Characterizing Stress-Induced Outer Membrane Vesicle Production in *Pseudomonas*

aeruginosa

by

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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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ABSTRACT

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Abstract

As an opportunistic Gram-negative pathogen, *Pseudomonas aeruginosa* must be able to adapt to changes and survive stressors in its environment during the course of infection. To aid survival in the hostile host environment, *P. aeruginosa* has evolved a myriad of virulence factors including the production of an exopolysaccharide capsule, as well as secretion of degradative proteases and lipases that also function as defense mechanisms. Outer membrane vesicles (OMVs) acts as a secretion system to disseminate virulence factors and function as a general bacterial stress response to remove accumulated periplasmic waste. Despite the growing insights of the field into the potential functions of OMVs, the mechanism for formation remains to be fully elucidated. The three proposed mechanisms for OMV formation in *P. aeruginosa* are mediated by the Pseudomonas quinolone signal PQS, the AlgU envelope stress response pathway, and the periplasmic chaperone MucD. This report investigates how *P. aeruginosa* responds to sublethal physiological stressors with regards to OMV production levels and whether the proposed mechanisms for OMV formation are required for stress-induced OMV formation. We concluded that exposure to cell wall directed stressors increased OMV production and activity of the sigma factor that controls MucD expression, AlgU. AlgU was shown to be sufficient to induced OMV production upon overexpression; however, stress-induced OMV production was not dependent on activation of AlgU as vesiculation could be induced in strains lacking

AlgU. Furthermore, MucD levels were not inversely proportional to OMV production under acute stress, and the ability to produce PQS was not required for OMV production. Finally, an investigation of the response of *P. aeruginosa* to oxidative stress revealed that hydrogen peroxide-induced OMV production requires the presence of B-band but not A-band lipopolysaccharide. We also demonstrated that the ability for *P. aeruginosa* to sense oxidative stress via OxyR, was important for hydrogen peroxide-induced OMV production, by a yet to be determined method. Together these results demonstrate that current proposed mechanisms for OMV formation do not universally apply under all stress conditions, and that additional mechanisms for OMV formation are still to be identified and fully elucidated during acute stress in *P. aeruginosa*.

To my beloved family and friends

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List of Abbreviations

AHL- Acyl homoserine lactone

AMP- Antimicrobial peptide

AQ- 2-alkyl-4-quinolone

BHI- Brain-heart infusion

CF- Cystic Fibrosis

CFTR- Cystic Fibrosis Transmembrane Receptor

CFU- Colony forming unit

HHQ- 2-heptyl-4-quinolone

IM- Inner membrane

LB- Luria-Bertani

LPS- Lipopolysaccharide

MIC- Minimum inhibitory concentration

MV- Membrane vesicle

OD- Optical density

OM- Outer membrane

OMPs- Outer membrane proteins

OMV- Outer membrane vesicle

PBP- Penicillin binding protein

PG- Peptidoglycan

PIA- Pseudomonas isolation agar

PQS- Pseudomonas quinolone signal

RFU- Relative fluorescence units

ROS- Reactive oxygen species

SDS-PAGE- Sodium dodecyl sulfate – polyacrylamide gel electrophoresis

TCA- Trichloroacetic acid

TLC- Thin-layer chromatography

T3SS- Type three secretion system

VAP- Ventilator-associated pneumonia

WT- Wild type

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1. Introduction

1.1 Pseudomonas aeruginosa, the opportunistic pathogen

Millions of years of evolutionary history have resulted in a vast diversity of life on Earth that has managed to coexist together in relative balance and harmony. As humans, we are often naïve to the complex intersection between our own daily lives and the microbial world. We should appreciate this hidden aspect of life because we would likely not exist today without the presence of bacteria and other microorganisms. Typically, our thoughts of the microbial world only come to mind when the balance between microbes and humans is disrupted, resulting in sickness. Fortunately, most of the interactions between mankind and bacteria do not have this result. In fact, there are more than ten times the amount of bacterial cells in the human body as there are human cells, yet we rarely develop infections (Savage, 1977). We even rely on bacteria to aid in food digestion and to produce necessary vitamins such as vitamin K. These commensal organisms are essential for our survival and help us to develop defense mechanisms against invading pathogens (Macpherson and Harris, 2004).

Despite the beneficial nature of commensal bacteria living in and on our bodies, certain opportunistic and pathogenic bacteria do find ways to colonize a host and cause infection. An opportunistic pathogen is a bacterium that commonly resides in the environment or as a commensal, but does not typically cause disease in healthy adults. The Gram-negative bacteria *Pseudomonas aeruginosa* is one such opportunistic pathogen.

Typically, *P. aeruginosa* is found ubiquitously in soil or water environments. Despite

living in the soil, *P. aeruginosa* does have the ability to become an infectious agent in a

host that suffers from a compromised immune system. Common instances of a

compromised immune system include a patient with H.I.V/A.I.D.S. (Human

Immunodeficiency Virus/ Acquired Immunodeficiency Syndrome), an organ transplant

recipient undergoing immunosuppressive therapy, and a patient suffering from the

genetic disorder Cystic Fibrosis (CF) (Cohen and Prince, 2012; Lyczak et al., 2000).

1.1.1 Clinical relevance

P. aeruginosa is one of six bacterial species that comprise the "E.S.K.A.P.E.

pathogens". E.S.K.A.P.E. pathogens include *Enterococcus faecium*, *Staphylococcus aureus*,

Klebsiella pneumoniae, *Acinetobacter baumannii*, *P. aeruginosa*, and *Enterobacter* species

(Boucher et al., 2009). These six organisms represent the largest threat that infectious

disease clinicians face due to very high incidence of antibiotic resistance. 40% of

intensive care patient infections and 65.6% of all ventilator-associate pneumonias (VAPs)

are caused by these organisms (Rice, 2010). Hospital acquired infections (HAIs) are

especially problematic as these bacteria have evolved resistance to many frontline

antibiotics. For example, greater than 20% of all VAP infections were reported to be

resistant to treatment (Sandiumenge and Rello, 2012). The combination of high antibiotic

resistance and a compromised patient population renders these E.S.K.A.P.E. pathogens

very dangerous.

P. aeruginosa is of particular concern for patients on ventilators or those suffering from CF as *P. aeruginosa* is the leading cause of morbidity and mortality in this patient group (Lyczak et al., 2000; Odeh and Quinn, 2000; Panmanee et al., 2008; Tummler and Kiewitz, 1999). Patients with CF have a mutation in the Cystic Fibrosis Transmembrane Receptor (CFTR), which is responsible for regulating chloride ion efflux into the extracellular matrix of the lung cells (Cohen and Prince, 2012). The epithelial goblet cells of the lung secrete mucin as a defense mechanism against particulate matter and infectious agents entering the lung (Burgel et al., 2007). In a healthy individual, the mucin is hydrated allowing for ciliary action to remove mucin and any contaminating material out of the lung. In a CF lung, the loss of ion efflux results in no water diffusing into the mucin. This causes the mucin layers to become dry and compacted on top of the epithelial layers (Pier, 2012). Dehydrated mucin is not able to be removed by ciliary action leading to mucin accumulation. Lack of mucin clearance allows bacteria to colonize and grow. *P. aeruginosa* is known to reside in the compacted mucin which prevents innate immune cells from reaching the bacterial invaders (Fig. 1). In the case of *P. aeruginosa*, inability to clear the infection will result in the formation of a communal biofilm commonly
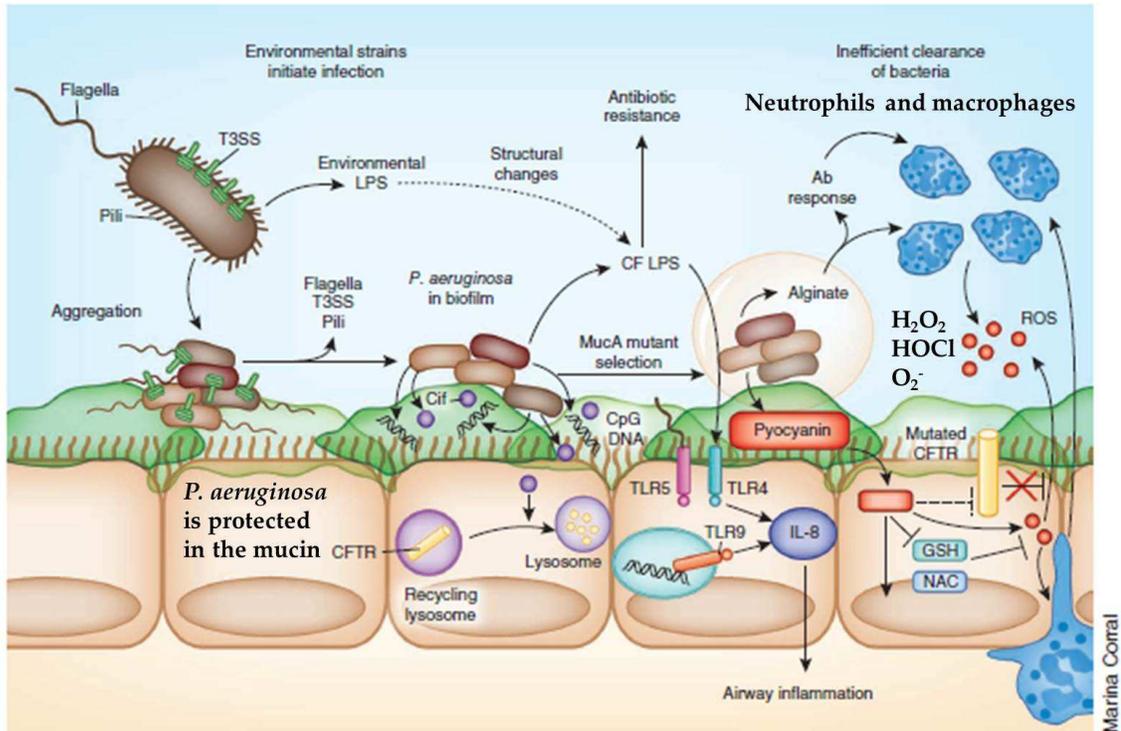
Figure 1: Adaptation of *P. aeruginosa* to the CF lung

Figure depicts the changes that occur to *P. aeruginosa* during infection of a CF lung. Bacteria expressing flagella, pilli, and T3SS enter into the lung and upon entering the mucin convert to a biofilm. Flagellin, pilli, and the Type 3 Secretion System (T3SS) are lost during this conversion, but alginate production increases. (Adapted from Cohen and Prince 2012 review in *Nature Medicine*)

associated with a chronic infection (Cohen and Prince, 2012). A chronic infection with *P.*

aeruginosa is very common for patient with CF, and chronic inflammation caused by the

continually stimulated immune system slowly damages the lung tissue until breathing is

no longer possible. A lung transplant is often the only long term treatment for these

patients (Burgel et al., 2007).

P. aeruginosa is also a common infectious agent in patients suffering from severe burn wounds. *P. aeruginosa* is classically found in warm, moist conditions which are typical of burns. Burns cause a loss of the skins protective layers allowing for very rapid infection and dissemination into the bloodstream. A recent study identified that *P. aeruginosa* was the most common Gram-negative infection in 44% of the burn units surveyed (Hodle et al., 2006). Despite the severity of this type of infection, treatment options are still available. For burns infected with non-resistant *P. aeruginosa*, aminoglycosides such as gentamicin were used in addition to aztreonam and piperacillin as front-line drugs. In the case of drug-resistant *P. aeruginosa* infections, antimicrobial peptides (AMPs) like polymyxin B and colistin have been reintroduced (after initial concerns of toxicity) in addition to the antibiotics ceftazidime and meropenem (Branski et al., 2009).

An additional problematic site for *P. aeruginosa* infection is the eye, causing corneal keratitis. Two of the virulence factors that aid *P. aeruginosa* in infecting the eye are the type three secretion system (T3SS) substrates ExoS and ExoU. ExoS is associated with an invasive phenotype while ExoU expressing bacteria typically remain extracellular but have a cytotoxic phenotype (Stapleton and Carnt, 2012). Corneal keratitis caused by *P. aeruginosa* has been linked with poor extended wear contact lens care. The ability of *P. aeruginosa* to form biofilms on plastic surfaces of contact lens cases exacerbates this problem. Removal of contact lenses is thought to disrupt the corneal

epithelial layers allowing for better invasion by *P. aeruginosa* (Klotz et al., 1990). It has

also been shown that *P. aeruginosa* attaches 2.5 fold better to corneal epithelial cells after

wearing contacts for an extended time (Fleiszig et al., 1992). These physical

perturbations of the epithelium, and the virulence factors unique to *P. aeruginosa*, allow

for rapid progression of disease and infection.

1.2 The stressful host environment

There are several obstacles and stresses that an opportunistic pathogen must

adapt and cope with in order to survive the transition from the environment to a hostile

host environment. Despite some of the most severe infections of *P. aeruginosa* being in

patients with a compromised immune system, the host still presents several distinct

stressors against an infectious agent.

1.2.1 Nonspecific stressors

The journey for *P. aeruginosa* to move from the environment to a host organism is

fraught with danger and challenging stresses from the host. The first challenge that *P.

aeruginosa* is faced with is the change in growth environment. For an organism found in

soil and water isolates, the environment of the host is very different.

Upon leaving the environment and entering a human host, there will be a large

change in temperature, nutrient availability, and osmolarity that the bacteria must adapt

to in order to survive. An increase in temperature will force the bacteria to increase its

metabolism and demands for nutrient acquisition due to increase in energy

6

consumption via protein production and DNA synthesis. *P. aeruginosa* grows well 37°C,

but an even further increase in temperature as a result of a fever could begin stressing

the bacteria to induce heat shock genes (Allen et al., 1988). As temperatures increase,

protein denaturation begins to occur and eventually results in cell death (Wood and

Ohman, 2006). Availability of food and nutrients also affects bacterial cell growth. In the

absence of amino acids, cells enter the "stringent response" which is associated with the

production of the alarmone, ppGpp (Poole, 2012). Cells also begin to favor alternative

sigma factor induced gene expression instead of maintaining housekeeping gene

expression which results in decreased growth rates (Poole, 2012). A lack of free ions is

also detrimental for bacterial membrane stability, and is known to occur in the host

tissue (Groisman, 2001). Mg^{2+} ions are used by *P. aeruginosa* to stabilize the outer

membrane lipopolysaccharide (LPS) through ionic interactions. Decreased availability of

these interactions may result in an increase susceptibility to cell wall targeted antibiotics

(Poole, 2012). Finally, changes in the osmotic environment occur when *P. aeruginosa*

moves into a host. It has been previously reported that the CF lung is a particularly

difficult osmotic environment to survive in due to limited water being present and

contested for by cells, dehydrated mucin, and electrolytes (D'Souza-Ault et al., 1993).

Imbalance in osmotic pressure will result in either cell lysis or dehydration resulting in

death.

In addition to the general environmental stressors that *P. aeruginosa* must face upon infection, the innate immune system presents its own general stress. The primary stress used by the immune system is the production of reactive oxygen species (ROSs). ROSs are highly reactive oxygen radicals that can damage cell machinery and function. ROSs are produced in an oxidative burst by neutrophils and macrophages within the phagosomal compartment by the NADPH-dependent phagocytic oxidase (Slauch, 2011). This enzyme pumps electrons into the phagosomal compartment which converts oxygen to superoxide anions. Superoxide can then dismute to hydrogen peroxide, which further can react to produce hydroxyl radicals (Slauch, 2011). These three forms of ROSs are very detrimental to cells as they can cause single strand breaks in DNA, alter amino acid structure, and cause lipid peroxidation (Cabiscol et al., 2000; Caldecott, 2008; Esterbauer et al., 1991). The ability to affect many different macromolecules is what makes ROSs so efficient at causing cell death.

1.2.2 Cell envelope-directed stressors

A common target for anti-bacterial therapeutics is the bacterial cell envelope because it possesses distinct architecture from eukaryotic cell membranes. Unlike the cell membrane of eukaryotic cells, Gram-negative bacteria have two lipid bilayers. The IM (IM) bilayer is similar to that of eukaryotes, while the outer membrane (OM) bilayer is distinct, in that it is composed of an inner leaflet of phospholipids and an outer leaflet of the endotoxin LPS (Fig. 2). The two membranes are separated by the periplasmic

O-antigens
B-band and
A-band LPS
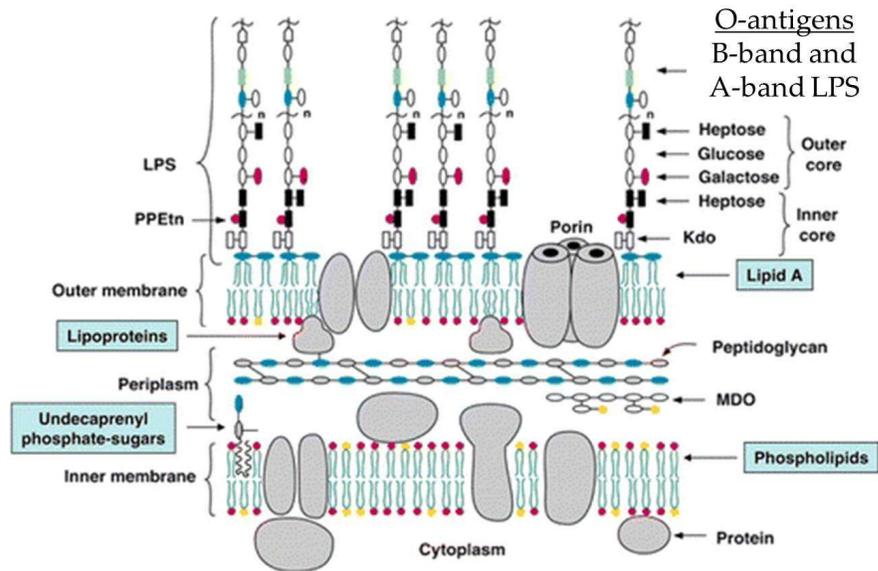
Figure 2: Schematic of the Gram-negative bacterial cell wall

Schematic depicts the Gram-negative bacterial cell wall. The IM is separated from the OM by the periplasmic space and a thin layer of PG anchored by lipoproteins. Integral OM proteins (OMPs) often function as pores (porins). The outer leaflet of the OM is composed of LPS. (Adapted from CRH Raetz *Lipopolysaccharide Endotoxins.* 2002)

space that contains thin layers of peptidoglycan (PG). PG interacts with the OM via

lipoprotein anchors to give the cell rigidity (Silhavy et al., 2010). Common antibiotics

which target the bacterial cell membrane include penicillin, and its derivative β-lactams.

Penicillin functions by interacting with the penicillin binding protein (PBP), which is

responsible for peptidoglycan assembly, by inhibiting peptidoglycan cross-linking. The

antibiotic D-cycloserine also functions as a peptidoglycan synthesis inhibitor, but by a

different mechanism. D-cycloserine competitively binds the alanine racemase and D-

alanine ligase required for peptidoglycan synthesis (Feng and Barletta, 2003). Inhibitors

of peptidoglycan synthesis disrupt the balance between peptidoglycan assembly and

degradation that occurs during normal cell growth. This imbalance results in degradation of the peptidoglycan layer leading to cell lysis caused by osmotic pressure (Silhavy et al., 2010).

AMPs are a rediscovered treatment option due to the ever increasing bacterial resistance to current and past generations of antibiotics. AMPs are small cationic molecules which directly target the bacterial OM. The charged nature of these molecules facilitate binding to negatively charged LPS on the OM and are typically thought to create pores in the membrane resulting in cell lysis (Daugelavicius et al., 2000). AMPs are not only synthetic molecules, but are also synthesized by eukaryotic and prokaryotic cells. Eukaryotic AMPs such as defensins are secreted by the epithelial cells of the lung as well as by innate immune cells. Human β-defensin-1 (HBD-1) and human β-defensin-2 (HBD-2) are known to be expressed by lung epithelial cells in response to bacterial LPS. These AMP similarly form pores in the bacterial membrane, and have been reported to have greater specificity at killing Gram-negative bacteria (Pazgier et al., 2006).

1.3 How P. aeruginosa copes with stress

Bacteria have coevolved with their hosts and have developed defense mechanisms directed towards mitigating the stressors of the host which we outlined above. A few of these defense mechanisms include envelope stress response pathways, production of antioxidants against reactive oxygen species, LPS modification systems to

prevent AMP binding, multidrug efflux pumps to remove antibiotics, quorum sensing, biofilm formation, and secreted enzymes.

1.3.1 Stress response pathways

There are two primary envelope stress response pathways in *P. aeruginosa*, the AlgU envelope stress response pathway and the AmgRS pathway. The AlgU envelope stress response pathway responds to misfolded OM proteins while the AmgRS pathway is thought to respond to accumulated aberrant proteins produced during aminoglycoside treatment. Most of our knowledge about the AlgU envelope stress response pathways comes from experiments done in the homologous RpoE envelope stress response pathway in *E. coli*. This system senses envelope stress by specifically recognizing the C-terminal domain of OM proteins (OMPs) that have become unfolded as a result of stress (Sohn et al., 2007; Walsh et al., 2003). When folded properly, the C-terminal domain is hidden within the OMPs beta barrel structure, but upon misfolding can be recognized by the enzyme DegS to begin activation of the RpoE pathway (Sohn et al., 2007). OMP binding to DegS initiates a regulated cleavage event of the anti-sigma factor RseA, which sequesters RpoE to the IM and blocks RpoE from activation. Upon RseA cleavage, RpoE interacts with RNA polymerase to upregulate genes responsible for membrane integrity such as the periplasmic chaperone/protease DegP (Dartigalongue et al., 2001). DegP recognizes misfolded OMPs and either degrades or refolds the proteins to reestablish membrane integrity.

The two component system AmgRS has recently been elucidated as a potential

envelope stress response mechanism during aminoglycoside treatment. It is thought that

this pathway functions to remove mistranslated proteins from causing membrane

damage (Poole, 2012). Several proteases and membrane transport proteins were

identified to be regulated by this two component system. Interestingly, AmgRS

regulated these membrane transporters and proteases in a way reminiscent of the

CpxRA envelope stress pathway in *E. coli* (Lee et al., 2009). Further characterization of

this pathway is required to determine if AmgRS functions as a general envelope stress

pathway like CpxRA in *E. coli*.

*P. aeruginos*a has evolved defense mechanisms against ROSs to mitigate cell

damage caused by oxidation. In *P. aeruginosa*, the primary response regulator for

oxidative damage is the transcription factor OxyR (Hassett et al., 2000). OxyR becomes

activated by oxidation and a disulfide bond is consequently formed in the protein. This

conformational change allows OxyR to interact with the RNA polymerase to upregulate

the expression of several genes with antioxidant activity. These genes include catalases

KatB, and KatE which detoxify hydrogen peroxide as well as the alkyl hydroperoxide

reductases AhpB and AhpCF (Ochsner et al., 2000; Storz et al., 1990). OxyR has also been

shown to be necessary for full virulence in a mouse model, required for exotoxin

expression, and linked to production of the phenazine pigment pyocyanin which is

known to possess redox capabilities (Lau et al., 2005; Melstrom et al., 2007; Vinckx et al., 2010).

1.3.2 Antibiotic resistance in *P. aeruginosa*

P. aeruginosa combats cell wall targeting AMPs through modifications to its LPS and lipid A structures. Canonically, lipid A modifications that confer AMP resistance are regulated by the PhoPQ and PmrAB two component systems. PhoPQ and PmrAB have both been shown to become activated upon low Mg^{2+} ion concentration and AMPs in *Salmonella* (Poole, 2012). Magnesium ions aid in stabilizing the LPS and OM, so a loss in magnesium ions prompts the cell to undergo membrane modifications. Both PhoPQ and PmrAB modify the membrane by upregulating the *arnBCADTEF* operon responsible for the addition of 4-aminoarabinose sugars to lipid A. 4-aminoarabinose is neutrally charged which decreases the binding of cationic AMPs (Gunn, 2001). Despite the conserved nature of these pathways in *P. aeruginosa*, it was not thought that PhoPQ and PmrAB would become activated under physiological conditions because the human body contains 1-2 mM divalent cations (Fernandez et al., 2010). This led to the identification of a novel two component system in *P. aeruginosa*, ParRS, that is responsible for responding to AMPs. ParRS was also shown to be responsible for activation of the *arnBCADTEF* lipid A modification operon upon subinhibitory challenge with polymyxin, colistin, and bovine peptide indolicidin (Fernandez et al., 2010).

P. aeruginosa is known for being extremely antibiotic resistant. A main

contributing factor to this resistance is the presence of multidrug efflux pumps that

function to remove harmful antibiotics from the cell. These pumps are often composed

of three interacting proteins which span the IM, PG, and OM. *P. aeruginosa* has four

characterized multidrug efflux pumps which vary slightly in function or expression.

MexAB-OprM is the primary efflux pump and is expressed at all times under laboratory

conditions. MexAB-OprM functions to remove fluoroquinolones, β-lactams, tetracycline,

macrolides, chloramphenicol, novobiocin, trimethoprim, and sulphonamides (Poole,

2001). MexCD-OprJ is not expressed at detectable levels when grown under laboratory

conditions. Expression of this system is observed upon a mutation in the *nfxB* repressor.

MexCD-OprJ is known to export macrolides, chloramphenicol, novobiocin, tetracycline,

trimethoprim, and some β-lactams (Poole, 2001). The MexEF-OprN efflux pump is also

not expressed under laboratory conditions unless there is a mutation in the *nfxC*

regulator. MexEF-OprN is known to export fluoroquinolones, chloramphenicol,

trimethoprim, and the carbapenem imipenem (Poole, 2001). Surprisingly, the operon of

the fourth multidrug efflux pump MexXY did not contain an associated OM porin. It

was later found that MexXY uses the OprM porin to remove aminoglycosides,

tetracycline, erythromycin, fluoroquinolone, and tetracycline (Poole, 2001). This efflux

pump was further reported to be regulated by oxidative stress and a primary

determinant of aminoglycoside resistance. The oxidative induction of this efflux pump is

clinically relevant because the environment of the CF lung is thought to contain many

ROSs, which would prompt the expression of this resistance pump facilitating bacterial

survival (Poole, 2012).

1.3.3 Quorum sensing in *P. aeruginosa*

Quorum sensing is a mechanism that microbes have evolved in order to better

sense changes in their environment associated with increasing bacterial cell density and

nutrient scarcity. Most quorum sensing pathways studied to date share a similar

paradigm in which a signaling molecule is secreted and increases in concentration as the

bacterial population increases, and upon reaching a threshold concentration, becomes

capable of eliciting transcriptional changes in the cell (Bassler, 2002). The transcriptional

changes can have a variety of functions, but may effect changes in colony morphology,

virulence factor expression, motility, attachment, and expression of secretion

apparatuses (Venturi, 2006).

P. aeruginosa utilizes quorum sensing to adapt to a changing environment in a

coordinated fashion, but is not limited to a single quorum sensing mechanism. Quorum

sensing in *P. aeruginosa* is mediated by three distinct, yet interconnected signaling

pathways: LasR/LasI, RhlR/RhlI, and the Pseudomonas Quinolone Signal PQS. The

LasR/LasI system is the primary quorum sensing system in *P. aeruginosa*. The specific

signaling molecule for the LasR/LasI system is the N-acylhomoserine lactone (AHL) *N*-

(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C_{12}-AHL) produced by the LasI enzyme

(Venturi, 2006). Similarly, the secondary RhlR/RhlI system is mediated by its specific

AHL N-(butanoyl)-$_L$-homoserine lactone (C$_4$-AHL) produced by the RhlI enzyme

(Schuster and Greenberg, 2006; Williams and Camara, 2009). The distinct signaling

molecules allows for specificity to be gained by each pathway, although there is also

known to be a great deal of overlap in genes regulated by these two mechanisms. The

Las system has been reported to alter gene expression of elastase, LasA protease, and

alkaline protease A while the Rhl system has been reported to alter gene expression for

rhamnolipids, elastase, LasA protease, hydrogen cyanide, pyocyanin, siderophores, and

the cytotoxic lectins PA-I and PA-II (Popat et al., 2008).

The third mechanism of quorum sensing used by *P. aeruginosa* is not mediated by

an AHL, but instead by a 2-alkyl-4-quinolone (AQ) molecule 2-heptyl-3-hydroxy-4-

quinolone termed the Pseudomonas quinolone signal, PQS. PQS is synthesized from

anthranilate and is converted to HHQ (2-heptyl-4-quinolone) by the operon of *pqsABCD*

(Diggle et al., 2006). HHQ is then thought to be secreted and internalized by other

neighboring bacterial cells which convert HHQ to PQS by the *pqsH* enzyme in an oxygen

dependent manner (Schertzer et al., 2010). PQS is then known to positively regulate RhlI

along with LasR to increase rhamnolipid production. Interestingly, PqsH is positively

regulated by LasR while PQS production is negatively regulated by RhlR. This

interconnectivity begins to demonstrate the very intricate temporal control of genes

which *P. aeruginosa* has evolved to adapt to its changing environment.

1.3.4 Biofilm formation

An explosion of recent studies has highlighted the protective benefits of community living for microbes that can organize into biofilms. Conversion to a biofilm lifestyle is typical of *P. aeruginosa* infections associated with CF, contact lenses, contaminated catheters, and ventilators. The components of the extracellular matrix that facilitate biofilm formation are not well defined, but are thought to contain a combination of OMVs, polysaccharides, proteins, lipids, and nucleic acids (Beveridge et al., 1997; Mashburn-Warren and Whiteley, 2006; Schooling and Beveridge, 2006; Whitchurch et al., 2002; Yonezawa et al., 2009). Three polysaccharides contribute to biofilm formation in *P. aeruginosa*: Psl, Pel, and alginate. In non-mucoid cells, Psl is the primary polysaccharide component of the biofilm matrix. Psl functions in cell to surface attachment and also mediates cell to cell attachment (Colvin et al., 2012). Pel appears to have a redundant role with Psl and has also been shown to function in cell to cell attachment in biofilms (Colvin et al., 2011). Interestingly, the two primary *P. aeruginosa* lab strains, PAO1 and PA14, differ in their preferred majority matrix component. PAO1 uses Psl while PA14 was reported to primarily use Pel for mature biofilm formation. Alginate, the third polysaccharide component of the matrix, is known to predominate above Psl and Pel in mucoid isolates of *P. aeruginosa* (Colvin et al., 2012).

In addition to polysaccharide components of the biofilm matrix, OMVs and extracellular DNA have also been indicated as major components of the biofilm matrix.

Although the exact contribution of OMVs to biofilm formation and maintenance remains unclear, it is thought that the charged nature of OMVs facilitates interaction with extracellular DNA, that extracellular DNA acts as a scaffold for additional extracellular polysaccharides and other compounds, and that these interactions result in a robust biofilm (Kulp and Kuehn, 2010; Schooling and Beveridge, 2006; Whitchurch et al., 2002).

Biofilms are known for their robustness against treatment and for conveying increased levels of resistance to a wide variety of antibiotics, AMPs, and phagocytosis by immune cells. A common way for bacterial communities to adapt to antibiotic stress would be to spread antibiotic resistant genes to neighboring cells. Evidence is mounting that, in addition to being a structural component of the biofilm matrix, OMVs can facilitate the horizontal transfer of antibiotic resistance between bacteria (Ciofu et al., 2000; Mashburn-Warren and Whiteley, 2006). OMVs from *M. catarrhalis* which carry β-lactamase were reported to promote survival of *Streptococcus pneumoniae* and *H. influenzae* in the presence of the antibiotic amoxicillin (Schaar et al., 2011b). Not only can OMVs transiently transfer resistance proteins to neighboring cells, but OMVs can also facilitate permanent lateral gene transfer and increase long-term drug resistance in a population. It has been demonstrated that resistance to antibiotics can spread from a resistant donor cell to a sensitive recipient cell via the fusion of OMVs and the transfer of DNA for *Acinetobacter baumannii*, *E. coli*, and *N. gonorrhoeae* (Dorward et al., 1989; Rumbo et al., 2011; Yaron et al., 2000). The spread of resistance plasmids and decreased efficacy

of the immune system against biofilms explains why *P. aeruginosa* infections tend to become chronic and difficult to eradicate.

1.3.5 *P. aeruginosa* virulence factors that combat stress

P. aeruginosa is well adapted to mitigate the threats of the host in order to grow and cause disease. A number of virulence factors have been identified which not only aid in disease progression, but in minimizing stress on the organism. Some of these virulence factors include the production of alginate, degradative enzymes, and the redox molecule pyocyanin.

