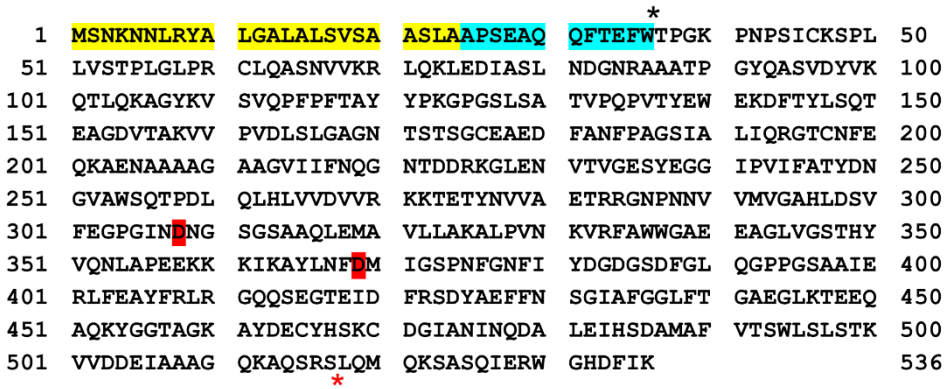

Figure 3: Annotated PaAP amino acid prosequence

PaAP is secreted as a propeptide, and the full sequence of this protein is shown. Important features of the propeptide are denoted as follows: yellow, signal sequence; blue, N-terminal prosequence; red, residues of Zn^{2+} coordination in the active site; black asterisk, site of N-terminal autoprocessing; red asterisk, site of C-terminal protease cleavage

The PaAP aminopeptidase is secreted as a full length propeptide that requires processing at both its N- and C-termini to become a fully active enzyme. **Figure 3** shows the full PaAP sequence and denotes its important features including: an N-terminal signal sequence (aa 1-24), an N-terminal prosequence (aa 25-36), a C-terminal epitope aa 521-536), active site residues (Asp-308, Asp-369), and an epitope near the active site required for activation (aa 294-309). After PaAP has been secreted into the extracellular environment, the C-terminal epitope is cleaved by third party proteases. In their work characterizing PaAP activation, Sarnovsky et al demonstrated that the C-terminal cleavage site is relatively promiscuous, and aminopeptidase activation can be completed by trypsin, chymotrypsin, or endogenous *P. aeruginosa* elastase (Sarnovsky et al. 2009).

However, as elastase is not required for PaAP activation in its 56 kDa form (Cahan et al. 2001), more study will be needed to determine what other endogenous proteases are able to complete this activation step. After C-terminal cleavage, the N-terminus prosequence interacts with a basic residue near the enzymatic active site. This allows for PaAP to undergo autoprocessing and use its own enzymatic activity to cleave aa 25-36. At this stage, PaAP is a 56 kDa, full-length, enzymatically active protein. Additional processing by elastase or induced by temperature-dependent degradation can further shorten the enzyme to form several smaller, enzymatically active products (Sarnovsky et al. 2009). The most common of these proteins is 28 kDa. The process of PaAP processing and activation process is graphically displayed in **Figure 4**.

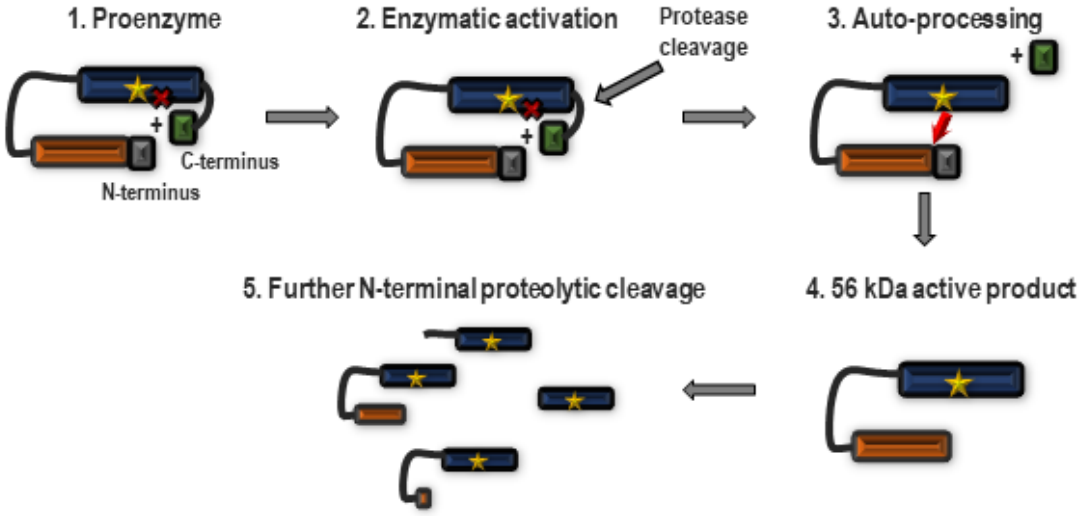

Figure 4: PaAP secretion, processing, and activation

PaAP undergoes several processing steps after its secretion into the extracellular environment. From its proenzyme state, the C-terminus is cleaved by other endogenous proteases secreted by *P. aeruginosa*, including elastase B. The active site then interacts with the N-terminus, allowing for autoprocessing that removes the prosequence and activates the enzyme. From this full-length 56 kDa product, the aminopeptidase can undergo additional cleavage by other proteases and form smaller, enzymatically active forms.

1.5.3 PaAP identification in *P. aeruginosa* OMVs

Multiple studies have noted that PaAP is one of the most abundant secretion products from a variety of *P. aeruginosa* strains (Cahan et al. 2001; Bauman and Kuehn 2006; Michel, Durand, and Filloux 2007). However, as detailed in section 1.1, bacterial secretomes contain both soluble secretion products as well as vesicle content. A previous member of our laboratory characterized vesicle content from various laboratory, environmental, and clinical *P. aeruginosa* isolates, and found PaAP protein, at varying expression levels, in the vesicle-containing fractions from these strains. Interestingly,

further analysis of fractionated cell-free supernatants reveled that PaAP is present in both vesicle-containing and soluble protein fractions. This indicates that, once secreted and activated, the aminopeptidase may associate with OMVs or remain soluble within the extracellular milieu. While more study is required to determine the localization of PaAP once associated with vesicles, the authors also state that preliminary evidence indicates the aminopeptidase associates with the surface of OMVs (Bauman and Kuehn 2009).

Toyofuku et al also noted significant packaging of PaAP into OMVs purified from biofilm matrices. In this study, vesicles were purified from both PAO1 biofilm colonies and planktonically grown cultures. PaAP was found to significantly associate with vesicles isolated from biofilm matrices but was notably absent from vesicles isolated from planktonic PAO1 (Toyofuku et al. 2012). These findings are generally consistent with work from our laboratory, which has shown a low level of PaAP association with OMVs from planktonic PAO1 cultures, likely due to relatively low expression of the aminopeptidase in this strain under standard growth conditions (Bauman and Kuehn 2009).

Due to the importance of OMVs in bacterial pathogenesis and biofilm formation, as well as the packaging of known virulence determinants into *P. aeruginosa* OMVs, the association of PaAP with these particles indicates the likely involvement of the aminopeptidase in pathogenesis phenotypes. Additionally, increased PaAP levels in

biofilm-associated OMVs suggests a possible role for the aminopeptidase in the process of biofilm formation.

1.5.4 PaAP's role in *P. aeruginosa* infection and biofilm formation

PaAP regulation by *P. aeruginosa* QS systems, its association with OMVs, and its enrichment in biofilm-derived OMVs all provide strong evidence for the aminopeptidase's involvement in processes relevant to both infection and overall *P. aeruginosa* biology. Further evidence additionally implicates PaAP in chronic CF infections, the association of *P. aeruginosa* and OMVs with host cells, as well as the development of a protective biofilm matrix.

In their characterization of *P. aeruginosa* OMVs, Bauman et al examined protein packaging into vesicles from several strains: PAO1 (a well-known laboratory strain), an environmental soil isolate, and three clinical isolates taken from chronically infected CF patients (CF2, CF3, and S470). While PaAP was observed at least at low levels in vesicles from each of these isolates, it was found to be dramatically higher in each of three clinical isolates. These data strongly indicate an important role for the aminopeptidase during chronic infection (Bauman and Kuehn 2006).

During infection, interactions between host cells and either bacterial cells or their secreted products can have important effects on the establishment of bacterial colonization and host immune responses. As such, the determination of changes in bacterial gene expression during infection or under infection-like *in vitro* conditions can

help inform on which bacterial products are involved in these processes. When *P. aeruginosa* was grown on a layer of polarized lung epithelial cells, PaAP expression was found to increase significantly along with other known virulence determinants (Chugani and Greenberg 2007). Additionally, *P. aeruginosa* OMVs were found to associate with and be internalized by A549 lung epithelial cells and human bronchial epithelial (HBE) primary cells. Internalization of these vesicles was increased when the OMVs were isolated from clinical strains with high PaAP expression, and deletion of the aminopeptidase significantly reduced vesicle uptake, providing further evidence that PaAP mediates the interaction of bacteria and bacterial products with host cells (Bauman and Kuehn 2009). Taken together, these data strongly suggest that the PaAP aminopeptidase plays a substantial role in mediating *P. aeruginosa* interactions with host epithelia.

Biofilm formation is another critically important aspect of *P. aeruginosa* biology, particularly during chronic infections, though it also plays a role in acute infections and environmental colonization. Not only has PaAP been shown to be more highly expressed in vesicles isolated from biofilm OMVs, but it was also found to be one of the top-ten most abundant proteins in the PAO1 biofilm matrix (Toyofuku et al. 2012). This is especially noteworthy due to low relative expression of PaAP in this strain and suggests that it may play an important role in modulation of biofilm matrix structure or protective properties. Additionally, expression of the PaAP-encoding gene was found to increase 21-fold under

microaerobic conditions, which is now believed to be a state that the bacteria experience during chronic CF lung infections (Alvarez-Ortega and Harwood 2007). While PaAP has been shown to be present in biofilms and biofilm-OMVs and upregulated under biofilm-formation conditions, a role for the aminopeptidase in biofilm development has not yet been established.

2. *P. aeruginosa* leucine aminopeptidase mediates early biofilm formation and affects matrix composition

2.1 Summary

While many bacteria are studied in their motile, or planktonic, state, it is now appreciated that the formation of sessile colonies, called biofilms, are the dominant mode of bacterial growth. The opportunistic pathogen *P. aeruginosa* is a model organism for the study of biofilms, but even still this process is incompletely understood. The aminopeptidase PaAP has been found as an important component of biofilm matrices and biofilm-associated vesicles, but its contribution to biofilm microcolonies remains unclear. In this chapter, we outline the development of a bacteria-host cell coculture model to study the effects of this aminopeptidase on biofilm formation under infection-like conditions. We show that deletion of the aminopeptidase led to microcolonies with increased cellular biomass and decreased matrix polysaccharides, creating a less-protective environment for the bacteria against antimicrobial challenge. We additionally demonstrated that, while PaAP may interact with both host and bacterial products, this phenotype applies to certain pellicle biofilms grown without a host cellular substrate, as well, implicating the aminopeptidase in early biofilm formation under multiple growth conditions.

Material in this chapter was adapted from the article "Pseudomonas aeruginosa leucine aminopeptidase influences early biofilm composition and structure via vesicle-associated anti-biofilm activity" published in *MBio* (Esoda and Kuehn, 2019).

2.2 Introduction

P. aeruginosa is considered a model organism for the study of biofilm formation, and many of the cellular and matrix components contributing to this mode of growth have been studied previously (Moradali, Ghods, and Rehm 2017; Davey and O'toole 2000; Mann and Wozniak 2012). Importantly, biofilm development requires that stationary bacteria secrete extrapolymeric substances (EPS) to form a dense matrix to surround and protect the developing colonies, as well as to aid in nutrient acquisition. As detailed above, the matrix contains a variety of polysaccharides, with Psl and Pel present in all strains, and alginate found in mucoid *P. aeruginosa* isolates (Franklin et al. 2011). It also contains proteins, lipids, extracellular outer membrane vesicles, and eDNA; however, the mechanism by which many of these secreted bacterial components specifically affect microcolony growth and development remain unclear.

As with other extracellular pathogens, *P. aeruginosa* secretes many of its virulence determinants into the extracellular milieu to affect interactions with the host, including those in the biofilm matrix. Interestingly, many of the virulence factors secreted specifically by *P. aeruginosa* demonstrate proteolytic activities (Hoge et al. 2010; Suleman 2016; Cahan et al. 2001). These enzymes, including elastases, protease IV, and alkaline protease, act on both bacterial and host proteins to directly impact host-pathogen interactions. For example, purified elastase has been found to cleave fibrin, laminin, various immunoglobulins, and components of the complement system (Galloway 1991).

The activity of these enzymes not only interferes with host defense mechanisms but may also compromise host epithelial junctions, allowing the bacteria into otherwise inaccessible tissues (Hoge et al. 2010). These proteases can additionally facilitate biofilm formation and antibiotic resistance, either through direct application of their proteolytic activity or through indirect regulation of other factors produced by the bacteria (Tielen et al. 2010). Based on the importance of known virulence-associated, secreted proteases in *P. aeruginosa* biofilm formation, it follows that other proteases in the secretome, many of which have not been fully characterized, may also play a role in this process.

With the goal of identifying factors that may contribute to chronic infections, our lab compared secretome protein expression profiles of clinical *P. aeruginosa* isolates from chronically infected CF patients to those of highly passaged laboratory and environmental strains. Several proteins were identified as being more highly expressed in all the clinical strains, indicating their potential involvement in virulence and chronic infection mechanisms. Among the proteins identified was the PA2939 aminopeptidase, known as PaAP. Outside of the cell, PaAP is found both soluble in the extracellular environment and as an abundant component of bacterial OMVs. It has also been shown to facilitate interactions between host cells and bacterial vesicles (Bauman and Kuehn 2009).

In this chapter, we report that the formation and composition of early biofilms are modulated by the secreted *P. aeruginosa* leucine aminopeptidase. Using a modified coculture model in which single-species bacterial biofilms are formed on a confluent layer

of host cells, we show that PaAP expression inhibits the formation of cellularly dense microcolonies, while promoting greater exopolysaccharide production and colistin resistance. We also provide preliminary evidence that the aminopeptidase interacts with proteins produced by both the host and bacteria. Finally, we show that under certain media and culture conditions, this phenotype is also observed during pellicle biofilm formation, affecting both matrix polysaccharide and extracellular protein content of the biofilms grown on abiotic substrates, allowing us to conclude that PaAP influences biofilm architecture without the need for host cell interactions. These data establish a novel role for the *P. aeruginosa* leucine aminopeptidase in biofilm formation and provide insight into how secreted bacterial products modulate clinically-relevant bacterial biofilms under infection-like *in vitro* conditions.

2.3 Development of a coculture model to study the effects of PaAP on biofilm formation under infection-like conditions

Based on previous studies demonstrating the high expression of the PaAP leucine aminopeptidase under specific growth conditions, we set out to develop a model to replicate these conditions which would also allow us to observe the direct effects of PaAP deletion on biofilm growth. Specifically, the aminopeptidase was found to be highly expressed both in biofilms (Toyofuku et al. 2012) and when the bacteria are grown on a monolayer of lung epithelial cells (Chugani and Greenberg 2007). To this end, we adapted a previously described coculture protocol in which fluorescent bacterial biofilms are

grown on a confluent layer of epithelial cells cultured in glass bottom dishes, allowing for microscopy imaging of the resultant biofilm microcolonies (Moreau-Marquis et al. 2010).

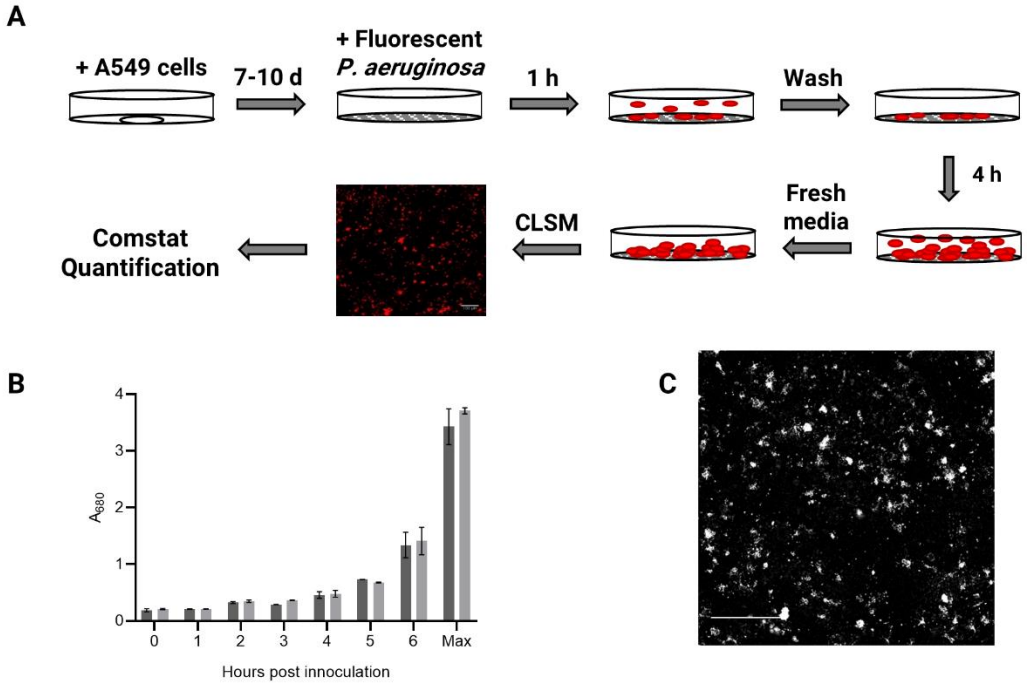

Figure 5: Establishment of a biofilm coculture assay

(A) In the coculture assay, A549 cells were grown to confluency, the cellular substrate was inoculated with fluorescent *P. aeruginosa*, the cocultures washed at 1 hpi, and biofilm formation was examined by CLSM at 5 hpi. Quantification was carried out on stacked images using the ImageJ plugin Comstat 2.1. **(B)** LDH release was measured in S470 WT (dark gray) and S470 ΔPaAP (light gray) cultures at 1 h intervals post inoculation. "Max" indicates the maximum level of LDH release by these cells. **(C)** A representative image taken of S470 WT coculture biofilms at 5 hpi. The punctate areas of fluorescence show microcolony-like structures adhered to the A549 cellular substrate. Scale bars: 200 μm.

In establishing this coculture protocol, we used A549 lung epithelial cells as a substrate for biofilm growth. When grown over the course of several days, these cells form fully confluent cell layers that partially model the polarized epithelial monolayers that the bacteria colonize during lung infection. Once grown to confluency, the A549 cells were inoculated with fluorescent *P. aeruginosa*, and after an hour-long incubation to allow for bacterial attachment to the host cell substrate, unattached cells were washed from the cocultures. After washing, the growth media was replaced with tissue culture media supplemented with 0.4% arginine to promote microcolony formation, and the colonies were allowed to develop over the course of several hours. At the final time point, bacterial growth was assessed by confocal laser scanning microscopy (CLSM). **Figure 5A** shows a graphic overview of this protocol.