1.3.5.1 Alginate

Alginate is an exopolysaccharide secreted by *P. aeruginosa*, and one of the primary external defense mechanisms for the cell. Alginate surrounds the bacterium in a protective capsule and is often a main component of the extracellular matrix in biofilms. Alginate production is regulated by the AlgU sigma factor and is known to be regulated by envelope and oxidative stress (Mathee et al., 1999). An increase in alginate production is often found in CF patients due to a mutation in the anti-sigma factor MucA. This mutation causes a constitutive production of alginate. Alginate has further been shown to protect against oxidative stressors by scavenging hypochlorite which can be produced by neutrophils allowing for greater survival of mucoid strains (Learn et al., 1987). Alginate also prevents phagocytosis by neutrophils, macrophages, and

protozoans and does not activate complement (Cabral et al., 1987; Leid et al., 2005; Oliver and Weir, 1985; Pedersen et al., 1990).

1.3.5.2 Secreted Enzymes

P. aeruginosa is also known to secrete a myriad of virulence factors to aid in infection and immune evasion. One secreted virulence factor that the cell employs to fulfill both these roles is the elastolytic zinc metalloproteinase elastase B. The role of elastase B is to damage host cells by disrupting tight junctions and by hydrolyzing the extracellular matrix. This allows for the epithelial and endothelial layers to be breached. Elastase B has also been shown to similarly hydrolyze components of the innate and adaptive immune systems including surfactant proteins A and D, cytokines and chemokines, antibacterial peptides, and immunoglobulin A and G (Kuang et al., 2011). Alkaline protease is another zinc metalloprotease secreted enzyme of *P. aeruginosa* which often functions to complement the effects of elastase. Like elastase, alkaline protease has also been shown to degrade C1q and C3 of complement as well as cytokines IFN-γ and TNF-α (Hong and Ghebrehiwet, 1992; Parmely et al., 1990). Neutrophil chemotaxis and the activity of natural killer (NK) cells were also inhibited by alkaline protease (Kharazmi et al., 1984; Pedersen and Kharazmi, 1987). Functioning in tandem, elastase and alkaline protease are a potent force again stressors generated by the innate and adaptive immune response (Laarman et al., 2012).

1.3.5.3 Pigment

One of the most distinguishing features of *P. aeruginosa* is the secretion of a

pigment that causes sputum and culture supernatants to turn green. The pigment

responsible for the green color is the redox molecule pyocyanin. Pyocyanin production

is regulated by OxyR and the Rhl quorum sensing system (Vinckx et al., 2010).

Paradoxically, pyocyanin is a pro-oxidant molecule capable of directly accepting

electrons from NADPH and reduced glutathione, then transfers the electrons to oxygen,

stimulating ROS production (Liu and Nizet, 2009). This pro-oxidant action is used

offensively as pyocyanin can easily diffuse through membranes to disrupt the host cells'

electron transport chain, vacuolar ATPases, cell growth, and Ca^{2+} ion transport

responsible for ciliary beat (Liu and Nizet, 2009). Defensively, pyocyanin was reported

to irreversibly interact with hydrogen peroxide which functioned to quench ROSs

(Reszka et al., 2004).

1.4 Gram-negative Outer membrane vesicles (OMVs)

The formation of OMVs from Gram-negative bacteria has been the subject of

ongoing study for nearly five decades. Since the 1960's, observations have been made

regarding spherical membrane blebs present in electron micrograph images of Gram-

negative bacteria (Birdsell and Cota-Robles, 1967; Knox et al., 1966). The content,

composition, and purpose of these structures were largely unknown. It was often

assumed that the observed membranous structures were cell debris fragments caused by

lysis. Since that time, the production and presence of OMVs has been ubiquitously

identified for a variety of Gram-negative species, including environmental, laboratory,

as well as clinical and pathogenic isolates (Ellis and Kuehn, 2010; Kuehn and Kesty,

2005; Unal et al., 2011).

1.4.1 Structure and composition of OMVs

OMVs produced by Gram-negative bacteria are typically 10-300 nm spherical

blebs derived from the OM and therefore are termed "OMVs." The Gram-negative

bacterial OM is distinct from the inner, cytoplasmic membrane (IM) and is separated

from the IM by the periplasmic space and a thin layer of peptidoglycan (PG). The OM is

composed of phospholipids and endotoxin (or lipopolysaccharide, LPS) as the lipid

constituent of the inner and outer leaflets, respectively, in addition to integral OM

proteins (OMPs) and lipid Anchored lipoproteins. As derivatives of the OM, OMVs

contain these OM components as well as soluble content inside and attached to their

outer surface of the vesicles. The contents of the vesicle lumen are derived from the

periplasm between the IM and OM. Additionally, proteins and DNA have been found

associated with LPS on the external surface of OMVs (Horstman and Kuehn, 2000;

Schooling and Beveridge, 2006; Schooling et al., 2009) (Fig. 3).

Proteomic analysis has revealed that the protein profiles of OMVs are distinct

from those of the cellular subfractions (Choi et al., 2011; Lee et al., 2007; Lee et al., 2008).

It was also demonstrated by McBroom *et al.*, that particular lumenal cargo are enriched

in OMVs as compared to the periplasm (McBroom and Kuehn, 2007). Also, particular

LPS subtypes are enriched in OMVs as compared to the OM and these can participate in

OMV cargo selection. The selective composition of OMVs and the lack of concurrent cell

lysis during their formation suggest that OMV production is a regulated secretion

mechanism (Bauman and Kuehn, 2006; Choi et al., 2011; Haurat et al., 2011;

Kadurugamuwa and Beveridge, 1995; Lee et al., 2007; Lee et al., 2008; McBroom et al.,

2006; McBroom and Kuehn, 2007; Nally et al., 2005).
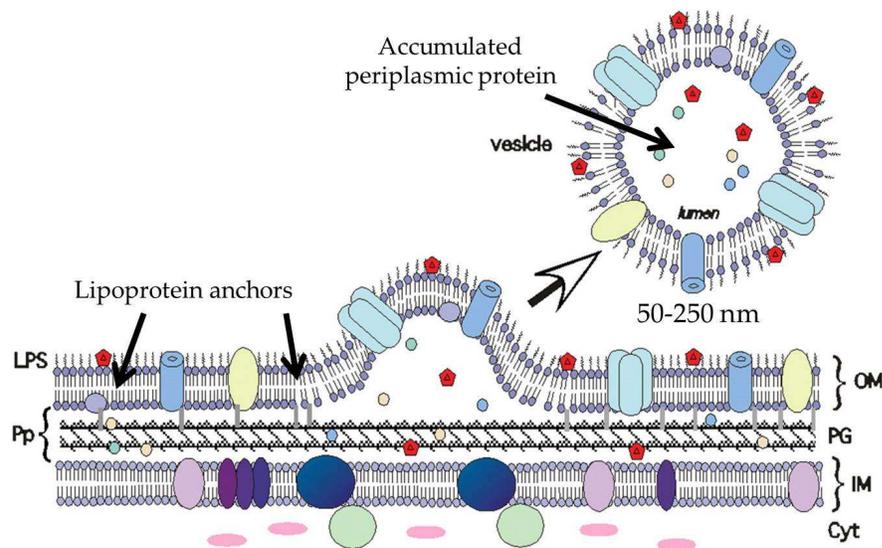
Figure 3: Overview of Gram-negative OMV production

Image depicts the budding and release of an OMV from the bacterial OM. OMVs are 10-300 nm in diameter and contain periplasmic content in their lumen. Integral OM proteins are throughout the OMV. Formation of the OMV likely coincides with local depletion of PG linkages with the OM. (Adapted from Kesty *et al.*, 2005)

1.4.2 OMVs as a stress response pathway

The function of OMVs was largely unknown until a screen for mutants with altered vesiculation phenotypes identified a number of genes associated with the σ^E envelope stress response pathway (McBroom et al., 2006). These mutants could not respond to envelope stress resulting in an accumulation of misfolded toxic proteins in the periplasmic space. With an increase in accumulated protein stress, an increase in OMV production was also observed. It was concluded that the amount of OMVs produced directly correlated with the amount of toxic proteins present in the periplasm (Tashiro et al., 2009). OMV cargo selection was determined to be a regulated mechanism to remove toxic proteins because a protein designed to mimic a misfolded OMP was preferentially sorted into OMVs (McBroom and Kuehn, 2007). This novel stress response mechanism was further shown to be independent of all other known envelope stress responses mechanisms (McBroom and Kuehn, 2007).

It was later demonstrated that OM vesiculation had a protective role in coping with external stress. Cells treated with polymyxin B or ethanol were shown to survive better if they overproduced OMVs (McBroom and Kuehn, 2007). Similarly, a hypervesiculating strain survived the expression of a toxic periplasmic protein better than wild type cells (McBroom and Kuehn, 2007). More recent studies have confirmed the role of OMVs as a stress response pathway because increases in OMV production have been observed upon oxidative stress, antibiotic treatment, and nutrient limiting

conditions (Manning and Kuehn, 2011; Nakajima et al., 1998; Sabra et al., 2003; van de Waterbeemd et al., 2013). Current studies indicate that the inability to increase OMV production in response to stress results in accumulated misfolded proteins and leads to cell death (Schwechheimer and Kuehn, manuscript in preparation).

1.4.3 Offensive functions of OMVs

Over the course of evolution, pathogenic bacteria have emerged by developing tools to colonize a host and cause infection and disease. Some of these tools serve to mitigate the stressful environment of a host organism and its defense mechanisms. OMV production is one of these tools, as OMVs can mediate bacterial attachment, biofilm formation, internalization, and virulence factor transmission, as well as modulate the immune response (Fig. 5).

The presence of OMVs during the infectious process has been observed in human patient samples and tissue biopsies. OMVs were observed in the cerebrospinal fluid of an infant diagnosed with *Neisseria meningitidis* as well as from a case of fatal septic shock caused by *N. meningitidis* serogroup B as determined by electron microscopy (Namork and Brandtzaeg, 2002; Stephens et al., 1982). Several reports of clinical isolates of *Helicobacter pylori* show OMVs in contact with host epithelial cells (Fiocca et al., 1999; Keenan et al., 2000). *Moraxella catarrhalis* was reported to also induce OMV production when in contact with leukocytes (Tan et al., 2007), suggesting that crosstalk between the pathogen and host cells can elicit regulated bacterial vesicle

production. Similarly, non-typeable *H. influenzae* (NTHI) released OMVs during

extended tissue co-culture (Ren et al., 2012).

The specific contribution to virulence of OMVs in an infected host is unknown

due to the lack of a strain or mutant that cannot produce OMVs. However, investigators

can speculate how OMVs might contribute to virulence, since the effect of purified

OMVs from pathogens can be readily observed on cultured and primary host cells.

Using *in vitro* assays, OMVs were often found to contact host epithelial or immune cells,

a finding consistent with observations of clinical and biopsy studies (Fiocca et al., 1999;

Keenan et al., 2000; Ren et al., 2012; Sharpe et al., 2011). Because receptor-binding

adhesins are associated with the bacterial OM, it is logical that OMVs would also contain

adhesins. Indeed, specific host cell interaction was observed for OMVs from several

bacterial species including *E. coli*, *H. pylori*, *Shigella*, *Actinobacillus*, and *Borrelia* (Heczko

et al., 2000; Horstman and Kuehn, 2002; Kadurugamuwa and Beveridge, 1998; Kesty et

al., 2004; Meyer and Fives-Taylor, 1994; Shoberg and Thomas, 1993). OMV adherence

has been studied in molecular detail for the heat-labile enterotoxin (LT) which is

associated with OMVs produced by enterotoxigenic *E. coli* (ETEC). LT binds the surface

of ETEC OMVs using an LPS binding pocket that is distinct from the host receptor G_{M1}

binding site, and thus LT functions as an attachment bridge between the ETEC OMV the

host cell. (Horstman et al., 2004; Horstman and Kuehn, 2002; Mudrak et al., 2009).

Similarly, *M. catarrhalis* OMVs attach directly to host cells by the adhesion and virulence

factor UspA1, since UspA1 interacts with the CEACAM-1 adhesion molecule on host cells (Schaar et al., 2011a; Slevogt et al., 2008). It was also demonstrated that the adhesins BabA, SabA, and VacA mediate *H. pylori* OMV attachment to epithelial cells (Olofsson et al., 2010).

After attachment to host cells, OMVs can sometimes fuse with the host cell membrane or become internalized by the host cell. Evidence for fusion of OMVs into the host membrane was demonstrated in a few studies and represents a means for soluble, lumenal OMV cargo to be directly deposited into the target cell. Kadurugamuwa *et al.*, demonstrated that OMVs derived from *Shigella flexneri* were able to entrap and deliver gentamicin into the eukaryotic host cytoplasm (Kadurugamuwa and Beveridge, 1998). OMV-mediated delivery of insoluble molecules into host cells has also been observed. LPS from *Salmonella enterica* serovar *typhimurium* was identified within the host cells vacuole compartments as determined by antibodies directed against the O-antigen (Garcia-del Portillo et al., 1997). Also, OMVs from *A. actinomycetemcomitans* transferred a lipid tracking dye to the host cell plasma membrane (Demuth et al., 2003). Although evidence for direct OMV fusion is growing, it is unknown how the asymmetric, LPS/phospholipid architecture of the bacterial OMV membrane is able to integrate into a symmetric phospholipid-based eukaryotic membrane without the help of some type of fusion machinery.

Examples are abundant which demonstrate the internalization of intact OMVs. LT-containing OMVs from ETEC were shown to enter epithelial cells via binding to the G_{M1} ganglioside and lipid raft-mediated endocytosis (Kesty et al., 2004). NTHI OMVs also enter epithelial cells via a caveolae-mediated process (Sharpe et al., 2011). OmpA is the common OM protein which interacts with the Ecrp receptor on brain microvascular endothelial cells and has been linked to host cell entry resulting in neonatal meningitis. Host receptor tropism has been observed to facilitate OMV uptake by particular cell types. For example, *N. meningitidis* OMV uptake was facilitated by the bactericidal/permeability-increasing protein but not by the LPS binding protein on dendritic cells (Schultz et al., 2007). Not only do OMVs become internalized inside host cells, but OMVs can facilitate bacterial invasion of host cells. Cytotoxic necrotizing factor 1 (CNF1) was recently shown to be associated with *E. coli* OMVs, and secreted CNF1 leads to host cell invasion (Yu and Kim, 2012).

Finally, it should be considered that the destination of all OMVs may not be merely the outer surface or interior of the host cell, but it may be through the cell, such as the basolateral side of the epithelial barrier. Evidence for this came from studies of *Treponema denticola* OMVs which can disrupt a cell monolayer using their OMV-associated dentilysin activity, thereby breaking through the barrier of an intact epithelium (Chi et al., 2003).

One of the benefits of OMV production to bacteria is their ability to function as a secretion mechanism for complex mixtures of soluble and insoluble factors. Due to the small size, complex composition, and protective aspect of OMVs, they are capable of facilitating the dissemination of a concentrated cohort of protease-protected virulence factors at a distance. The ability for OMVs to act distal to the parent organism is important especially for the establishment of chronic infections and biofilms.

The transport of toxins by OMVs is thought to be one of the primary purposes for OMVs, but this emphasis may be simply because the toxic activity of OMVs has been studied the most. For ETEC, 95% of the LT is associated with OMVs, and LT is trafficked into host cells by OMV internalization and subsequent trafficking of the active A subunit through the endoplasmic reticulum, resulting in toxicity (Horstman and Kuehn, 2002; Kesty et al., 2004).

In some cases, the toxin seemingly relies on the OMV compartment as a means to achieve proper folding or antigen presentation. The secreted *E. coli* toxin, ClyA, was found to require the reducing environment of OMV to oligomerize and become active (Wai et al., 2003). When oligomerized, ClyA forms observable pores in the OMVs. Interestingly, the periplasmic environment of the cell is an oxidizing environment, leaving ClyA inactive until it is secreted.

1.4.4 Defensive functions of OMVs

The secretion of OMVs as a mechanism for disseminating damaging virulence factors is a relatively obvious tool that can promote a bacterial infection, however, sometimes the best offense is a strong defense. Downregulating and diverting the host innate immune system are means by which OMVs can allow pathogens time to establish an infection in a host. It has also been considered that non-pathogenic species also produce OMVs, and so OMV production may also deflect chemical and biological environmental insults and thereby benefit microbial survival (Fig. 5).

1.4.4.1 Decoy activity

Cell wall-lysing compounds and membrane-disrupting cationic AMPs are common man-made and natural mediators of microbial cell death. Not only do microbes encounter synthetic antibiotic compounds during clinical treatment, but they are also attacked by natural antimicrobials produced by bacteria and host epithelial and innate immune cells. OMVs naturally mimic the outside of a microbe, and as such, released OMVs can function as decoys, absorbing antimicrobial compounds (Fig. 4). A recent report supports this decoy hypothesis by demonstrating that stressors directed at disrupting the cell wall were absorbed by the exogenous addition of OMVs, and OMV-hyperproducing mutants of *E. coli* survived treatment with AMPs better than wild type (Manning and Kuehn, 2011). These data demonstrate that producing large amounts of

OMVs can convey a selective advantage and may provide insight into why clinical

isolates often produce more OMVs than laboratory adapted strains.
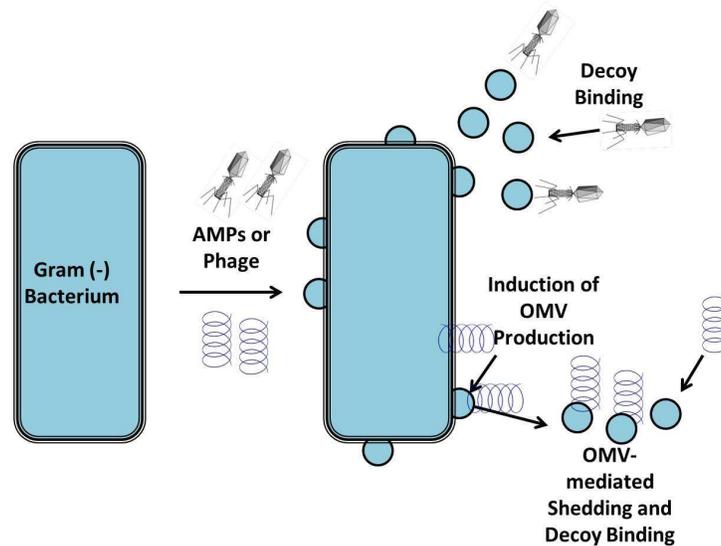
Figure 4: Decoy potential of OMVs against AMPs and bacteriophage

Image displays the defensive capability of OMVs. OMVs can function as decoys to absorb cell wall directed drugs as well as bacteriophage. Stress conditions further increase OMV production and allow OMVs to remove any stressors which do interact with the cell. Figure adapted from (MacDonald, 2012. *Research in Microbiology*)

Non-pathogenic bacterial species are also subjected to OMV-mediated protection

against a completely different class of antimicrobials, bacteriophage. Bacteria are

outnumbered by bacteriophage and thus are bound to encounter these potentially lethal

viruses during their lifespan. As mimics of the microbial surface, OMVs theoretically

should have a capacity to absorb free phage in the culture. Indeed, not only were *E coli*-

specific T4 phage found to bind to *E. coli* OMVs, but this occurred in an irreversible

manner, indicating that the phage has attached and injected its viral DNA into the OMV

(Manning and Kuehn, 2011). Attachment to an OMV, instead of a bacterial cell, removes

that virus from possible further infection and spread (Fig. 4). As with defense against

cell wall-directed AMPs, strains which produced more OMVs displayed higher

resistance when infected with bacteriophage.
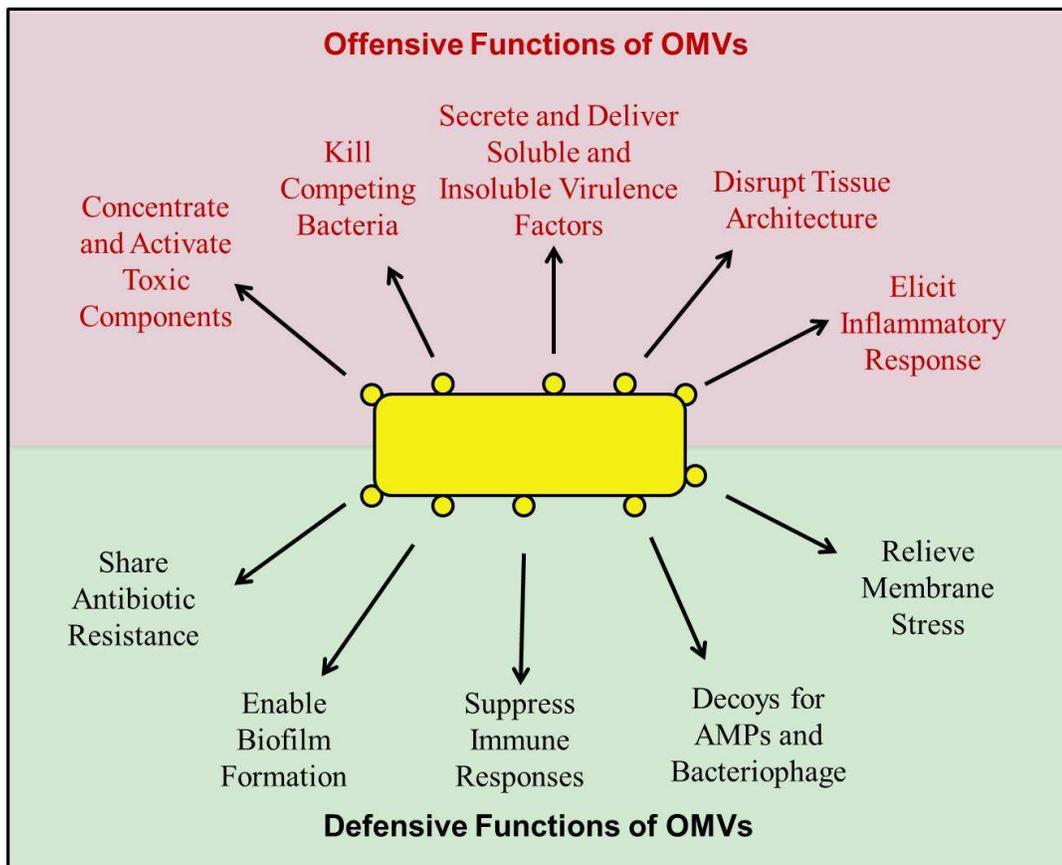
Figure 5: Offensive and defensive functions of OMVs

Offensive and defensive capabilities of OMVs are highlighted in the above figure. Figure
adapted from (Adapted from MacDonald, 2012. *Research in Microbiology*)

1.4.5 Roles of OMVs in infection and immunity

A response from host cells is often mediated through contact, and thus it was not surprising that OMVs, which have been observed to contain both immunomodulatory determinants and adhesins, generate host cell responses. One of the primary constituents of OMVs is LPS. LPS is recognized by the TLR4 ligand on host cells and leads to an inflammatory response. Porins, another abundant component of OMVs, are also important stimulators of proinflammatory cytokines, as observed in *H. pylori*, *Salmonella*, *Fusobacterium*, and *Yersinia* species (Galdiero et al., 1993; Galdiero et al., 1999; Takada et al., 1988; Tufano et al., 1994a; Tufano et al., 1994b). The host cell cytokine response to OMV challenge has been well-studied for many species including *Klebsiella pneumoniae*, *S. typhimurium*, *H. pylori*, ETEC, and *N. meningitides*, and commonly include upregulation of proinflammatory cytokines IL-8, IL-6, and IL-1β (Chutkan and Kuehn, 2011; Durand et al., 2009; Hong et al., 2009; Lapinet et al., 2000; Lee et al., 2012). It is important to note that the cytokine profiles for OMV challenges do not directly mimic that for whole cells or purified antigens due to the distinct protein profiles of OMVs as well as synergistic effects of OMV components (Chutkan and Kuehn, 2011; Ellis et al., 2010). Similar amounts of soluble and OMV-bound LT toxin stimulated different immune responses by different signaling pathways in polarized intestinal epithelial cells (Chutkan and Kuehn, 2011). Soluble and OMV context-dependent differences were also found for the adenylate cyclase toxin of Bordetella pertussis (Donato et al., 2012).

1.4.5.1 Suppression of host response

An uninhibited immune response can lead to septic shock and death of the host organism which is obviously not beneficial for the colonizing bacteria. As an apparent response to the potentially harmful but unavoidable consequence of a pathogen stimulating the immune system, certain bacterial strains have incorporated proteins into their OMVs which facilitate immune suppression. Examples of OMV production that mediate immune suppression or immune evasion can be found. The UspA1/A2 virulence factor found in *M. catarrhalis* OMVs was observed to bind and inactivate complement protein C3 in addition to its role in aiding attachment (Nordstrom et al., 2004; Nordstrom et al., 2005; Singh et al., 2010; Tan et al., 2007). Inactivation of complement causes the loss of one arm of the innate immune system. Like *M. catarrhalis*, *Neisseria* OMVs have been shown to remove bactericidal factors found in serum (Pettit and Judd, 1992). *P. gingivalis* causes CD14 degradation on O937 human macrophages by incorporating gingipain proteases in their OMVs (Duncan et al., 2004). Also, *P. gingivalis* OMVs induce the expression of ICAM-1 and E-selectin which function in inhibiting the synthesis of interferon γ MHC class II on vascular endothelial cells (Srisatjaluk et al., 1999; Srisatjaluk et al., 2002). Thus for certain pathogens, MV production may have evolved to benefit virulence by exerting immune suppressing effects.

1.5 P. aeruginosa OMVs

1.5.1 Structure and distinct composition of *P. aeruginosa* OMVs

OMVs derived from *P. aeruginosa* are very similar in structure (50-250 nm) and

composition to other Gram-negative species, though differences are present (Bauman

and Kuehn, 2006). A distinct characteristic of *P. aeruginosa* OMVs is that, of the two LPS

O-antigen subtypes *P. aeruginosa* expresses, native OMVs only contain B-band LPS

(Kadurugamuwa and Beveridge, 1995). Only under conditions of cell wall distress does

the second O-antigen, A-band LPS, become visible on OMVs (Kadurugamuwa and

Beveridge, 1997; Kadurugamuwa et al., 1993). A second distinct characteristic of *P.*

aeruginosa OMVs is that the quorum sensing molecule PQS has been demonstrated to be

tightly associated with OMVs (Tashiro et al., 2009). PQS is not only thought to associate

with the OM, but to actively participate in their formation (Mashburn-Warren et al.,

2009; Mashburn-Warren et al., 2008; Mashburn and Whiteley, 2005).

1.5.2 Virulence activity of *P. aeruginosa* OMVs

1.5.2.1 Cell-Host interactions

OMVs from a number of Gram-negative species have been reported to contain

virulence factors that mediate interactions with the host, and *P. aeruginosa* is no

exception. *P. aeruginosa* OMVs were previously reported to contain virulence

determinants such as phospholipase C, proteases, alkaline phosphatase, an

aminopeptidase, murein hydrolases, and hemolysins (Kadurugamuwa and Beveridge,

1995; Li et al., 1998). Phospholipase C and alkaline phosphatase are two degradative

enzymes that often work in tandem to scavenge inorganic phosphate. It was reported

that the host plasma is not conducive to *P. aeruginosa* growth due to a lack of inorganic

phosphate (Weinberg, 1974). As a result, phospholipase C and alkaline phosphatase are

secreted to generate inorganic phosphate from the environment. A primary target for

phospholipase C is the surfactant of the lung which is primarily composed of

phosphatidylcholine (Ostroff et al., 1989). Degradation of surfactant not only provides

nutrients for the bacterial cells to grow, but also facilitates colonization (Liu, 1974).

The OMV-associated aminopeptidase (PA2939) of *P. aeruginosa* is produced

during stationary phase growth under quorum sensing control. PA2939 has been

demonstrated to facilitate binding to A549 lung epithelial cells in tissue culture and lead

to OMV uptake (Bauman and Kuehn, 2009). However, it is unknown whether the *P.*

aeruginosa aminopeptidase mediates attachment directly, or whether the active enzyme

cleaves surface antigens on the host cell surface uncovering other ligands for binding.

With the advent of proteomics, the list of virulence factors for *P. aeruginosa*

OMVs has increased to include EstA, LasA, IcmP, OprG, and OprL (Bauman and

Kuehn, 2006; Choi et al., 2011). EstA is an esterase which was reported to induce nitric

oxide and cytokines in macrophages (Kang et al., 2009). LasA is a protease commonly

associated with invasive *P. aeruginosa* and chronic cystic fibrosis (Storey et al., 1998).

IcmP was demonstrated as a metalloprotease capable of cleaving fibrinogen (Fricke et

36

al., 1999). OprG and OprL are both OMPs which aid in toxicity and generating an immune response respectively (Gatypova et al., 2009; McPhee et al., 2009). Although the contribution of OMVs to virulence is unknown, disseminated enzymes and toxins are bound to impact the survival of *P. aeruginosa* when coping with stress.

1.5.2.2 Intermicrobial interactions

Establishing an infection in a host is not solely focused on the interaction between the microbe and the host. Interactions between competing commensal organisms and the invading pathogenic species are also important for the pathogen to survive and establish a niche in the host.

Just as OMVs can disseminate virulence factors to host cells to facilitate virulence, likewise OMVs can facilitate aggressive microbial attacks that benefit the parent microbe. The predatory nature of OMVs against other bacteria was demonstrated by a report in which OMVs derived from *P. aeruginosa* have the ability to kill other Gram-negative as well as Gram-positive bacterial species (Kadurugamuwa and Beveridge, 1996, 1999; Kadurugamuwa et al., 1998; Li et al., 1998). The mechanism of killing was shown to be by vesicle fusion or attachment leading to the delivery of murein hydrolases capable of degrading the murein sacculi of other, but not parental, species.

The predatory activity of OMVs may serve to clear out commensal flora so that the parent bacteria can colonize a particular host site, however it should also be

considered that OMV-mediated lysis of other microbes in the immediate environment of a pathogen can be a source for nutrition and novel genetic material for the pathogen, as well as an indirect source of inflammation of the infected host (Rice and Bayles, 2008). However, these roles for OMVs during host infection remain unconfirmed for the same reason that their role in virulence remains speculative: There is not yet a means to test the role of OMVs supplied *in trans* on the colonization or infection by a non-OMV producing mutant.

1.5.3 Immune response to *P. aeruginosa* OMVs

OMVs possess a number of immunostimulatory features including CpG DNA, flagella, LPS, and protein antigens on the OMV surface. It is no surprise then that OMVs derived from *P. aeruginosa* can elicit a potent immune response. *P. aeruginosa* purified OMVs were shown to elicit a strong IL-8 response when added *in vitro* to A549 and HEB lung cells. Clinical isolates of *P. aeruginosa* were shown to cause an even greater response highlighting the selective pressure to elicit a strong immune response *in vivo* (Bauman and Kuehn, 2006).

Similar proinflammatory responses were observed when RAW 264.7 macrophages were treated with *P. aeruginosa* OMVs. Flagellin is often associated with the outside of OMVs in motile Gram-negative species. OMVs containing flagellin stimulated TLR-5 to produce the proinflammatory cytokines IL-6 and MIP-2 (Ellis et al., 2010). LPS is a second major immune stimulator which binds to TLR-4 on host cells. LPS

matched OMVs were reported to elicit a greater immune response than LPS alone with increased expression of TNFα and IL-6 (Ellis et al., 2010). This indicated that despite the potent stimulatory ability of LPS through TLR-4, the synergistic effects of LPS and additional OMV components combines to create an even greater immune response.

In addition to immune stimulation through signaling proteins on the cell surface, OMVs from *P. aeruginosa* have been demonstrated to trigger immune signals upon internalization. NOD-1 is an intracellular sensor for the presence of Gram-negative PG (Magalhaes et al., 2005). *P. aeruginosa* OMVs were shown to trigger an increase in intracellular signaling by NOD-1 leading to the upregulation of NF-KB. The proinflammatory cytokines MIP-2, IL-8, IL-6, and KC were all significantly increased in response to OMVs (Kaparakis et al., 2010).

1.5.4 Mechanisms of OMV formation in *P. aeruginosa*

1.5.4.1 Conserved mechanism of OMV formation

The dynamics of OMV formation are not well understood despite the ubiquitous production of OMVs by Gram-negative bacteria; however, some mechanistic concepts have begun to emerge for *P. aeruginosa* OMV formation. Mutants lacking particular OM anchoring lipoproteins exhibit high levels of OMV budding, suggesting that OMV production requires regulated, local depletion or removal of such linkages by an as yet undefined mechanism (Kulp and Kuehn, 2010). Membrane-anchored lipoproteins covalently and non-covalently link the PG to the OM, giving the cell rigidity. Indeed,

strains lacking the genes (e.g. *tol-pal*, *lpp*, or *ompA*) or residues critical for the non-covalent and covalent OM–PG linkages exhibited increased OMV production (Bernadac et al., 1998; Deatherage et al., 2009; Moon et al., 2012; Walburger et al., 2002).

1.5.4.2 AlgU envelope stress response

      The AlgU envelope stress response pathway responds to misfolded OM proteins by increasing chaperone production. In *E. coli*, a second arm of the RpoE envelope stress response pathway was discovered, and it involved the production of OMVs. A transposon mutagenesis screen looking for mutants that had altered OMV production phenotypes identified several hits in the RpoE pathway (McBroom and Kuehn, 2007). It was further observed that stress conditions which induced RpoE activity, also led to an increase in OMV production. RpoE was also shown to be sufficient for OMV induction upon overexpression (unpublished data). These data prompted our lab concluded that induction of the RpoE envelope stress response pathway was a mechanism for OMV formation.

      The homologous sigma factor to RpoE in *P. aeruginosa* is AlgU. Differences have been noted between the two divergent species pathways. First, AlgU is not essential for growth while RpoE is essential (De Las Penas et al., 1997; Liberati et al., 2006). Second, while RpoE is thought to be almost entirely sequestered by RseA to the IM, it was reported that 33% of AlgU is cytoplasmic (Rowen and Deretic, 2000). This may increase background expression of AlgU in comparison to RpoE. Finally, AlgU is the sigma factor

responsible for alginate production in *P. aeruginosa* which functions as a primary defense

mechanism against stress. *E. coli* lacks such a defense mechanism. Despite these

differences, the AlgU envelope stress response pathway has been shown to be well

conserved in responding to membrane stress, so we hypothesize that the AlgU stress

response pathway may represent a mechanism for OMV formation in *P. aeruginosa*

similar to that observed in *E. coli*.

1.5.4.3 MucD the periplasmic chaperone

Upon induction of the RpoE envelope stress response pathway in *E. coli*, the

periplasmic chaperone/protease DegP increases expression in the periplasmic space to

mitigate the accumulation of misfolded OMPs (Alba and Gross, 2004; Walsh et al., 2003).

It was also observed in the transposon screen, that a mutant strain lacking DegP

overproduced OMVs (McBroom et al., 2006; McBroom and Kuehn, 2007). A similar

observation was made in *P. aeruginosa* indicating that a lack of the DegP homologue,

MucD, resulted in an increase of OMVs. It was also observed that overexpression of

MucD resulted in a decrease in OMV production (Tashiro et al., 2009). This result was

different from those observed in *E. coli* which indicated that an increase in DegP

expression could result in an increase in OMV production. From their observations, it

was suggested that the expression level of MucD is inversely proportional to the amount

of OMVs produced. Under high MucD expression, the periplasmic space is thought to

be clear of accumulated proteins resulting in a decrease in OMV production, while low

MucD expression correlates to an increase in accumulated protein waste. The authors

felt that MucD represents a novel regulatory mechanism for mediating OMV production

in *P. aeruginosa* (Tashiro et al., 2009).

1.5.4.4 Pseudomonas quinolone signal (PQS)

We describe above the three pathways that *P. aeruginosa* uses to facilitate quorum

sensing. In addition to its role in quorum sensing, the multi-functional Pseudomonas

quinolone signaling (PQS) molecule 2-heptyl-3-hydroxy-4-quinolone has been proposed

as a key reagent responsible for OMV formation (Mashburn and Whiteley, 2005). It was

reported that a mutant deficient in PQS formation resulting in little to no detectable

levels of OMV production as determined by absorbance at 220 nm. Exogenous addition

of PQS was able to recover the loss of OMV phenotype. This was the first suggested

instance of a bacterial strain that did not produce OMVs (Mashburn and Whiteley, 2005).

Further studies revealed that PQS was very adept at interacting with lipids including

LPS and the membrane of red blood cells. The addition of PQS to pure LPS facilitated

the formation of LPS micelles and to induce bleb formation in RBCs (Schertzer and

Whiteley, 2012). It was also demonstrated that PQS can bind and intercalate into the

bacterial OM (Mashburn-Warren et al., 2009; Mashburn-Warren et al., 2008). The

accumulation of negative charges by the addition of PQS to LPS is thought to create

charged repulsion and, thereby, a force sufficient to bend the membrane, ultimately

leading to vesicle budding (Mashburn-Warren et al., 2009). Despite PQS being

maximally expressed during late-log and stationary phase and little during log phase

growth, PQS is believed to be one of the primary mechanisms for OMV formation in *P.*

aeruginosa.

1.5.4.5 Lipopolysaccharide Structure in *P. aeruginosa*

Cell surface-linked sugars have been investigated as a mechanism for OMV

production. In the Gram-negative opportunistic pathogen, *P. aeruginosa*, two distinct

forms of LPS are present in the OM (Kadurugamuwa and Beveridge, 1995;

Kadurugamuwa et al., 1993). The O-specific antigen, or B-band LPS, is composed of

many tandem sugar repeats that vary depending on the specific strain serotype and is

highly charged. The common antigen, or A-band LPS, is composed of a limited number

of rhamnose sugar repeats and is uncharged. Beveridge and coworkers observed that a

majority, if not all, of the LPS in naturally-produced OMVs was B-band LPS

(Kadurugamuwa and Beveridge, 1995; Kadurugamuwa et al., 1993). It was proposed

that the charged nature of B-band LPS created repulsion forces in the membrane

facilitating OM blebbing. In contrast, when they analyzed OMVs isolated from *P.*

aeruginosa treated with the cell well perturbing agent, gentamicin, both A- and B-band

LPS were present. Further support for the correlation of B-band LPS and OMV

production was made by *Sabra et al.,* when they observed increased OMV production

with increased saturated oxygen levels, which increases B-band LPS levels (Sabra et al.,

2003). Additionally, not only was a particular outer polysaccharide component of LPS

found to be selected in OMVs, but the saccharide also appears to be important in OMV cargo selection for *Porphyromonas gingivalis* (Haurat et al., 2011). Finally, McMahon *et al.,* demonstrated that *Serratia marcescens* lacking polysaccharide-containing extracellular common antigen (ECA) resulted in an increase in OMV production (McMahon et al., 2012). The mechanism by which bacterial surface sugars serve to regulate OMV production and content remains unclear, but it is evident that LPS structure is an important mechanistic contributor to OMV formation.

1.6 Major questions in the field of P. aeruginosa OMVs

Over the last few decades of study we have gained insights into the structure and components of OMVs from many species of Gram-negative bacteria. We have also begun to determine how OMV associated virulence factors contribute to pathogenesis and the inflammatory response. Despite our increase in knowledge about OMVs in Gram-negative bacteria, fundamental questions still remain to be answered.

The first question that remains unanswered is by what mechanisms are OMVs being formed inside the host environment? Our mechanistic understanding of how OMVs are produced is limited to laboratory conditions in batch culture. These conditions are not relevant to the hostile environment of a host. Because the conditions that have been used to determine OMV production mechanisms do not replicate aspects of the native host environment, potential mechanisms for OMV production may remain undiscovered that are more physiologically relevant. We hope to determine how

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physiologically relevant general and cell wall directed stressor effect OMV production.

We also want to elucidate if the current proposed mechanisms for OMV formation apply

while the bacterium is under acute stress. We feel that using both general and cell wall

directed stressors recapitulates aspects of the host environment better than batch

culture, and that through this method we may gain insights into additional novel

mechanisms for OMV formation.

2. Stress-induced outer membrane vesicle production and the AlgU envelope stress response

2.1 Summary

The *E. coli* σ^E envelope stress response pathway functions to sense and relieve misfolded protein stress in the periplasmic space. A well-conserved, homologous pathway to the σ^E envelope stress response pathway exists in *P. aeruginosa* and is regulated by the alternative sigma factor AlgU. Previous work in our lab had established a link between outer membrane vesicle (OMV) formation and the σ^E envelope stress response pathway. It was thought that OMV formation represented a second, novel arm of this stress response mechanism and functioned to remove toxic, misfolded proteins from the cell by secreting them in OMVs. Although some work on the AlgU envelope stress response pathway had been conducted, those studies gave little insight into how this pathway may contribute to the production of OMVs.

In this chapter we investigated if the AlgU envelope stress response pathway contributes to OMV production in *P. aeruginosa* as was reported for the homologous σ^E pathway in *E. coli*. Further, we addressed whether the AlgU envelope stress response pathway was induced in response to general, and cell wall directed sublethal stressors. Our studies revealed that the cell wall directed stressors polymyxin B and D-cycloserine increased AlgU activity, MucD expression, and OMV production. In contrast, hydrogen peroxide stress resulted in an increase in OMV production, but showed no increase in MucD expression or AlgU activity. Further, temperature stress resulted in the opposite

effect as hydrogen peroxide stress, with AlgU activity increasing with no significant

increase in OMV production. These results demonstrated that the activity of AlgU did

not necessarily correlate with OMV production levels.

We also demonstrated that AlgU is sufficient to increase OMV formation upon

overexpression, but that it is not necessary for OMV production upon stress induction as

hydrogen peroxide stress resulted in an increase in OMV production in the absence of

AlgU. Finally, we observed that an increase in expression of the periplasmic chaperone

MucD did not decrease OMV production under non-stressed and oxidative stress

conditions. These data led us to conclude that activation of the AlgU envelope stress

response pathway can result in an increase in OMV production, but neither AlgU nor

MucD were solely responsible for mediating OMV production upon stress treatment.

2.2 Introduction

Opportunistic pathogens, like *P. aeruginosa*, encounter a variety of environmental

changes and toxic stressors during infection as the organism moves from the

environment into a host. To respond to these stressors, bacteria have evolved a number

of defense and stress response mechanisms. A major defense mechanism of *P. aeruginosa*

is the production of the exopolysaccharide capsule composed primarily of alginate.

Alginate protects *P. aeruginosa* from phagocytosis, oxidative stress, complement, and

facilitates biofilm formation allowing for persistence of chronic infections (Ramsey and

Wozniak, 2005; Schurr et al., 1996; Wood and Ohman, 2006, 2009). In addition to an

alginate capsule, *P. aeruginosa* produces several secreted virulence factors including degradative enzymes (proteases, lipases), and redox compounds (pyocyanin) (Ciofu et al., 2000; Kadurugamuwa and Beveridge, 1995, 1997; Kuehn and Kesty, 2005) which aid in countering host stressors. An additional defense mechanism that *P. aeruginosa* employs is the production of outer membrane vesicles (OMVs).

A role for OMVs as an envelope stress response mechanism was first elucidated in *E. coli*. Mutations in several σ^E envelope stress response pathway genes (*rseA*, *degP*, and *degS*) were discovered to cause constitutively high levels of OMVs (McBroom et al., 2006). The *E. coli* σ^E pathway responds to the presence of misfolded OM proteins (OMPs) located in the periplasmic space by triggering a proteolytic cascade resulting in the activation of the alternative sigma factor σ^E (encoded by *RpoE* in *E. coli*), and the consequent upregulation of the extensive σ^E regulon (Alba and Gross, 2004; Walsh et al., 2003). It was further demonstrated that a fusion protein mimicking an unfolded OMP that activated σ^E was 10-fold enriched in OMVs as compared with other periplasmic proteins (McBroom and Kuehn, 2007). Thus, OMVs were proposed to remove unfolded proteins from the periplasmic space as an independent envelope stress response. Mutants of *E. coli* that constitutively undervesiculate are hypersensitive to external stressors, demonstrating that OMVs are a critical bacterial stress response (McBroom and Kuehn, 2007).

P. aeruginosa contains a homologue of the *E. coli* σ^E envelope stress pathway, including the alternative sigma factor AlgU (also, named AlgT) and the periplasmic protease/chaperone MucD, although this pathway is not as well characterized (Ramsey and Wozniak, 2005; Schurr et al., 1996; Wood and Ohman, 2006, 2009). In addition to a potential role as a mediator of envelope stress homologous to RpoE in *E. coli*, AlgU has been well-studied for its role in activating alginate biosynthesis. Unlike *rpoE* in *E. coli*, *algU* is not essential for growth (Liberati et al., 2006). Also, RpoE in *E. coli* is thought to be almost entirely sequestered by the anti-sigma factor RseA, whereas 33% of AlgU in *P. aeruginosa* exists free in the cytoplasm despite the presence of a homologous anti-sigma factor, MucA (Rowen and Deretic, 2000). These apparent differences in the function of AlgU may provide examples for how evolutionarily divergent bacteria can use a well conserved pathway for slightly distinct functions.

Recently, Tashiro *et al.* studied the effect of an *algU* deletion on vesiculation by *P. aeruginosa* (Tashiro et al., 2009). Their results indicated that the absence of AlgU resulted in an increase in OMV production. It was hypothesized that without AlgU, the bacteria could not cope with the normal rate of OMP misfolding, resulting in envelope stress and an increase in OMV production. In line with this hypothesis, they also reported that the expression levels of the periplasmic chaperone MucD were inversely proportional to OMV production. This was due to their observations that a loss of MucD resulted in an

increase in OMV formation caused by protein accumulation in the periplasm, while increased expression of MucD resulted in a decrease in OMV formation.

The focus of this study was to determine whether *P. aeruginosa* increased OMV production in response to stress and, if so, whether the increase in OMV production was dependent on the previously identified regulatory mechanisms, AlgU and MucD. We used the peptidoglycan synthesis inhibitor D-cycloserine, and three physiologically relevant stressors (hydrogen peroxide, polymyxin B, and temperature) to assess OMV abundance, and we investigated whether AlgU or MucD played a role in these stress-regulated levels of OMVs. We concluded that the cell wall directed stressors increased AlgU activity, OMV production, and MucD expression levels while no correlation between AlgU activity, MucD expression, and OMV production was observed for the general stressors. We further demonstrated that AlgU was sufficient, but not necessary for stress induced OMV formation. Finally, we showed that overexpression of MucD can result in an increase in OMV formation in a strain dependent manner, but that MucD overexpression lacks the ability to rescue the increased OMV production phenotype induced upon hydrogen peroxide treatment.

2.3 Results

OMV production increases upon physiological stress. Based on our previous studies in *E. coli*, we hypothesized that *P. aeruginosa* would respond to envelope stress with increased OMV production. The PG synthesis inhibitor D-cycloserine functions as a

chemical mimic for D-alanine, an amino acid that is required for PG formation and

bacterial OM anchoring. In addition to perturbing cell wall assembly, D-cycloserine has

an indirect effect that results in envelope stress because it also causes AlgU activation in

P. aeruginosa PAO1 (Wood and Ohman, 2009). AlgU in *P. aeruginosa* is the homologue to

RpoE in *E. coli* (Alba and Gross, 2004; Wood and Ohman, 2006, 2009).

Figure 6: D-cycloserine induces OMV formation in *P. aeruginosa*

OMVs were collected and quantitated from cultures of PA14/pLW127 treated with 250 µg/mL D-cycloserine. OMV yields were averaged and normalized to untreated controls (Untreated) to calculate fold change. n ≥3, p ≤ 0.05 (*).

The amount of OMVs in the cell free supernatant produced after shift to the

stress condition was quantified using FM4-64. FM4-64 is a dye that fluoresces upon

incorporation into a lipid environment and was previously determined to report on the

amount of OMVs in a cell free culture supernatant (Manning and Kuehn, 2011;

McBroom et al., 2006; Pogliano et al., 1999). The other means to quantitate OMVs, by

measuring OMP content in the cell free supernatant was not feasible, as stress conditions altered the protein composition of the membrane. By comparing the quantity of OMVs in the supernatant of untreated and D-cycloserine treated late-log phase cultures of *P. aeruginosa* PA14, we determined that vesiculation increased by 9.2-fold (Fig. 6). These data demonstrate that D-cycloserine treatment significantly induced production of OMVs.

To determine if physiologically-relevant environmental stresses also stimulate OMV production by *P. aeruginosa*, we quantitated OMV levels after treatment with subinhibitory doses of three distinct stressors: hydrogen peroxide, increased temperature, and polymyxin B. Hydrogen peroxide (H_2O_2) treatment mimics the reactive oxygen burst that is produced by innate immune cells against bacteria during infections. Temperature shifts from 25°C-37°C and from 25°C-39°C are experienced by bacteria during colonization from the environment into a host and, particularly, during febrile conditions in an acute infection. The AMP polymyxin B, which targets the bacterial OM, is a clinically relevant chemotherapeutic stress encountered by *P. aeruginosa* during clinical treatment of infections (Furtado et al., 2007) and also mimics defensins produced by human tissues in response to infection (Moskowitz et al., 2004).

Treatments with 4 μg/mL polymyxin B and 250 μM H_2O_2 significantly increased OMV production by 6.3-fold and 2.7-fold, respectively (Fig. 7A,B). In contrast, neither a

12°C nor 14°C rise in culture temperature caused a significant increase in OMV

production (Fig. 7C). Bacterial viability following hydrogen peroxide and temperature

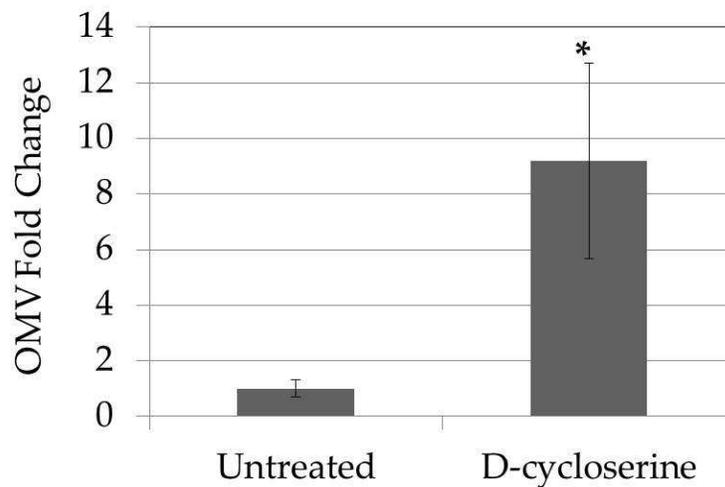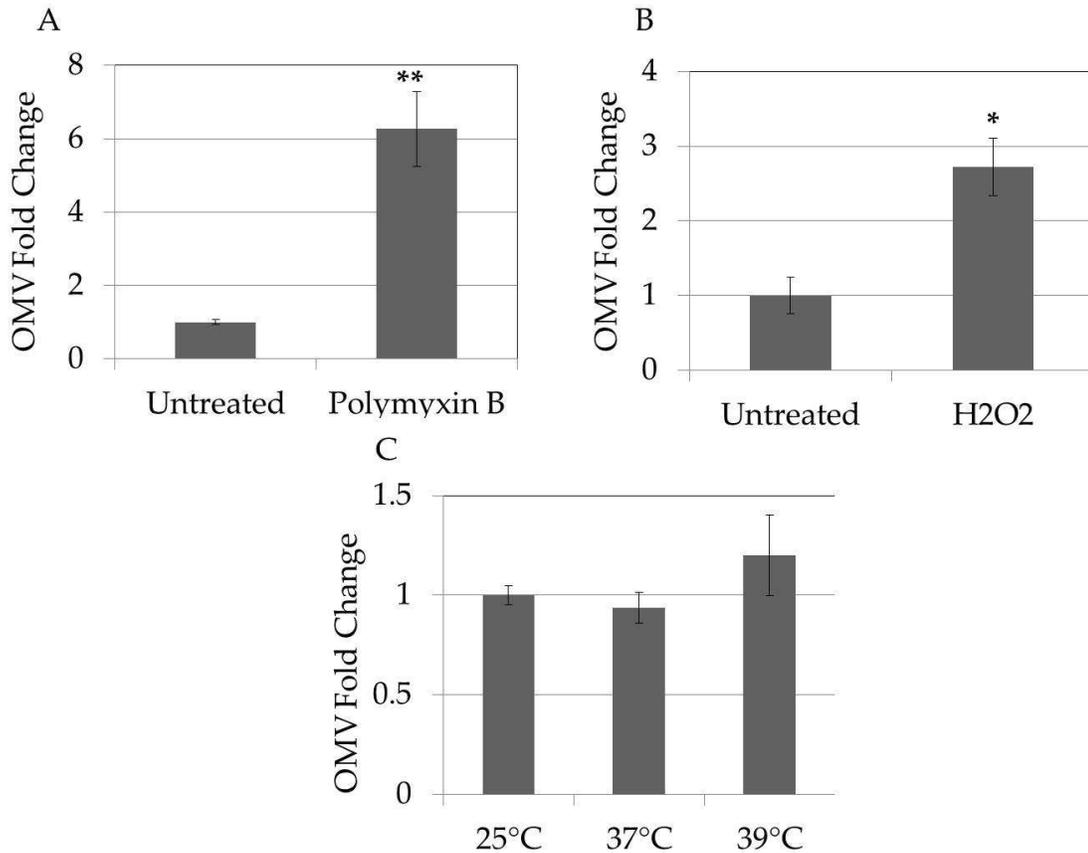**Figure 7: Physiological stressors induced OMV formation in *P. aeruginosa***

OMVs were collected and quantitated from cultures of PA14/pLW127 treated with 4 µg/mL polymyxin B (A), 250 µM hydrogen peroxide (H_2O_2) (B) or grown at 25°C and shifted to 37°C or 39°C (C). OMV yields were averaged and normalized to untreated controls (Untreated) (for parts A and B) or to a culture maintained at 25°C (for part C) to calculate fold change. n ≥3, p ≤ 0.05 (*), p ≤ 0.01 (**).

treatments was compared with untreated preparations and bacterial cell counts were not

significantly different (Table 1), suggesting cell death was not causing the increased

OMV levels. Although polymyxin B treatment resulted in 2.3 fold fewer cells compared

to untreated preparations, lysed cells did not account for the difference in relative OMV

yield since there were no significant differences in OMV profiles from untreated and

treated cultures and the presence of other cellular components would have been

noticeable (Fig. 8). From these results, we concluded that both hydrogen peroxide and

polymyxin B treatments increased OMV formation similar to the addition of D-

cycloserine, whereas an increase in temperature did not result in an increase in OMV

production over the course of the experiment.
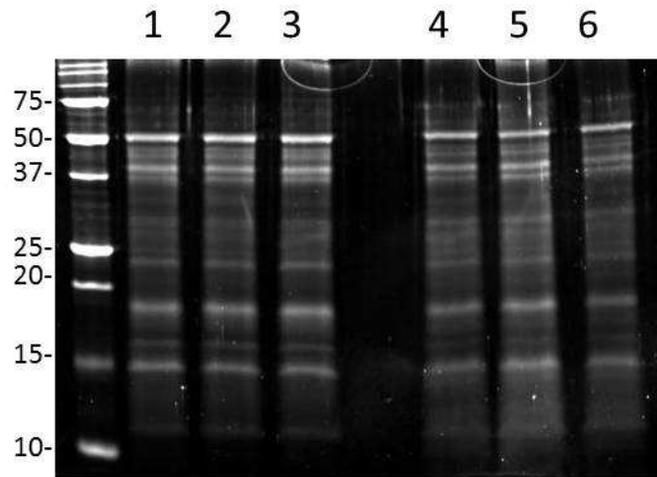
Figure 8: Polymyxin B treatment does not generate significant cell lysis and membrane fragment accumulation

PA14 was treated with or without 4 µg/ml polymyxin B and OMVs were purified. OMVs (1 µg total protein/lane) were analyzed using 15% SDS-PAGE. Lanes 1-3 are replicates of untreated OMVs from untreated PA14 cultures. Lanes 4-6 are replicates of OMVs from polymyxin B treated PA14 cultures. Molecular weight standards are indicated (kDa).

Stress-induced vesicle production is independent of AlgU activation. The alternative sigma factor σ^E (RpoE) is responsible for the activation of the σ^E envelope stress pathway in *E. coli*, and overexpression of RpoE resulted in high levels of vesiculation in *E. coli* and *Salmonella enterica* serovar Typhimurium (unpublished data). We monitored an AlgU-dependent promoter using a LacZ reporter and determined that, consistent with prior reports (Wood et al., 2006), treatment with 250 µg/mL D-cycloserine increased AlgU activity (Fig. 9A). These data demonstrate that D-cycloserine stress significantly induced production of OMVs concurrent with AlgU activation. We tested whether there was also a direct correlation between stress-induced vesiculation and activation of AlgU for the other stress treatments. Polymyxin B and the 12°C and 14°C temperature shifts all increased AlgU activity (2.4, 3.4, and 5.8-fold, respectively)(Fig. 9B,D). The temperature shift results were surprising as OMV production did not increase concurrently with the increase in AlgU expression. Also quite unexpectedly, hydrogen peroxide treatment did not result in increased AlgU activity despite causing increased vesiculation (Fig. 9C). From these data we concluded that OMV production is independent of AlgU activation for general environmental stressors.

AlgU activation is sufficient, but not necessary for OMV formation. In order to test directly whether there is a relationship between AlgU activation and OM vesiculation, we used strain PA14 *ΔalgU*/pIM001 in which the only copy of *algU* exists

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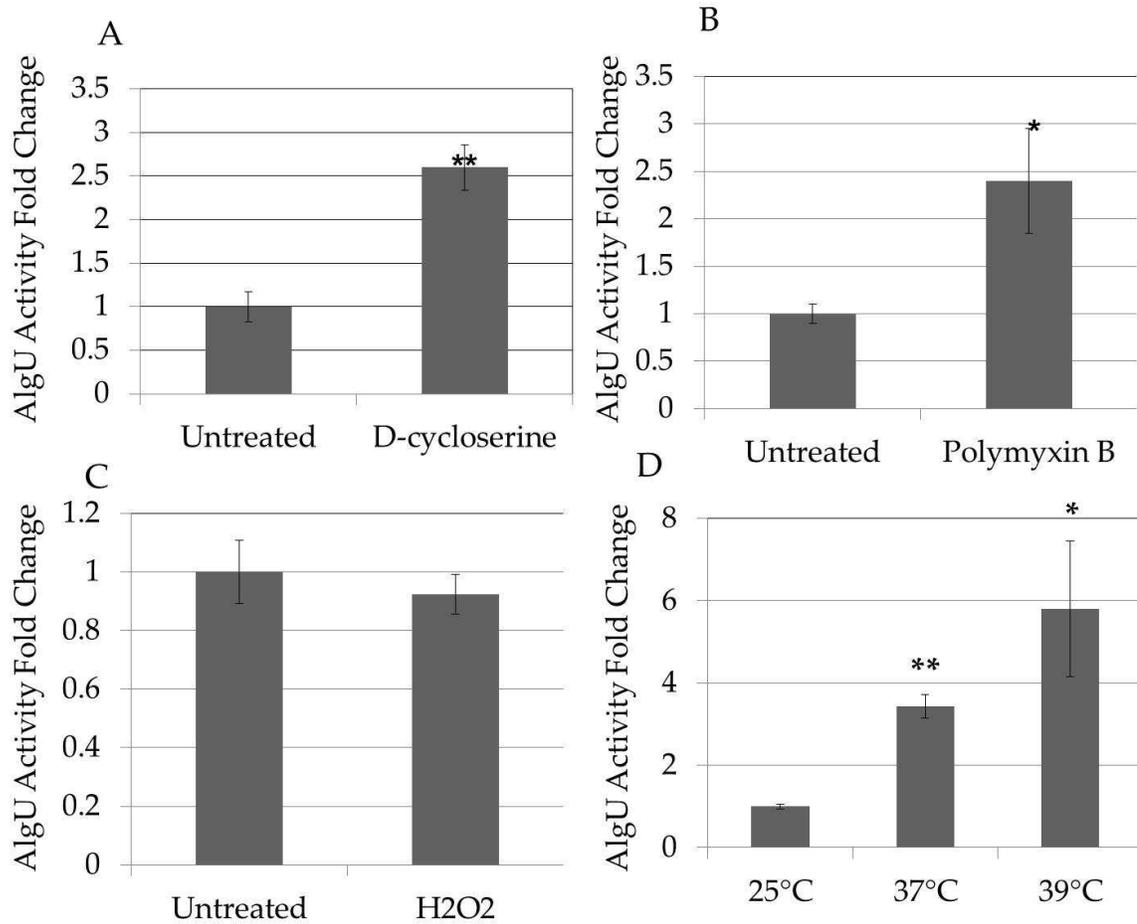
Figure 9: Stress induced AlgU activity

AlgU promoter activity was measured in cultures (37°C, OD_{600} 0.9-1.1) of PA14/pLW127 treated with 250 μg/mL D-cycloserine (A), 4 μg/mL polymyxin B (B) or 250 μM hydrogen peroxide (H_2O_2)(C) using a β-galactosidase assay. (D) AlgU promoter activity was measured in cultures (OD_{600} 0.9-1.1) of PA14/pLW127 grown at 25°C and shifted to 37°C or 39°C. Values were averaged and normalized to untreated controls (Untreated) (for parts A-C) or to a culture maintained at 25°C (25°C) (for part D) to calculate fold change. n ≥3, p ≤ 0.05 (*), p ≤ 0.01 (**).

on an inducible expression plasmid. We induced expression of AlgU during mid-log phase and quantitated OMVs once the cultures reached late-log phase. We found that AlgU overexpression did not correlate with a significant increase in OMV production, although some increase was noted (Fig. 10A). Since $\Delta algU$ mutants have a higher basal level of OMV production and overexpression of alginate could interfere with OMV purification (Tashiro et al., 2009; Yu et al., 1995), we also tested the overexpression of AlgU in PA14 $\Delta algD$. AlgD is a GDP-mannose dehydrogenase, and the first gene in the alginate biosynthesis operon (Wozniak and Ohman, 1994). Deleting AlgD prevents synthesis of alginate, while not affecting the envelope stress response function of AlgU. OMVs were quantitated following mid-log phase induction, and we found that increased levels of AlgU could significantly increase OMV production in the $\Delta algD$ background (Fig. 10B). These results indicates that like RpoE in *E. coli*, increased levels of AlgU are sufficient to stimulate OM vesiculation in *P. aeruginosa* , but that AlgU stimulation of OMV production depends on the background strain.