Based on previous studies from our lab which noted high PaAP expression in clinical strains of *P. aeruginosa*, we optimized this protocol using the clinical isolate S470, which was originally isolated form a chronically infected CF patient and passaged to obtain a non-mucoid clonal variant (Bauman and Kuehn 2009). Due to high inherent cytotoxicity in certain strains of *P. aeruginosa*, integrity of the epithelial cell substrate was assessed using a lactate dehydrogenase (LDH) assay (**Figure 5B**), and the cells were found to lose significant integrity after 6 hours post inoculation (hpi); therefore, final time points in this assay were taken at 5 hpi, when LDH measurements of lysis remained low. After this point, consistent with previous studies (Moreau-Marquis et al. 2010), the cytotoxic

bacteria infiltrated the host cell layer as the microcolony structure broke down and the bacteria both colonized the underlying tissue culture dish and migrated to the air-liquid interface. This time course is broadly consistent with results from previous studies of *P. aeruginosa* static biofilm growth on host epithelial cells, though the cytotoxicity of S470 against A549 cells is increased compared to documented cell killing by the PAO1 laboratory *P. aeruginosa* strain in similar assays which reduced the optimal imaging time point from 8 hpi to 5 hpi (Moreau-Marquis et al. 2010). However, even at this relatively early stage of biofilm development, microcolony-like structures were still observed in S470 cocultures (**Figure 5C**).

2.3.1 Confirmation of cocultures colonies as biofilms

To confirm that the bacterial colonies we observed constituted true early biofilms, rather than simply adherent cells attached to the A549 substrate, they were tested for gene expression patterns often seen during biofilm development (Whiteley et al. 2001). Due to inconsistencies in the biofilm models used in the field, studies examining expression changes between planktonic and biofilm cultures are quite variable. Several of these studies, however, have noted a logical decrease in the expression of motility-associated genes after the initial phases of biofilm cell attachment, as well as a relative increase in genes associated with the production of matrix components, including Psl. With this in mind, we used qRT-PCR to test for expression of five motility-related genes that had been previously shown to be downregulated in biofilm models (Whiteley et al. 2001) as well as

the Psl-encoding operon. Expression of these factors in S470 cocultures was compared to bacteria grown planktonically and in pellicle biofilms for the same incubation period. **Figure 6A** shows that, consistent with biofilm colonies, bacteria grown in cocultures showed decreased expression of the motility genes and increased expression of Psl and PaAP.

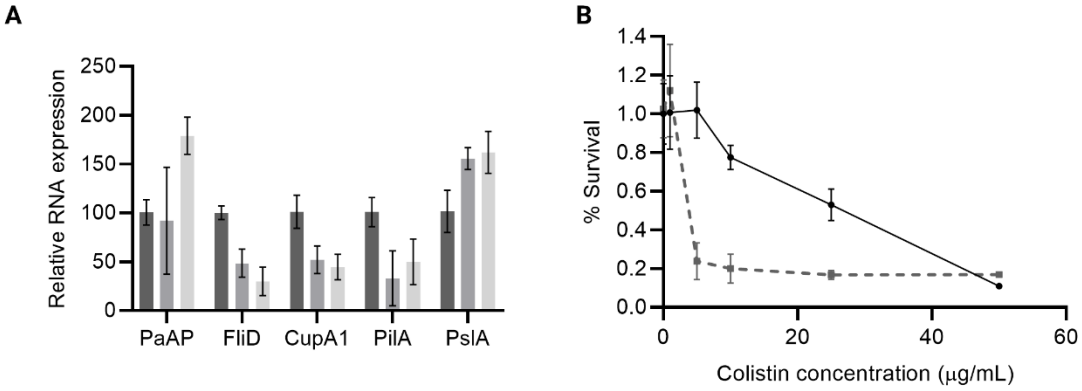

Figure 6: Gene expression and antibiotic resistance experiments confirm that cocultures exhibit biofilm-like properties

(A) RNA was isolated from cells grown planktonically (dark gray), as pellicle biofilms (medium gray) and coculture biofilms (light gray) and the samples were subjected to qRT-PCR to quantify relative expression of biofilm-associated genes. **(B)** S470 WT biofilm cocultures and planktonic cultures were treated with the indicated concentrations of colistin for 2h, and survival was determined by remaining attached fluorescent biomass (cocultures) or live/dead staining (planktonic). Cocultures: solid line. Planktonic cultures: dashed line.

Another key characteristic of biofilm formation is increased resistance to antibiotic treatment as compared to the same cells grown planktonically. This can result from decreased antibiotic infiltration through the biofilm matrix as well as differences in the metabolic state of biofilm-associated bacterial cells. We therefore compared the resistance of coculture and planktonically-grown S470 to antibiotics of three different classes: tobramycin, ciprofloxacin, and colistin. Interestingly, both biofilm and planktonic S470 displayed complete resistance to tobramycin and ciprofloxacin, with biofilm formation increasing significantly in cocultures subjected to very high concentrations of either antibiotic; however, planktonic and coculture grown cells were found to be sensitive to colistin, which is considered "last line of defense" antibiotic for the treatment of clinical biofilm-based infections (Lim et al. 2010). After a two-hour incubation with varying concentrations of colistin, planktonic cultures reached minimum survival at 10 μg/mL, while biofilms were able to tolerate concentrations just under 50 μg/mL (**Figure 6B**). Based on this observed increase in antibiotic resistance, as well as expression of documented biofilm-associated genes, we concluded that the microcolonies observed visually in our coculture assay qualify as biofilm structures.

2.4 PaAP deletion increases biomass and cellular organization in coculture biofilms

Once the bacterial coculture samples were confirmed as biofilms, the effect of the PaAP leucine aminopeptidase on *P. aeruginosa* biofilm microcolony growth was examined by comparing cocultures of fluorescent S470 WT to S470 ΔPaAP, a previously described

isogenic knockout strain that contains a disruption of PA2939, the gene encoding PaAP (Bauman and Kuehn 2009). At 5 hpi, we observed significant differences between the biofilms formed by the two strains; unexpectedly, the PaAP mutant strain formed biofilms with substantially greater cellular biomass compared to the WT parent strain. This effect was not only visible by direct examination of the microscopy images, but also quantifiable and statistically significant. **Figure 7A** and **B** show both representative images of these experiments taken at 10X magnification, as well as quantification of these images.

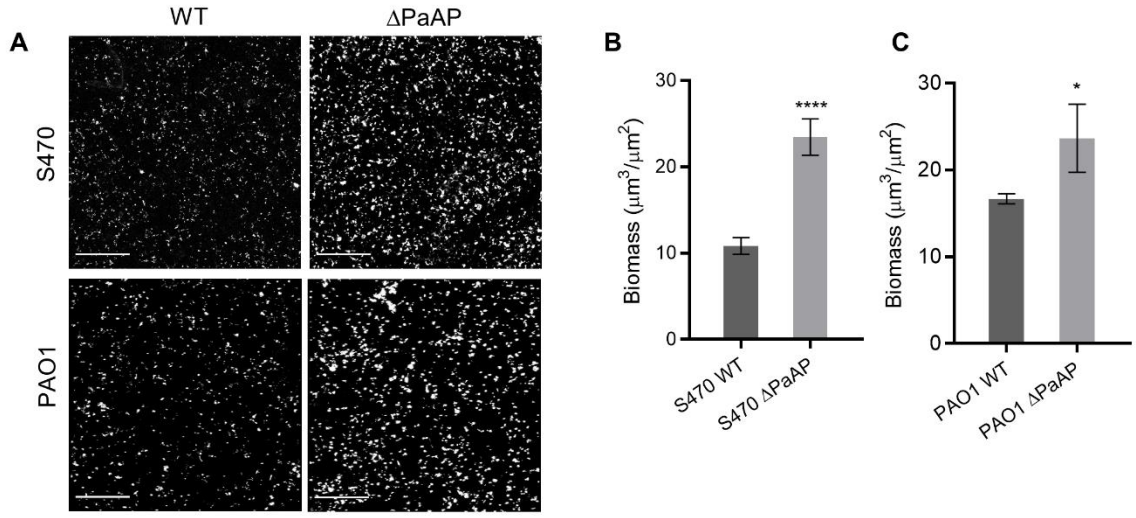

Figure 7: Deletion of the PaAP aminopeptidase increased the biomass of bacterial biofilms on host cells

(A) S470 and PAO1 WT and ΔPaAP strains were inoculated onto confluent A549 cell layers, and images were taken at 10X magnification, 5 hpi and **(B, C)** the images were quantified. Representative results are shown. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Scale bars: 200 μm.

To confirm this PaAP-dependent phenotype in a different strain background, we compared the biomass of PAO1, a laboratory strain of *P. aeruginosa* commonly studied *in vitro*, and the isogenic ΔPaAP strain that harbors a transposon insertion in PA2939. While PAO1 was originally isolated from an infected patient blood sample, it has been passaged countless times in laboratory conditions and displays less pathogenic characteristics than most clinical *P. aeruginosa* isolates. For example, PAO1 demonstrates significantly reduced cytotoxicity against host epithelial cells compared to clinical isolates (Finck-Barbançon et al. 1997). Additionally, our lab has previously shown that both supernatants and OMVs isolated from PAO1 show very low aminopeptidase levels compared to S470 and other isolates from CF patients (Bauman and Kuehn 2006). However, expression of the PaAP protein has been shown to increase in this strain when the cells are grown as biofilms, rather than planktonically, and the aminopeptidase was identified as a major component of PAO1 biofilm matrices (Toyofuku et al. 2012). As with the clinical S470 strain, PAO1 ΔPaAP coculture biofilms displayed significantly greater biomass as compared to the wild type counterpart (**Figure 7A, C**). These observations indicate that PaAP is important to biofilm development, even in strains with low endogenous planktonic and OMV-associated PaAP expression, and that its role may be relevant to a broad range of *P. aeruginosa* isolates.

While cellular biomass is an important characteristic of biofilm development, expression of extracellular material is also known to impact microcolony morphology. For

example, biofilm expression of rhamnolipids and production of eDNA have both been found to impact the formation of mushroom-shaped microcolonies, and reduction of either can result in a more homogenous, lawn-like biofilm and specifically influence the formation of mushroom caps (Allesen-Holm et al. 2006; Pamp and Tolker-Nielsen 2007). We therefore examined S470 WT and ΔPaAP microcolony morphologies in coculture biofilms by imaging bacterial structures at 100X magnification (**Figure 8**). In addition to greater quantitative biomass in these experiments, S470 ΔPaAP microcolonies displayed more distinct cellular organization. Characteristic mushroom-shaped microcolony structures were observed for the PaAP mutant even at this early point in biofilm development. The S470 WT microcolonies, by comparison, showed very little organization into mushroom stalks or caps and were packed less densely. However, while the cells appear to be "floating" in these images, the replacement of media directly prior to imaging ensured that they were in fact adhered to the substrate material, indicating that this extracellular space was likely filled by acellular matrix material. Based on these data, we can conclude that the lack of PaAP not only increases biomass in these samples, but also organization of colonies and cellular packing at the microcolony level during early biofilm development.

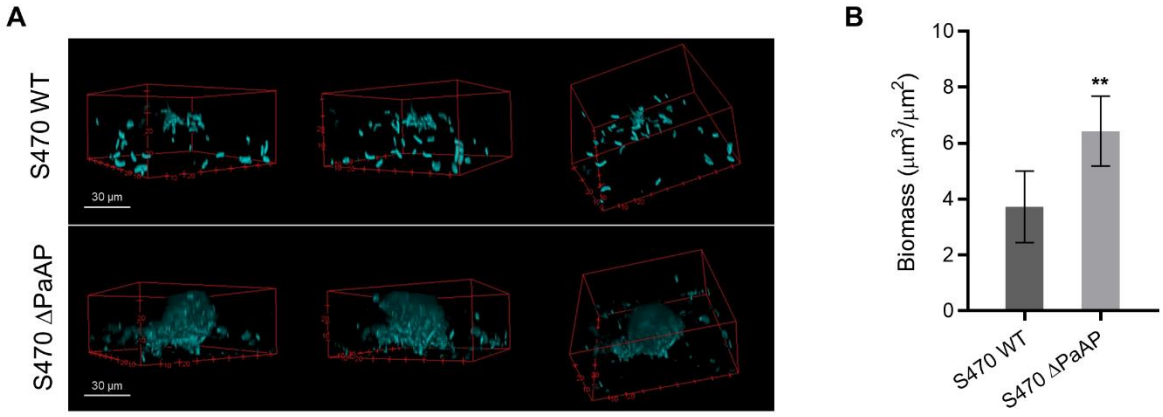

Figure 8: PaAP deletion leads to greater cell density and organization at the microcolony level in biofilm cocultures

(A) Images of S470 WT and ΔPaAP microcolonies were taken using 100X magnification at 5 hpi and **(B)** quantified. Representative results are shown. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Scale bars: 30 µm.

2.5 Differences between wild type and ΔPaAP biofilms develop late in the observed time course

While the coculture protocol used in these studies does not allow for examination of biofilms through complete maturation, several different stages of biofilm development are observed. To form biofilms, cells undergo a transition from their free-swimming planktonic state to a stationary mode of growth via stages of both reversible and irreversible attachment. They subsequently secrete EPS components to form a protective matrix, and the final stages of maturation involve construction of more intricate microcolony structures containing passageways for nutrient distribution throughout the colony and finally dispersal of certain cells from the colony to colonize new environments

(Moradali, Ghods, and Rehm 2017). Based on the results shown in Figure 6 demonstrating the downregulation of motility-associated genes and upregulation of the Psl operon, the coculture microcolonies described here have likely completed the first three stages of biofilm development, though have yet to fully mature and show little dispersal from the colony. Examination of these stages in more detail would therefore reveal insights to the mechanism behind PaAP-dependent biomass inhibition in coculture microcolonies.

With this in mind, we detailed the time course of the PaAP-dependent phenotype by imaging the cocultures at hourly intervals post-inoculation. Directly following the wash step at 1 hpi and up to 3 hpi, we observed a similar level of biomass from the S470 WT and ΔPaAP strains. A significant phenotype did not develop until 4 hpi (**Figure 9A**), suggesting that the aminopeptidase is critical in limiting microcolony growth and development rather than during the initial steps of host cell colonization and bacterial attachment.

Based on this timeline, it is expected that the observed phenotype would develop in parallel with the level of aminopeptidase expression. Indeed, previous reports found PaAP to be regulated by the Las quorum sensing system (Michel, Durand, and Filloux 2007; Nouwens et al. 2003), and thus PaAP expression would be expected to increase as bacterial density increases in our experimental system. Using qRT-PCR, we examined the expression of PaAP RNA in S470 WT biofilms over time under the coculture assay conditions. Consistent with quorum sensing density-dependent expression, our results

show expression of PaAP mRNA at 1 hpi, increasing steadily over the next several hours (**Figure 9B**). We also detected PaAP protein in cocultures at the 5 hpi time point by precipitating and concentrating coculture protein material and immunoblotting for the aminopeptidase (**Figure 9C**). Based on these data, it appears that the biofilm limiting phenotype may depend on either a threshold level of aminopeptidase or a specific microcolony developmental phase.

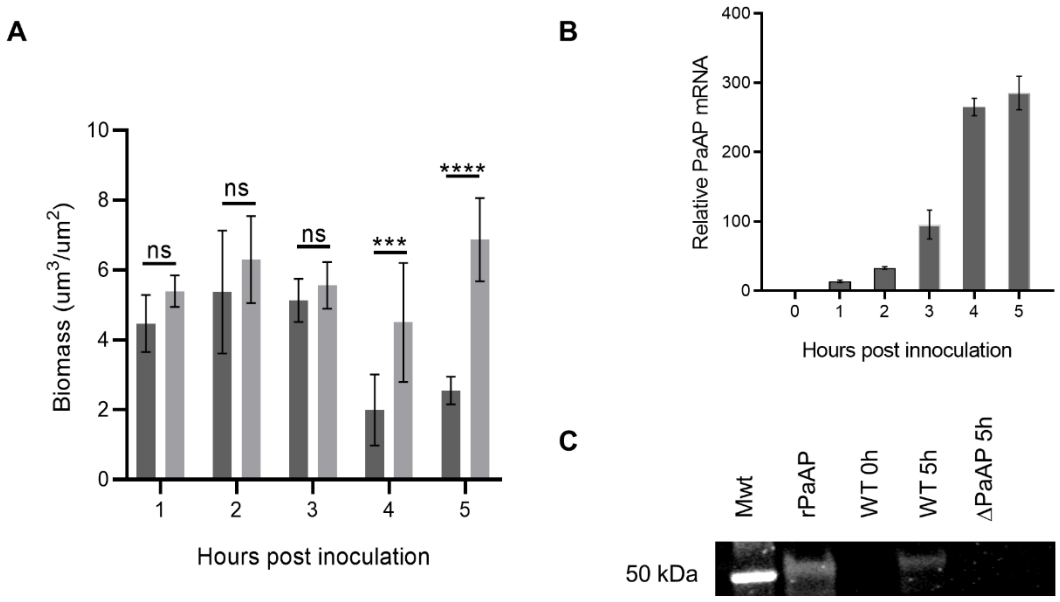

Figure 9: Differences in coculture biomass between the WT and ΔPaAP strains develop late in the observed time course, coincident with PaAP RNA and protein expression

(A) Bacterial biofilms cocultured on A549 cells were imaged by confocal microscopy at indicated time points. (S470 WT: dark gray, S470 ΔPaAP: light gray). **(B)** RNA samples were isolated from S470 WT cocultures at the indicated time points. qRT-PCR was performed to determine the levels of aminopeptidase expression during the time course. RNA levels are shown relative to PaAP expression in planktonic S470 WT cultures during log growth. **(C)** Total protein was extracted from S470 WT and ΔPaAP cocultures at indicated hpi and precipitated, and PaAP was detected in the samples using Western blot. Mwt, 50 kDa standard; PaAP, purified PaAP control. ; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns, not significant. For A and C, representative results are shown.

2.6 ΔPaAP biofilm matrices contain less of the exopolysaccharide Psl and display increased susceptibility to colistin

Bacterial fluorescence images form the coculture assay shown in Figures 7 and 8 revealed significant density and structural differences between the biofilms formed by wild type and ΔPaAP strains, but by using fluorescent strains, we had only detected

differences in the bacterial cells and not the matrix structures. We were also interested in whether PaAP affected the composition of the biofilm EPS, as this part of the colony structure contributes significantly to bacterial pathogenesis, interaction with the immune system, and resistance to antibiotics (Mann and Wozniak 2012).

The *P. aeruginosa* matrix is composed of polysaccharides, proteins, DNA, and vesicles secreted by cells in the microcolony. Three polysaccharides are known to form the majority of this matrix: Psl, Pel, and alignate (Mann and Wozniak 2012). The S470 isolate used in this study was non-mucoid and therefore does not express alginate. Of the two other polysaccharides, Psl has been studied extensively in *P. aeruginosa* biofilms and is known to contribute significantly to the maintenance of a protective matrix structure and play a role in chronic infections (Jones and Wozniak 2017). To image the Psl content of the matrix in S470 cocultures, we used a *Hippaestrum hybrid* lectin which binds α-mannose residues and has been shown to be specific for Psl (Ma et al. 2007). As seen in **Figure 10 A** and **B**, the S470 WT biofilms produced significantly greater Psl relative to cellular biomass. Notably, Psl in the wild type strain surrounds bacterial cells on all sides, whereas the Psl detected in the ΔPaAP biofilms was localized mainly at the top of the mushroom cap structure. Consistent with the microcolony images presented in Figure 8, these data suggested that PaAP expression in the WT samples helped to promote production of a biofilm with greater matrix content, securing the seemingly "floating" cells in these images to the colony architecture and host cell substrate.