We next examined the role of AlgU in induced vesicle production using a PA14 $\Delta algU$ strain. Experiments to test σ^E-dependent vesiculation cannot be performed in *E. coli*, due to *rpoE* being an essential gene. The $\Delta algU$ mutation was previously reported to significantly increase the constitutive levels of vesiculation by PA14 (Tashiro et al., 2009). We tried studying the effects of polymyxin B and D-cycloserine on OMV production in PA14 $\Delta algU$, however this strain was hypersensitive to these agents and the experiment

could not be interpreted. Since PA14 *ΔalgU* was not hypersensitive to oxidative stress, we were able to determine if oxidation-induced vesiculation was AlgU dependent. We found that PA14 *ΔalgU* increased significantly with hydrogen peroxide treatment (2.5-fold)(Fig. 11), similar to the increase observed using a WT background (Fig. 7B). These results indicate that AlgU activation is not necessary for the induction of OM vesiculation in response to oxidative stress.
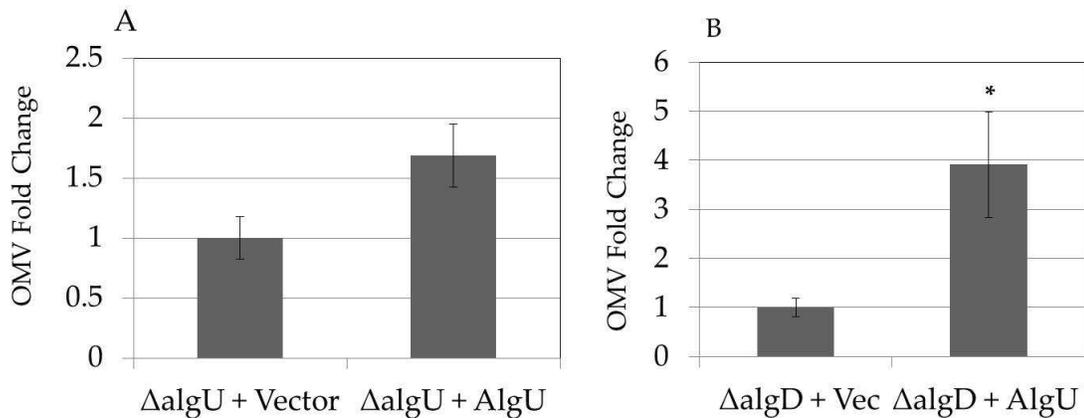
Figure 10: AlgU overexpression is sufficient to increase OMV production

(A) OMVs were collected and quantitated from cultures of PA14 *ΔalgU*/pMF54 (*ΔalgU* + vector) and PA14 *ΔalgU*/pIM001 (*ΔalgU* + AlgU) that were supplemented with 1 mM IPTG at mid-log phase. The difference was not significant, p=0.068. (B) OMVs were collected and quantitated from cultures of PA14 *ΔalgD*/pMF54 (*ΔalgD* + vector) and PA14 *ΔalgD*/pIM001 (*ΔalgD* + AlgU) that had both been supplemented with 200 μM IPTG at mid-log phase. OMV yield was normalized to that of the vector-containing culture to calculate fold change. n = 3, p< 0.05 (*).
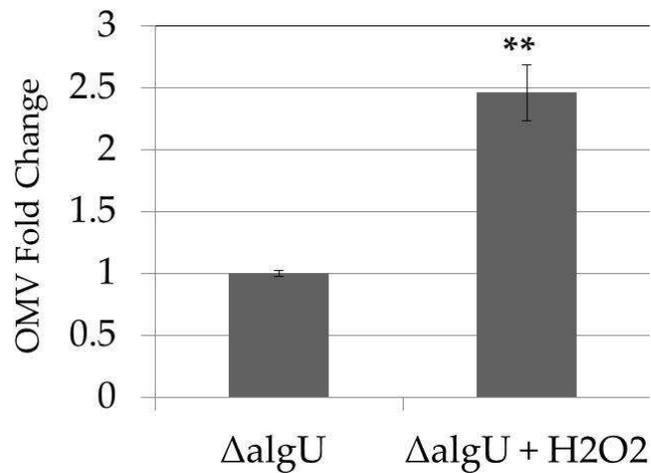
Figure 11: AlgU is not necessary hydrogen peroxide induced OMV production

(A) OMVs were collected and quantitated from cultures of PA14 *ΔalgU* (*ΔalgU*) treated with 250 μM hydrogen peroxide (*ΔalgU* + H_2O_2). OMV yield was normalized to that of untreated culture to calculate fold change. n = 3, p ≤ 0.01 (**).

Levels of periplasmic MucD are not inversely proportional to stress induced

OMV production. As an extension of the AlgU envelope stress response mechanism for

OM vesiculation, Tashiro *et al.*, concluded that OMV production is inversely

proportional to the amount of the periplasmic chaperone MucD (Tashiro et al., 2009). It

was suggested that low levels of MucD result in misfolded protein accumulation in the

periplasmic space, promoting OMV formation, whereas high levels of MucD result in

low levels of protein accumulation, decreasing OMV production. If this MucD-

dependent model holds for stress-induced vesiculation, then we would predict that the

increased OMV formation we observed upon treatment with polymyxin B, D-

cycloserine, and hydrogen peroxide should correlate with decreased levels of MucD in

the periplasm. Prior to stress treatment, we confirmed the specificity of the MucD antibody by isolating periplasmic contents and from both PA14 and PA14 *ΔmucD*. PA14 *ΔmucD* functions as the negative control for this assay. Periplasmic contents from both strains were equally loaded and run on SDS-PAGE prior to transfer onto a nitrocellulose membrane. Analysis of the periplasmic content by protein staining (Fig. 12A) and anti-MucD immunoblotting revealed multiple processed forms of MucD (Fig. 12B). It has been previously known that MucD undergoes autocleavage resulting in two ~43 kDa bands (Wood and Ohman, 2006). The band present in the PA14 *ΔmucD* negative control, we hypothesize is AlgW, a protein similar to MucD ~ 41 kDa.

To test whether stress-induced vesiculation is MucD-dependent, we measured MucD levels in the periplasm of untreated and treated *P. aeruginosa* PA14. Cultures were exposed to stressor for only 15-30 min prior to isolating periplasmic content so that we could assess immediate changes in MucD levels upon stress. Equivalent amounts of total periplasmic protein were then separated using an SDS-PAGE gel and MucD was detected by immunoblotting. As quantified by densitometry, periplasmic levels of MucD increased upon addition of D-cycloserine (Fig. 13A, B) and polymyxin B (Fig. 13C, D). Thus, these cells exhibited elevated levels of MucD and elevated levels of OMV formation upon cell wall-directed stress, a result which was not predicted from the proposed model. In addition, treatment with 250 μM hydrogen peroxide did not alter the expression of MucD in the periplasm, despite increasing OMV production (Fig. 13E,

F). Finally, we tested the consequence of MucD overexpression directly in unstressed

conditions. Upon substantial induction of MucD expression (Fig. 14A, B), no change in

OMV production was noted (Fig. 14C). We were able to significantly increase OMV
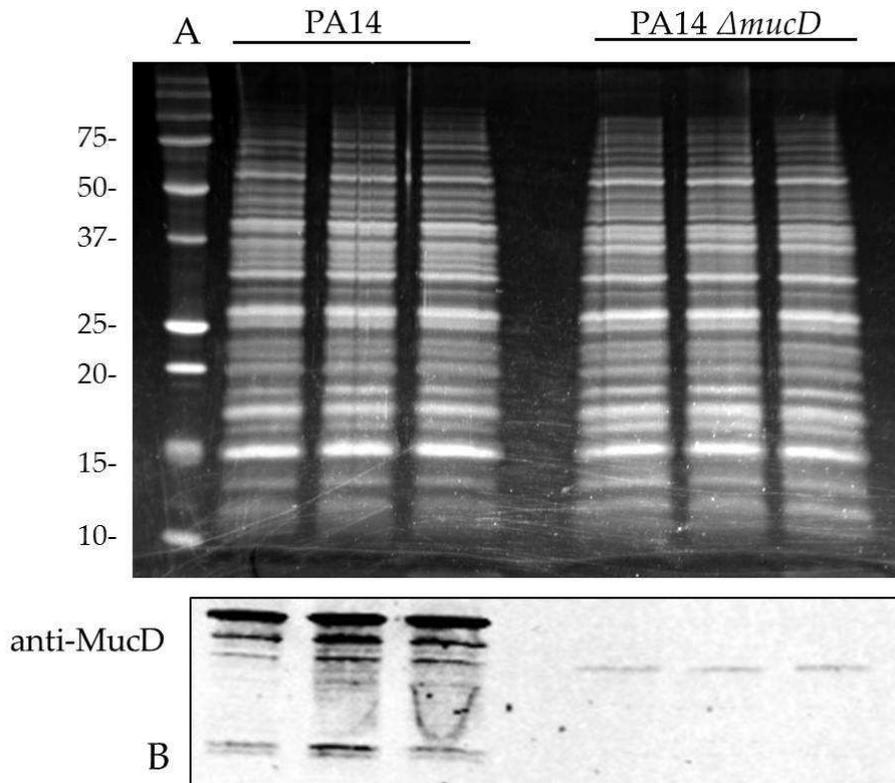
Figure 12: Total protein and anti-MucD immunoblot analysis of periplasm from PA14 and PA14 *ΔmucD*

A) PA14 and PA14 *ΔmucD* were grown to late log phase and periplasmic contents were isolated by osmotic/temperature shock. Periplasm (2μg total protein/lane as determined by Bradford protein assay) was analyzed using SDS-PAGE. B) A gel identical to that described in part A was transferred to nitrocellulose membrane and blotted with an anti-MucD antibody. Biological triplicates are shown.
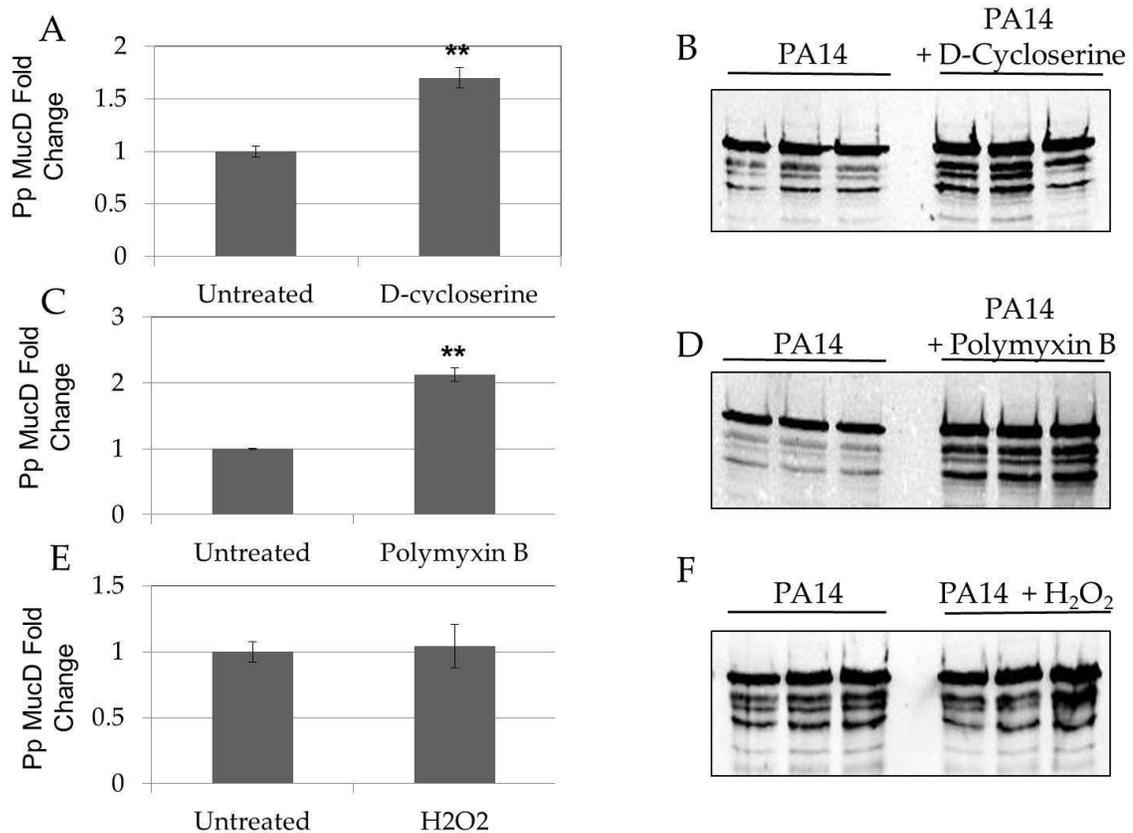
Figure 13: Periplasmic expression of MucD upon stress induction

Periplasmic MucD expression fold change in PA14/pLW127 15-30 min after treatment with or without 250 μg/ml D-cycloserine (A), 4 μg/ml polymyxin B (C), or 250 μM hydrogen peroxide (H_2O_2)(E) was determined by densitometry of anti-MucD immunoblots (B,D,F). 4 μg (B and D) and 10 μg (F) total periplasmic protein was loaded per lane. Values were averaged and normalized to samples from untreated cultures to calculate fold change. n ≥3, p ≤ 0.01 (**).

formation upon MucD overexpression in the PAO1 background (Fig. 14D)

demonstrating strain specific differences in MucD induction. These results together

demonstrate that upon stress induction, MucD levels are not a good indicator of OMV

formation. We further show that in our hands, overexpression of MucD never resulted

in a decrease in OMV production as expected by the proposed model.
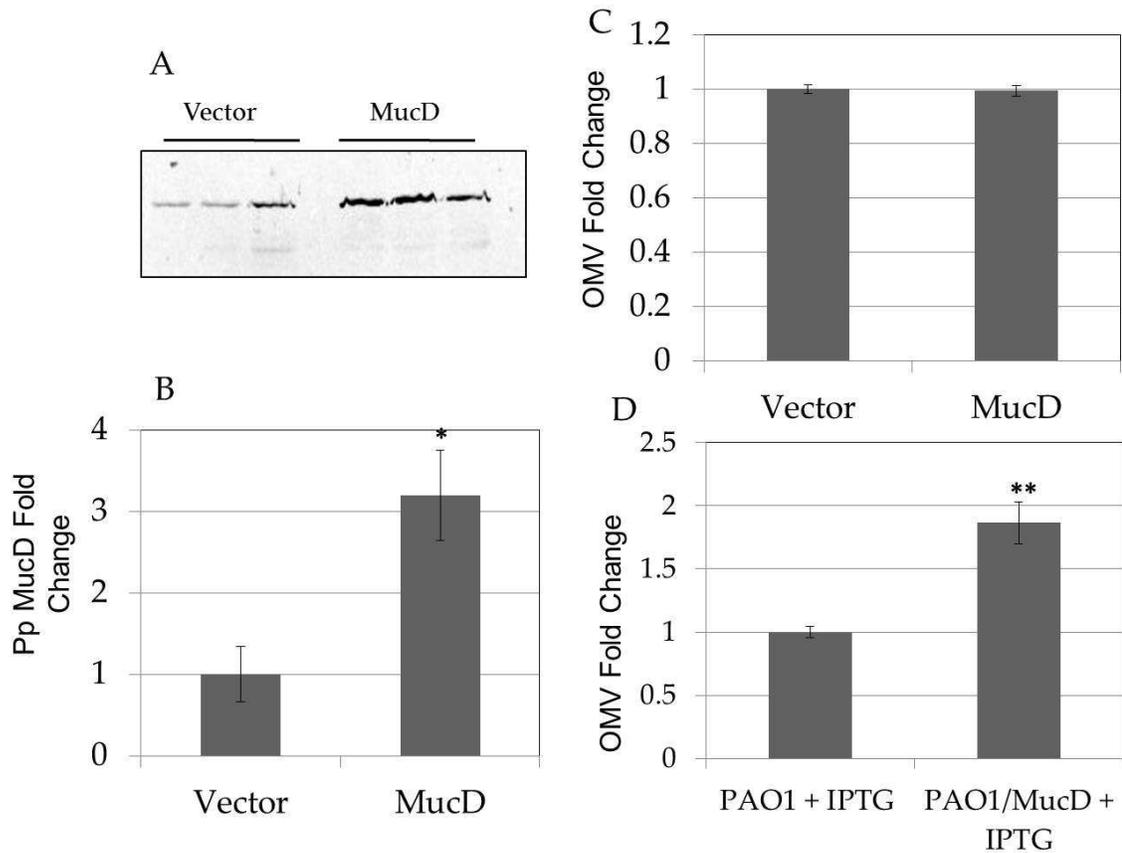
Figure 14: MucD overexpression increases OMV production in a strain dependent manner

(A) Periplasmic MucD expression in cultures of PA14 pMF54 (Vector) or PA14/pLW112 (MucD) induced with 1 mM IPTG was measured by densitometry of anti-MucD immunoblots (5 µg total protein was added per lane). Biological triplicates are shown. (B) Densitometry of the MucD bands in part A was used to determine fold change periplasmic MucD expression. (C) OMVs were collected and quantitated from cultures of PA14 pMF54 (Vector) and PA14/pLW112 (MucD) (part C), or PAO1pMF54 (PAO1 + IPTG) and PAO1/pLW112 (PAO1/MucD + IPTG) (part D) induced with 200 µM IPTG at mid-log phase. OMV yield was normalized to the respective vector control to calculate fold change. n=3, p ≤ 0.05 (*), p ≤ 0.01 (**).
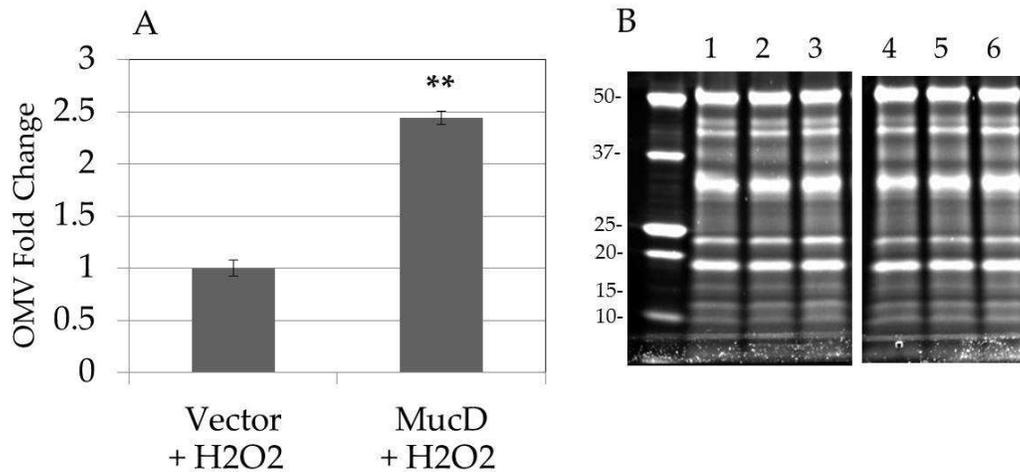
Figure 15: MucD overexpression does not decrease oxidative stress-induction of OMVs in PA14

(A) OMVs were collected and quantitated from cultures of PA14/pMF54 (Vector+H2O2) and PA14/pLW112 (MucD+H2O2), both induced with IPTG and treated with 250 μM hydrogen peroxide (H_2O_2) at mid-log phase. OMV yield was normalized to the induced vector control (Vector+H2O2) to calculate fold change. n=3, p ≤ 0.01 (**). (B) Purified OMVs were analyzed using a 4-20% gradient gel stained with Ruby stain to examine protein content. Lanes 1-3 are biological replicates of OMVs from treated cultures of PA14/pMF54 (vector+ H_2O_2). Lanes 4-6 are biological replicates of OMVs from treated cultures of PA14/pMF54 (MucD + H_2O_2). No significant differences in protein patterns were observed, suggesting no cell lysis occurred despite significantly different CFUs after the induction of MucD.

Oxidative stress-induced vesiculation cannot be rescued by MucD

overexpression. As described above, hydrogen peroxide-induced oxidative stress

increased OMV production. Since oxidative damage might cause the accumulation of

misfolded MucD substrates in the periplasm, we wondered if increasing MucD

expression could suppress the oxidative stress response. We tested this hypothesis by

examining OMV formation in cultures stressed with hydrogen peroxide and induced

Table 1: CFU values for stress-induced OMV production

Strain	Stress	Untreated (Avg. CFU $\pm$ STD)[6]	Treated (Avg. CFU $\pm$ STD)	P-value[7]
PA14	H_2O_2[1]	6.99 ± 2.16	3.71 ± 0.55	0.11
PA14	D-cycloserine[2]	0.52 ± 0.12	0.33 ± 0.33	0.24
PA14	polymyxin B [3]	6.2 ± 1.72	2.72 ± 1.49	0.006
PA14	25°C to 37°C shift[4]	10.56 ± 2.07 (25°C)	7.97 ± 1.91 (37°C)	0.08
PA14	25°C to 39°C shift[5]	10.56 ± 2.07 (25°C)	6.18 ± 4.02 (39°C)	0.07
PA14 $\Delta algU$	H_2O_2[5]	4.82 ± 0.65	3.73 ± 1.01	0.20
PA14/pLW112 + IPTG	H_2O_2	4.85 ± 0.37	2.4 ± 0.25	0.001

for MucD expression. We found that induction of MucD expression in cultures treated

with hydrogen peroxide resulted in a significant increase in OMVs beyond that induced

by peroxide alone (Fig. 15A). No significant differences were detected in the protein

profiles for the vector and MucD overexpression OMV preparations (Fig. 15B). The

similar OMV profiles showed that cell death and membrane leakage were unlikely to

[1]treatment with 250 μM H_2O_2 for 3h

[2]treatment with 250 μg/ml D-cycloserine for 3h

[3] treatment with 4 μg/ml polymyxin B for 3 h

[4]cultures were grown at 25°C for 8h then shifted to 37°C for 3h

[5]cultures were grown at 25°C for 8h then shifted to 37°C for 3h

[6]CFU were determined by dilution of the cultures, agar-plating, and colony counting after overnight growth.

[7]treated and untreated culture CFUs were compared.[1]

contribute to the increase in OMV quantitation for the difference in CFUs observed between the two treatments (Table 1). We conclude that overexpression of MucD cannot alleviate the stress caused by oxidative damage and that MucD overexpression is contributing to the increase in OMV production under hydrogen peroxide stress.

2.4 Discussion

Gram-negative bacteria must successfully react to environmental stressors in order to survive. For pathogenic bacteria, the hostile host environment is a critical challenge to overcome during the establishment of infection. We propose here that OMVs are induced by *P. aeruginosa* in response to physiological stressors, such as those encountered during infection, and that the mechanism of stress-induced OMV production is independent of AlgU and MucD.

We first examined the relationship between environmental stressors, AlgU activity, and induction of OMV production. The σ^E envelope stress response pathway in *E. coli* is activated upon sensing misfolded OMPs in the periplasmic space (Alba and Gross, 2004). Based on homology and conservation of this stress response pathway in *P. aeruginosa* (Alba and Gross, 2004; Wood and Ohman, 2006, 2009), it can be inferred that the AlgU-mediated response functions in a similar manner. These homologous pathways provide a means for the bacteria to manage accumulated misfolded proteins in the periplasm as a result of environmental stress.

Treatment with cell wall directed compounds, polymyxin B and D-cycloserine, resulted in an increase in OMV production with a concomitant (and previously reported for D-cycloserine (Wood and Ohman, 2009)) increase in AlgU activity. Similar responses to these treatments were expected because both compounds physically perturb the OM. Although the hypersensitivity of *ΔalgU* to these stressors prevented us from determining if AlgU was critical to the induction of OMVs by polymyxin B or D-cycloserine, it was noteworthy that the *ΔalgU* strain was hypersensitive. AlgU expression typically results in increased alginate production which provides the bacterium protection against phagocytosis, oxidative stress, and complement (Martin et al., 1993). Thus, the *ΔalgU* hypersensitive phenotype suggests that the activation and function of AlgU plays an additional, more general, protective role against agents that physically disrupt the cell wall. Additional stability may be mediated by the AlgU-controlled periplasmic MucD chaperone. In response to envelope stress, increased expression of MucD could help maintain the functionality of the periplasm as well as the membrane architecture.

Temperature change was examined as a general stressor that *P. aeruginosa* typically encounters during the course of infection. Shifting *P. aeruginosa* from growth at 25°C to 37°C or 39°C mimics the change in environment that the pathogen would encounter upon moving from the external environment into a human host. Increasing the temperature did not result in an increase in OMV production, but did increase the

amount of AlgU activity. The increase in AlgU is consistent with previous reports that AlgU is regulated by temperature and leads to the regulation of heat shock genes such as *rpoH* (Schurr and Deretic, 1997; Schurr et al., 1995). The fact that OMV production did not change, despite the change in AlgU, was surprising. However, this result may be explained if a threshold level of AlgU activation is needed to induce OMV production and this level was not achieved in the experiments. Indeed, heat shock genes are commonly expressed at temperatures higher than our experimental design. Nevertheless, we demonstrate that there is not a linear correlation between AlgU activation and OMV production in *P. aeruginosa*.

Oxidative stress was tested as another general stressor that *P. aeruginosa* would encounter during the course of infection, and we determined that oxidative stress resulted in a significant increase in OMV production. ROSs such as hydrogen peroxide are produced by innate immune cells and released upon contacting an infectious agent. Reactive oxygen is a very potent defense mechanism because it targets all aspects of the cellular machinery and bacteria cannot evolve a single mechanism to evade ROSs. Because overexpression of MucD could not mitigate peroxide-induced production of OMVs, the data suggest that oxidative stress is unlikely to result in the accumulation of misfolded periplasmic protein. Consistent with this, unlike either the responses to cell wall or temperature stressors, oxidative stress did not result in increased AlgU activity. PA14 *ΔalgU* cells were not hypersensitive to oxidative stress, therefore we were able to

address directly whether induction of OMV production depended on AlgU. Notably, these results demonstrated that AlgU was not necessary to produce constitutive or stress-induced levels of OMVs in *P. aeruginosa*.

Despite AlgU not being required for OMV production, overexpression of AlgU did prove to be sufficient to increase OMV production. This result was similar to our previous studies of RpoE in *E. coli*. The increase in OMV production in *P. aeruginosa* and *E. coli* due to overexpression of AlgU or RpoE, respectively, is possibly indirect, due to the downstream effects of changes in periplasmic chaperone and envelope component expression that cause crowding and or misfolding of envelope proteins.

Since hydrogen peroxide stress elicited AlgU-independent OMV induction, we were prompted to test another proposed regulatory mechanism for OMV formation in *P. aeruginosa*. Recently it was proposed that OMV production in *P. aeruginosa* inversely correlates with the level of the periplasmic chaperone, MucD. However, upon addition of hydrogen peroxide treatment, MucD levels remained unchanged despite an increase in OMV production. If MucD levels and OMV production were inversely proportional, MucD levels would have been decreased in treated cells. In addition, we observed that cell wall-directed stressors which caused elevated OMV and AlgU production also resulted in increase in MucD, again, contrary to the proposed model. Differences in experimental setup, sampling, and growth duration may account for some of the reported differences. Notably, it was previously reported that MucD overexpression in

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the *P. aeruginosa* strain PAO1 resulted in decreased OMV production; however, we observed a significant increase in OMV formation. We hypothesize, that like AlgU induced OMV formation, at high expression levels, MucD itself may become toxic to the cell resulting in a necessary increase in OMV formation to mitigate the accumulated protein in the periplasmic space. A decrease in OMV formation upon MucD overexpression may be possible with an induction that is very minimal, allowing periplasmic stress to be removed, without MucD itself becoming a burden to the cell. Our vector used for overexpression, has leaky expression even without inducing conditions, which may account for our inability to replicate the previous reports.

2.5 Acknowledgements

3. PQS is not required for constitutive or stress-induced OMV production

3.1 Summary

Quorum sensing is an important adaptation for several species of bacteria to sense the density of bacterial communities. Quorum sensing molecules allow the bacteria to react to its changing environment and enact transcriptional regulation of virulence factors, nutrient usage, and community morphology such as the conversion to biofilms. *P. aeruginosa* has three interconnected quorum sensing pathways, two of which are mediated by distinct *N*-acylhomoserine lactones (AHL). These two pathways are regulated by LasR/LasI and RhlR/RhlI. The third quorum sensing mechanism that *P. aeruginosa* uses to facilitate interbacterial signaling is via 2-heptyl-3-hydroxyl-4-quinolone, also termed the Pseudomonas Quinolone Signaling molecule, PQS. Unlike the AHL based quorum sensing system, PQS has been reported as a key requirement for OMV formation in *P. aeruginosa*

In this study, we wanted to determine if PQS was required for acute stress-induced OMV formation. We found that OMV production increased upon treatment of a PQS deficient strain (PA14 *ΔpqsA*) with subinhibitory levels of D-cycloserine, polymyxin B, and hydrogen peroxide. We further demonstrate that, not only is PQS not required for stress-induced OMV production, PQS is also not required for constitutive OMV formation during log-phase and stationary phase growth, despite previous reports. We

conclude that production of stress-induced OMVs is independent of PQS, although PQS levels can somewhat modulate OMV production depending on growth phase.

3.2 Introduction

Cell-cell communication was an important evolutionary adaptation that allowed bacteria to interact as a community, instead of as a single isolated bacterium. The most common form of bacterial cell-cell communication is mediated by quorum sensing. Quorum sensing allows for bacteria to sense the density of their population through diffusion, and accumulation of small signaling molecules (Bassler, 2002). Upon reaching a critical threshold of signaling molecules, specific transcriptional changes occur which allow the bacteria to modulate genes responsible for virulence, motility, community morphology, and nutrient acquisition (Venturi, 2006). These global transcription changes also provide the cells a means of coping with a stressful environment.

P. aeruginosa has evolved three distinct, yet interconnected pathways for cell-cell communication. The three quorum sensing systems in *P. aeruginosa* consist of the Las, Rhl, and Pseudomonas quinolone signal, PQS. Both the Las and Rhl systems use *N*-acylhomoserine lactones (AHL) as their signaling molecules, though each pathway has a specific AHL. LasR/LasI represents the primary quorum sensing pathway in *P. aeruginosa* as it is first to be turned on during cell growth. LasI synthesizes the AHL *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C_{12}-AHL) which is specific for activation of Las dependent genes. Upon activation, LasR will positively regulate RhlI to

begin the second level of quorum sensing control mediated by the Rhl system. Similar to

Las, the secondary RhlR/RhlI system is mediated by its specific AHL N-(butanoyl)-L-

homoserine lactone (C_4-AHL) produced by the RhlI enzyme (Schuster and Greenberg,

2006; Venturi, 2006; Williams and Camara, 2009). Together, with the addition of other

host genes, these two quorum sensing systems will turn on gene expression of elastase,

LasA protease, alkaline protease A, rhamnolipids, hydrogen cyanide, pyocyanin,

siderophores, and the cytotoxic lectins PA-I and PA-II (Popat et al., 2008).

PQS is the third arm of quorum sensing control in *P. aeruginosa*. Unlike Las and

Rhl, PQS is not mediated by an AHL, yet the three quorum sensing systems are

intertwined. The signaling molecule PQS is in the family of 2-alkyl-4-quinolones. PQS (2-

heptyl-3-hydroxy-4-quinolone) is synthesized from anthranilate by the *pqsABCD* operon

to produce the molecule HHQ (2-heptyl-4-quinolone) (Diggle et al., 2006). HHQ is then

secreted and internalized as a signaling molecule by neighboring cells, and converted to

PQS by the enzyme *pqsH* in a LasR dependent manner (Diggle et al., 2006). PQS is then

known to enhance synthesis of the Rhl regulon, especially the production of

rhamnolipids. Enhancing rhamnolipid production was found to be essential for PQS

because PQS is not very soluble in aqueous solution. It is thought that production of the

biosurfactant rhamnolipid, aids in solubilizing PQS so that it can diffuse and act as a

quorum sensing molecule (Calfee et al., 2005). In addition to rhamnolipids increasing the

activity of PQS in solution, PQS is also thought to use OMVs as a means for secretion

into the environment. In support of this hypothesis is that PQS has been reported to be tightly associated with OMVs (Mashburn-Warren et al., 2009; Mashburn and Whiteley, 2005; Tashiro et al., 2009).

In addition to its role in quorum sensing, PQS was reported to be required for OMV formation (Mashburn and Whiteley, 2005). Strains lacking PQS (PA14 *ΔpqsA*, PA14 *ΔpqsH*) were found to produce few to undetectable levels of OMVs after overnight growth as determined by the absorbance at 220 nm of 0.22 μm-filtered cell culture supernatant. The lack of PQS production by this strain was shown to be responsible for the decrease in OMV production as exogenous addition of PQS partially rescued the loss in OMV production (Mashburn and Whiteley, 2005). PQS was also demonstrated to be sufficient to induce LPS to form micelles (Mashburn-Warren et al., 2008). Later, PQS was further demonstrated to interact with both bacterial and eukaryotic cell membranes. The interaction with the bacterial cell membrane was shown to be specific for PQS, and not for other related compounds with shorter acyl chains (Mashburn-Warren et al., 2009). It was hypothesized that the charged nature of LPS interacts with PQS as it intercalates into the membrane, resulting in membrane curvature induced by charge repulsion. Similar PQS-mediated blebbing was observed to occur for erythrocyte membranes (Schertzer and Whiteley, 2012).

As one of the few proposed mechanisms for OMV formation in *P. aeruginosa*, we wanted to determine if PQS production was required for *P. aeruginosa* to respond to

acute stress via OMV production. Our data demonstrates that PQS is not only not required for OMV production in response to acute stress for all three of the treatments studied in Chapter 2, but despite previous reports, a PQS deficient strain does produce OMVs. Interestingly, we found that the PQS deficient strain produces OMVs at a higher level during log-phase growth compared to the wildtype control, whereas overnight cultures of the PQS mutant produce fewer OMVs compared to wildtype. In conclusion, mechanisms besides PQS must exist for the production and suggests a growth phase-dependent regulation of OMVs in *P. aeruginosa*.