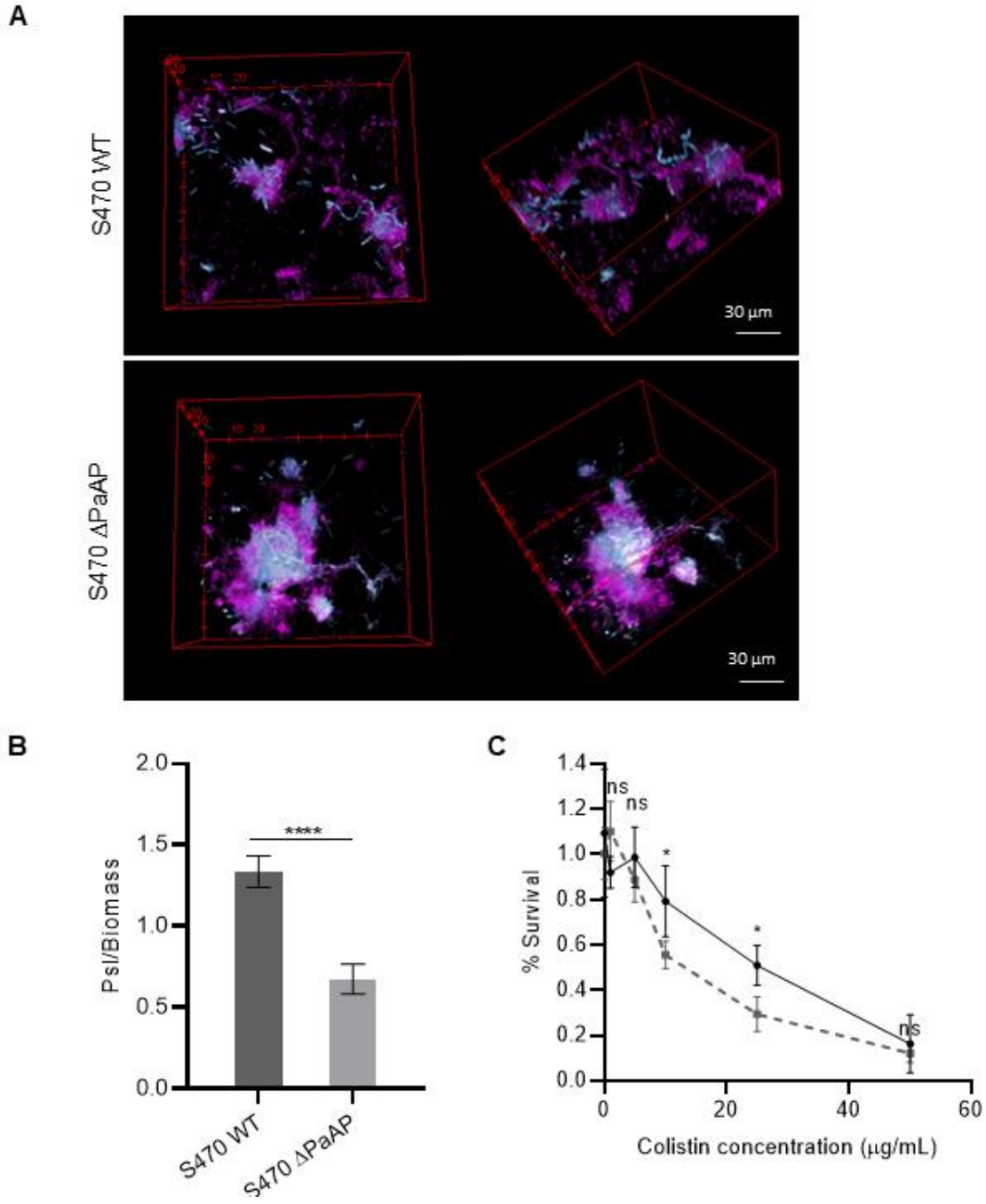

Figure 10: PaAP deletion decreases relative Psl levels and antibiotic resistance in coculture biofilms

(A) Bacterial biofilms (cyan) were cocultured on A549 cells for 5 h, stained with HHA-FITC lectin (magenta) and imaged by confocal microscopy and **(B)** the ratio of Psl/cellular biomass was calculated. **(C)** At 3 hpi, S470 WT (solid line) and ΔPaAP (dashed line)

cocultures were treated with the indicated concentrations of colistin. Survival indicates the percent of biomass remaining attached to the host cell substrate. For all experiments, representative results are shown. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns, not significant.

As noted above, the biofilm matrix is critically important for protection of the biofilm-associated cells from attack by both the host immune system and clinically administered antibiotic treatments. While the mechanisms behind this antibiotic resistance have not been fully elucidated, the prevailing theories in the field predict that the physical and biochemical properties of matrix EPS may prevent cells deep within the colony structure from ever being exposed to these antibiotics. To determine if the differences in matrix and biomass content of the WT and ΔPaAP biofilms affected their susceptibility to antibiotics, the S470 WT and ΔPaAP coculture samples were treated with colistin. **Figure 10C** shows that the increase in Psl to biomass ratios in the wildtype cocultures also correlated with a modest, though statistically significant, increase in colistin resistance in the S470 WT biofilms, suggesting that aminopeptidase-dependent modulation of matrix can result in an overall more robust biofilm structure.

2.7 Under certain growth conditions, PaAP deletion can also increase cellular biomass in P. aeruginosa pellicles

While our coculture model provided strong evidence that PaAP affects the growth and matrix development of biofilm microcolonies grown on a host epithelial cell surface, *P. aeruginosa* is also known to form other biofilm structures. Under certain growth

conditions, such as in flow cells, the bacteria can adhere to plastic substrates to form microcolonies. When grown in liquid media, *P. aeruginosa* forms more homogenous biofilm communities called pellicles at the air-liquid interface. Pellicles are studied extensively in the biofilm field, and they are often used for reproducible, high-throughput experiments due to the relatively simple experimental model and the ability to grow them in multi-well microplates. During pellicle formation, the bacteria form a layer of cells at the surface of the culture media and attach in a ring to the culture container. Pellicle development can be quantitatively assessed using a Crystal Violet stain to measure cellular attachment to the culture container (O'Toole 2011) or direct live-dead staining and imaging of the pellicle itself (Zhao et al. 2018). By using pellicle biofilms grown on an abiotic substrate, we were additionally able to test whether the host cell layer used in the coculture assays, and the resulting bacteria-host cell interactions, was required to observe PaAP-mediated changes in biofilms.

We first assessed pellicle development using a standard approach in the field (O'Toole 2011), in which biofilms are grown in M63 minimal media in multi-well dishes and Crystal Violet-stained (**Figure 11A**). Under these conditions, PaAP deletion had no effect on pellicle development (**Figure 11B**). Additionally, pellicles grown in LB + glycerol, fresh tissue culture media, or tissue culture media conditioned during coculture experiments failed to show a difference in Crystal violet staining of S470 WT and S470 ΔPaAP biofilms (**Figure 11B**).

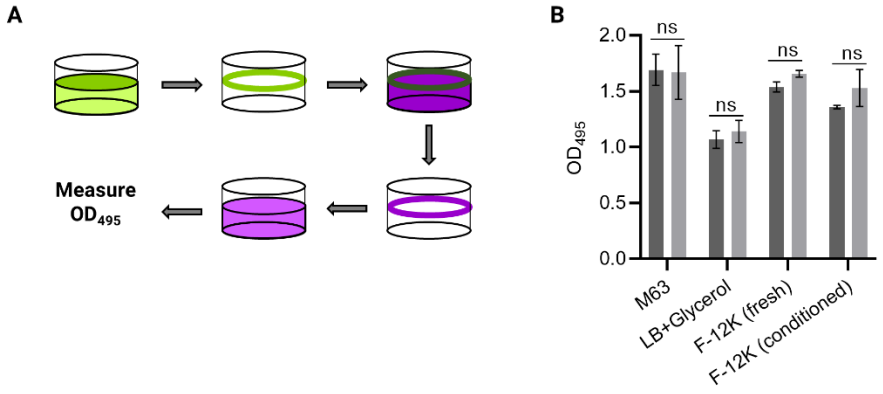

Figure 11: PaAP does not affect Crystal violet staining of pellicle formation under most tested growth conditions

(A) On overview of the Crystal violet staining protocol. Briefly, pellicles were grown in multi-well dishes and formed biofilms at the air-liquid interface. The wells were washed, leaving a ring of attached cells in the well. These cells were stained, and the stain was solubilized and quantified using OD measurements. **(B)** Pellicles were grown for 6 h in under the indicated media conditions and quantified using the Crystal violet staining assay. (S470 WT: dark gray, S470 ΔPaAP: light gray). ns, not significant.

Recently, however, Zhao et al published results showing that PaAP can affect PAO1 *P. aeruginosa* biofilm pellicles grown in cultures using Jensen's media at 30°C (Zhao et al. 2018). While the body of this work focused on a late-stage phenotype, they also observed that PaAP deletion led to increased biomass in early pellicle biofilms. Jensen's media was originally formulated by Jensen et al (Jensen, Fecycz, and Campbell 1980) to optimize the expression of bacterial proteases during planktonic growth and has since been used by various groups studying *P. aeruginosa* as a standard growth media for their experiments (Ma et al. 2012, 2009; Landry et al. 2006). By growing the bacteria planktonically in Jensen's media to mid-log phase and diluting prior to inoculation of the

culture containers, we were able to replicate this phenotype with the S470 clinical strain and corresponding PaAP deletion mutant. Under these growth conditions, the ΔPaAP strain formed pellicles with significantly greater biomass, as determined by Crystal Violet staining (**Figure 12A**). We additionally imaged these pellicles using a live/dead bacterial stain and quantified the resultant images, confirming the results of the Crystal Violet assay (**Figure 12B, C**). Notably, no differences were seen in the live/dead ratios between the two strains. These data lead us to conclude that the PaAP-dependent effects that we observe in early biofilms may also be relevant to pellicles, but only under certain nutrient and growth conditions. Importantly, these data provide crucial evidence that PaAP-dependent changes in coculture biofilm architecture and composition are not entirely due to microcolony interaction with the host cell substrate, as this phenotype can be observed in certain biofilms grown on abiotic substrates.

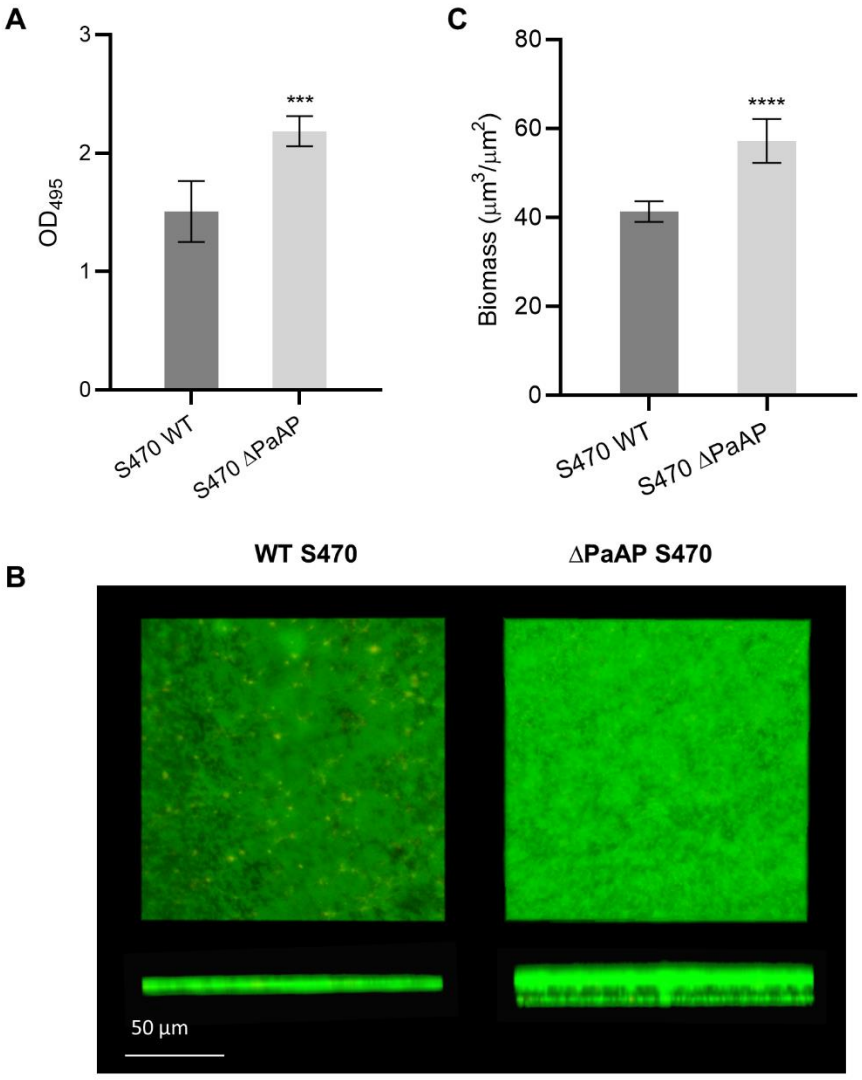

Figure 12: PaAP deletion modifies pellicle cellular biomass

(A) S470 WT or ΔPaAP were grown in 35mm dishes for 6 h and pellicle biofilms were quantified by Crystal Violet staining (OD_{495}). **(B)** Pellicles formed by S470 WT or ΔPaAP after 6 h of growth were live/dead stained and imaged by confocal microscopy **(C)** and their biomass calculated. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns, not significant. For microscopy experiments, representative results are shown.

2.8 PaAP deletion increases Psl and decreases matrix protein content in pellicles grown in Jensen's media

While pellicle biofilms exhibit different architecture and colony morphology than the microcolonies examined in the coculture assay, they still demonstrate many of the same basic characteristics, including the secretion of EPS to form a protective matrix structure. To determine whether the matrix phenotypes observed in the coculture biofilms were consistent in pellicles, we utilized the same Psl-specific lectin described above to quantify matrix Psl in Jensen's media pellicles. As with the coculture biofilms, the S470 WT pellicles consisted of significantly greater exopolysaccharide relative to cellular biomass as compared to the ΔPaAP pellicles (**Figure 13A**).

While polysaccharides are considered potentially the most important component of biofilm matrices, EPS also includes proteins, eDNA, and outer membrane vesicles. Due to the complexity of the coculture system and the presence of the underlying host cell substrate, detection of other matrix components was unfeasible in this model. The relative simplicity of pellicle biofilms, however, allowed for staining of matrix proteins using a specifically formulated biofilm matrix protein stain. Compared to the ΔPaAP strain, the wild type pellicles exhibited a significantly lower protein to Psl ratio (**Figure 13B**). Together, we showed that PaAP deletion leads to increases in cellular biomass and matrix protein, while decreasing matrix Psl. These data provide further evidence for our conclusion that, in wild type biofilms, PaAP aids in the formation of a polysaccharide-rich protective matrix structure.

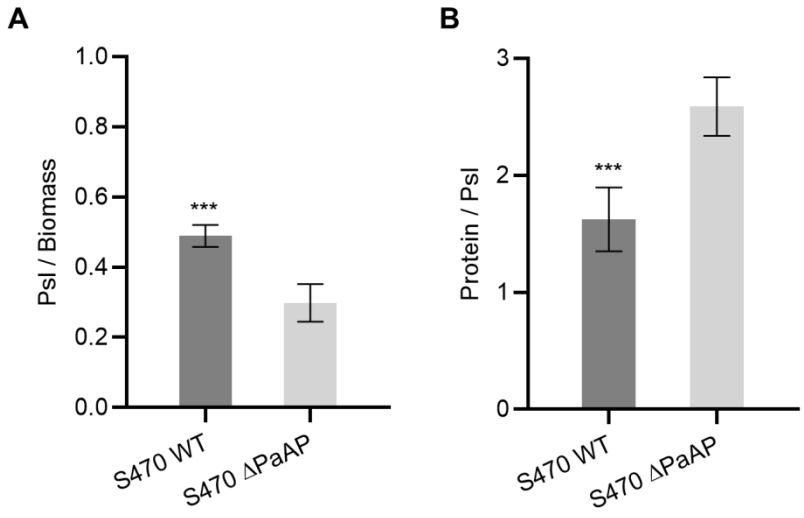

Figure 13: PaAP deletion mediates matrix Psl and protein content in pellicles grown in Jensen's media

Pellicles were stained for **(A)** matrix Psl and **(B)** total protein, and the results quantified and normalized to cellular volume and Psl volume, respectively. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns, not significant. Representative results are shown.

2.9 Discussion

In this chapter, we set out to investigate the role of the PaAP leucine aminopeptidase in biofilm development, as PaAP is one of the most abundant proteins in the *P. aeruginosa* biofilm matrix and one of the major protein components of OMVs from clinical *P. aeruginosa* isolates (Toyofuku et al. 2012; Bauman and Kuehn 2006). When we compared the coculture biofilms of wild type *P. aeruginosa* and a PaAP deletion mutant, we found that the mutant developed early biofilms with significantly higher cellular biomass, denser cell packing, and increased mushroom-shaped microcolony

organization. Additionally, the deletion mutants displayed lower relative expression of the matrix polysaccharide Psl and included a less robust biofilm architecture that rendered the cellular community more susceptible to the antibiotic colistin. We also confirmed the biomass and Psl phenotypes under specific pellicle biofilm growth conditions. This indicated that responses within the bacterial biofilm, rather than direct interaction with the host cell substrate, were responsible for the observed changes.

Perhaps the most interesting of the results presented in this chapter was the finding that PaAP deletion not only led to a matrix with less Psl (and in pellicles, more protein) but also increased susceptibility to the antibiotic colistin. In *P. aeruginosa*, as well as other biofilm-forming bacteria, antibiotic resistance is mediated not only by genetic mechanisms but also by the secretion of EPS (Davies 2003). By providing a physical barrier between microcolony bacteria and the extracellular environment, EPS can prevent direct association between antibiotics and target bacterial cells. We have shown that wild type biofilms not only contain more Psl, but also that the polysaccharide is distributed more evenly throughout these biofilms, surrounding what appears to be a greater proportion of the colony's cells. Colistin, also known as polymyxin E, functions via interactions with LPS and phospholipids in the bacterial OM, where it displaces divalent cations from the phosphate heads of these membrane lipids. This results in disruption of the OM, leakage of bacterial cytosolic contents, and eventual cell death (Poirel, Jayol, and Nordmann 2017). Based on the evidence we provided, we propose a model in which Psl content and

distribution in the wild type biofilms prevents the antibiotic from reaching the bacterial OM, resulting in increased colistin resistance. The ΔPaAP biofilms, on the other hand, localize much of their Psl to the mushroom cap, leaving other portions of the colony susceptible to antibiotic attack.

While some aminopeptidases have been implicated in pathogenesis phenotypes (Jarocki, Tacchi, and Djordjevic 2015), these enzymes are generally thought to act during starvation responses, in which they can provide necessary amino acids for cellular processes. Recently, Zhao et al published data demonstrating a role for PaAP in late-stage pellicle biofilms, in which the PaAP deletion mutant dispersed earlier than the counterpart wild type strain (Zhao et al. 2018). Under such conditions, the aminopeptidase is likely acting in its traditional role in providing nutrients to cells embedded more deeply within the biofilm structure. This is supported by their conclusion that lack of the aminopeptidase led to cell lysis, causing release of intracellular PslG, which degrades matrix Psl and causes detachment of biofilm cells. Our study, in contrast, demonstrated that PaAP impacts biofilm formation processes early in development and under nutrient-rich conditions, without any detectable differences in bacterial or host cell death. This work therefore represents a potentially novel role for a bacterial aminopeptidase in the modulation of biofilm growth, architecture, and matrix composition.

3. PaAP^{+} OMVs mediate cellular detachment and matrix remodeling in bacterial biofilms

3.1 Summary

The biofilm matrix contains a diverse array of molecules, including polysaccharides, proteins, eDNA, and bacterial membrane vesicles. PaAP, a major protein component of *P. aeruginosa* biofilm matrices, can be found as both a soluble enzyme and associated with bacterial OMVs. In this chapter, we show that OMVs from a PaAP-expressing strain, but not soluble aminopeptidase, can complement the phenotypes observed upon PaAP deletion. We additionally demonstrate that these vesicles display anti-biofilm activity against biofilms formed by the vesicle-producing strain, non-self *P. aeruginosa* isolates, and the lung pathogen *Klebsiella pneumoniae*. Finally, we provide evidence that vesicle-associated proteases, not the aminopeptidase itself, are directly responsible for this activity and cause cellular detachment from the microcolony structure. These results demonstrate a novel role for *P. aeruginosa* leucine aminopeptidase in biofilm remodeling and regulation of vesicle packaging of endogenous proteases.

Material in this chapter was adapted from the article "Pseudomonas aeruginosa leucine aminopeptidase influences early biofilm composition and structure via vesicle-associated anti-biofilm activity" published in *MBio* (Esoda and Kuehn, 2019).

3.2 Introduction

Once it has been secreted into the extracellular environment, PaAP is found both soluble in the extracellular milieu and as a major component of *P. aeruginosa* outer

membrane vesicles (OMVs). OMVs are formed from the envelope of Gram-negative bacteria and are small, discrete extracellular structures containing distinct membrane protein, lipid, and soluble periplasmic content (Orench-Rivera and Kuehn 2016). They are known to interact with the external environment, including host tissues (Ellis and Kuehn 2010). Vesicles produced by many Gram-negative pathogens, including *P. aeruginosa*, can elicit host immune responses, and many also serve as delivery mechanisms for bacterial virulence factors (Kuehn and Kesty 2005).

Of particular importance to our studies, OMVs have been implicated in the process of biofilm formation (Wang, Chanda, and Zhong 2015; Orench-Rivera and Kuehn 2016; Cecil et al. 2019). Schooling *et al*. demonstrated that OMVs are present in *P. aeruginosa* biofilm matrix and that bacteria in biofilms exhibit higher OMV production rates as compared to planktonic cells (Schooling and Beveridge 2006). When these biofilm-derived vesicles were analyzed, PaAP was found to be one of the most abundant proteins (Toyofuku et al. 2012).

Due to both the prevalence and emerging importance of OMVs in bacterial biofilms, as well as their documented importance in bacterial pathogenesis, we hypothesized that *P. aeruginosa* OMVs, which are associated with high concentrations of PaAP, may be responsible for the differences we observed between WT and ΔPaAP biofilms in the previous chapter. In this chapter, we show that WT OMVs display biofilm remodeling activity consistent with phenotypic differences observed between the WT and

ΔPaAP biofilms, and that WT OMVs can complement all of the knockout phenotypes we observe. Additionally, through the use of native secreted soluble PaAP (sPaAP) and purified recombinant aminopeptidase (rPaAP), we show that the aminopeptidase itself is not responsible for this activity, but rather non-PaAP vesicle cargo. We observe OMV-induced detachment of bacterial cells from pre-formed colony structures and trace this activity back to vesicle-associated proteases. Not only do our data implicate the *P. aeruginosa* aminopeptidase in a larger regulatory network governing secreted protease activity in OMVs, they also provide novel insight into how secreted bacterial products modulate clinically-relevant bacterial biofilms of diverse species.

3.3 Cell-free supernatant from wild type P. aeruginosa complements the biomass phenotype observed in ΔPaAP cocultures

Having established the effects of PaAP deletion on biofilm architecture and composition, we pursued complementation of these phenotypes. Endogenously, PaAP expression is governed by a tightly-controlled quorum sensing system. In *P. aeruginosa*, quorum sensing responses can be affected by any number of nutritional, environmental, and host signals (Shrout et al. 2006; de Kievit 2009). Additionally, once secreted, the aminopeptidase is cleaved and activated by other endogenous proteases (Sarnovsky et al. 2009) which are also likely subject to quorum sensing-regulated expression and activation. Because of this, complementation using plasmid-based PaAP expression proved to be challenging and unfeasible. We therefore chose to perform biochemical complementation

experiments to test the ability of natively secreted PaAP to reconstitute wild type properties in ΔPaAP biofilms.

Complementation was first tested using cell-free supernatant (CFS) harvested from mid-log phase planktonic cultures. The CFS contains a large number of bacterial secretion products, including secreted proteins and proteases, OMVs, and small molecules. CFS was isolated from both the wild type PaAP-containing strains (WT CFS) as well as the ΔPaAP strain (ΔPaAP CFS) to control for the effects of non-PaAP secreted material. Briefly, the cultures were grown, and supernatants were collected, concentrated, and filter sterilized to ensure only secreted material was present. PaAP was then confirmed as present or absent in the WT and ΔPaAP samples, respectively, by Western blot (**Figure 14A**) and by activity assay (**Figure 15C**).

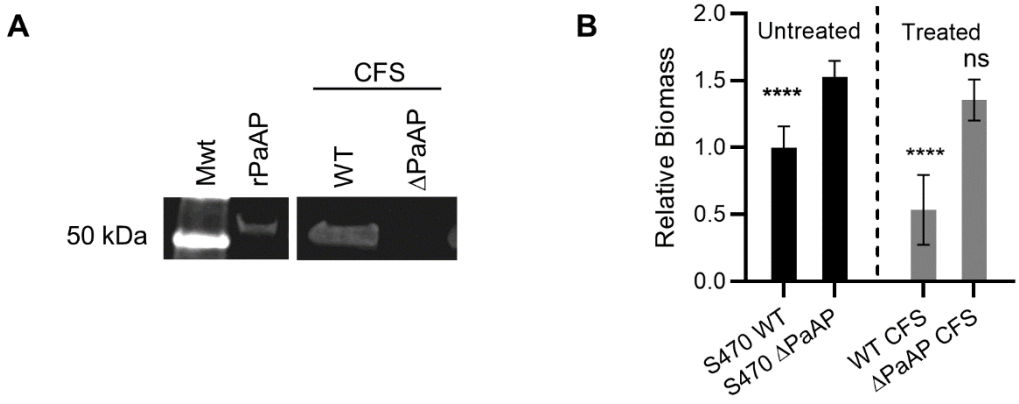

Figure 14: WT cell-free supernatants complemented the biomass changes observed in PaAP deletion mutants

(A) Cell free supernatants (CFS) from S470 WT and S470 ΔPaAP cultures were TCA-precipitated, and PaAP was detected by immunoblotting after separation of samples by SDS-PAGE. The relative migration of rPaAP and the 50 kDa molecular weight standard (Mwt) are shown. Samples were run on the same gel, and an intervening lane was excised in this figure. **(B)** S470 ΔPaAP biofilms cocultured with A549 cells were treated with S470 WT or ΔPaAP CFS at 1 hpi, imaged at 5 hpi, and biomass in these images was quantified. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns, not significant. Representative results are shown.

Complementation was first tested on coculture ΔPaAP biofilms. Based on the time course shown in Figure 9B, we concluded that PaAP does not directly affect the initial attachment stages of coculture biofilm development. Therefore, CFS was added after the 1 hpi wash step outlined in the coculture protocol. Equivalent amounts, measured by total protein, of CFS were added to washed S470 ΔPaAP coculture biofilms 1 hpi, and the bacteria were imaged 5 hpi. As seen in **Figure 14B**, addition of the S470 WT CFS, but not S470 ΔPaAP CFS, resulted in decreased biomass levels after 4 h, indicating that WT CFS

was able to complement the phenotype of ΔPaAP biofilms. Based on the significant and substantial difference between biofilms treated with WT and ΔPaAP CFS, we conclude that *P. aeruginosa* CFS harbors PaAP-dependent biofilm modulating activity.

3.4 PaAP+ OMVs, but not soluble aminopeptidase, complement changes in ΔPaAP coculture biofilm formation

Once PaAP has been secreted by the *P. aeruginosa* T2SS and activated by proteolytic cleavage and autoprocessing, it can be found both as a soluble protein (sPaAP) and associated with OMVs (Bauman and Kuehn 2006). While we hypothesized that PaAP itself was responsible for this activity, we also considered the possibility that PaAP deletion caused an indirect effect either on other material secreted into the CFS or on vesicle cargo. Therefore, to gain further insight into the molecules responsible for WT CFS-mediated complementation of the ΔPaAP phenotype, CFS samples were separated into vesicle and soluble secreted material.

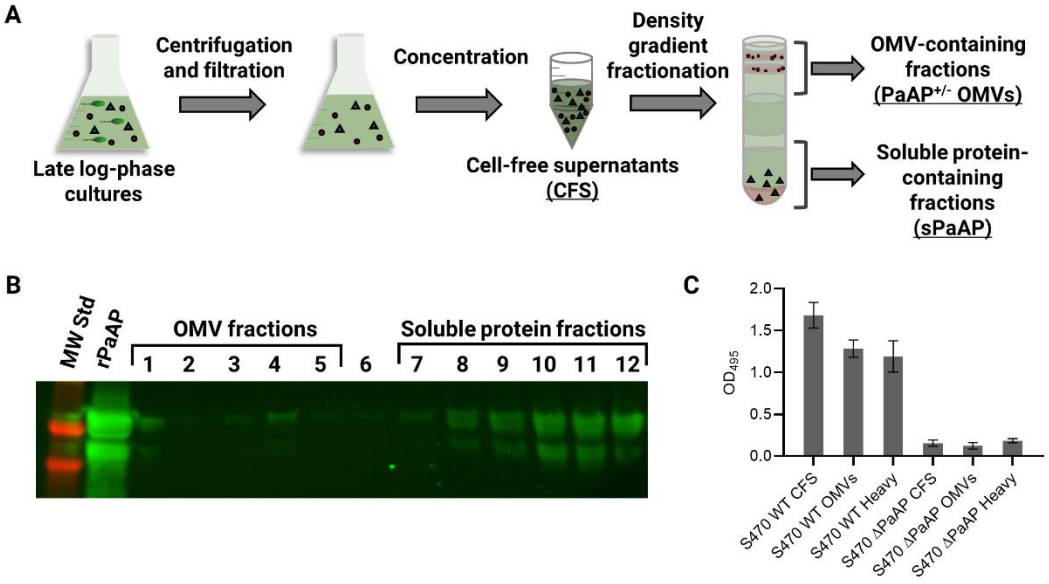

Figure 15: Fractionation of CFS into OMV- and soluble protein-containing fractions

(A) An overview of the protocol used for fractionation of CFS. Briefly, cultures were grown to mid-log phase and cells were removed using centrifugation and filtration. The resulting CFS was concentrated and loaded onto an iodixanol density gradient. Light (OMV-containing) and heavy (soluble protein-containing) fractions were separated and pooled, respectively. **(B)** S470 WT CFS fractions were separated using SDS-PAGE and PaAP was detected by Western blotting. **(C)** Aminopeptidase activity of purified cell free supernatants (CFS), vesicle fractions (OMVs), and dense fractions (Heavy) from S470 WT and ΔPaAP OMVs. 10μg total protein was used for each sample.

To fractionate CFS, the samples were loaded into the bottom an iodixanol density gradient. Upon centrifugation, this gradient separates the bacterial material, with high density soluble proteins and other molecules equilibrating in heavier fractions and vesicle-associated material floating to light density fractions. Fractionation of S470 WT CFS yielded results similar to those previously reported (Bauman and Kuehn 2006), with

PaAP detected in varying amounts across all 12 fractions (**Figure 15B**). The light (1-5) and heavy (7-12) density fractions were pooled, and aminopeptidase activity was detected in both of the pooled preparations (**Figure 15C**). S470 ΔPaAP CFS was also fractionated and pooled to serve as a negative control for the effects of non-PaAP secreted material.

Pooled OMV and soluble fractions of the CFS were each added to S470 ΔPaAP cocultures at 1 hpi to test their ability to complement the knockout phenotype. For initial experiments, the samples were matched by total protein concentrations. Interestingly, treatment with the OMV-containing S470 WT fractions (WT OMVs) significantly inhibited cellular biomass development by S470 ΔPaAP, while the heavier, sPaAP-containing fractions (WT Heavy) had no noticeable effect on biomass (**Figure 16A, B**). As expected, neither treatment at 1 hpi with OMV-containing (ΔPaAP OMVs) or heavy fractions (ΔPaAP Heavy) from S470 ΔPaAP cultures altered the ΔPaAP coculture biomass at 5 hpi (**Figure 16A, B**). Additional complementation experiments in which the amount of WT OMVs and sPaAP in the WT Heavy fractions were standardized to the level of aminopeptidase activity found in each also failed to show complementation by the heavy, soluble protein-containing fractions (**Figure 16C**). These data suggested that the OMVs from the wild type, PaAP-expressing cultures, but not soluble secreted aminopeptidase, were responsible for PaAP-dependent biofilm modulating activity.

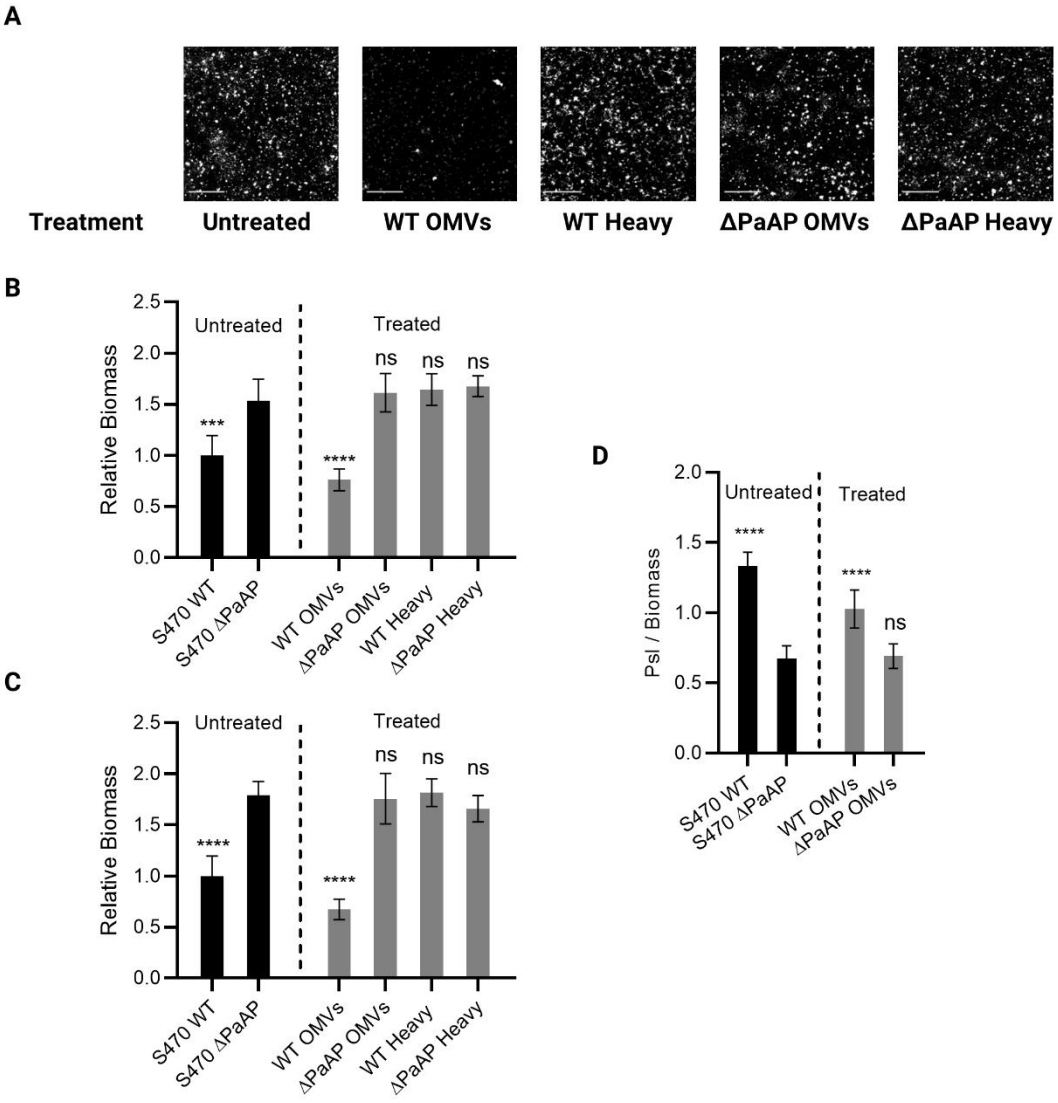

Figure 16: Addition of PaAP⁺ OMVs, but not soluble aminopeptidase-containing fractions, complements PaAP deletion

(A) S470 ΔPaAP biofilms cocultured with A549 cells were treated with fractions containing either light (OMVs) or dense (heavy) material from S470 WT or S470 ΔPaAP CFS, standardized to total protein content. **(B)** The biomass of these treated biofilms and the S470 WT untreated control were quantified at 5 hpi and were compared with untreated S470 ΔPaAP biofilms. **(C)** The same experiment as **B** was performed with samples standardized to aminopeptidase activity levels (with ΔPaAP controls normalized to equivalent protein amounts relative to their comparable CFS fraction from the WT samples); **(D)** Biofilm cocultures were grown, stained for Psl, and quantified. Psl:biomass volume ratios are shown. For treated samples, OMVs were added 1 hpi. Untreated WT

and treated ΔPaAP pellicles were stained as above, quantified, and values compared to untreated ΔPaAP pellicles ; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns, not significant. Scale bars: 200 μm.

While the complementation experiments detailed above demonstrate that WT OMVs can mediate changes in cell aggregate size of coculture biofilms, we had also shown that biomass increases in the ΔPaAP strain are accompanied by an important decrease in relative levels of Psl. To decipher whether WT vesicles could complement all of the phenotypic changes we had observed upon PaAP deletion, ΔPaAP cocultures were treated with WT OMVs at 1 hpi, stained for Psl using the previously described lectin, and imaged at 5 hpi. **Figure 16D** shows that WT OMV treatment partially, but significantly, increased the Psl/biomass ratio of the ΔPaAP coculture, while ΔPaAP OMVs had little to no effect on this ratio. In sum, WT vesicles not only mediate changes in microcolony biomass, but also matrix content, and we therefore conclude that these particles mediate remodeling of multiple aspects of biofilm architecture.

3.4.1 Recombinant aminopeptidase and co-additions of soluble PaAP with ΔPaAP OMVs are unable to complement the ΔPaAP phenotype

The data above present strong evidence that that the OMVs derived from a $PaAP^+$ strain rather than aminopeptidase activity itself are responsible for changes in biofilm composition between the WT and ΔPaAP strains. However, the sPaAP found in the heavy density gradient fractions used in these experiments was not fully purified. We therefore decided to perform the complementation experiments using a purified recombinant form

of the aminopeptidase, which we refer to as rPaAP (Sarnovsky et al. 2009). This construct was expressed in *E. coli* and purified from inclusion bodies. Because this protein is not natively secreted from *P. aeruginosa* and *E. coli* lacks the enzymes known to activate endogenous forms of the aminopeptidase, we used a construct that lacked both the C and N termini that would normally be cleaved during processing. Once purified and refolded, the rPaAP protein was fully active (**Figure 17C**).