3.3 Results

Stress-induced OMV production is independent of PQS. The quorum sensing molecule PQS has been demonstrated to increase OM blebbing of *P. aeruginosa* by interacting with LPS and was proposed to be "required and sufficient" for OMV formation in *P. aeruginosa* (Mashburn-Warren et al., 2009; Mashburn-Warren et al., 2008). To examine the mechanism of stress-induced OMV production, we wanted to determine whether OMV induction upon stress treatment was PQS-dependent. PA14 *ΔpqsA* was chosen for these analyses because it is unable to synthesize the precursor molecules in the biosynthesis pathway of PQS (Mashburn and Whiteley, 2005). OMVs were quantitated from cultures of PA14 *ΔpqsA* treated with subinhibitory concentrations of the peptidoglycan (PG) synthesis inhibitor D-cycloserine, the cationic peptide polymyxin B, and oxidative stress in the form of hydrogen peroxide. Because strains

A. Log Phase OMV Prep

Cells were grown in 250 ml LB broth to 0.9-1.1 OD_{600} → Cells are pelleted → Supernatants are filtered through a 0.45 µm filter → Supernatant was precipitated in 75% $(NH_4)_2SO_4$ → Precipitated supernatants were spun down

Ppt. sup. was dialyzed overnight and concentrated

0.45 µm filtered OMVs quantitated by FM4-64 ← OMP fractions were pooled and spun down ← SDS-PAGE and staining of fractions to determine which fractions contain OMPs ← Opti gradients were fractionated in 1 ml fractions ← Concentrated sups. were fractionated on a density gradient overnight

B. Stationary Phase OMV Prep

Cells were grown in 25 ml BHI broth overnight → Cells are pelleted → Supernatants are filtered through a 0.45 µm filter → 220,000 xg spin to isolate OMVs → 0.45 µm filtered OMVs quantitated by FM4-64, OMPs, or A_{220}

Normalize data to CFU

C. Mashburn- Stationary Phase OMV Prep

Cells were grown in 25 ml BHI broth overnight → Cells are pelleted → Supernatants are filtered through a 0.22 µm filter → 265,000 xg spin to isolate OMVs → OMVs quantitated by A_{220}

Normalize data to CFU

Figure 16: Methods for OMV isolation and determination

Log phase (A), stationary phase (B), and Mashburn-stationary phase (C) OMV prep methodologies for isolating OMVs from *P. aeruginosa*.

lacking PQS reportedly made very few OMVs, the cell-free supernatant from PA14 and

the PQS mutant cultures at late log-phase growth were concentrated using ammonium

sulfate precipitation to enrich for OMVs (Fig. 16A). Precipitated supernatants were

dialyzed and concentrated prior to being floated on an Opti-prep gradient. OMV-

containing fractions were pooled and isolated by centrifugation. OMV production was

quantitated using the fluorescent probe FM4-64 as well as by measuring OM proteins

(OMPs) by densitometry of SDS-PAGE-separated samples of OMV preparations.

The addition of D-cycloserine, polymyxin B, and hydrogen peroxide treatments

to PA14 $\Delta pqsA$ increased OMV production by 5.2, 6.4, and 2.2 fold, respectively (Fig.

17A-C), similar to the increases seen using the WT strain (Fig. 6, 7A,B). Therefore, stress-

induced OMV production by *P. aeruginosa* during log phase growth does not require

either the production or presence of PQS in the culture.

Constitutive OMV production independent of PQS. Because we demonstrated

PQS-independent OMV production upon acute stress treatment and because our OMV

quantitation was different from those used in the earlier report, we wondered if PQS

was, in fact, required for constitutive production of OMVs. To address this question, we

isolated OMVs from late-log phase cultures by floating concentrated cell-free culture

supernatants on a density gradient in the absence of stress. The presence of OMVs

isolated from PA14, PA14 $\Delta pqsA$, and PA14 $\Delta pqsH$ (PqsH is the final enzyme converting

HHQ to PQS) were confirmed by negative stain electron microscopy (Fig. 18 A-C).
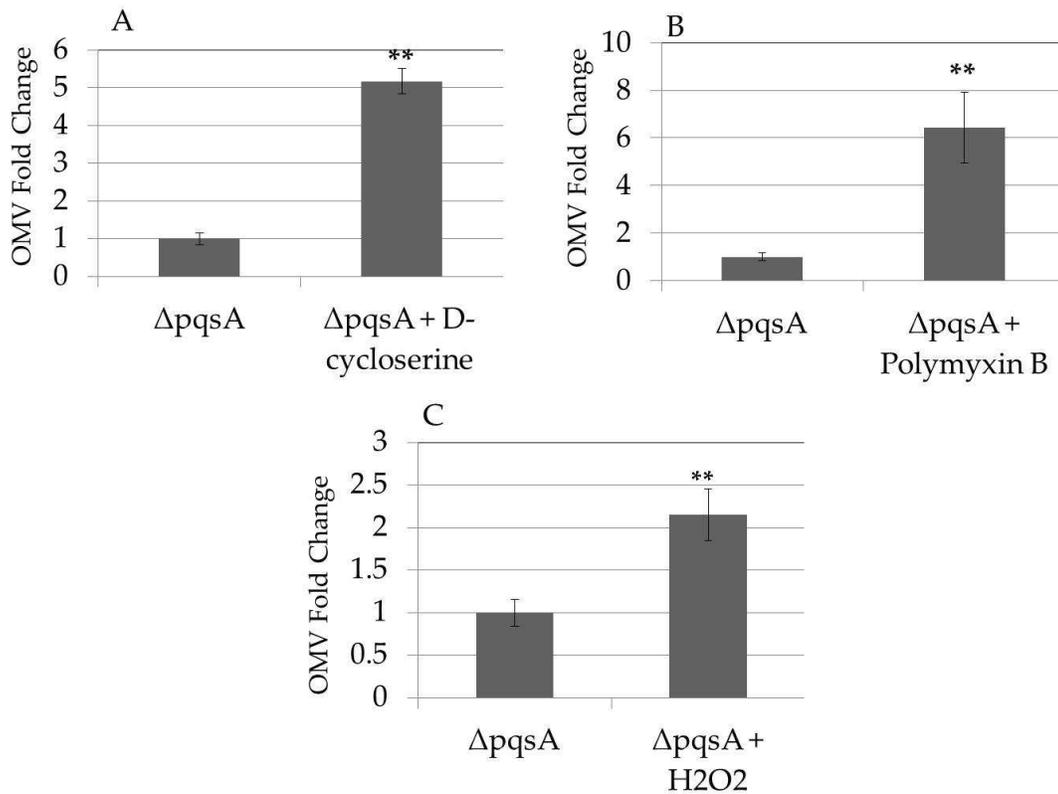
Figure 17: Acute stress induces an increase in OMV production in PA14 *ΔpqsA*

PA14*ΔpqsA* was grown to late-log phase and the cell free supernatants were precipitated using ammonium sulfate. OMVs were purified by floating concentrated supernatants on a density gradient and quantitated from cultures of PA14 *ΔpqsA* treated with 250 µg/mL D-cycloserine (A), 4 µg/mL polymyxin B (B), or 1 mM hydrogen peroxide (H_2O_2)(C). OMV yield was normalized to untreated controls (*ΔpqsA*) to calculate fold change. n ≥3, p ≤ 0.01 (**).

A	B	C

PA14 OMVs   *ΔpqsA* OMVs   *ΔpqsH* OMVs
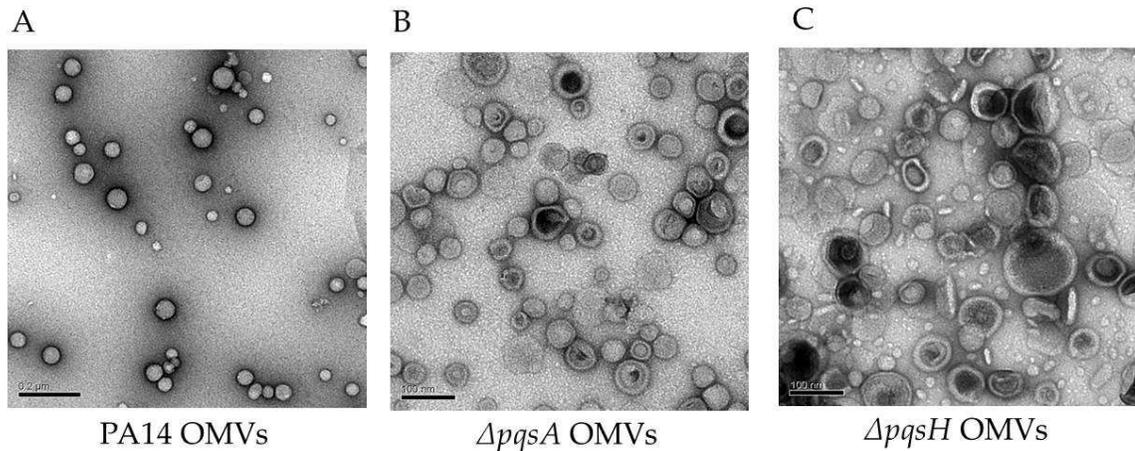
Figure 18: Negative stained electron micrograph of PA14, PA14 *ΔpqsA*, and PA14 *ΔpqsH* density gradient-purified OMVs

Negative stained electron micrograph of (A) PA14, (B) PA14 *ΔpqsA,* and (C) PA14 *ΔpqsH* OMVs purified using a density gradient from log phase growth cultures (images taken by A. Manning). Size bar: 200 nm (A), 100 nm (B,C).

We then examined whether there were differences in the amounts of OMVs

produced by wild-type and PQS-deficient strains using our quantitative methods. We

determined that not only did the PQS mutant strains constitutively produce OMVs, but

OMV production in PA14 *ΔpqsA* was actually ~3 fold greater and PA14 *ΔpqsH* ~6.0 fold

greater than PA14 as determined by FM4-64 normalized to CFU (Fig. 19A). To confirm

that the FM4-64 measurements of OMV production were not misleading, we also used

SDS-PAGE OMP analysis and densitometry to quantitate differences in OMV

production between PA14 and PA14 *ΔpqsA*. Consistent with the lipid data, we found

OMV production by PA14 *ΔpqsA* was ~2 fold greater than for PA14 as quantitated by

OM protein densitometry normalized to CFU (Fig. 19 B,C). From these data we
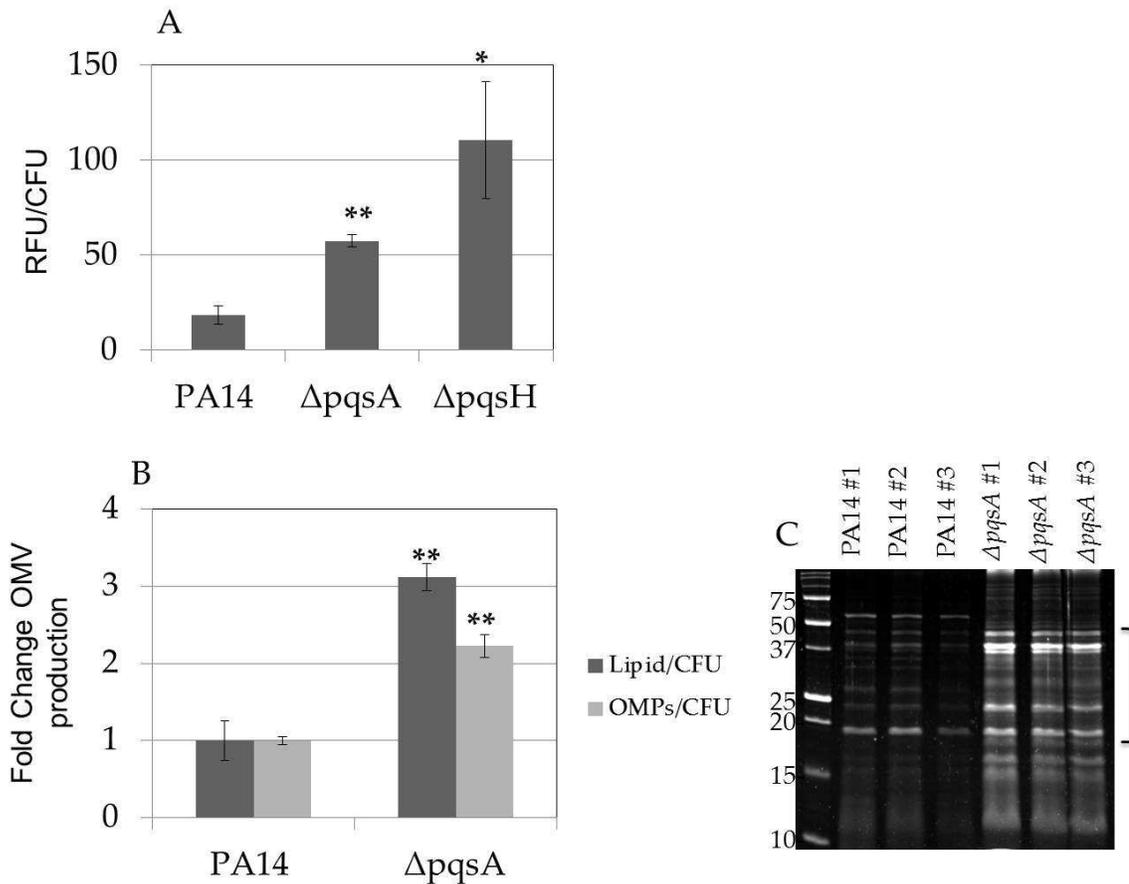
Figure 19: PQS mutants constitutively produced more OMVs than PA14 during log phase growth

(A) PA14, PA14 *ΔpqsA*, and PA14 *ΔpqsH* were grown to late log phase and OMVs were purified using a density gradient. OMV production was quantified by FM4-64 and normalized by CFU (RFU/CFU). n≥3. Statistical comparisons were made with OMV production by the wild type PA14, $p \leq 0.05$ (*), $p \leq 0.01$ (**). (B) PA14 and PA14 *ΔpqsA* were grown to late log phase and OMVs were purified using a density gradient. OMV production was quantified by FM4-64 normalized by CFU in the culture at the time of harvest (Lipid/CFU) and by protein band densitometry normalized by CFU (OMPs/CFU). OMV production by the mutant was divided by that of the wild type (PA14) to determine fold change. (C) SDS-PAGE of equal volumes of three biological replicate preparations of density gradient-purified PA14 and PA14 *ΔpqsA* OMVs used for densitometry in part B. Protein size standards are indicated (in kDa).

concluded that OMVs are constitutively produced by both PA14 and PA14 $\Delta pqsA$ during log phase growth, and that the PQS deficient strain produced more OMVs than the PA14 control.

OMV production by PA14 $\Delta pqsA$ suggests a growth phase dependent regulation. Our data demonstrated PQS mutants not only produce OMVs, but they are produced at a level above that of wild type PA14. This result was in direct contrast with previous published work. To reconcile our data with previously published work we attempted to replicate their experimental conditions (Fig. 16 B,C) by changing the growth media to brain-heart infusion (BHI) broth, decreasing the culture size to 25 ml, growing the cultures overnight, and isolating the OMVs by ultracentrifugation at 50,000 rpm for 70 min. With these conditions, we found that OMV production by PA14 $\Delta pqsA$ was 0.43-fold that of PA14 (Fig. 20 A,B). This decrease was confirmed by OMP densitometry, with PA14 $\Delta pqsA$ producing 0.81-fold the OMVs of the wild-type strain (Fig. 20 A,C). We also measured absorbance at 220 nm and found that PA14 $\Delta pqsA$ produced approximately 2-fold fewer OMVs than PA14 (Fig. 20A).

Together, these data demonstrate that OMVs are produced in both log and stationary phase growth by both wild-type and PQS mutant strains, but that PQS production can affect OMV production and suggests a growth phase dependent mechanism.

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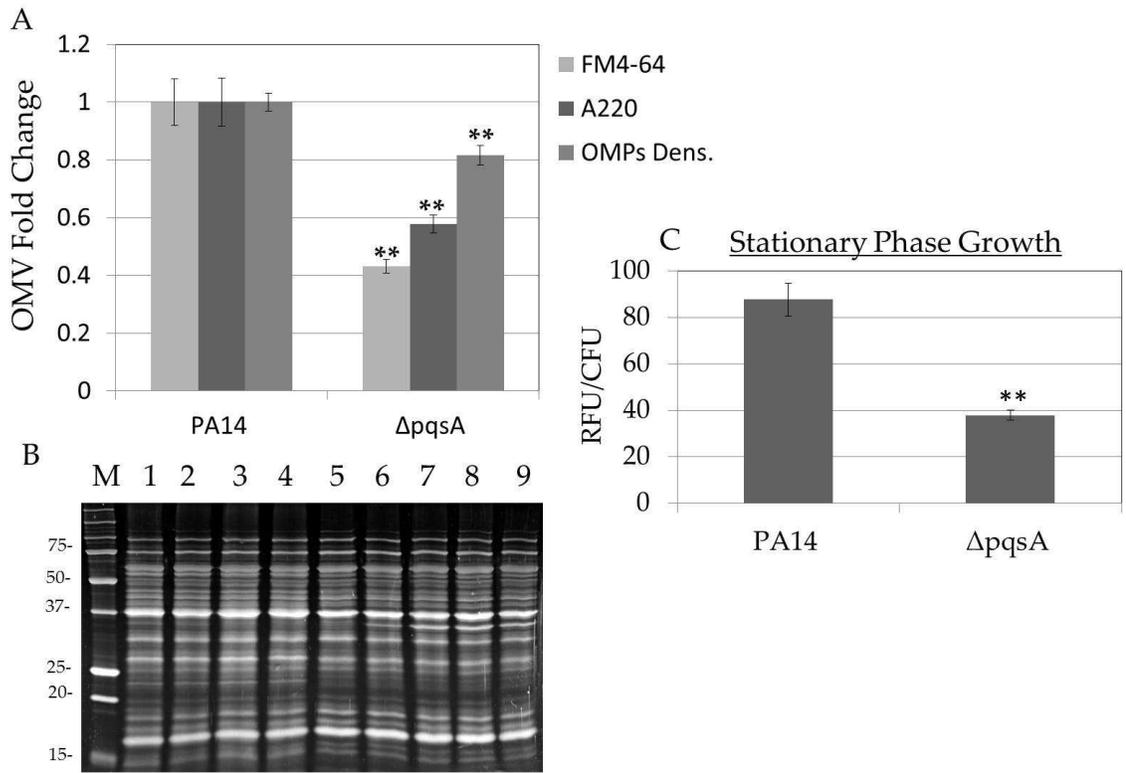
Figure 20: OMV production of PA14 *ΔpqsA* decreases during stationary phase

(A) PA14 and PA14 *ΔpqsA* were grown overnight for 16 hours and cell-free supernatants were pelleted by ultracentrifugation at 50,000 rpm. OMV production was quantified by FM4-64, absorbance at 220 nm, and by OMP densitometry normalized by CFU taken at the time of harvest. (B) SDS-PAGE of PA14 and PA14 *ΔpqsA* OMVs loaded by equivalent CFUs and stained with Sypro Ruby stain. Lane M, protein molecular weight standards, kDa are indicated. Lanes 1-4 are biological replicates of PA14 OMVs. Lanes 5-9 are biological replicates of PA14 *ΔpqsA* OMVs. (C) Raw data from FM4-64 quantitation of OMVs displayed in RFU/CFU. n≥3, p ≤ 0.01 (**).

3.4 Discussion

Our understanding of OMV formation mechanisms is limited to those observable under laboratory conditions in broth culture. OMVs have also been well characterized as a stress response pathway. Unfortunately, we have yet to fully elucidate if OMV

formation mechanisms in broth culture are relevant under stress conditions.

Understanding the mechanisms behind stress-induced OMV production could be key

for revealing how OMV production is regulated in a host during infection. This

information could also be useful for targeting by chemotherapeutics, since increased

OMV production has been linked to decreased antibiotic sensitivity (Manning and

Kuehn, 2011). Therapeutics which bypass the absorption capability of OMVs, or limit

OMV production could represent novel mechanisms to treat infections. Therefore, we

were very interested in determining whether the quorum sensing molecule, PQS, which

was previously reported to be required and sufficient for OMV formation, would be

responsible for the stress-induced OMV production we observed in Chapter 2.

Previously, a "null vesiculation" phenotype was reported for a *P. aeruginosa*

mutant lacking PQS (PA14 *ΔpqsA*) (Mashburn and Whiteley, 2005). However, we were

skeptical that PQS was, in fact, responsible for the increase in OMV formation upon

stress, because our cultures were examined during log-phase, when PQS is not highly

expressed (Fuqua et al., 2001; Venturi, 2006). PQS, as a quorum sensing molecule, is

maximally expressed during late-log and stationary phase mediated by the LasR/LasI

and RhlR/RhlI quorum sensing systems. Indeed, we observed equivalent increases in

OMV production for D-cycloserine, polymyxin B, and hydrogen peroxide for PA14

wild-type and PQS-deficient PA14 *ΔpqsA* cultures. These data confirm that PQS cannot

represent the sole driving force for OMV formation in *P. aeruginosa* when responding to acute stress.

The parity between vesiculation levels of our stressed PA14 and PA14 *ΔpqsA* cultures supports our hypothesis that PQS is likely also not contributing to OMV formation during log-phase growth. If PQS was contributing, we would have expected the fold change in OMV production upon acute stress to be lower in the PQS deficient strain. We therefore directly examined whether PQS-dependent differences in constitutive OMV formation (without stress) could be observed during log-phase growth. Our results demonstrated a surprising 2-3 fold increase in OMV production in PA14 *ΔpqsA* compared to PA14 during log-phase growth.

Our results appear to be in contrast to those previously published demonstrating that OMVs produced from a PQS mutant strain has decreased OMVs production. However, there were several differences. Mashburn and Whiteley quantitated OMVs by the absorbance at 220 nm of pelleted cell-free supernatant, whereas we quantitated ammonium-sulfate concentrated, density gradient-purified OMVs. In addition, the previous study analyzed cultures of PA14 *ΔpqsA* following overnight growth, whereas our analysis was conducted for log-phase cultures. To elucidate if the growth phase and measurement criteria were responsible for the discrepancy in the conclusions, we recreated the experimental setup of previous reports. After overnight growth in BHI media, a ~2-fold decrease in OMV production was observed for PA14 *ΔpqsA* compared

to PA14 based on FM4-64, absorbance at 220 nm, and protein densitometry. These results indicate that PA14 *ΔpqsA* did exhibit an undervesiculation phenotype, although not as substantial as reported earlier, and suggests that regulation of OMV production by PQS is dependent on growth phase.

In sum, constitutive production of OMVs was readily observed for the PQS mutant during all phases of growth and during conditions of acute stress, suggesting that other mechanisms besides the quorum sensing molecule are critical for OMV production and regulation in *P. aeruginosa*.

3.5 Acknowledgements

4. LPS structure is critical for stress-induced OMV production

4.1 Summary

The outer membrane (OM) bilayer of Gram-negative bacteria is structurally distinct. The OM is asymmetric, with phospholipids in the inner leaflet, and the endotoxin lipopolysaccharide (LPS) in the outer leaflet. LPS is composed of the lipid anchor portion, Lipid A, and the core sugars which are highly conserved, and the O-antigen portion, which is composed of oligosaccharide and is more variable in composition. LPS in *P. aeruginosa* contains two forms of O-antigen known as A-band and B-band LPS. Previous work had demonstrated that B-band LPS alone was present in natively produced outer membrane vesicles (OMVs), and that exposure of cultures to saturated oxygen increased B-band production. Whether the structural differences of *P. aeruginosa* LPS contributed to acute stress-induced OMV production was unknown..

We used the A-band mutant (PA14 *Δrmd*), B-band mutant (PA14 *ΔwbpM*), and the double A-B-band mutant (PA14 *ΔwapR*) to address the contribution of LPS structure to stress-induced OMV production. RMD is responsible for D-rhamnose production which is required for A-band synthesis. WbpM is required for B-band sugar synthesis in all 20 known serotypes of *P. aeruginosa*. WapR adds an essential D-glucose to the outer core of LPS, and as a result cannot ligate O-antigens to the core region. We determined that A-band LPS is not required for response to oxidative stress by OMV production, but that B-band LPS is required for oxidative stress-induced OMV production. These data

provided us with the first mechanistic insight into how oxidative stress leads to induction of OMV production in any Gram-negative organism.

4.2 Introduction

The membrane structure of Gram-negative bacteria is distinct from eukaryotic and Gram-positive bacteria cells because they have two distinct lipid bilayers. The IM is composed largely of phospholipids, similar to the cell membrane of eukaryotes. The periplasmic space between the two membranes is separated by a thin layer of peptidoglycan (PG) which forms a sacculus around the cell aiding in membrane rigidity. Peptidoglycan is also linked to the OM by lipoproteins to function as a connective anchor for the OM (Bouveret et al., 1999). The Gram-negative OM, is composed primarily of phospholipids in the inner leaflet, and the endotoxin lipopolysaccharide (LPS) in the outer leaflet. Integral membrane proteins are also common in the Gram-negative OM and often function as pores (Sugawara et al., 2006).

LPS is a complex sugar moiety composed of three primary components: lipid A, an inner and outer core, and O-antigen. Lipid A is composed of a disaccharide backbone of doubly phosphorylated diglucosamine. These sugars can be penta-, hexa-, or heptaacylated. Lipid A is ligated to two KDO sugars and two heptose sugars which create the inner core structure. Further sugar additions form the outer core which is the site for O-antigen ligation (Raetz and Whitfield, 2002). *P. aeruginosa* expresses two distinct types of O-antigen known as A-band and B-band LPS. A-band, or the common

antigen, is shorter and uncharged, composed primarily of repeating rhamnose

trisaccharides. A-band is not very immunogenic (Rocchetta et al., 1999). The second type

of O-antigen is responsible for the serotype specificity of *P. aeruginosa* and is commonly

known as B-band LPS. *P. aeruginosa* has been identified to have 20 distinct serotypes. B-

band LPS varies widely between serotypes but are typically longer, highly charged,

sugar repeats that are immunogenic (King et al., 2009). Because B-band LPS is so

immunogenic, *P. aeruginosa* isolated from patient with a chronic infection tend to no

longer express B-band LPS (Lam et al., 1989). The loss of B-band LPS provides a means

of immune evasion for the bacteria and typically result in antibodies being generated

against the remaining A-band LPS that is present. As an additional host defense

strategy, B-band LPS also confers serum resistance, whereas A-band LPS remains

sensitive to serum (Rocchetta et al., 1999).

As an endotoxin, LPS has a large contribution to Gram-negative bacterial

virulence. LPS is known to signal through TLR-4 and elicit a robust immune response

which can lead to septic shock (Ellis and Kuehn, 2010; Ellis et al., 2010). The number and

length of the acyl chains is responsible for the immunoreactivity of lipid A. LPS from *P.*

aeruginosa is typically less immunoreactive than *E. coli* or *Salmonella* because its acyl

chains are shorter (Backhed et al., 2003). In certain CF isolates, *P. aeruginosa* is

hyperacylated which is thought to elicit a stronger inflammatory response (Ernst et al.,

2003). Additionally, LPS can mediate attachment between host cells and the bacterium.

LPS was shown to be the ligand for type-II pneumocytes expressing CFTR, and this

attachment led to *P. aeruginosa* being ingested by the cells. Additional studies revealed

that LPS was important for corneal lens epithelial attachment and invasion by binding to

the ganglioside GM1 (Fletcher et al., 1993; Gupta et al., 1994).

Particularly pertinent to our studies, the distinct types of LPS structures were

discovered to be differentially incorporated into OMVs. Immunoblotting of naturally

produced *P. aeruginosa* OMVs revealed that these OMVs possessed only the B-band type

of LPS (Kadurugamuwa and Beveridge, 1995). By contrast, treatment with a cell wall

acting antibiotic (gentamicin), resulted in the release of OMVs containing both A-band

and B-band LPS. Later, a different group showed that cultures grown with increased

saturated oxygen conditions, exhibit increased B-band LPS expression and a

concomitant increase in OMV production (Sabra et al., 2003). Finally, it was also noted

that *P. aeruginosa* expressing O-antigen was more resistant to oxidative stress than a

mutant lacking O-antigen (Berry et al., 2009). These experiments indicate a potential

protective role for O-antigen in response to stress in *P. aeruginosa*, and that the specificity

of the LPS subtype may impact OMV production in response to stress.

The focus of this study was to test the hypothesis that B-band LPS in *P. aeruginosa*

is required for oxidative stress-induced OMV formation. We quantitated OMV

formation by isogenic mutants lacking A-band, B-band, and both A and B-band LPS and

determined whether OMV production by these strains changed upon treatment with

hydrogen peroxide. The data suggest that increased OMV production in response to

oxidative stress requires B-band, but not A-band LPS. These results present the first

identified mechanistic determinant for induced OMV formation.

4.3 Results

Confirmation of LPS phenotypes. *P. aeruginosa* OM LPS is composed of both B-

and A-band subtypes. Previously, it was demonstrated that increased levels of saturated

oxygen resulted in increased B-band LPS expression compared to an anaerobic

environment. OMV production was also noted to increase in response to increasing

saturated oxygen concentrations (Sabra et al., 2003). We reported previously an increase

in OMV production in response to hydrogen peroxide stress that was independent of

known mechanisms. Therefore, we wanted to investigate whether B-band LPS was

required for the induction of OMVs by hydrogen peroxide stress. We used LPS mutants

which are unable to synthesize A-band (PA14 Δ*rmd*), B-band (PA14 Δ*wbpM*), or both A-

and B-band (PA14 *ΔwapR*) LPS (King et al., 2009). Differences in A- and B-band LPS

structures were confirmed by silver stain after hot phenol or ethanol/Mg^{2+} extraction

(Fig. 21). PA14 (A+B+) and PA14 Δ*rmd* (A-B+) had visible laddering indicative of B-band

LPS, while PA14 Δ*wapR* (A-B-) had no laddering and only a band to indicate the KDO-

core region. Due to our inability to visualize A-band LPS well via silver stained gels,

corroborating evidence for the presence of A-band LPS in the PA14 Δ*wbpM* was

indicated by a shifted KDO absorbance spectrum to a peak at A$_{535}$ instead of the typical

A_{552} (data not shown). This shift is indicative of deoxysugars such as a rhamnose being

present (Hofstad, 1974).
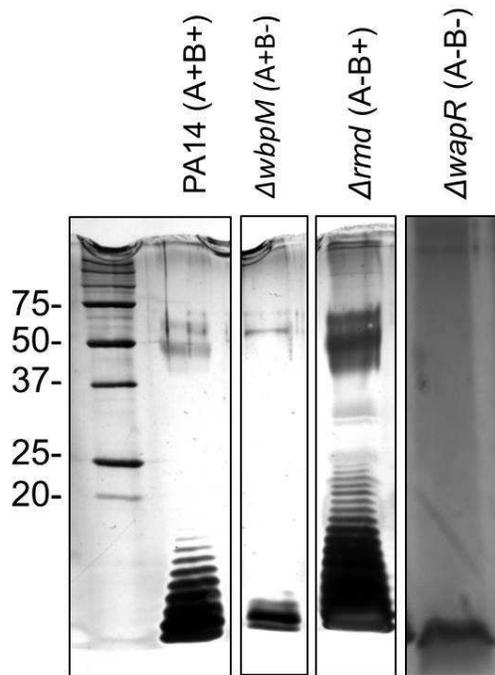
Figure 21: Silver stained SDS-PAGE of LPS from wild-type and O antigen mutant *P. aeruginosa*

LPS was isolated by organic extraction from PA14, PA14 *ΔwbpM* (B-band mutant), PA14 *Δrmd* (A-band mutant), PA14 *ΔwapR* (A-B-band mutant). LPS was analyzed using a 15% SDS-PAGE and the gel silver stained to visualize LPS bands. Gels were aligned according to the reference lane of protein size standards shown and molecular weights (kDa) are indicated.

OMV production by LPS O-antigen mutants. We first determined whether

distinct LPS subtypes affect the ability of *P. aeruginosa* to produce OMVs using our

quantitative assays. The constitutive level of OMV production by the A-mutant, B-

mutant, and A-and B-band double mutant (PA14 *ΔwapR*) was determined for log-phase

grown cultures (Fig. 22). The A-B-double mutant strain was the only strain to produce

significantly different levels of OMVs from the PA14 wild-type. The double mutant

produced 2.2-fold more OMVs than wild-type.

These results were surprising, because we anticipated that the lack of B-band LPS

might reduce OMV production since OMVs are primarily composed of B-band LPS.

This indicates that OMVs can be produced without B-band LPS during log-phase

growth and that additional mechanisms contribute to OMV formation in the absence of

B-band LPS. These results support the hypothesis that OMV production is not mediated

by a single mechanism.
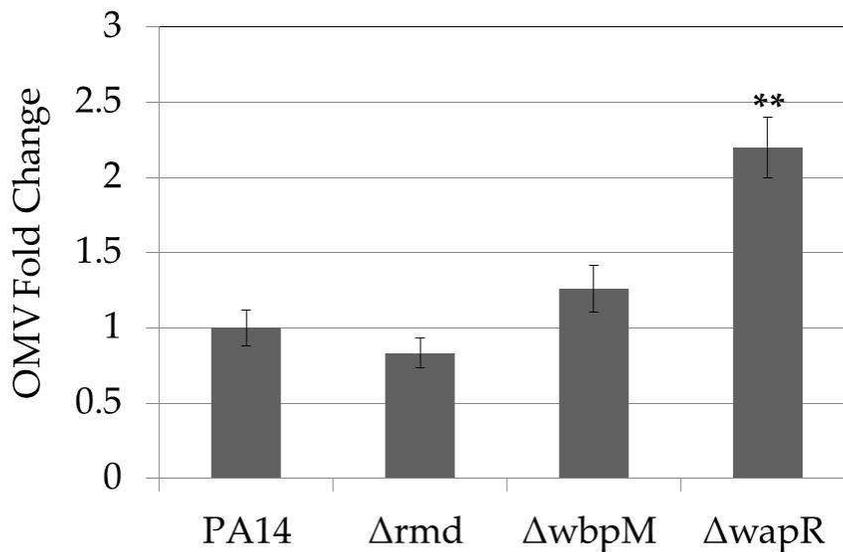
Figure 22: OMV production by O-antigen LPS mutants

OMVs were collected and quantitated from cultures of PA14, PA14 *Δrmd* (A-band
mutant), PA14 *ΔwbpM* (B-band mutant), and PA14 *ΔwapR* (A- and B-band mutant).
OMV yield was normalized to that of an untreated culture of PA14 (PA14). n ≥3, p ≤0.01
(**).

Oxidative stress-induced vesicle production is B-band dependent. We next

determined whether distinct LPS subtypes affect the ability of *P. aeruginosa* to induce

OMV production in response to stress. Cultures were grown to mid-log phase (OD_{600}

0.4) and cells pelleted. The supernatant was decanted to remove any OMVs produced

prior to treatment, and the cell pellet was resuspended in fresh media containing

hydrogen peroxide. Cultures were incubated again until they reached late-log phase,

OMVs were isolated from cell free supernatant and quantitated using the fluorescent

probe FM4-64. We found that 250 µM hydrogen peroxide treatment reduced OMV

production of the B-band mutant (PA14 Δ*wbpM*) by >60%, whereas peroxide stress

induced the A-band mutant (PA14 Δ*rmd*) to produce ~7-fold fold more OMVs. The

addition of oxidative stress did not induce vesiculation above the basal level for the A-B-

double mutant (Fig. 23). Together, the data show that hydrogen peroxide-induced OMV

production depends on the presence of B-band, but not A-band LPS. Also, oxidative

stress caused an undervesiculating phenotype in cells only expressing A-band LPS. In

addition, we found that cells expressing only A-band LPS exhibit reduced vesiculation

in response to oxidative stress.
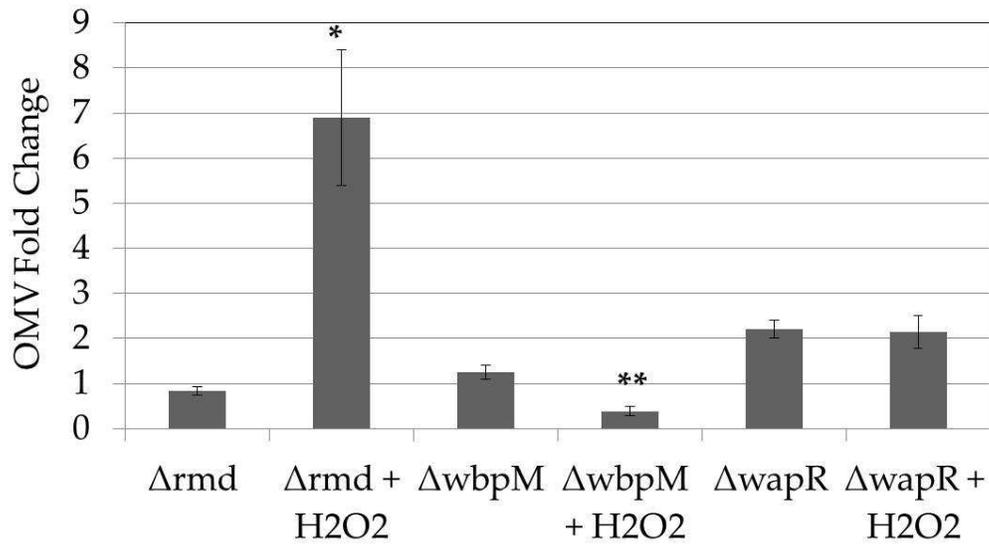
Figure 23: B-band LPS is required for H_2O_2 induced OMV formation in PA14

OMVs were collected and quantitated from untreated cultures of PA14, PA14 *Δrmd* (A-band mutant), PA14 *ΔwbpM* (B-band mutant), and PA14 *ΔwapR* (A- and B-band mutant), and from cultures treated with 250 µM hydrogen peroxide (+H_2O_2). OMV yield was normalized to that of an untreated culture of PA14 (PA14). n ≥3, p ≤0.05 (*) p ≤0.01 (**).

4.4 Discussion

OMV production in *P. aeruginosa* has been linked to the presence of B-band LPS in two prior studies. Natively produced OMVs from wild-type cells were reported to selectively contain B-band LPS (Kadurugamuwa and Beveridge, 1995). Also, cellular B-band LPS levels as well as OMVs increased in response to increased levels of saturated oxygen although it was not known whether the presence of B-band LPS was required for the induction of OMV production (Sabra et al., 2003). To examine the involvement of LPS types in OMV production, we first examined constitutive levels of OMV production by A- and B-band deficient mutants as well as the double mutant and then investigated

whether specific subtypes of LPS were required to induce OMV production in response to hydrogen peroxide. B-band LPS was found to be critical for *P. aeruginosa* to produce OMVs in response to oxidative stress, whereas A-band LPS was not required for oxidative stress-induced OMV formation.

The B-band mutant, PA14 *ΔwbpM* did not increase OMV production in response to stress, and in fact exhibited a significant reduction in OMV formation. This result was intriguing and may indicate that membrane rearrangement or tightening, to decrease OMV formation, is occurring upon oxidative stress in the absence of B-band LPS. Little is known about how oxidative stress affects the membrane in bacteria. In red blood cells, oxidative stress has been linked to rearrangement of the cell membrane which resulted in a decrease in phosphatidylserine and an increase in phosphatidylcholine. Shedding of microparticles was also observed in response to oxidative stress (Freikman et al., 2008). In *P. aeruginosa*, interactions between oxidative stress and the bacterial membrane is potentially mediated by the oxidative stress sensor, OxyR. OxyR was demonstrated to bind to the promoter of region of LpxC upon stress (Wei et al., 2012). LpxC is the enzyme which catalyzes the committed step of lipid A biosynthesis (Barb and Zhou, 2008). Perhaps upon oxidative stress, induction of lipid A biosynthesis results in a thickening of the bacterial membrane to protect against further oxidative stress. In the A-band LPS mutant, B-band LPS is present, and hydrogen peroxide stress increased OMV production. This suggests that an accumulation of lipid A may further stimulate

the promotion of OMV formation by B-band LPS as was previously reported in *P. aeruginosa* and analogous to the remodeling of red blood cell membranes (Freikman et al., 2008; Kadurugamuwa and Beveridge, 1995). B-band mutants have only A-band LPS and do not increase OMV production upon hydrogen peroxide stress, so an increase in lipid A biosynthesis without membrane blebbing could provide further stabilization against stress. Finally, the double A-B-band mutant was observed to have a higher basal level of OMV production in comparison to PA14. This result corroborated other data in our lab which demonstrates that deep rough LPS mutants (lacking O-antigen) of *E. coli* have higher levels of OMV production (Kulp et al, unpublished data). It is thought that the presence of full length LPS may act as a barrier for OMV shedding, which is not present in the deep rough mutants.

This report has provided a causal link between the previously observed increase in OMV formation upon oxidative stress, and the distinct sub-types of *P. aeruginosa* LPS. Further study is required to elucidate the complex interplay between the oxidative sensor OxyR, lipid A biosynthesis via LpxC, and OMV formation (Fig. 24A,B).

4.5 Acknowledgements

C.R.H. Raetz for providing necessary equipment and reagents for the LPS purification procedures.
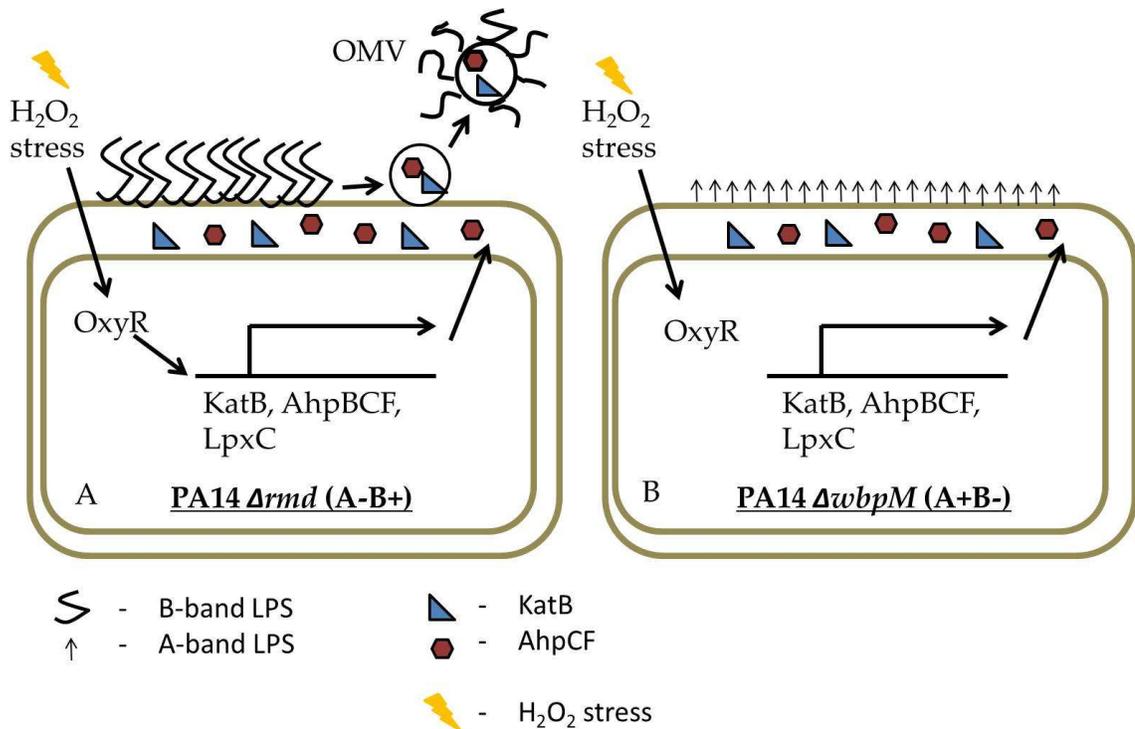
Figure 24: Summary of hydrogen peroxide-induced OMV production in LPS mutants

Schematic of PA14 *Δrmd* and PA14 *ΔwbpM* stress-induced OMV production. Hydrogen peroxide is sensed by OxyR resulting in the upregulation of oxidative defense genes and lipid A biosynthesis. Defense proteins localize to the periplasm increase accumulated protein and increase lipid synthesis resulting in a release of stress by OMV production. (A) The presence of highly charged B-band LPS provide an environment that facilitated OMV formation. (B) The presence of uncharged A-band LPS provide an environment that did not facilitated OMV formation.

5. The oxidative stress sensor OxyR, and OMV production

5.1 Summary

P. aeruginosa is commonly associated with respiratory illness, especially in patients suffering from the genetic disease cystic fibrosis (CF). Upon entering the lung, the organism must immediately cope with the innate immune response. Neutrophils and macrophages represent the primary line of defense in the lung, and in addition to phagocytosis, attempt to kill infectious agents by secreting a reactive oxygen burst. In addition to the direct sources of reactive oxygen species (ROS) targeted against *P. aeruginosa*, a second source of ROS is generated in response to antibiotic treatment. Cell envelope-directed antibiotics perturb the bacterial cell membrane resulting a disruption of the electron transport chain allow free radicals to form within the cell. Reactive oxygen radicals are very toxic due to their ability to cause damage to lipids, proteins, and DNA.

In order for a bacterium to survive the harsh oxidative burst by immune cells, a cell must be able to sense, and respond to the presence of free oxygen radicals. OxyR is the transcriptional activation responsible for sensing hydrogen peroxide stress within a cell. Upon oxidation, OxyR becomes active which allows it to interact with the RNA polymerase. Subsequent DNA binding of the RNA polymerase/OxyR complex results in the activation of several oxidative stress defense genes such as catalases and alky hydroperoxide reductases.

Our previous experiments demonstrated that hydrogen peroxide treatment resulted in an increase in outer membrane vesicle (OMV) production from *P. aeruginosa* PA14. We hypothesized that OxyR was required to sense oxidative stress in order for the cell to respond with an increase in OMV production. We tested this and found that, in the absence of OxyR, hydrogen peroxide was unable to increase OMV production. To examine the mechanistic link between OMV formation and OxyR, we assessed OMV production of mutants in genes known to be downstream of, or interaction partners with OxyR. We further examined whether OxyR was also required to mediate OMV formation when treated with the cell wall directed stressors D-cycloserine and polymyxin B and found differential effects.

We conclude that the transcriptional activator, and oxidative stress sensor OxyR, is required for hydrogen peroxide induced OMV formation, but that it is independent of known downstream effector genes as well as the Rhl quorum sensing system. Further experimentation is needed to fully understand the mechanism by which OxyR is mediating OMV production in response to hydrogen peroxide, and polymyxin B stress.

5.2 Introduction

ROSs are some of the most detrimental stressors that *P. aeruginosa* must cope with during the course of infection. ROSs are especially detrimental to the bacterial cell because instead of a single cellular target, oxidation affections many molecules and processes. DNA, proteins, and lipids can all be modified by free radicals. Free radicals

are known to cause DNA lesions and an increase in genes associated with the SOS response were upregulated in response to 1 mM hydrogen peroxide treatment (Chang et al., 2005). Three order of magnitude more single strand breaks in DNA were reported to be caused by hydrogen peroxide stress, compared with double strand breaks (Caldecott, 2008). The combination of DNA damage, lipid peroxidation, and amino acid modification can quickly destroy necessary cell functions, resulting in cell death.

Macrophages and neutrophils of the innate immune system have evolved to take advantage of the bactericidal effects of ROSs. These innate immune cells produce a burst of reactive oxygen to kill invading cells. ROSs are produced within the phagosomal compartment by the NADPH-dependent phagocytic oxidase. This enzyme pumps electrons into the phagosomal compartment which converts oxygen to superoxide anion (Slauch, 2011). Superoxide can then be degraded to hydrogen peroxide and further react to form the very reactive and damaging hydroxyl radical. Hydroxyl radicals indiscriminately attack all macromolecules quickly causing cell death (Dwyer et al., 2009).

The innate immune system is not the only source of ROSs that a bacterium must contend with. Although there is a constant low level amount of oxidative stress in the cell at all times, antibiotics and cell envelope-directed antimicrobial peptides (AMPs) have been linked to generating ROSs within the cell. This is thought to occur as a result of the electron transport chain being disrupted as these drugs disrupt the bacterial cell

membranes. Molecular oxygen can because oxidized to superoxide through redox chemistry with flavoenzymes generating superoxide and hydrogen peroxide. Both species can be produced, but it was reported that superoxide predominates (Dwyer et al., 2009).

Although the host and antimicrobials are well suited to challenge *P. aeruginosa* with oxidative stressors, *P. aeruginosa* maintains a number of different stress response pathways and enzymes to mitigate the effects of ROSs. Superoxide radicals are generated by compounds such as paraquat and are combated by the superoxide dismutase (SOD) family of proteins. *P. aeruginosa* contains both a Fe-SOD (*sodB*) and Mn-SOD (*sodA*) (Hassett et al., 1999). SOD enzymes are responsible for converting superoxide radicals into hydrogen peroxide. Typically, the response to superoxide would be regulated by the SoxR/S regulators. Interestingly, the SoxR gene in *P. aeruginosa* does not seem to function like the SoxR/S paradigm described in *E. coli*. *P. aeruginosa* is lacking a functional version of the SoxS gene, and SoxR maintains only 62% sequence identity and 77% sequence similarity to SoxR in *E. coli* (Palma et al., 2005). Despite these differences, SoxR in *P. aeruginosa* was still reported to elicit some protective effects in response to superoxide, although it is not a key factor in the defense response.

Hydrogen peroxide stress is canonically sensed by the transcriptional activator OxyR. Upon oxidation, OxyR is capable of interaction with RNA polymerase. This

interaction now facilitate the expression of several defense genes against hydrogen peroxide include catalases, and alkyl hydroperoxide reductases (Panmanee et al., 2008). It is these enzymes which convert damaging hydrogen peroxide radicals to water. In addition to OxyR, the repressor of the MexAB-OprM efflux pump, MexR has been demonstrated to respond to peroxide stress. MexR, upon oxidation, is released from DNA, allowing for the MexAB-OprM efflux pump to be expressed (Chen et al., 2008).

In addition to oxidative stress response pathways, alginate production has been linked to protection against oxidative stress. Mutations were observed in the *mucA* gene causing an increase in alginate production when exposed to hydrogen peroxide stress which facilitated bacterial survival (Mathee et al., 1999). As alginate expression was increased, a concomitant increase in Mn-SOD was noted, further supporting a link between alginate and an oxidative defense mechanism (Hassett et al., 1993).

The focus of this study was to gain insight into additional mechanisms by which hydrogen peroxide stress results in increased OMV production. We determined that the transcriptional activator, and oxidative stress sensor OxyR, was required for hydrogen peroxide induced OMV formation. This effect was shown to be independent of known downstream effector genes as well as the Rhl quorum sensing system. We tried to confirm this effect with the superoxide inducer paraquat, but no increase in OMV production was observed in PA14 or PA14 *ΔoxyR*. We did determine that DNA damage caused by mitomycin C or a lack of the DNA helicase RecG resulted in an increase in

OMV production, but this induction was independent of OxyR. We also determined the

effect that cell wall directed antimicrobial have on OMV production in PA14 *ΔoxyR* and

observed an increase in OMV production upon D-cycloserine treatment, but were

surprised to see an undervesiculation phenotype when PA14 *ΔoxyR* was treated with

polymyxin B. TLC analysis of lipid A and antibiotic sensitivity analysis revealed that

despite a similar undervesiculation phenotype, PA14 *ΔoxyR* was not resistant to

polymyxin B. Further study is still needed to fully elucidate the mechanism by which

OxyR facilitates OMV production.

5.3 Results

OxyR is required for oxidative stress induced OMV production. In Chapter 2

we demonstrated that oxidative stress strongly induces OMV formation by *P. aeruginosa*.

We wanted to test the hypothesis that the ability to sense hydrogen peroxide stress is

required for stress-induced OMV formation. Since OxyR is the transcriptional activator

required for hydrogen peroxide sensing in *P. aeruginosa* and is commonly known for

inducing catalase production, we decided to compare OMV production in PA14 *ΔoxyR*

and the isogenic wild type PA14. For these experiments, PA14 *ΔoxyR* cultures were

grown in a modified *Pseudomonas* isolation media because it was previously shown that

growth in LB produces free oxygen radicals resulting in cell death (Hassett et al., 2000).

We first examined whether there was a difference in OMV production between PA14

and PA14 *ΔoxyR* and found no significant difference (Fig. 25).

To quantitate stress-induced OMV production by the Δ*oxyR* strain, PA14 Δ*oxyR* cultures were pelleted when they reached mid-log phase and resuspended in fresh, temperature-adjusted media. For treated samples, hydrogen peroxide was added upon resuspension, and additional hydrogen peroxide was replenished every hour to counteract peroxide degradation. Cultures were re-incubated until they reached late-log phase, when OMVs were isolated from cell-free supernatants, and quantitated using the lipophilic dye FM4-64. We determined that the addition of 500 μM H_2O_2 did not induce OMV production in the absence of OxyR (Fig. 25).

It is thought that accumulation of misfolded proteins in the periplasmic space may cause an increase in OMV production as localized sites of protein accumulation could push the membrane into forming an OMV to resolve the envelope stress (Kulp and Kuehn, 2010). Because PA14 Δ*oxyR* did not result in an increase in OMV production upon stress, we hypothesized that it was because there was no periplasmic protein accumulation. To analyze protein accumulation, periplasmic contents were isolated by osmotic/temperature shock and sterile filtered for peroxide treated and untreated PA14 and PA14 Δ*oxyR*. Periplasmic protein content was quantified by a standard Bradford assay. Surprisingly, both PA14 and PA14 Δ*oxyR* exhibited increased periplasmic protein content (by 2.1 and 2.4 fold, respectively) upon addition of hydrogen peroxide (Fig. 26 A,B)(Table 2). From these protein concentration data and the previous OMV production data, we conclude that for PA14, periplasmic accumulation of protein and OMV
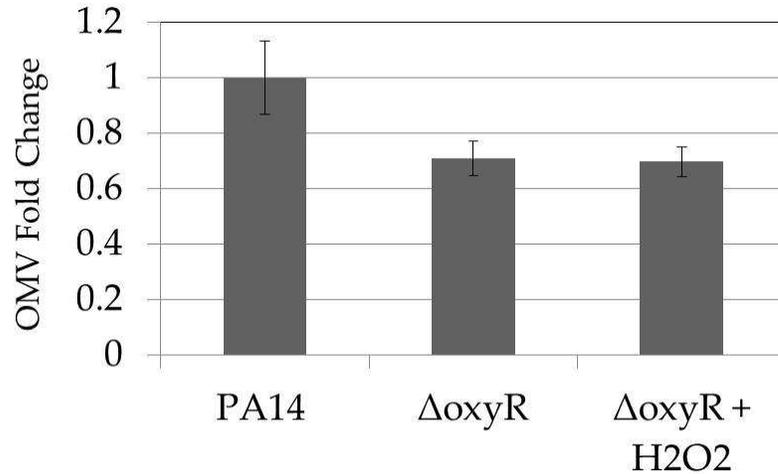
Figure 25: *oxyR* is required for hydrogen peroxide induced OMV formation

PA14 and PA14 *ΔoxyR* were grown to mid-log phase and the cells were pelleted. Cells were then resuspended with (+H2O2) or without the addition of 500 μM hydrogen peroxide, and grown until late log-phase with or without the addition of hydrogen peroxide every hour. OMVs were isolated from cell free supernatant and quantified by FM4-64. n≥3 p=0.086.

Table 2: Periplasmic protein concentrations with or without hydrogen peroxide stress

	PA14 1 mg/ml	PA14 2 mg/ml	PA14 3 mg/ml	ΔoxyR 1 mg/ml	ΔoxyR2 mg/ml	ΔoxyR 3 mg/ml
Untreated	0.320	0.313	0.352	0.220	0.221	0.182
Hydrogen Peroxide	0.847	0.651	0.857	0.500	0.338	0.596

production levels correlated well, but for PA14 *ΔoxyR*, there was no correlation.

Therefore, PA14 *ΔoxyR* may have a defect in protein sorting which prevents the

accumulated protein from being released as OMVs or a membrane which is too rigid to

release OMVs despite the increased periplasmic load.
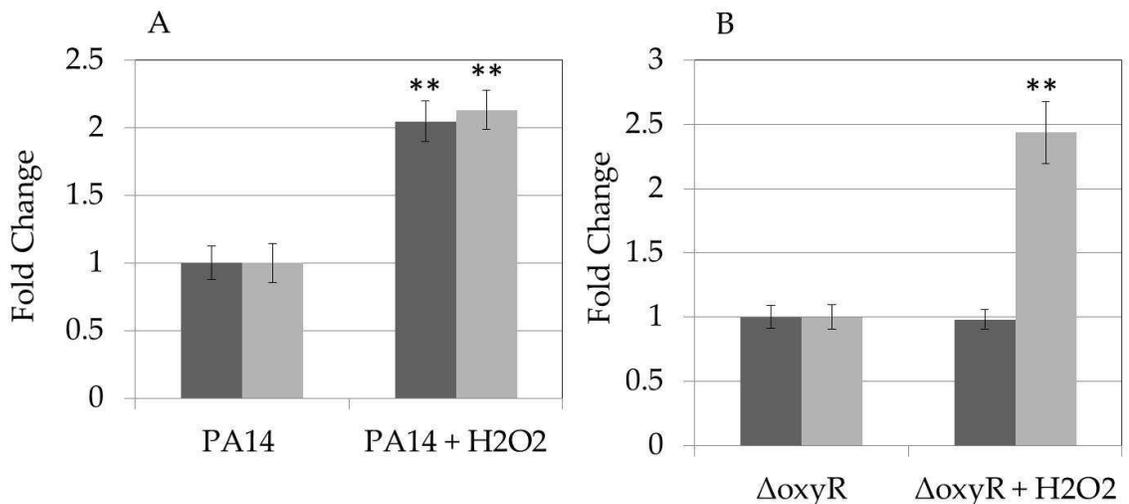
Figure 26: Fold change in OMV production and and periplasmic content for hydrogen peroxide-treated PA14 and PA14 *ΔoxyR*

OMV/CFU production (dark bars) and periplasmic protein concentration (gray bars) for PA14 (A) and PA14 *ΔoxyR* (B) treated with 500 µg/ml hydrogen peroxide. Fold change was determined by dividing values for the treated cultures (+H2O2) by those of the respective untreated cultures (PA14 or *ΔoxyR*). n ≥3 , p ≤ 0.01 for comparisons with untreated cultures.

Superoxide stress does not increase OMV production. To gain insight into the

potential mechanism for oxidative stress-induced OMV formation, we sought to

determine if the observed phenotype in PA14 *ΔoxyR* could be recapitulated upon

addition of a different oxidative stressor. We chose the superoxide generating

compound methyl viologen (MV, or paraquat). We first determined a sublethal, but

stressful dose of MV to use by analyzing growth curves on PA14 and PA14 *ΔoxyR*

treated with increasing concentrations of MV (0-6 mM) (Fig. 27 A,B). No discernible

difference in MV susceptibility was observed between the two strains. We determined

that a 1-2 mM dose provided adequate growth attenuation while remaining a

subinhibitory level of stress. We then assayed OMV production of PA14 and PA14

ΔoxyR in response to superoxide stress. For both PA14 and PA14 *ΔoxyR*, no significant

increase in OMV formation was noted upon addition of superoxide stress (Fig. 28 A,B).

We conclude that different oxidative stresses cause different reactions within the cell

and do not equally result in an increase in OMV production.

DNA damage increases OMV production independent of OxyR. Hydrogen

peroxide and free radicals are known to cause DNA damage. We were curious as to

whether the increase in OMV production upon oxidative stress was due to a DNA

damage response, and whether DNA damage is dependent on OxyR to mediate this

response. To determine if DNA damage was responsible for the increased OMV

production upon hydrogen peroxide stress, we treated mid-log phase cultures of PA14

and PA14 *ΔoxyR* with mitomycin C. Mitomycin C is known to cause DNA damage via

DNA crosslinking (Matsumoto et al., 1989). Treatment with 0.05 µg and

0.1 µg mitomycin C induced OMV production in PA14 by 2.9 and 8.3 fold respectively

(Fig. 29A) and for PA14 *ΔoxyR* by 2.2 and 4.8 fold respectively (Fig. 29B). From these

data we conclude that DNA damage can induce OMV formation, but that DNA damage-induced OMV formation was independent of the hydrogen peroxide sensor, OxyR.
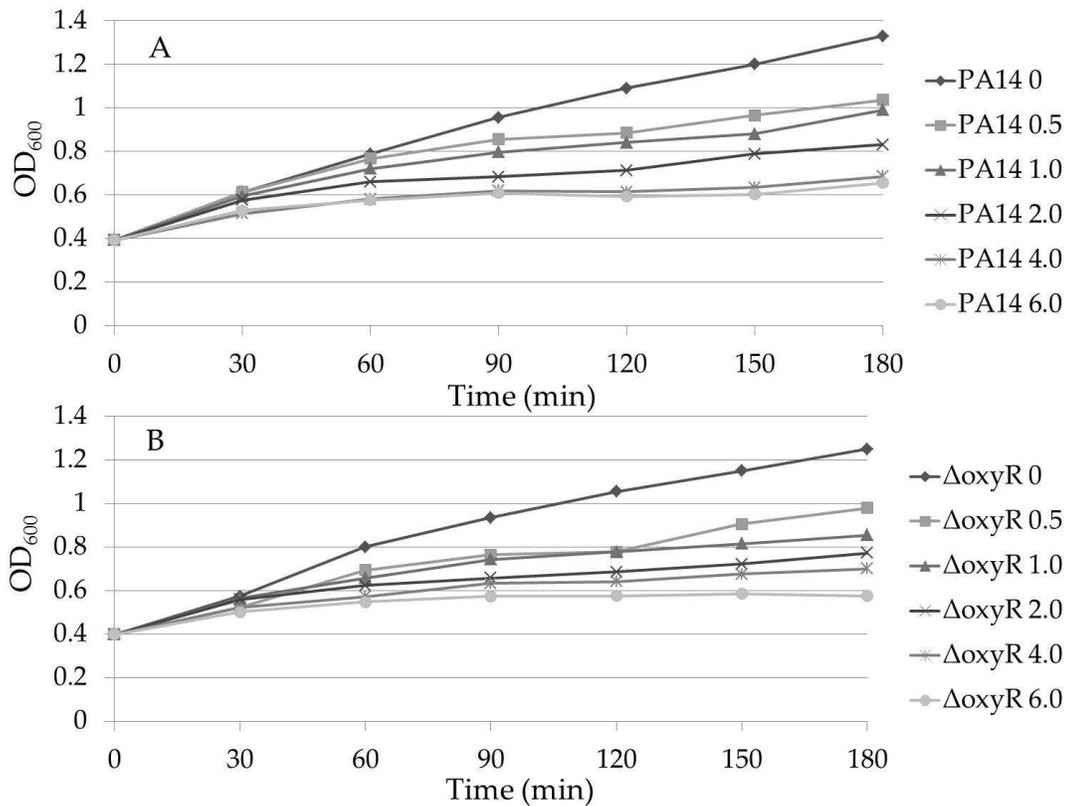
Figure 27: Methyl viologen (MV) treatment equally impacts growth of PA14 and PA14 *ΔoxyR*

Growth of PA14 (A) and PA14 *ΔoxyR* (B) treated with 0-6 mM MV. Cultures were grown (shaking at 37°C) to mid-log phase (OD_{600} 0.4) at which point (t=0) 25 mL aliquots were removed and treated with indicated doses of MV. Cultures were incubated at 37°C with shaking and OD_{600} was measured every 30 minutes for 3 h.
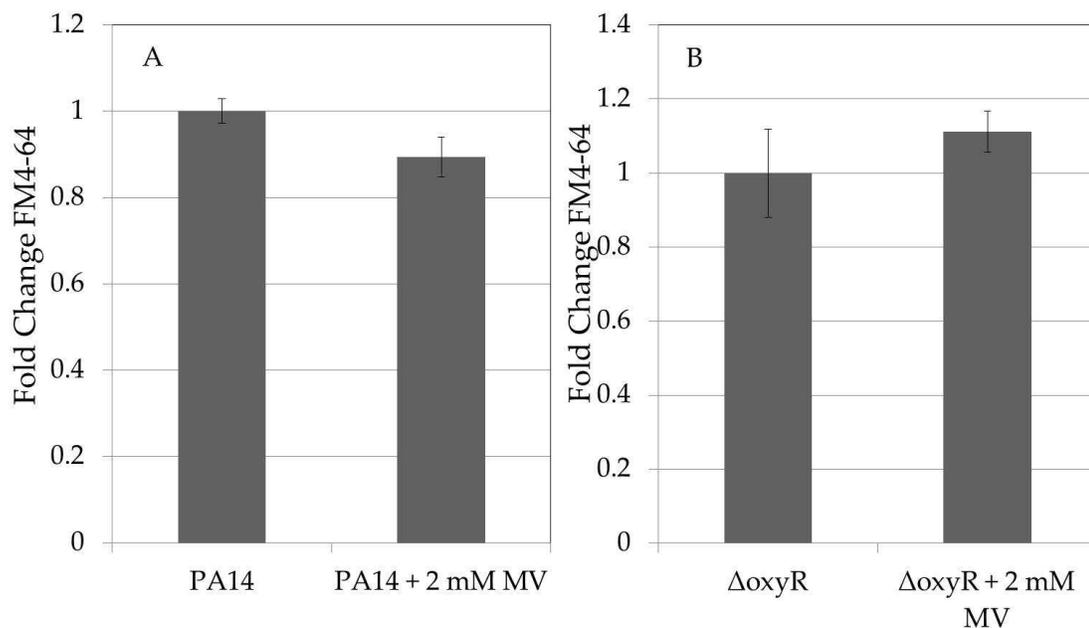
Figure 28: Addition of methyl viologen (MV) to PA14 and PA14 *ΔoxyR* does not elicit a stress response inducing OMV production

A) PA14 and B) PA14 *ΔoxyR* were grown (37°C, shaking) to mid-log phase at which point the cells were pelleted, supernatant decanted, and the cells resuspended in fresh media containing 2 mM methyl viologen (MV). Growth was continued (37°C, shaking) until the cultures reached late-log phase, then OMVs were isolated from cell-free supernatants and quantified by FM4-64. Fold change was determined by dividing the OMV/CFU of the treated (+2 mM MV) cultures by the OMV/CFU of the respective untreated (PA14 or *ΔoxyR*) cultures. $n \geq 3$, $p \geq 0.05$.
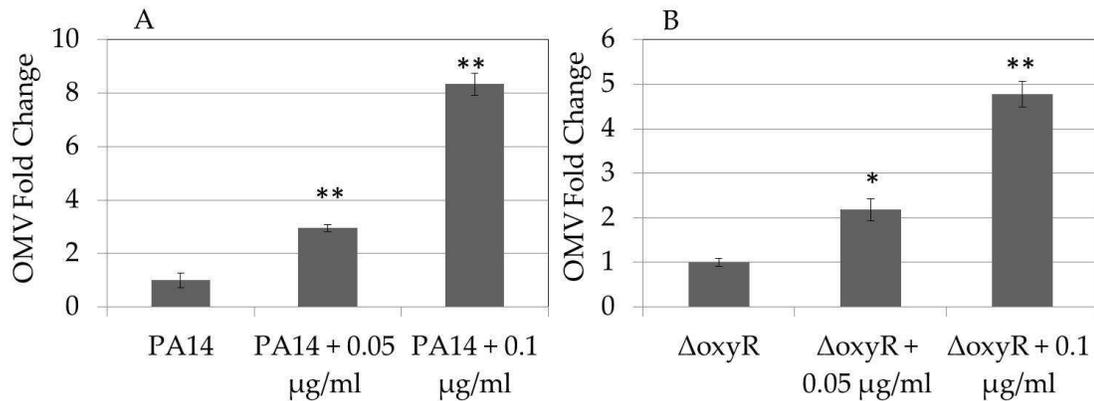
Figure 29: Mitomycin C treatment induces OMV production in both PA14 and PA14 ΔoxyR.