For recombinant protein complementation experiments, purified rPaAP was standardized to the level of aminopeptidase activity found in WT OMVs and added to ΔPaAP cocultures at 1 hpi, with imaging at 5 hpi. Consistent with the results using sPaAP, purified rPaAP was also incapable of disrupting the formation of coculture biofilms by the ΔPaAP strain, confirming that soluble aminopeptidase was not responsible for PaAP-mediated biofilm remodeling (**Figure 17A, B**).

Finally, we tested whether we could reconstitute WT OMV remodeling activity by adding rPaAP or sPaAP to ΔPaAP OMVs prior to the treatment of the ΔPaAP cocultures with vesicles. These rPaAP/ΔPaAP OMV and sPaAP/ΔPaAP OMV preparations had demonstrable aminopeptidase activity (**Figure 17C**) but failed to inhibit biomass formation by the S470 ΔPaAP strain (**Figure 17A, B**). This suggests that the aminopeptidase is not directly causing changes in biofilm architecture, but rather modifying OMV content, leading to the observed vesicle associated activity. Because this activity cannot be reconstituted with exogenous addition of PaAP to the OMV samples,

we can conclude that PaAP alters OMV cargo prior to native vesicle biogenesis. Taken together, these data led us to conclude that PaAP-dependent OMV activity, but not PaAP directly, mediates cell aggregation and matrix composition at early stages of biofilm microcolony development.

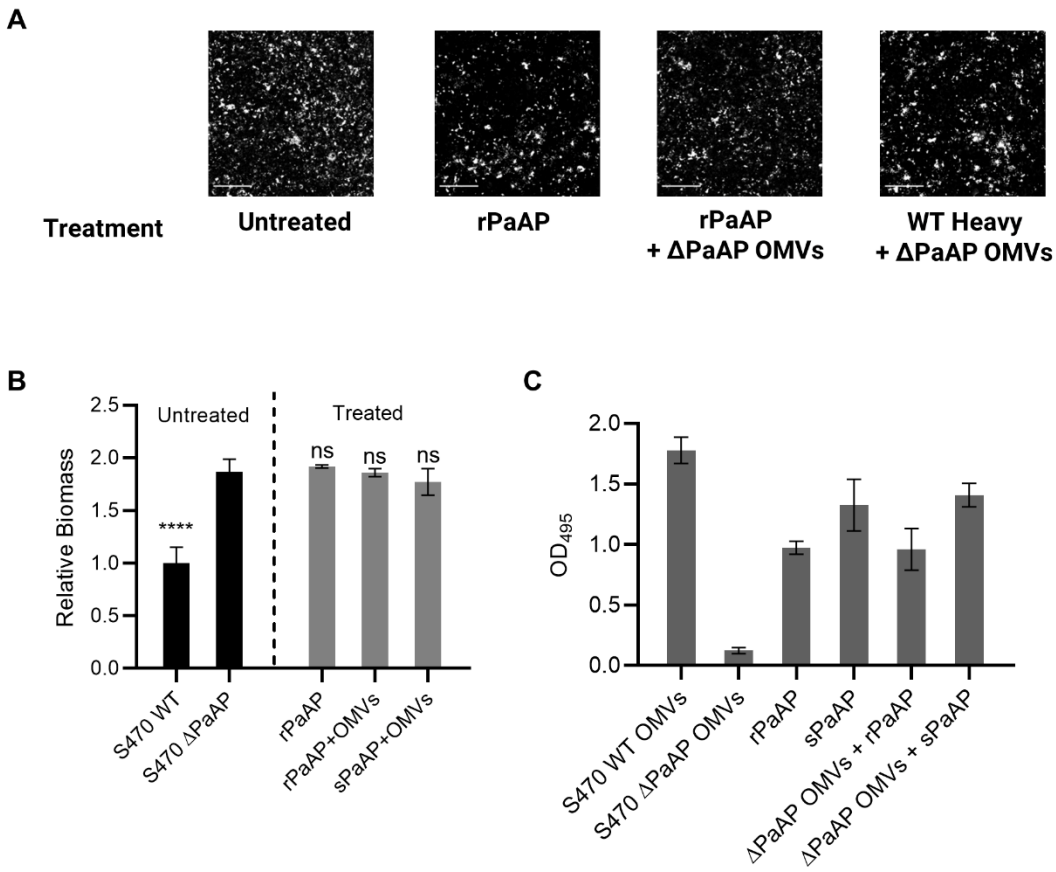

Figure 17: rPaAP and coadditions of rPaAP and sPaAP with ΔPaAP OMVs all fail to complement the knockout biomass phenotype

(A) S470 ΔPaAP biofilms cocultured with A549 cells were treated at 1 hpi with rPaAP, rPaAP with ΔPaAP OMVs, or WT Heavy (sPaAP-containing fractions) with ΔPaAP OMVs and imaged at 5 hpi. **(B)** The biomass of these treated biofilms and the S470 WT untreated control were quantified at 5 hpi and were compared with untreated S470 ΔPaAP biofilms. **(C)** Aminopeptidase activity was also confirmed in the appropriate samples. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns, not significant. For all experiments, representative results are shown. Scale bars: 200 μm.

3.5 WT OMVs also complement ΔPaAP biomass and matrix composition phenotypes in pellicle biofilms

While we had shown that the biomass and matrix remodeling phenotypes that present upon PaAP deletion were not specific to biofilms grown on a host epithelial substrate, we were curious as to whether WT OMVs could also complement ΔPaAP pellicle biofilms. We first tested this on pellicles grown in Jensen's media, which was the only media condition to present a biomass and matrix composition phenotype. Vesicles were added to S470 ΔPaAP pellicles at 1 hpi, and biofilm characteristics were assessed. WT OMV treatment restored wild type biomass levels to these pellicles, as determined by both Crystal violet staining and live/dead pellicle imaging (**Figure 18 A-C**). Additionally, to determine whether the vesicles affected pellicle matrix composition we stained OMV-treated pellicles for Psl and protein content. As with biomass, addition of WT OMVs to ΔPaAP pellicles at 1 hpi increased Psl/biomass ratios and decreased protein/Psl ratios to wild type levels (**Figure 18D, E**). As expected, the ΔPaAP OMVs had no significant impact on any of these phenotypes. These data confirmed that WT OMVs modulate pellicle biofilm composition in the same way observed for coculture biofilms, increasing matrix polysaccharide and reducing microcolony biomass.

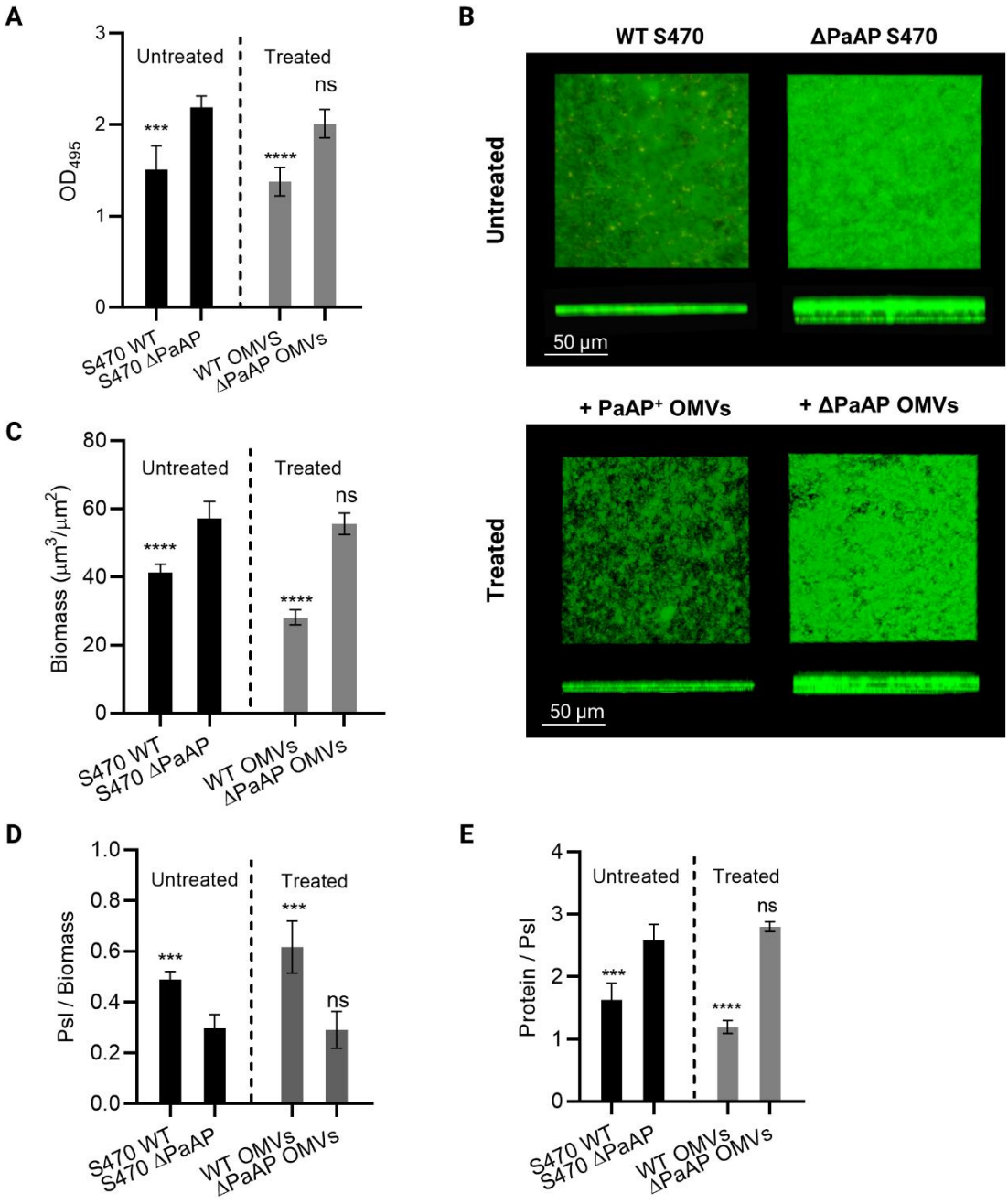

Figure 18: WT OMVs complement PaAP deletion in pellicle biofilms

Untreated: **(A)** S470 WT or ΔPaAP were grown in 35mm dishes for 6 h and pellicle biofilms were quantified by Crystal Violet staining (OD_{495}). **(B)** Pellicles formed by S470 WT or ΔPaAP after 6 h of growth were live/dead stained and imaged by confocal microscopy **(C)** and their biomass calculated. Pellicles were stained for **(D)** matrix Psl and **(E)** total protein,

and the results quantified and normalized to cellular volume and Psl volume, respectively. Treated: **(A -E)** ΔPaAP pellicles were treated at 1 hpi with S470 WT or ΔPaAP OMVs and imaged at 5 hpi. Untreated WT and treated ΔPaAP pellicles were stained as above, quantified, and values compared to untreated ΔPaAP pellicles; *p<0.05, **p<0.01, ***p<0.001, ****p<.0001, ns, not significant. For microscopy experiments, representative results are shown.

3.6 WT OMVs act on later stages of biofilm development and exhibit anti-biofilm activity against pre-formed microcolonies

During the various stages of biofilm development, bacterial communities exhibit very different characteristics that may modify the interaction of OMVs with microcolony structures. While bacterial interactions with the abiotic or host substrate and appropriate expression of motility machinery are important for biofilm attachment, cell aggregation and production of matrix molecules mediate later stages of biofilm formation. Later in the biofilm lifecycle, specific signaling cues or physical disruptions can affect bacterial cell release from the biofilm structure. Therefore, to better characterize the biofilm-remodeling mechanism of WT OMVs, we first studied the kinetics of vesicle-mediated anti-biofilm activity. OMVs were added to cocultures either before inoculation (pre-treatment), at 1 hpi, or at 4.5 hpi (0.5 h before the final imaging time point), and biomass was monitored at various time points after treatment. The results of these experiments are summarized in **Table 2**.

Table 2: PaAP+ OMVs inhibit microcolony development and disrupt pre-formed microcolonies

OMV Addition[1]	Imaging Time[2]	OMVs	Biomass[3]	Interpretation
Bacterial Attachment				
0 hrs (pre-treatment)	1 hr	None (control)	ns	OMV pretreatment of the A549 cells does not affect initial bacterial cell attachment to the host surface. This suggests PaAP's mechanism does not affect bacteria/host cell initial adhesion
		S470 WT	ns	
		S470 ΔPaAP	ns	
Microcolony Inhibition				
0 hrs (pre-treatment)	5 hrs	None (control)	ns	Addition of PaAP-containing OMVs at any point early in the coculture experiment results in lower bacterial biomass. This effect is more pronounced at later time points, suggesting PaAP-mediated biofilm inhibition acts on a bacterial pathway important to microcolony growth and formation, rather than initial attachment.
		S470 WT	- -	
		S470 ΔPaAP	ns	
1 hr	3 hrs	None (control)	ns	
		S470 WT	-	
		S470 ΔPaAP	ns	
1 hr	5 hrs	None (control)	ns	
		S470 WT	- -	
		S470 ΔPaAP	ns	
Microcolony Detachment				
4.5 hrs	5 hrs	None (control)	ns	PaAP bound OMVs can disperse established microcolonies, further suggesting that the aminopeptidase affects microcolony growth and dispersal mechanisms.
		S470 WT	- - -	
		S470 ΔPaAP	ns	

[1]S470 ΔPaAP cocultures were treated with the indicated OMVs at the indicated times. For pre-treatment, A549 cells were incubated with vesicles for 0.5 h before bacterial inoculation.
[2]Samples were imaged at the indicated times and assessed for biomass.
[3]Biomass is represented relative to the untreated S470 ΔPaAP controls for each experiment (ns: no significant difference; -: 5-25% decrease; - -: 25-50% decrease; - - -: >50% decrease).

Consistent with the late development of a biomass phenotype shown in Figure 9, pre-treatment of ΔPaAP biofilms with WT OMVs had no significant effect on bacterial attachment at 1 hpi. These results strengthen our conclusion that biofilm remodeling dependent on WT OMVs is not related to the initial phases of bacterial adhesion to a substrate. When pre-treated biofilms were imaged at 5 hpi, however, we found that complementation was achieved. These results were additionally recapitulated when the ΔPaAP cocultures were treated with vesicles at 1 hpi. In this case, a small relative reduction in biomass was observed at 3 hpi but did not fully complement the knockout until 5 hpi, suggesting that the vesicles are affecting cell aggregation or matrix characteristics later in biofilm development.

Based on the previous results of early vesicle treatment of coculture biofilms, we hypothesized that treatment of fully formed microcolonies with vesicles could lead to an immediate reduction of biomass at later time points. Indeed, treatment of S470 ΔPaAP cocultures with S470 WT OMVs at 4.5 hpi, 0.5 h before the final imaging timepoint, confirmed this hypothesis. We observed a rapid, significant loss of biomass when we treated with S470 WT OMVs, and no difference with S470 ΔPaAP OMV treatment (**Figure 19A**, upper panels, and **B**). These results indicate that the vesicles initiate a loss of bacteria from the microcolony, rather than inhibiting their aggregation, suggesting that they have anti-biofilm properties.

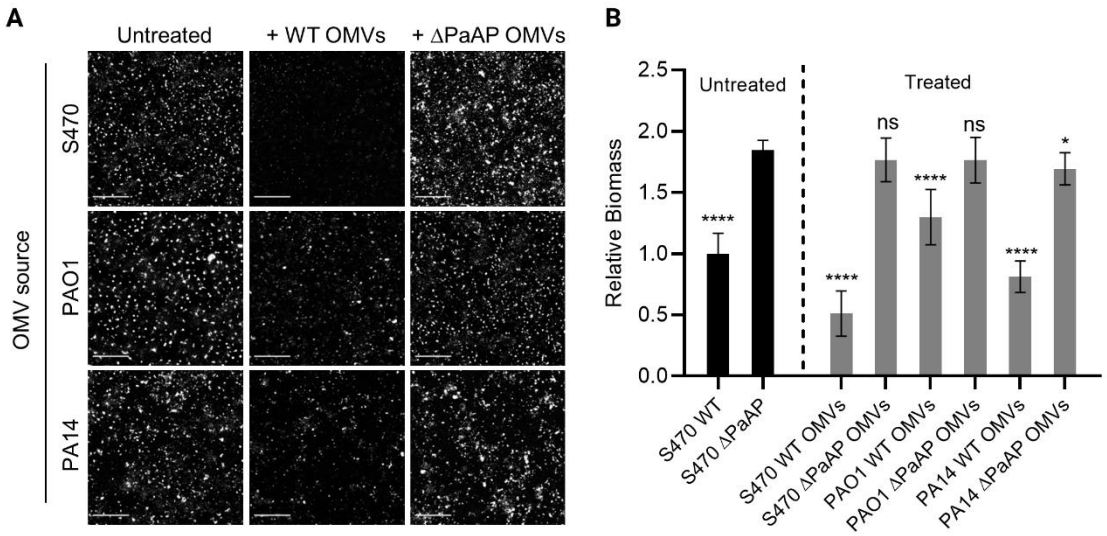

Figure 19: WT OMVs cause a rapid loss of biomass from pre-formed S470 ΔPaAP cocultures

(A) Top row: OMVs from WT or ΔPaAP cultures of the indicated P. aeruginosa strains were added to S470 ΔPaAP biofilm cocultures at 4.5 hpi, 30 minutes prior to imaging and **(B)** these results were quantified. The biomass of treated biofilms and the S470 WT untreated controls were quantified and were compared with untreated S470 ΔPaAP biofilms. For all experiments, representative results are shown. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns, not significant. Scale bars: 200 μm.

To examine whether OMV-related biofilm reducing activity was specific to S470 strain-derived vesicles, we purified and tested OMVs from PAO1 and PA14 WT strains and their corresponding ΔPaAP mutants. As outlined previously, PAO1 is a laboratory strain of *P. aeruginosa* that, while originally isolated from a patient blood sample, retains few characteristics of clinical *Pseudomonas* and exhibits low PaAP expression. PA14, a chronic wound isolate, is another *P. aeruginosa* strain commonly used in laboratory research, but it has retained many of its clinical properties, including enhanced

cytotoxicity. As seen with the S470-derived vesicles, WT OMVs from both PA01 and PA14 disrupted the preformed coculture biofilms, whereas OMVs from the isogenic mutants did not (**Figure 19A, B**). We can therefore conclude that OMV-associated anti-biofilm activity is not specific to S470-derived OMVs, but rather relevant to a range of *P. aeruginosa* strains.

3.6.1 *P. aeruginosa* OMVs cause detachment of *Klebsiella pneumoniae* coculture biofilms

During respiratory infections biofilms are often found as polymicrobial communities, and components secreted by one species can impact the microcolonies of other strains or species in nearby environments. To determine whether the anti-biofilm activity of the OMVs is species-specific, A549 cell layers were inoculated with *K. pneumoniae*, a bacterium which is often found with *P. aeruginosa* in cases of ventilator-associated pneumonia (Riquelme, Ahn, and Prince 2018). S470 WT or ΔPaAP OMVs were added at 4.5 hpi, and the cocultures were stained at 5 h with Congo Red to examine both cellular and matrix biomass. As seen in **Figure 20**, S470 WT vesicles significantly reduced *K. pneumoniae* total biomass, while ΔPaAP OMVs had very little effect. These data suggest that the OMVs target a conserved feature of biofilm architecture, allowing them to impact the colonization ability of multiple clinically relevant pathogens.