A) PA14 and B) PA14 *ΔoxyR* were grown (37°C, shaking) to mid-log phase at which point the cells were pelleted, supernatant decanted, and the cells resuspended in fresh media containing 0.05 or 0.1 μg/ml mitomycin C. Growth was continued (37°C, shaking) until the cultures reached late-log phase then OMVs were isolated from cell free supernatants and quantified by FM4-64. Fold change was determined by dividing the OMV/CFU of the treated (+0.5 and 0.1 μg/ml) cultures by the OMV/CFU of the respective untreated (PA14 or ΔoxyR) cultures. n ≥ 3, p ≤ 0.05 (*),p ≤ 0.01 (**) for comparisons with untreated cultures.

OxyR exists in an operon with the DNA helicase RecG which is known to

function in DNA damage response. To ensure that the phenotype we were observing

was from OxyR and not cause by polar effects of the transposon on RecG, we

determined the ability of a *recG* mutant to respond to hydrogen peroxide induced OMV

formation. We first determined the constitutive level of OMV production by PA14 *ΔrecG*

and found that PA14 *ΔrecG* yielded two fold fewer OMVs than wild-type PA14 (Fig.

30A). Since PA14 *ΔrecG* did not recapitulate the previously reported PA14 *ΔoxyR*

phenotype, we concluded that PA14 *ΔrecG* expression was not affected by the

transposon insertion in PA14 *ΔoxyR*. Addition of 250 µM and 500 µM hydrogen

peroxide stress caused an increase in OMV production by PA14 *ΔrecG* by 12.8 and 26.6

fold, respectively, compared to untreated PA14 (Fig. 30B). These data were consistent

with our previous mitomycin C data by demonstrating that DNA damage control is an

important inducer of OMV production.
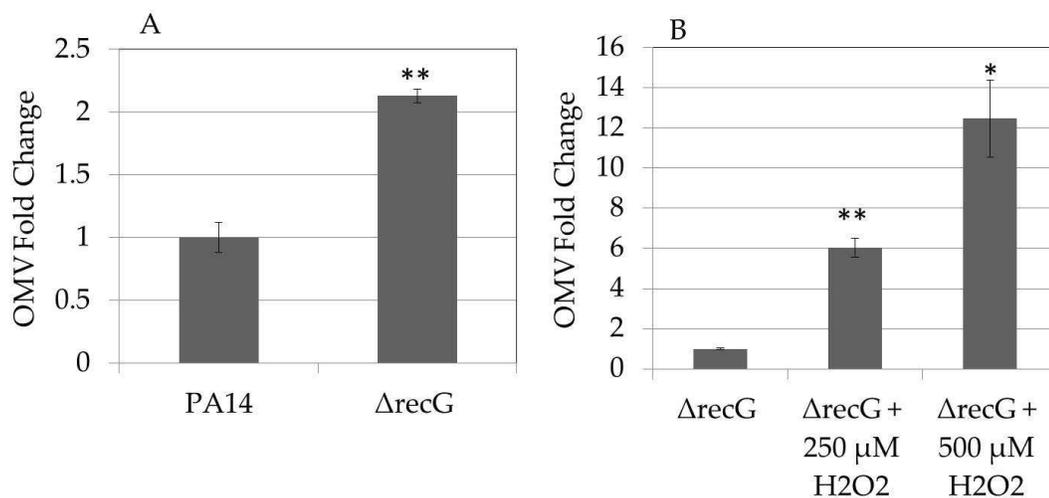
Figure 30: PA14 and PA14 *ΔrecG* OMV production increases upon addition of hydrogen peroxide stress

A) PA14 and PA14 *ΔrecG* were grown to late-log phase. OMVs were isolated from cell free supernatants and quantified by FM4-64. Fold change was determined by dividing the OMV/CFU of the PA14 *ΔrecG* culture by the OMV/CFU of the wild-type (PA14) culture. B) PA14 *ΔrecG* were grown to mid-log phase at which point the cells were pelleted, supernatant decanted, and the cells resuspended in fresh media containing 250 or 500 µM H_2O_2. Growth was continued until the cultures reached late-log phase, then OMVs were isolated from cell free supernatants and quantified by FM4-64. Fold change was determined by dividing the OMV/CFU of the treated (+250 H_2O_2 or +500 µM H_2O_2) cultures by the OMV/CFU of the respective untreated (PA14 or *ΔrecG*) cultures. n ≥ 3, p ≤ 0.05 (*),p ≤ 0.01 (**) for comparisons with wild-type (A) or untreated (B) cultures.

Differential dependence on OxyR-regulated genes for peroxide induction of OMVs. OxyR is known to regulate a number of downstream genes responsible for defense against oxidative stress. These genes include *katB*, encoding catalase, and *ahpB* and *ahpCF*, encoding defensive alkyl hydroperoxide reductases. By determining if mutations in these OxyR-regulated genes recapitulated the inability to respond to oxidative stress by PA14 *ΔoxyR*, we hoped to gain mechanistic insight into how PA14 *ΔoxyR* affects OMV production. We first determined the unstressed OMV phenotypes for PA14 *ΔahpB and PA14 ΔkatB*. The KatB mutant showed no significant difference compared to PA14, while the AhpB mutant had an undervesiculating phenotype 0.47 fold of the PA14 control (Fig. 31A). We then determined that upon addition of 500 μM hydrogen peroxide that OMV production increased 2.5 fold as compared to the untreated PA14 *ΔahpB* (Fig. 31B). We similarly tested the mutant in the OxyR-dependent catalase, *katB,* and observed no significant change in OMV production for stressed and non-stressed ΔkatB cultures, although these experiments were only repeated twice (Fig. 31C). From these data, we concluded that the loss of *ahpB* did not recapitulate the *ΔoxyR* phenotype in its OMV production response to oxidative stress. *katB* remains a promising candidate for further investigation as the results indicate that ΔkatB phenotype could mimic that of the *oxyR* mutant. As no clear mechanistic insight had been gained by examining the downstream and OxyR dependent effectors, our search expanded to include the Rhl quorum sensing system. It was previously reported that a mutation
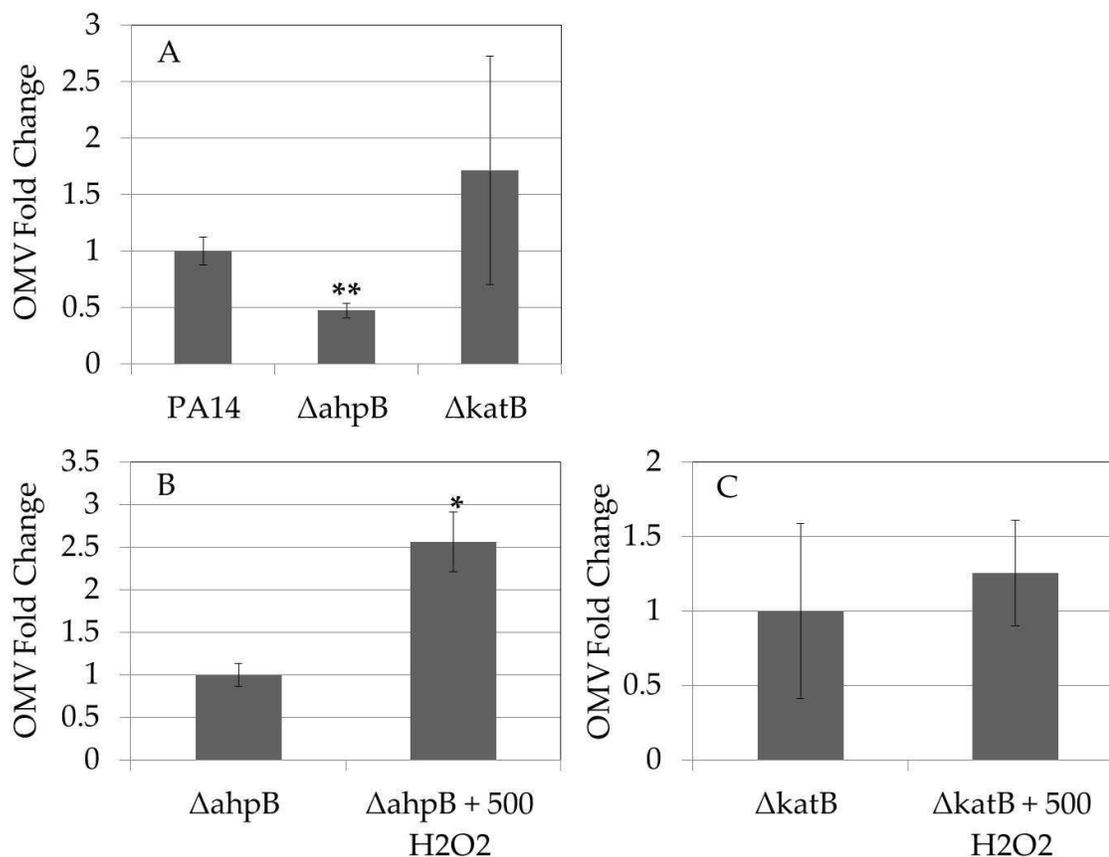
Figure 31: Differences in peroxide-induced OMV production by _ΔahpB_ and _ΔkatB_, mutants in genes regulated by OxyR

A) PA14, B) PA14 _ΔahpB,_ and C) PA14 _ΔkatB_ were grown (shaking, 37°C) to mid-log phase at which point the cells were pelleted, supernatant decanted, and the cells resuspended in fresh media containing 500 μM H_2O_2 (B,C). Growth was continued (shaking, 37°C) until the cultures reached late-log phase, then OMVs were isolated from cell free supernatants and quantified by FM4-64. Fold change was determined by dividing the OMV/CFU of the treated (+500 H_2O_2) cultures by the OMV/CFU of the respective untreated (_ΔahpB_ or _ΔkatB_) cultures. n ≥ 3 for (A,B);. n = 2 for (C_)_, p ≤ 0.05 (*) p ≤ 0.01 (**). .

in OxyR results in a loss of rhamnolipid and increase in pyocyanin production (Vinckx et al., 2010). Rhamnolipids are a biosurfactant produced by *P. aeruginosa* under quorum sensing regulation and is known to solubilize insoluble compounds such as PQS to facilitate OMV formation (Calfee et al., 2005). This connection to quorum sensing led us to investigate whether a mutant deficient in rhamnolipid production (PA14 *ΔrhlA*) could recapitulate the previously reported *ΔoxyR* peroxide-induced OMV production phenotype upon addition of hydrogen peroxide. We tested this hypothesis by assaying for OMV production in PA14 *ΔrhlA* with and without addition of 500 μM hydrogen peroxide through log phase growth. We observed no change in OMV production without peroxide stress and a 2.8 fold increase in OMV production by PA14 *ΔrhlA* with treatment (Fig. 32A,B). From this we conclude that the quorum sensing Rhl system was not responsible for the OxyR mutant's inability to respond to oxidative stress with increased OMV production.

OMV production in PA14 *ΔoxyR* upon addition of cell wall directed stressors. In addition to direct oxidative stress provided by external compounds such as methyl viologen and hydrogen peroxide, antibiotics and cell wall perturbing compounds have been reported to cause oxidative stress responses in cells. This effect is typically a result of a disruption the cell membrane electron transport chain being disrupted. To determine if OxyR is required to respond to antibiotic generated oxidative stress, we treated PA14 and PA14 *ΔoxyR* with D-cycloserine and polymyxin B. As for the

previously described stress induction assays, stressors were added when cultures

reached mid-log phase and OMV production was quantified by FM4-64 when the

stressed cultures reached late-log phase growth. Upon addition of 250 μM D-

cycloserine, OMV production increased by 2 fold (Fig. 33). From these data we

concluded that OMV production induced by the perturbation of peptidoglycan growth

is *oxyR* independent. However, addition of 4 μg/mL polymyxin-B to PA14 *ΔoxyR*

resulted in a suprising and dramatic undervesiculation phenotype, 0.14-fold that of
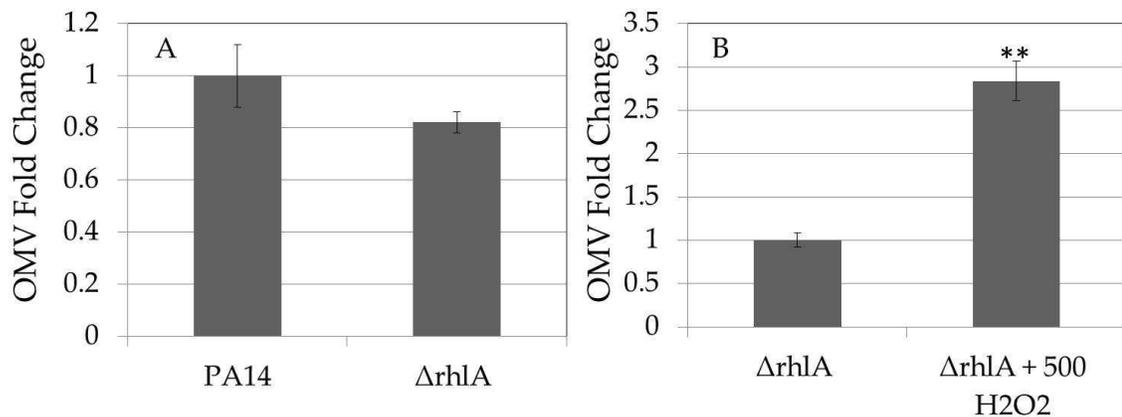
Figure 32: Hydrogen peroxide stress-increased OMV production by PA14 *ΔrhlA*

PA14 and PA14 *ΔrhlA* were grown (shaking, 37°C) to mid-log phase at which point the cells were pelleted, supernatant decanted, and the cells resuspended in fresh media (B) with or (A) without 500 μM H_2O_2. Growth was continued (shaking, 37°C) until cultures reached late-log phase, then OMVs were isolated from cell free supernatants and quantified by FM4-64. Fold change was determined by dividing the OMV/CFU of the treated (+500 H_2O_2) cultures by the OMV/CFU of the untreated (ΔrhlA) cultures. n ≥ 3, p ≤ 0.01 (**).
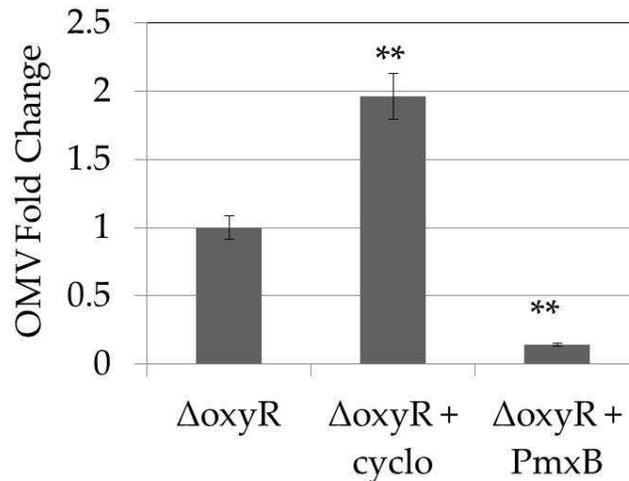
Figure 33: Envelope-directed antibiotics differentially modulate OMV production in PA14 *ΔoxyR*

PA14 *ΔoxyR* was grown (shaking, 37°C) to mid-log phase at which point the cells were pelleted, supernatant decanted, and the cells resuspended in fresh media containing 250 μM D-cycloserine (+ cyclo) or 4μg/ml polymyxin B (+PmxB). Growth was continued (shaking, 37°C) until the cultures reached late-log phase, then OMVs were isolated from cell free supernatants and quantified by FM4-64. Fold change was determined by dividing the OMV/CFU of the treated (+cyclo or +PmxB) cultures by the OMV/CFU of the untreated (ΔoxyR) cultures. $n \geq 3$, $p \leq 0.01$ (**) for comparisons with untreated cultures.

PA14 (Fig. 33). Therefore, polymyxin B treatment somehow suppressed OMV

production in the *ΔoxyR* strain. The surprising decrease in OMV production by PA14

ΔoxyR upon polymyxin B treatment led us to examine whether potential acquisition of

resistance to the effects of polymyxin B could be responsible for this phenotype. We first

generated a positive control, a polymyxin B resistant strain of PA14 (PmxB-R PA14), by

continually passaging PA14 cells with increasing concentrations of polymyxin B. We

then assayed the OMV production phenotype of PmxB-R PA14 upon treatment with or

without 4 µg/mL polymyxin B. In the absence of stressor, PmxB-R PA14 produced 0.35-fold the OMVs compared to the wild-type PA14 strain (Fig. 34). This was a similar to the phenotype of the OxyR mutant compared to PA14. Notably, even when this culture was treated with polymyxin B, OMV production was similar, at 0.25-fold that of wild type levels (Fig. 34). Thus, as expected, polymyxin treatment did not induce OMV production in the polymyxin B resistant strain, similar to the lack of OMV induction for PA14 *ΔoxyR* upon polymyxin B treatment. This provided us with the impetus to further explore whether PA14 *ΔoxyR* had evolved resistance to polymyxin B.
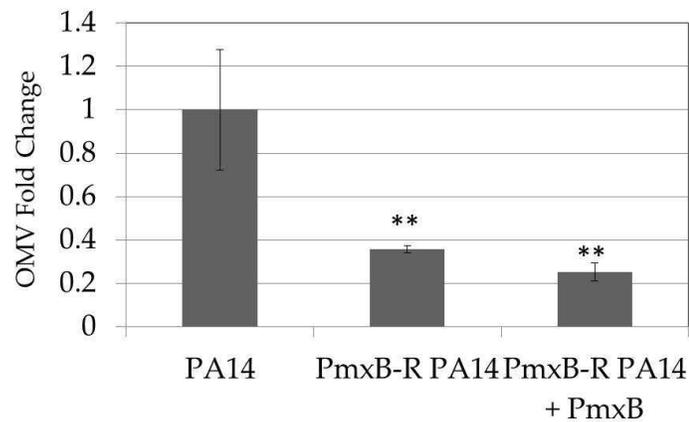
Figure 34: A polymyxin B resistant strain of PA14 undervesiculates and OMV production in not induced with polymyxin B treatment

PA14 and a polymyxin B resistant strain of PA14 (PmxB-R PA14) were grown (shaking, 37°C) to mid-log phase at which point the cells were pelleted, supernatant decanted, and the cells resuspended in fresh media with (+PmxB) or without 4 µg/ml polymyxin B. Growth was continued (shaking, 37°C) until cultures reached late-log phase, then OMVs were isolated from cell free supernatants and quantified by FM4-64. Fold change was determined by dividing the OMV/CFU of the resistant (PmxB-R PA14)or treated resistant (PmxB-R PA14+PmxB) cultures by the OMV/CFU of the untreated wild-type (PA14) cultures. n ≥ 3, p ≤ 0.01 (**) for comparisons with PA14.

PA14 *ΔoxyR* is not resistant to polymyxin B. Resistance to AMPs such as

polymyxin B is often caused by 4-aminoarabinose moieties replacing the phosphates on

the lipid A portion of LPS (Gunn, 2001). The consequent decrease in the negative charge

of the modified LPS decreases the binding affinity of the cationic AMPs. To determine if

PA14 *ΔoxyR* lipid A is modified, we isolated lipid A from both PA14 and PA14 *ΔoxyR*

LPS by acid hydrolysis and Bligh/Dyer extraction. Samples of lipid A were then

separated using thin layer chromatography (TLC). No discernible differences in the

migration of the lipid A species were observed, leading us to conclude that no

modifications had been made to the lipid A structure (Fig. 35).

P10   P5   O10   O5
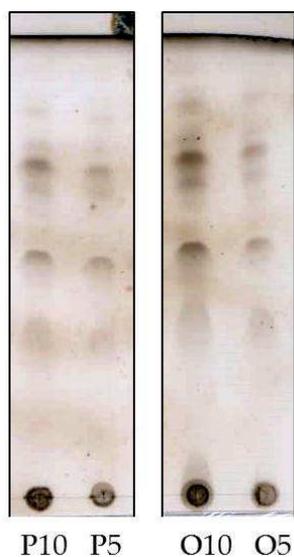
Figure 35: Similar TLC migration patterns for PA14 and PA14 *ΔoxyR* lipid A

PA14 and PA14 *ΔoxyR* LPS were purified by hot phenol extraction. Preparations, 10 μl
and 5 μl of PA14 lipid A (P10, and P5) and 10 μl and 5 μl of PA14 *ΔoxyR* lipid A (O10
and O5), were run on a TLC plate and scorched with sulfuric acid to visualize.

We further confirmed a lack of resistance to polymyxin in PA14 *ΔoxyR* by comparing the polymyxin B minimum inhibitory concentration (MIC) of PA14, PA14 *ΔoxyR*, and the resistant isogenic PA14 strain, PmxB-R PA14. Triplicate overnight cultures of each strain were inoculated to a low starting density, and then grown overnight (16h) in the presence of polymyxin B ranging in concentration from 0-32 µg/ml, and culture turbidity was scored (Tables 3, 4). The data show that there was no difference in resistance between PA14 and PA14 *ΔoxyR,* whereas PmxB-R PA14 was able to grow in all of the polymyxin B concentrations tested.

Table 3: MIC Determination of triplicate cultures of PA14, PA14 ΔoxyR, and PmxB-R PA14 (0-32 µg/ml polymyxin B)

	Polymyxin B (µg/ml)						
	0	1	2	4	8	16	32
PA14 1	+[2]	+	+	+	+	+/-[3]	-[4]
PA14 2	+	+	+	+	+	+/-	-
PA14 3	+	+	+	+	+	+/-	-
ΔoxyR 1	+	+	+	+	+	+/-	-
ΔoxyR 2	+	+	+	+	+	+/-	-
ΔoxyR 3	+	+	+	+	+	+/-	-
PmxB-R PA14 1	+	+	+	+	+	+	+
PmxB-R PA14 2	+	+	+	+	+	+	+
PmxB-R PA14 3	+	+	+	+	+	+	+

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[2] +, Wild-type turbidity
[3] +/-, Intermediate turbidity
[4] -, No turbidity

Table 4: MIC Determination of triplicate cultures of PA14, PA14 ΔoxyR, and PmxB-R PA14 (8-32 μg/ml polymyxin B)

	Polymyxin B (μg/ml)						
	8	12	16	20	24	28	32
PA14 1	+[5]	+	+	+	-	-	-
PA14 2	+	+	+	+/-[6]	-	-	-
PA14 3	+	+	+	+/-	-[7]	-	-
ΔoxyR 1	+	+	+	+/-	-	-	-
ΔoxyR 2	+	+	+	+	+/-	-	-
ΔoxyR 3	+	+	+	+/--	-	-	-
PmxB-R PA14 1	+	+	+	+	+	+	+
PmxB-R PA14 1	+	+	+	+	+	+	+
PmxB-R PA14 1	+	+	+	+	+	+	+

5.4 Discussion

Oxidative stress was previously shown to increase OMV production in *P. aeruginosa*, however it was unknown by what mechanism hydrogen peroxide induced formation of OMVs. We sought to answer this question by hypothesizing that the bacterial cell may need to sense the oxidative stress in order to elicit an increase in OMV formation. The primary hydrogen peroxide sensor in *P. aeruginosa* is the oxidative defense regulator, OxyR. Upon activation by oxidation, OxyR activates the expression of several oxidative defense genes. We used a strain of PA14 deficient in OxyR to elucidate if the ability to sense oxidative damage was required to respond at the membrane by

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[5] +, Wild-type turbidity
[6] +/-, Intermediate turbidity
[7] -, No turbidity

increasing OMV production. We demonstrated that OxyR was required for hydrogen peroxide stress-induced OMV formation.

Although we discovered that the increase in OMVs in response to hydrogen peroxide stress was dependent on OxyR, these results did not address how OxyR was eliciting a response at the membrane. One proposed mechanism for OMV formation for *E. coli* and *P. aeruginosa* is the presence of accumulated proteins in the periplasmic space (McBroom and Kuehn, 2007; Tashiro et al., 2009). Because PA14 *ΔoxyR* did not increase OMV production upon stress, we investigated whether periplasmic protein did not accumulate in these conditions. We found the opposite to be true: upon oxidative stress in both PA14 and PA14 *ΔoxyR,* there was a significant increase in the periplasmic protein concentration. From these data, we conclude that OxyR may have an indirect role on protein sorting into OMVs or a membrane structural defect that prevents OMV formation despite envelope stress.

OxyR regulates a number of oxidative defense genes including *katB*, *ahpB*, and *ahpCF*. AhpCF has been previously identified to be present in *P. aeruginosa* OMVs by proteomic analysis (Choi et al., 2011), and it is possible that KatB, a periplasmic protein, may also be present in OMVs. We investigated whether the inability for PA14 *ΔoxyR* to increase OMV production in response to hydrogen peroxide was dependent on its downstream target genes *ahpB* and *katB*. In limited experimental studies, *ΔkatB* had a similar OMV phenotype to PA14 *ΔoxyR* when treated with hydrogen peroxide. We

hypothesize that upon oxidative stress, defense products such as KatB localize to the periplasmic space resulting in an increase in periplasmic protein. Potentially, the accumulation of these defense proteins may cause an increase in OMV formation. This would be similar to the impact of increased sigmaE regulon factors in the periplasm in response to induction of the sigmaE stress response in *E. coli* (McBroom and Kuehn, 2007). In addition to KatB, KatA had previously been shown to be a very stable catalase produced by *P. aeruginosa* and found to be essential for PA14 *ΔoxyR* growth in LB broth (Hassett et al., 2000). The authors concluded that at low cell densities, initial cell lysis releases KatA. KatA remains stable in the media, providing protection from addition oxidative stressors that may be present, and allowing the remaining cells to survive and grow. We did not rule out that catalase activity, independent of OxyR, may be present in PA14 *ΔoxyR* culture media. The presence of catalase could detoxify the hydrogen peroxide that we intended to act as a stressor.

An *oxyR* mutant was reported to be deficient in swarming capability. This was caused by a loss of rhamnolipids production in the OxyR mutant strain (Vinckx et al., 2010). As rhamnolipids are a biosurfactant and believed to help solubilize insoluble molecules such as PQS, we wanted to investigate whether the inability for OxyR to respond to oxidative stress was functioning through a rhamnolipid dependent mechanism. We determined that oxidative stress induced OMV production in PA14 *ΔrhlA*. This result was not surprising because, as a quorum sensing-regulated

compound, rhamnolipids are not expressed until stationary phase. Our experiment was conducted during late-log phase growth when quorum sensing induced genes are not maximally expressed. This explains why the PA14 *ΔrhlA* strain would respond similar to PA14 in response to oxidative stress.

In addition to the oxidative stress added exogenously to the culture in the form of hydrogen peroxide, oxygen radicals can be generated by the bacteria themselves in response to cell membrane disruption by antibiotics and AMPs. We stressed PA14 *ΔoxyR* with the peptidoglycan synthesis inhibitor, D-cycloserine, and found that OMV production increased, whereas treatment with polymyxin B resulted in a suppression of OMV production. We confirmed that, despite having a similar suppressive OMV production phenotype as a polymyxin B resistant strain of PA14. PA14 *ΔoxyR* did not appear to be resistant to polymyxin B nor express modified lipid A. From these data we concluded that cell envelope directed compounds affect OMV production differently in PA14 *ΔoxyR* due to their distinct mechanisms of action at the OM or PG layers. D-cycloserine as a chemical mimic of D-alanine and functioning as a peptidoglycan synthesis inhibitor may not be subject to the same resistance mechanisms as other antibiotics. This could explain why OMV production still increased in the *oxyR* mutant strain (Fig. 36A,B).
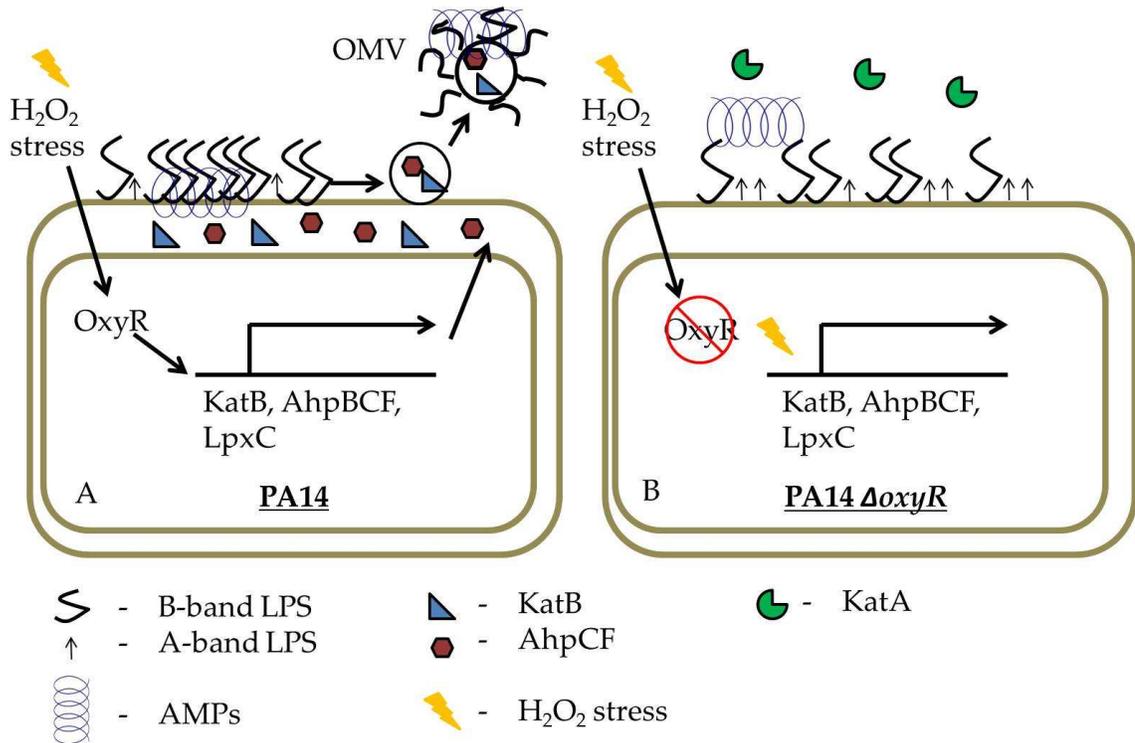
Figure 36: Summary of PA14 and PA14 *ΔoxyR* stress-induced OMV production

(A) Schematic of PA14 stress-induced OMV production. Hydrogen peroxide is sensed by OxyR resulting in the upregulation of oxidative defense genes and lipid A biosynthesis. Defense proteins localize to the periplasm increase accumulated protein and increase lipid synthesis resulting in a release of stress by OMV production. (B) Schematic of PA14 *ΔoxyR* stress-induced OMV production. Lack of OxyR results in no new lipid A synthesis in response to stress as well as the potential presence of KatA and an increase of A-band LPS. These factors contribute to the lack of OMV production increase in response to stress.