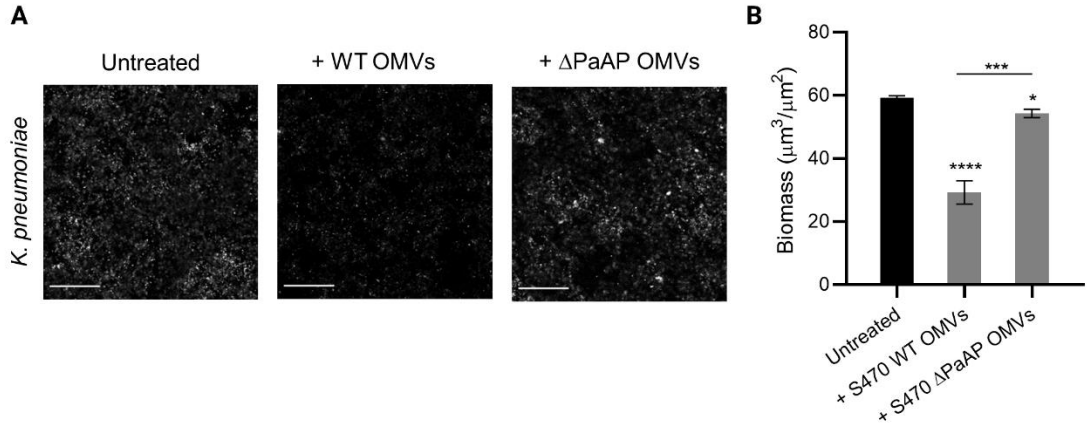

Figure 20: *P. aeruginosa* WT OMVs disrupt pre-formed *Klebsiella pneumoniae* microcolonies

(A) *K. pneumoniae* cocultures were treated with S470 WT or ΔPaAP vesicles at 4.5 hpi, biofilm formation was assessed by Congo Red staining at 5 hpi, and **(B)** quantified. The biomass of treated *K. pneumoniae* biofilms were quantified and were compared with untreated biofilms. For all experiments, representative results are shown. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns, not significant. Scale bars: 200 μm.

3.6.2 *P. aeruginosa* OMVs rapidly disrupt pellicle biofilms

As WT OMVs were found to also disrupt pre-formed microcolony structures in the coculture assay, we were also curious as to whether pellicles could be disrupted by relatively short incubations with the vesicles. We therefore added WT or ΔPaAP OMVs to pellicles grown under the different media conditions previously tested at 4.5 hpi and imaged them at 5 hpi. As seen in **Figure 21**, WT OMVs, but not ΔPaAP OMVs, displayed significant anti-biofilm activity against S470 ΔPaAP pellicles grown in Jensen's media; however, the other media conditions which did not present the original phenotype (M63 minimal, fresh and conditioned tissue culture media, and LB + glycerol) were unaffected

by any OMV treatment. These results confirm that WT OMVs exhibit anti-biofilm activity against biofilm grown without a host cellular substrate, and therefore that this phenotype is independent of host cell-bacterial interactions.

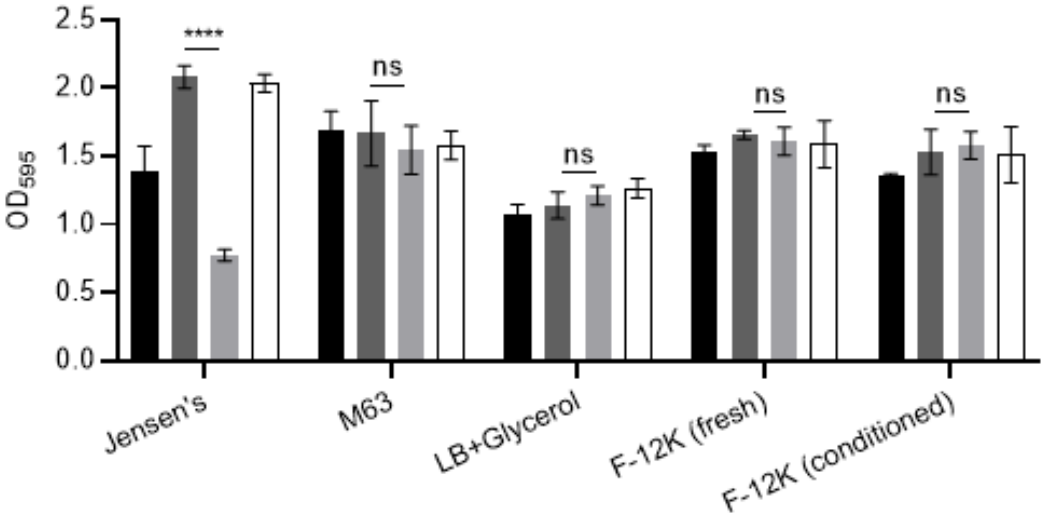

Figure 21: WT OMVs disrupt pre-formed ΔPaAP pellicle biofilms under certain growth conditions

Pellicle biofilms were grown in 96 well dishes for 5 h and biofilms were stained with Crystal Violet. For vesicle treated samples, OMVs were added at 4.5 hpi and stained at 5 hpi.. Black: S470 WT untreated; dark gray: S470 ΔPaAP untreated; light gray: S470 ΔPaAP + WT OMVs; white: S470 ΔPaAP + ΔPaAP OMVs; *p<0.05, **p<0.01, ***p<0.001, ****p<.0001, ns, not significant.

3.7 The anti-biofilm activity of OMVs causes cellular detachment from the biofilm structure

During the final stages of biofilm development, bacterial cells are released form the microcolony structure via a process known as dispersal. As nutrients become limited to cells in the center of the microcolony, specific signaling cues lead to a decrease in the

intracellular regulator cyclic-di-GMP. In turn, cells from the center of the microcolony begin to release from the greater matrix and cellular structure and transition back to a planktonic mode of growth. With renewed expression of motility factors, including flagella, the bacteria can travel away from the parent microcolony and colonize new areas (Petrova and Sauer 2016).

While regulated dispersal from the parent colony is considered an established stage in the biofilm lifecycle, this is not the only way that bacteria can leave the biofilm colony. Exogenous factors that the biofilm may encounter from other cells present in the environment, the immune system, or clinical treatments can interfere with matrix and cell aggregation factors. As these connections degrade, single cells or cellular aggregates can release from the microcolony structure in a process known as detachment. Detachment is often caused by physical or biochemical interference with *P. aeruginosa* molecules that hold the biofilm together, and known detachment factors include Proteinase K and DNAse, as well as polysaccharide hydrolases that degrade EPS (Petrova and Sauer 2016). Other substances known to cause detachment of *P. aeruginosa* biofilms are listed in **Table 3**.

Table 3: Agents known to cause detachment of cells from *P. aeruginosa* biofilms

Agent	Notes	References
Alginate lyase	Degrades alginate in the matrix and leads to detachment under certain biofilm growth conditions	Boyd and Chakrabarty, 1994
PslG	Degrades Psl in the matrix and leads to detachment during flow cell biofilm growth	Zhao et al., 2018 Yu et al., 2015
DNase I	Degrades eDNA in the matrix. Leads to a reduction in biomass in early biofilms but late biofilms are often unaffected.	Whitchurch et al., 2002
Proteases	Reduces cell aggregation, leading to disruption of biofilm-like structures. Polysaccharides in the matrix can protect against protease-induced detachment.	Reichhardt et al. 2018
Chelating agents	EGTA or EDTA can cause rapid cellular detachment and weakening of alginate structure in the EPS	Turakhia et al., 1983 Gordon et al., 1991
Surfactants	Causes a reduction in total biofilm protein, potentially due to disruption of hydrophobic interactions involved in matrix cross-linking	Chen and Stewart, 2000
Urea	Reduces viscosity of biofilm suspensions, potentially due to disruption of hydrogen bonding in matrix cross-linking	Chen and Stewart, 2000

To better characterize the mechanism by which WT OMVs lead to the release of bacterial cells from ΔPaAP cocultures, we utilized the antibiotic resistance characteristics of cells released by each process. When bacteria are dispersed from biofilms, they transition back to planktonic cells and, as such, lose many of the characteristics they displayed as biofilms, including resistance to antibiotics. In fact, several studies have

found that bacteria that undergo regulated dispersal are often more susceptible to antibiotics than they would be had they never been part of a biofilm. Cells detached by physical or biochemical disruption, on the other hand, retain many characteristics of the biofilm, including resistance to antibiotics. This is not only due to the retention of regulated biofilm characteristics, but the cells may be released as aggregates and/or maintain association with some features of the matrix, such as proteins and polysaccharides, that help prevent antibiotic delivery directly to the cells.

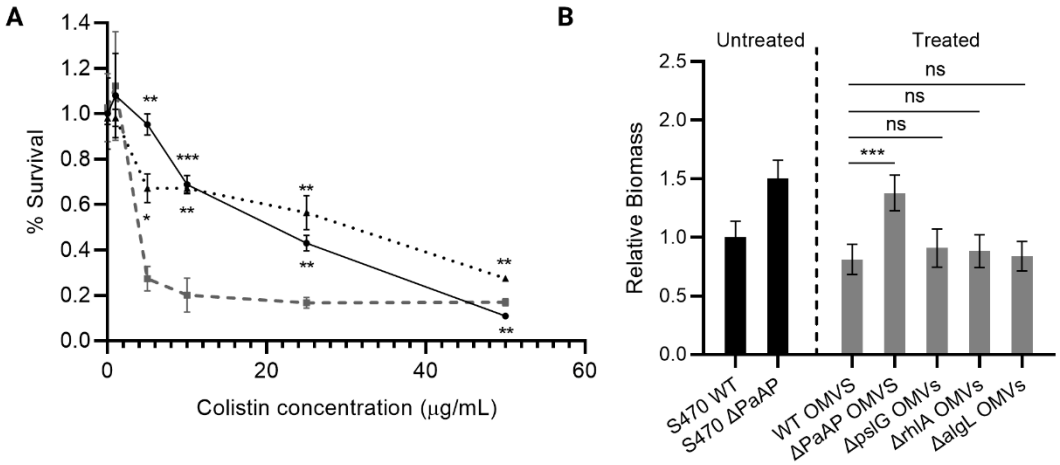

Figure 22: PaAP$^+$ OMVs cause the detachment of biofilm cells

(A) S470 ΔPaAP cocultures (solid line) were challenged with colistin at 3 hpi and examined for remaining biomass at 5 hpi. Additional S470 ΔPaAP cocultures were treated with S470 WT OMVs at 4.5 hpi, the detached cells (dotted line) were collected at 5 hpi and challenged with colistin for 2 h. These samples were compared to planktonically grown S470 ΔPaAP cells (dashed line) challenged with colistin at 3 hpi with live/dead assessment at 5 hpi. **(B)** S470 ΔPaAP biofilm cocultures were treated with OMVs isolated from PA14 WT and the indicated PA14 transposon insertion mutants at 4.5 hpi, and the biomass was quantified at 5 hpi. The biomass of treated biofilms and the S470 WT untreated control were quantified, and treated samples were compared to biofilms treated with S470 WT OMVs. For microscopy-based experiments, representative results are shown. For all other experiments, n=3. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns, not significant.

We therefore compared the antibiotic resistance profile of cells released from the coculture colony structure after WT OMV treatment to untreated cocultures and planktonic cells. **Figure 22A** shows the response of each of these cell types to the antibiotic colistin. Notably, while the cells collected after OMV treatment experienced a slight decrease in colistin resistance at high antibiotic concentrations, the profile of these cells matches closely with those from the S470 cocultures, with significantly greater colistin resistance than the planktonic cells. These data allow us to conclude that the vesicles are not leading to a programmed dispersal response from the biofilm structure, but rather that the microcolonies are being disrupted by an enzymatic or biochemical process elicited by vesicle cargo.

As noted above, many of the factors that have been documented as leading to bacterial detachment from biofilms interfere with the matrix or other factors facilitating bacterial aggregation, such as surface expressed proteins (Reichhardt et al. 2018). Several factors that are specifically expressed by *P. aeruginosa* during various stages of biofilm or planktonic cell development have been shown to cause detachment, and, because we had shown that the aminopeptidase itself was not directly responsible for this activity, we hypothesized that one of these factors may be responsible for the observed vesicle phenotype. Most notably, two specific polysaccharide hydrolases have been shown in previous studies to lead to matrix degradation and cell release from the biofilm. Alginate lyase degrades alginate, a major component of mucoid biofilm colonies, and PslG

degrades Psl, another major polysaccharide in *P. aeruginosa* microcolonies (Zhao et al. 2018; Petrova and Sauer 2016). While the S470 strain used in the majority of this work is a non-mucoid isolate, and therefore does not express alginate, alginate lyase is a well-documented mediator of detachment, and we therefore felt that it was important to include in these studies. PslG has recently been shown to play a role in biofilm remodeling in a different study of the PaAP leucine aminopeptidase. Zhao et al demonstrated that without PaAP expression during starvation conditions, cell lysis occurs during the final stages of biofilm maturation, leading to the release of intracellular PslG and dissolution of the biofilm (Zhao et al. 2018). Additionally, rhamnolipids have been implicated in both biofilm formation and programmed biofilm dispersal (Solano, Echeverz, and Lasa 2014; de Kievit 2009).

To test whether any of these molecules contributed to OMV-associated bacterial detachment from biofilms, we utilized the PA14 transposon insertion mutant library (Liberati et al. 2006) and isolated OMVs from mutants lacking alginate lyase, PslG, or rhamnolipids. Interestingly, the vesicles from each of these mutant strains were able to recapitulate the anti-biofilm activity observed in the wild type PA14 vesicles (**Figure 22B**), leading us to conclude that none of these previously documented factors are involved in OMV-mediated biofilm detachment. Further study of the activity of these WT OMVs was therefore required to determine what PaAP-regulated component caused this activity.

3.8 WT OMVs display higher protease activity compared to ΔPaAP OMVs, and this activity is responsible for OMV-induced detachment

While known detachment factors were not found to contribute to OMV-associated anti-biofilm activity, proteases may also contribute to this process. The most well studied example of this is Proteinase K, which, in *P. aeruginosa*, has been shown to interfere with CdrA, a surface expressed cellular adhesin that mediates cell aggregation (Reichhardt et al. 2018). Due to the high number of proteases expressed in *P. aeruginosa*, it was impractical to test each mutant individually, and we therefore examined overall protease activity in WT and ΔPaAP OMVs. Using a standard protease activity assay, which would capture much of the activity caused by various proteases produced by *P. aeruginosa*, we found that while both WT and ΔPaAP vesicle samples contained substantial protease activity, it was significantly higher in the WT OMVs (**Figure 23A**). PaAP itself would also cause a protease response in this assay and were therefore utilized purified rPaAP to control for PaAP activity. Using the aminopeptidase-specific activity assay, we titrated the rPaAP control to match the leucine aminopeptidase activity found in the WT OMV samples tested in the protease assay. The final column in **Figure 23A** shows the amount of overall protease activity associated with the amount of aminopeptidase activity present in the WT OMV sample. This activity was less than the difference between the WT and ΔPaAP OMVs, allowing us to conclude that the difference in protease activity between these vesicle samples is caused by non-PaAP proteases.

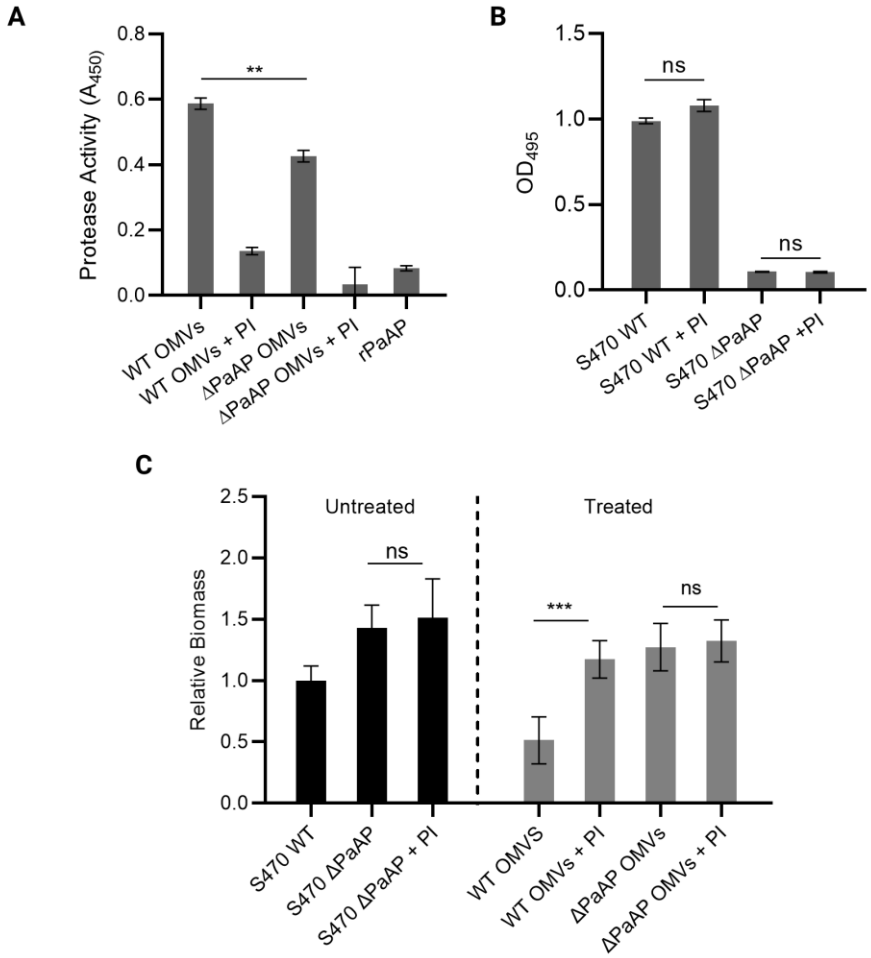

Figure 23: Increased protease activity in PaAP⁺ OMVs mediates OMV-associated biofilm detachment activity

(A) General proteolytic activity was measured in the indicated S470 WT and ΔPaAP OMV preparations with and without protease inhibitor cocktail (PI) and for the amount of rPaAP that matched the aminopeptidase activity found in the WT OMV sample. **(B)** S470 WT and ΔPaAP OMVs were treated with a protease inhibitor cocktail (PI) or buffer only and incubated at 37°C. Aminopeptidase activity was measured. **(C)** S470 ΔPaAP biofilm cocultures were treated with the indicated S470 WT and ΔPaAP OMVs with and without PI at 4.5 hpi and the resulting biomass was quantified at 5 hpi. The biomass of treated biofilms, the S470 WT untreated control, and the S470 ΔPaAP biofilm incubated with PI were quantified and samples were compared to the corresponding samples without PI.

For microscopy-based experiments, representative results are shown. For all other experiments, n=3. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns, not significant.

While we had shown that WT OMVs have higher overall non-PaAP protease activity than their ΔPaAP counterparts and previous studies have demonstrated that proteases can cause cellular detachment from biofilms (Petrova and Sauer 2016; Hoge et al. 2010), we wished to determine whether the activity we observe was directly responsible for WT OMV anti-biofilm activity. To do so, OMV samples were pretreated with a cocktail of protease inhibitors (PI) that fully reduced all protease activity (**Figure 23A**). Of note, this cocktail did not affect PaAP activity in the PaAP$^+$ OMV samples (**Figure 23B**). Protease-inhibited vesicles were added to ΔPaAP cocultures at 4.5 hpi, and biomass was determined at 5 hpi. PI-treatment significantly reduced the anti-biofilm activity of WT vesicles, and biomass in the cocultures treated with these samples returned nearly to untreated ΔPaAP coculture levels **Figure 23C**. While we were unable to determine exactly which endogenously produced proteases were responsible for this activity, these data allowed us to conclude that WT OMV-mediated biofilm detachment is not caused by the aminopeptidase itself, but rather by increased expression of proteases packaged into vesicles when PaAP is present.

3.9 Discussion

In this chapter, we set out to investigate the mechanism behind biofilm matrix and architectural remodeling which we had observed upon PaAP deletion. Upon fractionation

of bacterial supernatants into soluble protein and OMV-containing samples, we found that OMVs isolated from wild type cultures, but neither soluble proteins nor OMVs secreted by ΔPaAP cells, were able to complement the effects of PaAP deletion. Additionally, after examining the kinetics of vesicle-associated anti-biofilm activity and the properties of cells released after vesicle treatment, we determined that WT OMVs induce detachment from both coculture and pellicle biofilm structures. We additionally found that this activity could cause the detachment of *K. pneumoniae* biofilms, demonstrating that this activity was not specific to a *P. aeruginosa* substrate. The effect of PaAP expression was indirect, as neither recombinant nor purified soluble aminopeptidase alone or in combination with ΔPaAP OMVs possessed detachment activity. Our data further lead us to conclude that PaAP influences changes in biofilm architecture through proteolytic activity, as WT OMVs exhibited higher proteolytic activity than ΔPaAP OMVs and WT OMV-mediated detachment was sensitive to protease inhibition

OMVs have been implicated in the process of biofilm formation (Schooling and Beveridge 2006; Wang, Chanda, and Zhong 2015) but while their presence in these bacterial communities has been documented, active roles for these particles and the bacterial products they carry have not been extensively studied. Recently, Zarnowski *et al.* have reported a role for vesicles in the biogenesis of antimicrobial-resistant *Candida albicans* biofilm matrices and have provided evidence that vesicle cargo proteins are

"functional passengers" that can affect biofilm structure (Zarnowski et al. 2018). Similarly, our results suggest that vesicle-associated enzymes functionally impact *P. aeruginosa* biofilms.

Detachment of cells from the biofilm structure is a process previously shown to be able to be mediated by a variety of enzymes and biosurfactant molecules native to *P. aeruginosa*. Polysaccharide hydrolases, including both PslG and alginate lyase, have been described by multiple studies as mediating the release of cells from biofilm structures (Petrova and Sauer 2016). Zhao et al reported on the involvement of PslG in biofilm disruption, which was also, intriguingly, PaAP-dependent (Zhao et al. 2018). However, in this case, biofilm disruption was found to take place in late log-stage biofilms under nutrient-limiting conditions such that the lack of PaAP leads to cell lysis, releasing the periplasmic PslG enzyme. By contrast, the biofilm phenotype we observed not only does not depend on starvation, it is mediated by OMVs and is independent of PslG expression. Additionally, AlgL and rhamnolipids, which have biosurfactant properties that can cause the release of biofilm cells, were shown not to be involved in this effect. These results demonstrate a distinct role for both PaAP and OMVs in the modulation of bacterial communities.

Other described mechanisms to influence bacterial detachment involve protease activity, which is important in many aspects of *P. aeruginosa* biology (Petrova and Sauer 2016; Hoge et al. 2010). Previous studies have implicated endogenous proteases in biofilm

detachment and the dissolution of cellular aggregates, specifically those with a protein-rich matrix (Reichhardt et al. 2018). Based on our data we can add new details to this model: endogenous PaAP expression helps regulate protease activity in secreted vesicles which, in turn, lead to the remodeling of biofilm architecture (**Figure 24**). The changes induced by OMVs ultimately increase Psl/biomass ratios in early biofilms and help to protect the developing colony from disruption by antimicrobials.

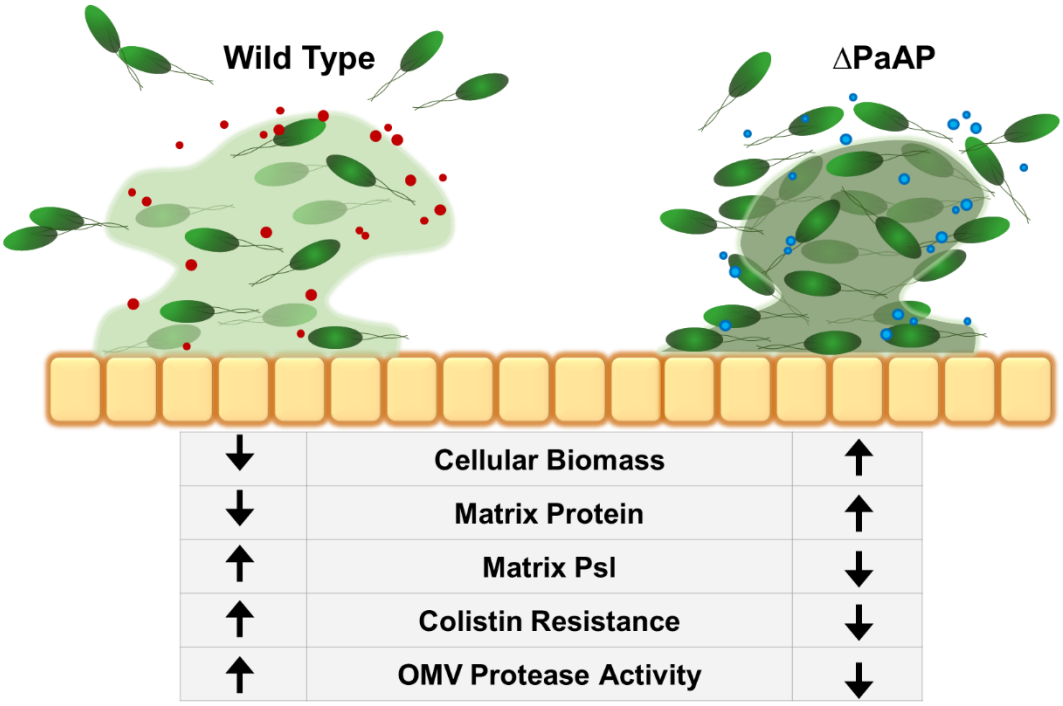

Figure 24: Summary of the effect of PaAP⁺ OMVs on early biofilm development

During WT growth, bacterial microcolonies secrete PaAP⁺ OMVs with increased endogenous protease activity. This leads to increased cell detachment from the colony structure and allows for increased matrix Psl polysaccharide production and protection for the cells against antibiotic treatment. Orange squares: A549 epithelial cells; Green cells: *P. aeruginosa* cells; Red circles: PaAP⁺ OMVs; Blue circles: ΔPaAP OMVs; Green haze: matrix Psl. Darker matrix color indicates a higher concentration of protein.

As for the specific protease(s) and substrate(s) involved in this mechanism, protease expression and activation during *P. aeruginosa* development is a highly complex, regulated process with many steps, including expression, secretion, and processing. Additionally, many of these proteases are involved in the activation and expression of

others (Sarnovsky et al. 2009; Hoge et al. 2010; Oh et al. 2017). This complex system makes it challenging to specify which protease(s) are affected by PaAP expression, whether this is the effect of one or several endogenous proteases, and how PaAP increases vesicle-associated protease activity. The protein substrate(s) for the OMV activity are also unknown and will require further study. One interesting observation, however, is that both early biofilms of *P. aeruginosa* and *K. pneumoniae* contain a substrate for the activity.

Additionally, because we have shown that this phenotype is not directly caused by the vesicle-associated aminopeptidase, but rather other vesicle-associated proteases, we have implicated PaAP in a more complex regulatory network that influences the activity of bacterial molecules and structures important to the formation of robust biofilm structures. Future work on this project will seek to elucidate the mechanism by which PaAP influences OMV-mediated biofilm maturation.

4. Future directions

In this dissertation, we have provided strong evidence that *P. aeruginosa* OMVs have functional significance in the biofilm matrix directly related to the expression of the PaAP aminopeptidase. However, work on this subject is far from complete and many directions remain open for future research. The following sections detail proposed future experiments and research considerations on this topic.

4.1 Determining the proteases responsible for OMV-mediated biofilm remodeling

As shown in **Figure 23**, PaAP expression leads to a significant increase in vesicle-associated protease activity, which we found to be responsible for OMV-mediated biofilm detachment. This brings up the interesting question of how the aminopeptidase aids in OMV-associated protease regulation as well as what protease or proteases are directly responsible for OMV-associated detachment activity. A logical first step toward deciphering this activity may be to establish proteomic profiles of WT and ΔPaAP OMVs, which would likely allow for the identification of differently packaged or expressed vesicle-associated proteases.

The question of how PaAP directly causes this increase in OMV-associated protease activity is far more complex. Most proteases, including PaAP, are subject to several regulatory steps before becoming fully functional enzymes. The gene encoding PaAP, PA2939, is under QS regulation, the propeptide is secreted by the QS-regulated T2SS, and the enzyme is activated by other endogenous proteases that likely also

experience similar expression and secretion regulation. Notably, in *P. aeruginosa* many secreted proteases are activated via cleavage by other similarly expressed proteases or aminopeptidases, creating a very intricate regulatory network. Additionally, the protease(s) of interest must then be packaged into vesicles for delivery to the biofilm matrix, and PaAP may act on any of these processes to influence OMV-associated protease activity. Once the protease(s) responsible for the observed biofilm phenotypes have been identified, experiments designed to elucidate differences in the activation or association of these enzymes with vesicles between WT and ΔPaAP strains may also shed light on the direct role of PaAP in this mechanism.

4.2 Characterizing PaAP interactions with host and bacterial cell substrates

Both evidence presented here, as well as previous research on *P. aeruginosa* OMVs, implicate roles for the aminopeptidase in bacteria-host cell interactions as well as intrabacterial interactions. Specifically, we have shown that the aminopeptidase can influence biofilm formation both in the presence of host cells and during pellicle biofilm growth. Additionally, work from our lab previously showed that PaAP, thought to be localized to the external surface of OMVs, mediated the interactions between vesicles and A549 lung epithelial cells. (Bauman and Kuehn 2009). During our study of the aminopeptidase, we collected preliminary evidence that PaAP may interact with both host and bacterial cell products.

To test for interactions between PaAP and A549 cell factors, we used Far-Western blot and co-immunoprecipitation experiments. Fractionated A549 cell lysates were separated by SDS-PAGE and incubated with PaAP in a Far Western blot. As shown in **Figure 25B**, at least one prominent, high density protein was found to bind recombinant aminopeptidase (rPaAP). We also confirmed these results by co-immunoprecipitation, and several potential A549 interaction partners were identified during these experiments (**Figure 25C**), indicating that the aminopeptidase may directly bind or cleave A549 substrates.

We additionally studied interactions of the aminopeptidase with bacterial cell products. S470 ΔPaAP whole cell lysates were separated using SDS-PAGE, and checked for interactions with rPaAP using a Far-Western blot. As seen in **Figure 25D**, rPaAP was found to bind multiple protein bands from the bacterial cell lysates. Identification of these interaction partners, from both S470 and A549 lysates, would provide an interesting direction for future experiments, and may help to shed light on PaAP's influence over OMV-host cell associations. It may also help to elucidate PaAP's direct functional role in mediating biofilm formation and/or vesicle-associated protease activity.

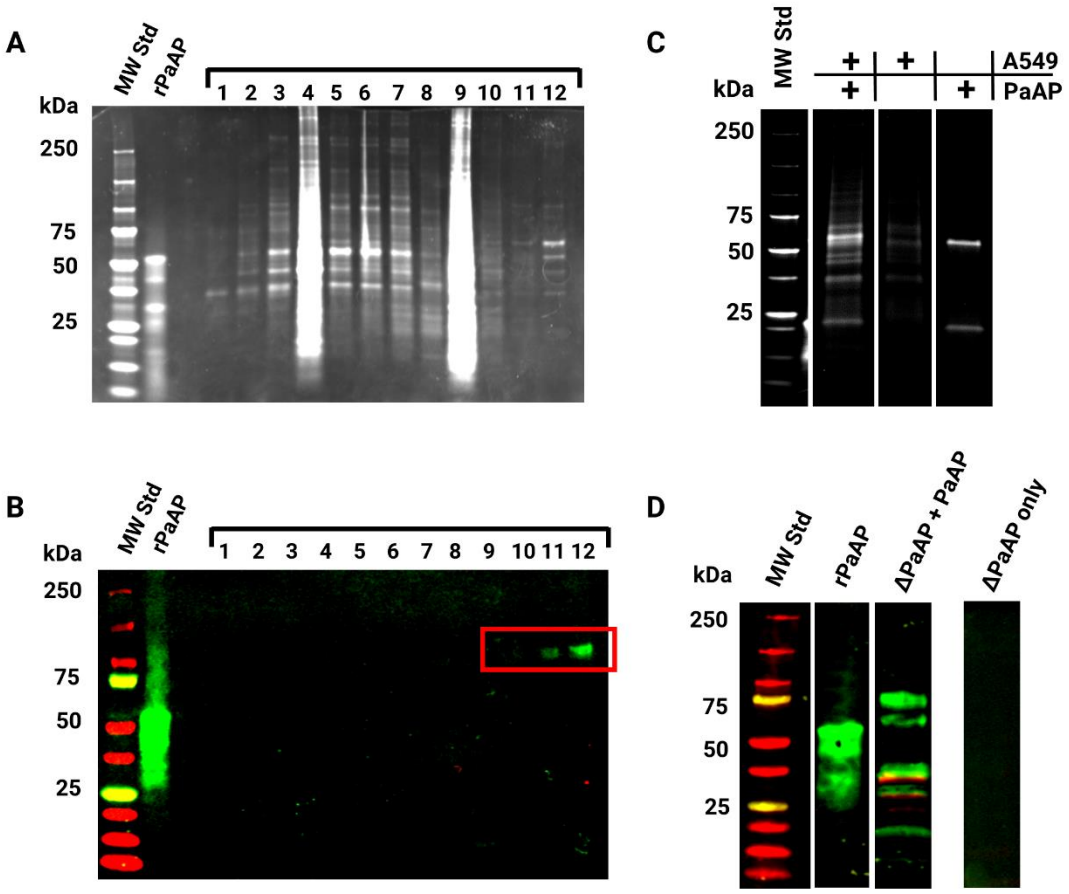

Figure 25: PaAP displays reactivity with both A549 and S470 ΔPaAP proteins
(A) Iodixanol density gradient fractionation of A549 cell lysates were separated using SDS-PAGE gel and imaged using a total-protein Ruby stain. **(B)** In a "Far-Western," the cell lysate fractions shown in **A** were separated by SDS-PAGE, transferred to a PVDF membrane, and the blot incubated with purified recombinant aminopeptidase. rPaAP binding to the membrane was imaged using α-PaAP antibody detection. **(C)** A549 lysate co-immunoprecipitated with rPaAP immobilized on Dynabeads was separated using SDS-PAGE and imaged using a Ruby stain for total protein. A549-lysate only and rPaAP only bands are also shown, as indicated. The migration of protein standards is also shown. **(D)** ΔPaAP cell lysate was separated using SDS-PAGE gel and rPaAP binding was detected using the same Far-Western blot method described in **B**. The final lane (from a different gel) shows the lack of αPaAP antibody reactivity with ΔPaAP lysates.

4.3 Exploiting P. aeruginosa OMVs as a biofilm detachment strategy

The ability of WT OMVs to disperse pre-formed microcolony structures provides another interesting topic for future study. *Pseudomonas aeruginosa* has well documented intrinsic resistance to a wide range of clinically relevant antimicrobials, contributing to its designation as an ESKAPE pathogen by the CDC and a bacteria of high research importance by the WHO (Skariyachan et al. 2018). The bacterium's ability to form biofilms additionally increases resistance as many antimicrobials are unable to penetrate the dense biofilm matrix and others are ineffective against the relatively dormant cells found at the center of mature microcolonies. While traditionally biofilm formation was only thought to occur during chronic *P. aeruginosa* infection, new evidence suggests that biofilms may also form during shorter acute infections. Additionally, the NIH estimates that 65-80% of all bacterial have the ability to form biofilms (Jamal et al. 2018). As such, the discovery of novel anti-biofilm treatments has become a subject of rapid study within the field of *P. aeruginosa* and other bacterial pathogen research. A subset of these emerging therapies are listed in **Table 4**. Interestingly, many of these treatments involve the modulation of endogenous bacterial processes, such as adhesion, dispersion, and QS regulation, to either initiate biofilm disruption or prevent attachment. Several also involve the use of nanoparticles with either intrinsic antimicrobial properties or loaded with anti-biofilm or antibiotic agents.

During our characterization of OMV-associated biofilm detachment activity, we tested the ability of these particles to disrupt pre-formed microcolonies from the S470 WT strain, and our preliminary results showed that exogenous addition of vesicles to WT cocultures led to significant decreases in cellular biomass. Additionally, as shown in Figure 20, WT *P. aeruginosa* OMVs cause the detachment of *K. pneumoniae* coculture biofilms, proving their efficacy against non-pseudomonads. If the full mechanism behind this activity is better characterized, it may provide opportunities for research into novel anti-biofilm agents, either through the stimulation of endogenously-produced OMVs which can induce microcolony detachment, or through the exogenous addition of engineered nanoparticles containing the identified detachment agents.

Table 4: Emerging treatments for *P. aeruginosa* biofilm-based infections

Treatment	Notes	References
Phage therapy	Large, localized populations of bacterial cells in biofilms result in high phage titers, promoting efficient bacterial killing by lytic phages Anti-biofilm phage therapy also disrupts matrix integrity	Ahiwale et al., 2011 Sulakvelidze et al., 2001 Glonti et al., 2010
Antimicrobial peptides	High-throughput screening has identified peptides with increased activity against biofilm-grown cells LL-37 also displays increased toxicity against cells in biofilms Certain *Pseudomonas*-derived pyocins display antimicrobial activity against clinical *P. aeruginosa* strains	de la Fuente-Núñez et al., 2012 Michel-Briand and Baysse, 2002 Overhage et al., 2008
QS inhibition	Inhibition of QS systems at multiple points in regulation (reduction of AHL synthase, inhibition of AHL production, AHL degradation, AHL receptor competitive binding) can disrupt biofilm formation and virulence mechanisms Efficacy against clinical biofilms has not been conclusively shown	Pang et al., 2018 Taylor et al., 2014
Dispersal enhancement	Treatments such as DNase or NO can trigger the dispersal of cells from biofilms, allowing for them to be more easily killed by traditional antibiotics	Barraud et al., 2009 Allesen-Holm et al., 2006
Adherence inhibition	New materials for medical implants, especially catheters, decrease initial attachment of bacteria, preventing biofilm formation Inhibition of bacterial LecA/LecB can prevent bacterial adhesion to host epithelium	Li et al., 2010 Krachler and Orth, 2013
Nanoparticle therapies	Nanoparticle packaging of antibiotics and small molecules increases bioavailability and half life, reduces host toxicity, and leads to more accurate delivery to infection sites Antimicrobial particles (such as silver nanoparticles) can effectively eliminate biofilms Nanoparticles can also cause host toxicity and inflammation, and they have not yet been introduced clinically	Jiang et al., 2018 Wang et al., 2017 Mohanty et al., 2012 Pang et al., 2018

5. Materials and methods

This section contains detailed descriptions of the experimental methods and reagents used in this study.

5.1 Bacterial strains and culture methods

P. aeruginosa strains used include the laboratory strain PAO1 (Pf1 phage-cured from our lab collection), PA14 and associated mutants from a previously described transposon insertion library (Liberati et al. 2006), and minimally passaged, non-mucoid cystic fibrosis clinical isolate S470 along with its previously-described isogenic ΔPaAP (PA2939 deletion) mutant (Bauman and Kuehn 2006; Bauman and Kuehn 2009). To generate fluorescent strains, we transformed bacteria with the dTomato expression plasmid, p67T1 (Singer et al. 2010). *Klebsiella pneumoniae* strain 43816 was obtained from ATCC. A549 human lung epithelia carcinoma cells (ATCC CCL-185) were grown in F-12K media containing 10% fetal bovine serum plus penicillin/streptomycin/fungizone. Unless specifically mentioned, all reagents were obtained from Sigma-Aldrich.

5.2 Coculture assay

A549 cells were grown in MatTek glass bottom 3.5 mm tissue culture treated dishes for 10 days at 37°C in 5% CO_2, with media changes every two days. Bacterial cultures were grown overnight in LB (lysogeny broth) with the appropriate antibiotics, 1 mL of the culture was pelleted and resuspended in 1 mL sterile PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). To fully resuspend the bacterial

samples and reduce aggregation, each was passed through a 1 inch, 26-1/2G needle tip, 10 times. The OD_{600} was measured and conversion values were calculated to determine the number of cells/OD_{600} for each strain. For all following experiments, the OD_{600} was used to determine cell counts and multiplicity of infection (MOI). The bacterial samples were diluted in F-12K media (without antibiotic/antimycotic) and added to the A549-containing wells at MOI 30. The cocultures were incubated for 1h (37°C, 5% CO_2) to allow the bacteria to attach to the cell surface. The cocultures were then washed three times with sterile PBS, and the media was replaced with F-12K containing 0.4% L-arginine. The cultures were incubated for an additional 4 h at 37°C and the media was replaced with microscopy-grade media (Sigma DMEM without Phenol Red, supplemented with 0.4% arginine). LDH release was measured using the CyQUANT LDH Cytotoxicity Assay kit (Thermo Fisher). Images were taken on a Zeiss 780 inverted confocal microscope. To ensure unbiased image collection, imaging fields were separated into evenly spaced sections and images were taken from a randomly selected spot within each section. To prevent photobleaching, no section was imaged more than once. Biomass calculations were completed using Comstat 2.1, and 3D colony models were created using the 3D viewer plugin for Image J. This protocol was adapted from previously published work (Moreau-Marquis et al. 2010).

5.3 Immunoblotting

To quantify PaAP protein in coculture biofilms, the cocultures were lysed with 100 μL RIPA buffer (VWR) and homogenized by repeated pipetting. For detection of PaAP in CFS by immunoblotting, 1 mL of CFS was used. All samples were precipitated with 250 μL trichloroacetic acid (TCA), incubated on ice for 1 h, pelleted by centrifugation (12,500 x g), washed with 1 mL acetone, re-pelleted (12,500 x g) and resuspended in 0.75 M Tris-Cl, pH 8.8, and 1x SDS-PAGE sample buffer (1% β-mercaptoethanol, 0.004% bromophenol blue, 6% glycerol, 2% sodium dodecyl sulfate (SDS), 50 mM Tris-Cl, pH 6.8), prior to SDS-PAGE on BioRad 4-20% Tris-HCl gels. For anti-PaAP immunoblotting, gels were transferred to Amersham Hybond 0.45 μm polyvinyldifluoride (PVDF) membranes (GE Healthcare), blocked with 1% non-fat dry milk in TBS (50 mM Tris-Cl, pH 7.6; 150 mM NaCl), and then incubated (4°C, overnight) with anti-PaAP antibody[15] diluted 1:1000 in TBS with 0.1% Tween (TBST). Blots were washed three times with TBST, incubated (room temperature, 1 h) with goat anti-rabbit Li-Cor secondary antibody (Odyssey) diluted 1:40,000 TBST + milk, washed, and analyzed using an Li-Cor Odyssey CLx imaging system with Li-Cor Image Studio software. ImageJ was used to quantify the anti-PaAP reactive bands.

5.4 qRT-PCR

Pellicle and coculture biofilms were grown for the indicated times as described. For coculture biofilms, supernatants were removed and 1 mL of Trizol was added to each

sample to lyse both bacterial and A549 cells. For pellicle biofilms, 3 mL/well of cultures incubated in 6-well polystyrene dishes (VWR) were used to obtain sufficient biofilm material for analysis over the given time course. All samples were pipetted extensively to partially homogenize them and transferred to 15 mL conical tubes, in which the bacteria were pelleted. For planktonic culture comparisons, bacterial cultures were prepared as described for the coculture assay and grown in LB at 37°C with shaking for 5 h. Trizol (1 mL, Invitrogen) was used to lyse each sample. A standard Trizol extraction was performed as described by the Invitrogen protocol, and RNA was extracted from the aqueous phase. The RNA was reprecipitated using 3 M sodium acetate for further purification and resuspended in dH_20. RNA samples were then DNase treated and reverse transcribed using the Applied Biosystems High-Capacity cDNA Reverse Transcription kit protocol. Applied Biosystems Power Sybr Green PCR Master Mix was used to prepare the samples, and they were analyzed on a StepOne Plus real-time PCR machine (Applied Biosystems). ΔC_T values were calculated relative to *proC* as a housekeeping control, which was confirmed to have similar expression patterns in both biofilm and planktonic cultures at the concentrations of RNA used. Relative expression was calculated against planktonic S470 WT samples. Forward (F) and Reverse (R) primers used in this study are listed in **Table 5**.

Table 5: qRT-PCR Primers used in this study

Target	Sequence (5′-3′)		Reference
proC	F	CAGGCCGGGCAGTTGCTGTC	Savli et al., 2003
	R	GGTCAGGCGCGAGGCTGTCT	
PaAP	F	GTGGTACGCAAGAAGACCGA	Mellbye and Schuster, 2014
	R	ATCACCACGTTGTTCGGGTT	
FliD	F	CCAGTTCAAGAGTGCGATCA	Deng et al., 2014
	R	ACGGAGATGTTCAGCGTACC	
CupA1	F	GCGGCAAACACTATCACATTC	Qaisar et al., 2013
	R	AACAGGGTGGTGAAATGCTC	
PilA	F	AACCTGAACCTGGACTGTGG	Qaisar et al., 2013
	R	TTGCCTTCGCCATCTTTT	
PslA	F	AAGATCAAGAAACGCGTGGAAT	Colvin et al., 2012
	R	TGTAGAGGTCGAACCACACCG	

5.5 Antibiotic resistance

To determine antibiotic resistance of coculture biofilms and planktonic cultures, samples were grown as described above, and treated with the indicated concentrations of colistin hydrochloride (Sigma-Aldrich) for 2 h at 37°C. For cocultures, viability was determined by calculating fluorescent biomass retained at the cellular substrate, based on our finding that dead cells lost fluorescent activity. For planktonic cultures, viability was determined using the Live/Dead BacLight Bacterial Viability Kit (ThermoFisher Scientific). For detachment experiments, cocultures were treated with vesicles as described below, and supernatants were collected. The detached cells were pelleted,

resuspended in PBS, incubated with the indicated colistin concentrations for 2 h at 37°C, and viability determined using BacLight staining.

5.6 Pellicle biofilm quantification

Bacterial cultures were grown overnight in LB (37°C, 200 rpm) with antibiotics if appropriate. The cultures were vortexed for 15 sec and diluted 1:1000 in Jensen's media[27], M63 minimal media (supplemented with 1 mM magnesium sulfate, 0.2% glucose, and 0.5% casamino acids as outlined previously (Jensen, Fecycz, and Campbell 1980)), LB+ 0.4% glucose, or tissue culture media (described above) with no antibiotics. Cultures were grown to an OD_{600} of 0.5 and diluted to OD_{600} of 0.1. Aliquots of the diluted culture (100 µL/well) were incubated in five 96-well flat bottom polystyrene dishes (VWR) for 0, 2, 6, 10, or 24 h at 37°C with no shaking and evaluated using a static biofilm quantitation assay[28]. Briefly, at the incubation endpoint, the plate was washed out using distilled water (dH_20). After three washes, 150 µL of 1% aqueous Crystal Violet solution was added to each well and the samples were incubated for 10 min at room temperature. The stained wells were washed three times using dH_20, 200 µL of 30% aqueous acetic acid was added to solubilize the dye, and the OD_{495} was measured.

5.7 Pellicle biofilm imaging

Cultures were prepared as described above and diluted cultures were grown in 35 mm glass bottom dishes for 5 h at 30°C. Media was removed and the pellicles were stained

using BacLight. The pellicles were imaged using a Zeiss 780 inverted confocal microscope and biomass was calculated using Comstat 2.1.

5.8 Psl and matrix protein staining

To stain the biofilm extrapolymeric substance, 100 μg/mL HHA-FITC lectin (USA Biologicals) was added to cocultures 2 h before the imaging timepoint, and the samples were incubated at 37°C with 5% CO_2. Pellicle samples were treated with 100 μg/mL HHA-TRITC (USA Biologicals) lectin for 2 h before the final imaging timepoint. For protein staining, pellicles were stained with Ruby FilmTracer SYPRO Ruby Biofilm Matrix Stain (ThermoFisher Scientific) for 5 min before imaging, as described in the reagent protocol. All samples were imaged using a Zeiss 780 inverted confocal microscope and staining was quantified using Comstat 2.1.

5.9 OMV isolation and supernatant fractionation

Bacterial cultures were grown overnight in LB at 37°C, diluted 1:100 into 1.5 L LB, and grown (37°C, shaking) to OD_{600} 0.9-1.1. Cells were pelleted (10,000xg for 15 min 4°C), and supernatants were collected and concentrated to 100 mL using tangential flow with a 10 kDa MWCO filter (Pall). The retentate was then filtered (0.22 μm, Pall) to generate cell-free supernatant (CFS). To concentrate soluble proteins and OMVs from the CFS, ammonium sulfate was added to a final concentration of 90%, dithiothreitol added to 0.1 M, and the samples incubated overnight at 4°C with stirring. The precipitate was pelleted (15,000xg, 15 min, 4°C) and resuspended in 10 mL 20 mM HEPES, pH 8.0 (HEPES). The

samples were then dialyzed in a ThermoFisher G2 cassette (10,000 MWCO) against HEPES, overnight at 4°C. The samples were further concentrated using Millipore 10,000 MWCO centrifugal filters, and the soluble proteins were separated from the OMVs using an iodixanol (Optiprep, Sigma) density gradient. Concentrated proteins and OMVs were adjusted to 50% Optiprep/HEPES in 2 mL, and applied to the bottom of the gradient tube, and subsequently layered with 2 mL of 40%, 2 mL of 35%, 4 mL of 30%, and 2 mL of 25% Optiprep/HEPES. The gradient was centrifuged overnight at 40,000xg, 4°C in an ultracentrifuge (SW 40 Ti swing bucket rotor, Beckman Coulter). Gradient fractions were removed from the top in 1 mL aliquots, and the lipid and protein content for each fraction were identified using FM4-64 and Bradford assays as described previously (MacDonald and Kuehn 2013). The OMV-containing fractions (typically 1-5) and soluble protein fractions (typically 7-12) were pooled and diluted with HEPES, then centrifuged at 40,000xg for 2 h to pellet OMVs and proteins and to remove Optiprep. Protein and OMV pellets were resuspended in HEPES, and sterile-filtered (0.4 μm PVDF, VWR).

5.10 rPaAP purification

rPaAP expression, purification, and refolding were carried out as described previously (Sarnovsky et al. 2009), and the refolded protein was concentrated using 10,000 MWCO Amicon filter units (Millipore-Sigma). The concentrated material was then dialyzed using the same method described above, applied to a S200 Sephadex ion exchange column, eluted with a gradient of 0-2 M NaCl, pH 7.6, at a flow rate of 0.02

mL/min, and 18- 2 mL fractions were collected. Fractions were assayed for leucine aminopeptidase activity as described (Sarnovsky et al. 2009) and analyzed by SDS-PAGE and Ruby staining as described above. Fractions containing pure rPaAP were pooled and concentrated using Amicon 10,000 MWCO filter units to ~0.5 μg/mL.

5.11 Complementation and detachment experiments

For biofilm complementation assays, CFS, OMV fractions, soluble fractions, and rPaAP samples were prepared as described above, total protein was measured by Bradford assay, and aminopeptidase activity was measured as described above. For standard complementation experiments, samples were added to cocultures with new media at 1 hpi. 50 μg total protein was used for CFS samples, and 25 μg total protein was used for OMVs, soluble fractions, and rPaAP samples. For co-addition experiments, 25 μg of ΔPaAP OMVs were incubated with 25 μg of sPaAP-containing WT Heavy fractions or rPaAP for 10 min at room temperature prior to coculture treatment. In detachment experiments, 25 μg of OMV samples were added to cocultures at 4.5 hpi. For OMV pretreatment of A549 cell layers, 25 μg of OMV samples were added to A549 cells for 15 min prior to inoculation. For experiments matched by PaAP activity, samples were standardized to the aminopeptidase activity found in 25 μg (by total protein) of $PaAP^+$ OMVs.

5.12 Protease activity and inhibition

Protease activity was determined using the Pierce Protease Activity Kit (ThermoFisher Scientific). 10 μg of each OMV sample was used, and the rPaAP control was matched to the aminopeptidase activity found in this concentration of OMVs. Protease activity was inhibited by incubating the samples with PI (2X cOmplete Protease Inhibitor Cocktail (Millipore Sigma) and 10 mM phosphoramidon (Sigma) in 20 mM HEPES) for 30 min at 37°C.

5.13 Statistics

For all experiments, statistics were completed using GraphPad Prism t-tests unless otherwise indicated and the average +/- SEM is shown. For microscopy images and imaging quantifications, representative results are shown. Figures show a single biological replicate calculated from at least 5 technical replicate images. Images chosen for publication were the closest to the mean value for each experiment. For all other experiments, results are calculated from 3 biological replicates and the average +/- SEM is shown.

5.14 Acknowledgements

We thank David FitzGerald (National Cancer Institute, Bethesda, MD) for anti-PaAP antibody and rPaAP constructs, and acknowledge the contribution of the dTomato plasmid by Carol Kim (University of Maine, Orno ME).

6. Conclusion

The secretion of bacterial products into the extracellular milieu is a function critical to bacterial biology, as it provides a mechanism for the bacteria to interact with their extracellular environments and influence survival, competition with other microbes, and host-pathogen interactions. Outer membrane vesicles are produced by every Gram-negative bacterium studied to date and allow for the extracellular delivery of a large number of diverse molecules, including many bacterial virulence factors. The study of OMV composition, cargo, and functions is a field of ongoing research, however the impact of these particles on bacterial pathogenesis and under various growth conditions remains poorly understood. This is particularly true of biofilm growth. During biofilm formation, OMVs have been observed as a significant portion of the matrix material, alongside other secreted molecules, but their contributions as both structural components and potential functional mediators of biofilm composition have not been widely studied.

In this dissertation, we have provided evidence that vesicles derived from a wild type strain of *Pseudomonas aeruginosa* actively mediate the overall architecture of biofilm colonies and provide a more protective environment for biofilm cell growth, thus establishing a novel role for OMVs in bacterial biofilm development. Recently, Zarnowski et al published preliminary evidence that vesicles from the pathogen *Candida albicans* may also play an important role in fungal microbe biofilm development. They demonstrated that *C. albicans* strains deficient in vesicle production produced less matrix

polysaccharides and were more susceptible to the antifungal fluconazole. Exogenous addition of vesicles complemented these phenotypes but was dependent on vesicle packaging of certain cargo proteins, establishing a role for cargo-dependent vesicle activity within the fungal biofilm matrix. Similarly, our research shows that remodeling of *P. aeruginosa* biofilms is dependent on OMV packaging of proteases, which can induce cellular detachment and promote the production of *P. aeruginosa* polysaccharide, which in turn leads to enhanced protection of biofilm cells from the antibiotic colistin. While more work will need to be done to elucidate the full impact of microbe-derived vesicles on microbial biofilms, these data strongly suggest that OMVs and their cargo have functional impacts on biofilm development.

We have additionally shown that PaAP expression influences OMV-associated proteases activity. Traditionally, bacterial aminopeptidases are thought to provide minimal contributions to overall bacterial biology, especially during pathogenesis. Because their enzymatic functions have such a small overall effect on target proteins - the removal of a single amino acid from the N-terminus - most are only known to aid in nutrient acquisition or the final stages of digesting misfolded proteins. However, more rigorous study of these molecules has revealed that they can serve additional functions with larger impacts on both bacterial biology and host-pathogen interactions, both through enzymatic activity and "moonlighting" functions carried out by non-enzymatic domains. For example, the C5a peptidase produced by *Streptococcus agalactiae* can bind

fibronectin and disrupt immune signaling via degradation of the C5a component of the complement system (Jarocki, Tacchi, and Djordjevic 2015). Studies such as these accentuate the necessity of deeper analysis of the structure and function of previously underrecognized bacterial aminopeptidases. Previous studies have provided strong foundational evidence that PaAP may have significant impacts on *P. aeruginosa* biofilm formation and pathogenesis (see **Table 1**). The data we present here builds upon this foundation, establishing a novel role for the aminopeptidase in regulating OMV cargo and biofilm composition. Further research will be necessary to determine the mechanism behind PaAP-dependent regulation of OMV-associated proteases, and whether this activity requires PaAP enzymatic function or is a result of a yet undescribed function of the aminopeptidase.

Recently, Zhao et al published data detailing a different function for PaAP during biofilm formation that may be related to traditional roles during starvation. In their experiments using Jensen's media pellicles, they found that during late stage biofilm growth, PaAP deletion mutants dispersed earlier than the wild type strains. They found that this correlated with cell lysis in the mutants, leading to the release of intracellular PslG, which is known to cause degradation of the biofilm matrix (Zhao et al. 2018). In contrast, we showed that PslG was not directly involved in the observed biofilm-remodelling phenotype. Instead, cellular detachment was caused by PaAP-dependent vesicle-associated protease activity, which can, in turn, directly mediate the composition

of the biofilm at large, as well as the levels of Psl polysaccharide and protein in the biofilm matrix. This remodeling leads to an increase in the biofilm's resistance to the antibiotic colistin, which may have significant clinical implications. While we have not elucidated the mechanism behind this regulation, the fact that the aminopeptidase is involved in such a complex regulatory network counters the traditionally held belief that these proteins are merely used by the bacteria for nutrient acquisition.

In this dissertation, we have shown that the PaAP aminopeptidase regulates biofilm composition and matrix architecture, and that it influences the content of bacterial OMVs. Through this work, we have additionally demonstrated that OMVs serve more than a simply structural role in bacterial biofilms, and that they can have significant functional implications for biofilm communities. Many questions remain about mechanism behind PaAP regulation of OMV-associated proteases and the ability of these proteases to disrupt microcolony structures to remodel biofilm composition. However, the data we have presented not only establishes the PaAP aminopeptidase as an important mediator of *P. aeruginosa* biofilms, it provides a strong foundation for future work into the biofilm-mediating properties of bacterial vesicles.

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Biography

Caitlin Noel Esoda graduated from Loudoun Valley High School in 2009 and began her studies at Duke University in August of the same year. She graduated from Duke University in 2013, earning a Bachelor of Science in Biology with a concentration in Evolutionary Biology and a minor in Evolutionary Anthropology. After graduation, she continued her education at Duke University in the Cell and Molecular Biology graduate program. In her second year of graduate work, she joined the lab of Meta Kuehn, PhD and affiliated with the Department of Molecular Genetics and Microbiology. During her time in this lab, she authored an article titled "*Pseudomonas aeruginosa* leucine aminopeptidase influences early biofilm composition and structure via vesicle-associated anti-biofilm activity," which was published in the journal *mBio*. She is expected to receive her Ph.D. in Molecular Genetics and Microbiology with a Certificate in Cell and Molecular Biology in Winter 2019.