5.5 Acknowledgements

6. Concluding Remarks and Future Directions

6.1 Future directions for study of OxyR and its contribution to stress-induced OMV formation in P. aeruginosa

We reported in Chapter 2 that hydrogen peroxide increased OMV production in

P. aeruginosa. The mechanism for how oxidative stress increased OMV formation

remained elusive. We took two approaches to determine the answer to this question. In

the first approach we asked whether B-band LPS, which is linked to oxidative stress,

was required for hydrogen peroxide-induced OMV formation (Chapter 4). In the second

approach, we tested the hypothesis that the oxidative stress sensor OxyR is required to

mediate an increase in OMV production in response to oxidative stress. This was shown

to be true, and the results are outlined in Chapter 5. Further study is required to fully

elucidate the mechanism by which OxyR is effecting OMV formation. We outline here

potential future directions for experimentation.

It was previously reported that PA14 *ΔoxyR*, when inoculated at a low cell

density, required release of the catalase, KatA, from dying cells to allow for growth of

the population (Hassett et al., 2000). KatA is very stable, as a result we discussed the

possibility that cultures of PA14 *ΔoxyR* have residual catalase activity present in the

media. Catalase could remove the added hydrogen peroxide from the culture media,

removing the stressor from the environment resulting in little to no stressor contacting

the PA14 *ΔoxyR* cells. To determine if KatA is present in the media, an anti-KatA western

blot analysis should be performed on concentrated or TCA precipitated cell free

supernatants. The presence of KatA would not mean that no stress is being felt by the cells in the culture, but could contribute as a mechanism to reduce hydrogen peroxide stress.

Our previous work (Chapter 4) indicated that B-band LPS was required for OMV production in response to hydrogen peroxide stress. OxyR was shown to regulate lipid A biosynthesis by interacting with the LpxC promoter (Wei et al., 2012). Little is known about how cells regulate the ratio of A-band and B-band LPS. It was reported that growth at elevated temperatures resulted in a reduction in O-antigen chain length and a loss of B-band LPS expression when grown at 45°C (Rocchetta et al., 1999). It may be possible that oxidative stress, like temperature stress, results in a decrease in B-band LPS expression. It would be important for future experimentation with PA14 *ΔoxyR* to determine what the ratio of A-band and B-band LPS is under stressed and unstressed conditions. A decrease in B-band expression may impair the cells inability to increase OMV production in response to hydrogen peroxide.

With the advent of systems biology and whole genome arrays, great advances could be made in determining the response of PA14 *ΔoxyR* to hydrogen peroxide on a whole organism level. By comparing the gene expression profiles of hydrogen peroxide treated PA14 and PA14 *ΔoxyR*, insights could be gained to explain the lack of increased OMV production of PA14 *ΔoxyR*. Target genes in LPS biosynthesis, membrane remodeling, stress response pathways, drug efflux pumps, and a variety of previously

unknown genes could be identified to help scientists parse out what mechanistic contributions OxyR is making to OMV formation.

In addition to whole genome arrays, proteomic analysis has begun to be a primary source of information for identifying the components of OMVs and elucidating potentially mechanisms for OMV formation. Proteomic analyses of OMVs from *E. coli* and *P. aeruginosa* have already been performed and increased our knowledge of OMV associated virulence factors (Choi et al., 2011; Lee et al., 2007; Lee et al., 2008). We have not yet analyzed the changes in OMV proteomes derived under stress conditions. As OMVs are a documented stress response, determining the proteome of OMVs during stress may aid in identifying unknown mechanisms for OMV formation. We know that certain inducible enzymes during stress are localized to the periplasmic space. KatB and AhpCF, in particular, are known to localize to the periplasmic space. It would be interesting to determine whether these proteins became enriched in OMVs upon hydrogen peroxide treatment, and if they contribute to OMV formation by accumulating in the periplasmic space.

6.2 Stress-induced vesiculation is important and clinically relevant

We discuss at length the mechanisms previously thought to play a role in OMV formation in *P. aeruginosa*, and challenged those mechanisms with the addition of general and envelope-directed stressors. We demonstrated that typically, *P. aeruginosa* responds to stressors by increasing OMV production as a defensive stress response

mechanism. Rarely did the proposed models appear to be relevant for stress induced

OMV formation, although there was an indirect correlation found between the

envelope-directed stressors and the AlgU envelope stress response pathway. From these

experimental observations, we conclude that additional mechanisms for OMV formation

must be present in *P. aeruginosa*, but that those mechanisms only become activated upon

entering a stressful environment.

It is important to note that the previously reported mechanisms for OMV

formation were assessed in batch broth culture in ideal laboratory conditions. Although

these experiments were the springboard for our current understanding of OMV

formation mechanisms, laboratory conditions do not replicate the hostile environment of

a host and the myriad of stressful circumstances that a bacterium is must adapt to. We

argue that our experiments to determine OMV formation mechanisms under stress

conditions more accurately represent aspects of the host environment. Previous

scanning electron microscopy data from our lab demonstrated an increase in bleb

formation on the surface of enterotoxigenic *E. coli* that had been recovered from the

intestinal tract of a mouse as compared to a laboratory broth-grown control culture (Ellis

and Kuehn, 2010). Although technically challenging to perform, quantitating OMV

production of bacteria in an *in vivo* or *ex vivo* setting would represent an ideal system to

determine the physiologically relevant mechanisms responsible for OMV production.

In addition to stress-induced OMVs representing a more accurate picture of OMV formation, there may be great clinical consequences to further understanding *P. aeruginosa* OMV formation. With bacteria evolving increased resistance to antibiotics, novel mechanisms for therapeutics are needed. Manning et al., described the innate immune response of Gram-negative bacteria in utilizing OMVs as cellular decoys to remove AMPs through absorption (Manning and Kuehn, 2011). One possible approach to increase the efficacy of AMPs would be to decrease the ability for OMVs to absorb antimicrobial compounds. Identifying compounds which can actively suppress OMV biogenesis would also result in decreased absorption potential for OMVs. Suppression of vesiculation may also prove useful, because OMV production has been observed in all Gram-negative bacteria studied to date and no null-vesiculation mutant has been found. This may indicate that the process of vesiculation is essential for life. If OMV production cannot be prevented, we can use our knowledge of OMVs binding capability to modify our therapeutics so that they only interact with the bacterial cell instead of the highly curved spherical nature of OMVs. Knowing that OMVs bind therapeutics may also provide further impetus to create compounds which do not act at the membrane because OMVs do not absorb non cell wall directed compounds.

OMVs not only function defensively as decoys, but as nucleation sites for biofilm formation due to interaction with extracellular DNA (Schooling and Beveridge, 2006; Schooling et al., 2009). Antibiotic resistance conferred by biofilms is a large clinical

burden, and the ability for OMVs to increase horizontal gene transfer in communities only intensifies the clinical problem. Because OMVs may function as a starting site for biofilm formation (Grenier, 2013), therapeutics could be developed that would decrease the interaction of OMVs with the biofilm matrix. It is thought that the charge interactions with LPS and extracellular DNA facilitate binding leading to biofilm formation (Schooling and Beveridge, 2006). A strategy to coat OMVs, or negate the charged nature of the LPS on OMVs could be a viable option to prevent biofilms from establishing robust resistance to drug treatment.

Finally, it is worth noting that stress and antibiotic treatments do elicit an increased production of OMVs by pathogens such as *P. aeruginosa*. In addition to the defensive capabilities of OMVs, the OMV associated virulence factors such as toxins and degradative enzymes are well documented. Understanding that subinhibitory treatment may cause a greater concentrated release of virulence factors is important, and the potential risks associated with an increased inflammatory response should be assessed prior to drug selection, dosage, and treatment.

6.3 Future directions for OMV experimentation

Despite our increasing knowledge of bacterial OMV production and their contribution to cell-cell and cell-host interaction, a great deal of research remains to be done. Major questions still to be address include identifying what is upregulated in the

cell under OMV inducing conditions, and what contribution specific cargo plays in virulence.

The regulation and production mechanism for bacterial OMVs remains elusive. Conserved pathways and mechanisms of OMV biogenesis have been outlined above, but there is little empirical evidence for a unifying mechanism for OMV biogenesis which consistently apply under all conditions. To gain insight into this question, it will be important for future work to determine what genes and proteins are being upregulated in the entire bacterial cell, and not focused solely on the OMVs themselves. Focusing on the OMVs specifically has granted us the mechanistic insights we have today, but the result remains unsatisfying. As more mechanistic studies are performed and proteomic analyses and whole genome microarray data are incorporated, systems biology may allow us to identify unifying themes for OMV formation which our myopic focus prevented us from seeing before.

A second unresolved question that requires further investigation is determining what contribution OMVs make towards virulence. This issue is complicated because no strain lacking OMVs has been found. We assume that because all bacterial species produce OMVs that they are important, but without a null OMV producing strain, this question is difficult to test. And without a known mechanical basis for OMV production, such a null mutant has yet to be identified. As an extension of this question, we also need to understand better how cargo is selected into OMVs as it is the contents that

contribute to virulence. Cargo selection was demonstrated by the protein profiles of

OMVs being distinct from other cellular compartments, as well as directly determined

by identifying tagged proteins enriched in OMVs (McBroom and Kuehn, 2007). Selective

packaging of toxins and virulence factors into OMVs as a means of secretion has only

been demonstrated for a limited number of toxins and virulence factors such as LT from

ETEC, anthrax toxins from *B. anthracis*, and gingipains from *P. gingivalis* (Haurat et al.,

2011; Horstman and Kuehn, 2000; Rivera et al., 2010). Further studies would need to be

conducted to demonstrate if additional virulence factors, toxins, and defensive enzymes

were being selectively packaged into OMVs resulting in "toxin bombs". To elucidate the

contribution of specific factors associated with bacterial OMVs to the inflammatory

response, individual mutagenesis studies would need to be performed. These

experiments would help to determine the importance of each component on the immune

response instead of the collective synergistic effects of the OMV antigens which may be

too complicated to parse apart.

7. Materials and Methods

7.1 Bacterial Strains and Plasmids

The sources for the *E. coli* and *P. aeruginosa* strains and plasmids used in this

work are presented in Table 5. *P. aeruginosa* PA14 (wild type), and individual gene

mutants the transposon insertion mutant collection (Liberati et al., 2006) were obtained

from the Broad Institute at the University of Massachusetts. The AlgU reporter plasmid

pLW127 containing the AlgU dependent OsmC promoter fused to β-galactosidase was

generously provided by the Ohman Lab (Wood and Ohman, 2009). Plasmid pMF54 was

provided by the Ohman Lab and used for protein expression. pLW127 was transferred

into the appropriate background by triparental mating using the pMT616 helper

plasmid (provided by C.R.H. Raetz). PA14 mutant strains, PA14/pLW127, and *E.

coli*/pMT616 were grown in Lauria Bertani (LB) broth at 37°C containing gentamicin (25

μg/ml), carbenicillin (100 μg/ml), and chloramphenicol (25 μg/ml) respectively. Selection

was done by plating on Pseudomonas Isolation Agar (BD) containing carbenicillin. PA14

pIM001 was created by triparental mating between *E. coli*/pIM001, PA14, and *E.

coli*/pMT616. (Table 5)

7.1.1 Media and Growth Conditions

P. aeruginosa and *E. coli* were cultured in Luria-Bertani (LB) Miller broth (10 g

tryptone, 5 g yeast extract, and 10g NaCl) purchased from Novagen. Overnight growth

cultures contained 5 ml of sterile LB Miller broth in a test tube and were inoculated from

glycerol freezer stocks and grown at 37°C with shaking at 200 rpm for 12-18 hours.

Table 5: Table of strains and plasmids

Strain or Plasmid	Description	Reference
E. coli		
DH5α	*E. coli* cloning strain	Gibco
DH10β	*E. coli* cloning strain	Gibco
Ec233	*E. coli*/pMT616, Cm^R	(Karbarz et al., 2003)
Ec822	*E. coli*/pLW112, Amp^R	(Wood and Ohman, 2006)
Ec869	*E. coli*/pLW127, Amp^R	(Wood and Ohman, 2009)
Ec870	*E. coli*/pMF54, Amp^R	(Mathee et al., 1997)
Ec887	*E. coli*/pIM001, Amp^R	This work
P. aeruginosa		
Pa725	*P. aeruginosa* strain PAO1	(Wood and Ohman, 2009)
Pa831	PA14 $\Delta pqsH$, Gm^R	(Mashburn and Whiteley, 2005)
Pa866	*P. aeruginosa* strain PA14	(Liberati et al., 2006)
Pa867	PA14 $\Delta algU$, Gm^R	(Liberati et al., 2006)
Pa872	PA14/pLW127, Cb^R, Amp^R	This work
Pa874	PA14/pMF54 Gm^R, Cb^R, Amp^R	This work
Pa876	PA14 $\Delta algU$/pMF54, Gm^R, Cb^R, Amp^R	This work
Pa877	PA14 $\Delta algU$/pIM001, Gm^R, Cb^R, Amp^R	This work
Pa879	PA14 $\Delta pqsA$, Gm^R	(Liberati et al., 2006)
Pa891	PA14/pLW112, Cb^R, Amp^R	This work
Pa892	PA14/pIM001, Gm^R, Cb^R, Amp^R	This work
Pa895	PA14 $\Delta oxyR$, Gm^R	(Liberati et al., 2006)
Pa896	PA14 $\Delta oxyR$/pLW127, Gm^R, Cb^R, Amp^R	This work
Pa1300	PA14 $\Delta algD$/pMF54, Gm^R, Cb^R, Amp^R	This work
Pa1301	PA14 $\Delta algD$/pIM001, Gm^R, Cb^R, Amp^R	This work
Pa1302	PA14 $\Delta wbpM$, Gm^R	(Liberati et al., 2006)
Pa1303	PA14 $\Delta wapR$, Gm^R	(Liberati et al., 2006)
Pa1304	PA14 Δrmd, Gm^R	(Liberati et al., 2006)
Pa1305	PA14 $\Delta algD$, Gm^R	(Liberati et al., 2006)
Pa1306	PA14 polymyxin B Resistant	This work
Pa1307	PA01 pLW112, Cb^R, Amp^R	This work
Pa1308	PA14 $\Delta rhlA$, Gm^R	(Liberati et al., 2006)
Pa1309	PA14 $\Delta katB$, Gm^R	(Liberati et al., 2006)
Pa1310	PA14 $\Delta recG$, Gm^R	(Liberati et al., 2006)
Pa1311	PA14 $\Delta ahpB$, Gm^R	(Liberati et al., 2006)
Plasmids		
pMT616	Triparental mating helper plasmid, Cm^R	(Karbarz et al., 2003)
pLW127	pOsmC::LacZ, AlgU-dependent promoter fused to LacZ, Amp^R	(Wood and Ohman, 2009)
pMF54	pMF54 expression vector, Amp^R	(Mathee et al., 1997)
pLW112	pMF54-based MucD expression plasmid, Amp^R	(Wood and Ohman, 2006)
pIM001	pMF54-based expression vector for AlgU, Amp^R	This work

Larger scale growth was performed by inoculating sterile media at a 1:100 or 1:50 dilution from an overnight culture and grown at 37°C with shaking at 200 rpm until the desired growth stage was reached unless otherwise stated. For growth on agar plates, *P. aeruginosa* and *E. coli* can be cultured on LB Miller broth containing 1.5% agar. To selectively grow and isolate *Pseudomonas* from *E. coli*, Difco™ Pseudomonas Isolation Agar (PIA) was used. PIA contains 20 g/L tryptone, 1.4 g/L $MgCl_2$, 10 g/L K_2SO_4, 25 mg/L Irgasan, 20 ml/L glycerol, and 13.6 g/L agar and can be purchased from BD. Experiments containing the PA14 *ΔoxyR* strain were not able to be grown in LB broth or LB plates due to sensitivity to oxygen radicals produced in the media, but could be grown on PIA plates. PA14 *ΔoxyR* was grown in a modified Pseudomonas Isolation broth containing the same ingredients as PIA, but without Irgasan and agar. Brain-heart infusion (BHI) broth (37 g/L) was used for overnight PA14 *ΔpqsA* studies.

7.1.2 Triparental Mating

Plasmids were moved into the *P. aeruginosa* PA14 receptor strain by triparental mating as described (Goldberg and Ohman, 1984). Donor plasmids were typically carried in the *E. coli* DH5α or DH10β background, and the MT616 plasmid served as the helper strain. 500 µl of each strain were combined in a 1.5 ml microfuge tube and pelleted at 16,000 rpm for 1 min and the supernatant was decanted. The cell pellet was resuspended in 500-1000 µl of sterile saline and centrifuged. The cell pellet was resuspended in 50 µl sterile saline and spotted on a LB plate and incubated overnight at

30°C. The bacterial growth was removed with a sterile cotton swab and resuspended in 3 ml sterile saline. Serial dilutions of the suspension were done at 10^{-2} and 10^{-3}. 100 μl of suspension, or serial dilutions were then plated on PIA media with appropriate antibiotics to select for *P. aeruginosa* strains containing the conjugated plasmid and grown overnight at 37°C.

7.2 Stress Treatment

Overnight cultures were used to inoculate 250 ml of LB broth at a 1:50 dilution and grown at 37°C with shaking (200 rpm) to mid-log phase (OD_{600} ~0.4). Cells were then pelleted (10,000 xg, 10 min), and resuspended in fresh 37°C LB media to a final volume of 250 ml. Hydrogen peroxide, D-cycloserine, and polymyxin B were added to the indicated final concentrations (250-1000 μM, 250 μg/ml, and 4 μg/ml, respectively). No stressor was added to negative control cultures. Fresh hydrogen peroxide was added every hour to counteract peroxide degradation. Cultures were grown until an OD_{600} ~0.9-1.1. A portion of the culture was assessed for growth on agar plates to determine colony forming units (CFU/mL).

For temperature stress assays, bacterial cultures were inoculated as described above and grown overnight at 25°C until mid-log phase. Cells were pelleted, resuspended in fresh, pre-warmed (25, 37, or 39°C) LB media, and grown to an OD_{600} of ~0.9-1.1. A portion of the culture was assessed for growth on agar plates to determine CFU/ml.

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7.3 Periplasmic Protein Isolation

PA14/pLW127 was grown at 37°C in 250 ml of LB broth containing 100 μg/ml carbenicillin with shaking (200 rpm) to mid-log phase (OD_{600} ~0.4). Cells were then pelleted (10,000 xg, 10 min), and resuspended in fresh 37°C LB media to a final volume of 250 ml. Hydrogen peroxide, D-cycloserine, and polymyxin B were added to the indicated final concentrations (250 μM, 250 μg/ml, and 4 μg/ml respectively). Cells were pelleted and periplasmic protein was isolated 15-30 min after treatment by osmotic and temperature shock, as follows. The cell pellet was washed in sterile saline and resuspended in periplasmic shock buffer (50 mM Tris-Cl, 200 mM $MgCl_2$ pH 7.3) with alternating incubations at 37°C and 4°C (Jensch and Fricke, 1997). Cells were re-pelleted and the supernatant containing periplasmic contents were filtered through a 0.22 μm syringe filter. Protein concentrations were determined by Bradford assay.

7.4 OMV quantitation

7.4.1 Hard spin protocol

After completion of log phase or overnight growth, the cultures were centrifuged (10,000 x g, 10 min) and the supernatant filter-sterilized through a 0.45 μm membrane to remove any remaining cell debris. OMVs in the cell free supernatants were then pelleted by centrifugation (38,000 x g, ≥1 h) (Horstman and Kuehn, 2000). The OMVs were resuspended in sterile PBS and filtered again through a 0.45 μm filter. Samples were then concentrated by pelleting (100,000 x g, 1 h) and resuspended in 100 μl sterile PBS.

For 25 ml small scale preps, log phase or overnight growth cultures were centrifuged (10,000 x g, 10 min) and the supernatant filter-sterilized through a 0.45 μm syringe filter to remove any remaining cell debris. Cell free supernatants were spun at 50,000 rpm in a Beckman ultracentrifuge rotor Ti50.2 for 80 minutes. The pellets were resuspended in sterile PBS and filtered again through a 0.45 μm spin column filter. Samples were then concentrated by pelleting (100,000 x g, 1 h) and resuspended in 100 μl sterile PBS.

7.4.2 Ammonium sulfate precipitation

After log phase or overnight growth, the cultures were centrifuged (10,000 x g, 10 min) and the supernatant filter-sterilized through a 0.45 μm membrane to remove any remaining cell debris. Cell free supernatants for these cultures were precipitated overnight using 71-75% ammonium sulfate at 4°C with stirring. Precipitated supernatants were centrifuged for 20 min at 10,000 xg and the supernatant was decanted. The precipitate was resuspended in 50 mM HEPES (pH 6.8) buffer by gentle rocking and injected into a 10,000 MWCO dialysis cassette (Thermo Scientific). Samples are dialyzed overnight against 100x volume 50 mM HEPES (pH 6.8) buffer to remove ammonium sulfate. Dialyzed samples were then concentrated to a final volume of 500 μl using 10 kD Amicon centrifugal concentrators (Millipore). 1.5 ml 60% Optiprep was added to each sample to yield a 45% optiprep fraction. Samples were floated in Optiprep gradients (45%, 40%, 35%, 30%, and 20% Optiprep w/w in 10 mM HEPES, 0.85% NaCl

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pH 7.4) as described (Bauman and Kuehn, 2009). A portion of each fraction was analyzed by SDS-PAGE to identify the fractions containing OMPs. These OMV-containing fractions were combined, diluted in 200 ml sterile PBS to remove any remaining Optiprep from the samples, pelleted as described in the above section, and resuspended in sterile PBS.

7.4.3 FM4-64 quantitation

The quantity of OMVs in the resuspended material was assessed using the fluorescent lipophilic dye FM4-64 (Molecular Probes). Probe alone and sample alone were used as controls. Fluorescence was measured at 506 nm (excitation) and 750 nm (emission) to obtain relative fluorescence units/mL (RFU/ml). RFU/ml was then divided by colony forming units (CFU/ml) determined at the time of vesicle harvest to generate the OMV yield (RFU/CFU). The RFU/CFU of OMV preparations from treated cultures was then divided by the RFU/CFU of OMV preparations from untreated cultures to determine fold OMV induction.

7.4.4 Outer membrane protein quantitation

OMV samples were run on 15% SDS-PAGE until completion and stained with Sypro Ruby Stain as described. Previously identified OMPs in *P. aeruginosa* OMVs include OprD, PA1288, OprF, OprG, and OprH (Bauman and Kuehn, 2006). Using the ImageJ software, the densitometry of each band was quantitated. If OMVs were density gradient purified, quantitation of the entire lane could also be performed. Hard spun

samples were assay by performing densitometry on OprF, OprD, and PA1288. The band densitometry of samples loaded by equal volume was normalized to OD_{600} or CFU. If CFU was known prior to determining OMP densitometry, samples were loaded by equal CFU.

7.5 AlgU reporter assay

To determine the activity of AlgU, 25-50 μl of each culture containing the pLW127 vector were lysed in a buffered solution containing 0.1% SDS and chloroform. The chromogenic substrate ONPG (*ortho*-Nitrophenyl-β-galactoside) (4 mg/ml) was used for this assay. The assay reaction was quenched by adding 1 M Na_2CO_3. AlgU reporter activity was quantified by measuring the absorbance of the solution at 420 nm and 550 nm. Each sample was done in triplicate to account for assay error and normalized to CFU/ml and assay duration in minutes.

7.6 Protein expression

7.6.1 AlgU expression

AlgU was amplified from PA14 genomic DNA by PCR and added restriction endonuclease sites for (Xba1 and Xho1) using the forward primer (5′-AATCTAGAATGCTAACCCAGGAACAGGATCA-3′) and the reverse primer (5′-ATCTCGAGTCAGGCTTCTCGCAACAAAGGCT-3′). The AlgU fragment was then ligated into the pMF54 expression vector provided by the Ohman Lab to create pIM001. pIM001 was then transformed into *E. coli* DH10β by electroporation and grown on LB

ampicillin plates to select for transformants. The pIM001 plasmid was then moved into

both the PA14 $\Delta algD$ and PA14 $\Delta algU$ background by triparental mating. AlgU

expression was induced with 200 µM IPTG at mid-log phase growth. AlgU induction

was assessed by immunoblotting with the cross-reactive rabbit anti-RpoE primary

antibody (kindly provided by C. Gross). To determine relative fold AlgU induction,

equal volumes of treated and untreated cultures were separated by SDS-PAGE,

immunoblotted, and the intensity of the immunoreactive AlgU bands in the samples

compared by densitometry.

7.6.2 MucD expression

PA14/pLW112 was grown at 37°C in 250 ml of LB broth containing 100 µg/ml

carbenicillin with shaking (200 rpm) to mid-log phase (OD_{600} ~0.4). Cells were then

pelleted (10,000 xg, 10 min), and resuspended in fresh 37°C LB media to a final volume

of 250 ml. Cultures were induced with 1 mM IPTG and stressed with 250 µM H_2O_2 (and

added hourly as indicated previously) when appropriate.

7.7 LPS purification

7.7.1 Ethanol precipitation

P. aeruginosa LPS was isolated by ethanol/Mg^{2+} precipitation as described

(Darveau and Hancock, 1983). Dried, cell pellets were resuspended in 15 ml 10 mM Tris-

HCl (pH 8.0) buffer containing 2 mM $MgCl_2$, DNase I and RNase A, and passed twice

through a French pressure cell at 15,000 lb/in². To ensure complete cell breakage, the cell

lysate was sonicated for two 30 sec bursts. Samples were incubated at 37°C for two hours with additional RNase A (50 μg/ml) and DNase I (200 μg/ml). Tetrasodium EDTA and SDS were added to reach a final volume of 25 ml and concentration of 2% SDS-0.1M tetrasodium EDTA, dissolved in 10 mM Tris-HCl (pH 8.0). Samples were vortexed to solubilize LPS. To remove peptidoglycan, the samples were centrifuged at 50,000 xg for 30 min at 20°C. The supernatant was decanted and incubated with pronase at 37°C overnight with shaking.

Two volumes of 0.375 M $MgCl_2$ in 95% ethanol were added and cooled to 0°C in a -20°C freezer. After cooling, the samples were centrifuged at 12,000 xg for 15 min at 0-4°C. The pellet was resuspended in 2% SDS-0.1M tetrasodium EDTA, dissolved in 10 mM Tris-HCl (pH 8.0) and sonicated to resuspend. Samples were incubated at 85°C for 10-30 min to denature protein. After cooling, pronase (25 μg/ml) was added and incubated at 37°C overnight with shaking. After incubation, LPS was precipitated in 0.375 M $MgCl_2$ in 95% ethanol and pelleted as described above. The pellet was resuspended in 10 mM Tris-HCl (pH 8.0) by sonication and centrifuged at 1,000 rpm for 5 min to remove insoluble Mg^{2+}-EDTA crystals. Finally, the supernatant was centrifuged at 200,000 xg for two hours at 15°C in the presence of 25 mM $MgCl_2$. If Mg^{2+}-EDTA crystals contaminated the pellet, the pellet was resuspended the pellet in buffer containing 25 mM $MgCl_2$ and centrifuged repeatedly until crystals were gone. The final LPS pellet was resuspended in deionized water.

7.7.2 Phenol/chloroform/light petroleum extraction

LPS was also purified by hot phenol/chloroform extraction as described (Brade and Galanos, 1982). *P. aeruginosa* cell pellets were frozen and dried by lyophilization prior to organic LPS extraction. Cell pellets were suspended in 1 volume 90% aqueous phenol at 65°C and homogenized by vortexing. 1.6 volumes of light petroleum and 1 volume of chloroform were added and warmed to 65°C and homogenized by vortexing for one minute. The samples were cooled at room temperature with continuous rotation. The suspension was centrifuged at 5,000 xg for 30 min and the supernatant was decanted. Light petroleum and chloroform were removed by evaporation in a heated vacuum container overnight. LPS was precipitated from the phenol solution by addition of deionized water and centrifuged to pellet the precipitate. LPS was washed twice with 80% aqueous phenol and several times with acetone to remove residual phenol. The remaining LPS was resuspended in deionized water.

7.8 Lipid A extraction and TLC plating

Lipid A was extracted from 500 μl purified LPS by acid hydrolysis. LPS was added to a sealed glass tube and hydrolyzed at 100°C for one hour in the presence of 2% acetic acid to cleave Kdo-lipid A linkages. Lipid A was then extracted in a two phase Bligh/Dyer system using $CHCl_3$/MeOH/H_2O (2:2:1.8 v/v). After thorough mixing, samples were centrifuged at 1,000 rpm for 5 min to separate aqueous and organic phases. The lower organic phase was removed and washed with fresh upper phase and

dried under nitrogen. Samples were dissolved in $CHCl_3$/MeOH (4:1 v/v). 10 μl and 5 μl of each sample was spotted on glass backed silica gel 60 thin-layer chromatography (TLC) plates (EMD) and run in a solvent solution of $CHCl_3$/pyridine/88% formic acid/water (50:50:16:5 v/v). TLC plates were sprayed with sulfuric acid and charred on a hot plate to visualize lipid A species (Brade and Galanos, 1982; Gibbons et al., 2005).

7.9 KDO assay

LPS quantities were normalized by determining the KDO present in each sample. 50 μl of each sample was added to 50 μl 0.5 N H_2SO_4 in a microfuge tube and vortexed. Samples were heated on a heating block for 10 min at 100°C. After cooling, 50 μl H_5IO_6 (2.28 g in 100 ml H_2O) was added, vortexed, and allowed to sit for 10 min. 200 μl arsenite reagent (2.0 g in 50 ml 0.5N HCl) was added and vortexed followed by 800 μl thiobarbituric acid reagent (150 mg in 25 ml H_2O) and mixed. Samples were moved to 15 ml conicle tubes and placed in 100°C water bath for 10 min. Samples were cooled to room temperature and 1.5 ml of butanol reagent (5.0 ml of HCl added to 95 mL n-butanol) was added and vortexed. Samples were centrifuged at 2,000 rpm for 5 min in a clinical centrifuge. 800 μl was taken from the upper butanol layer and placed in a cuvette to be read at OD_{552}.

To create a standard curve, 0, 10, 20, 30, 40, and 50 μl samples of a 200 μg/ml standard were used. All starting volumes were brought to 50 μl by adding deionized water.

7.10 SDS-PAGE and immunoblotting

Samples were prepared by boiling in standard denaturing and reducing SDS-PAGE sample buffer, and separated using a 15% polyacrylamide gel at 120 Volts until complete. Lanes were equally loaded according to total protein content as determined by Bradford Assay.

Gels were transferred to nitrocellulose for immunoblotting for a minimum of 30 min at 15 volts. After blocking with Odyssey Blocking Buffer (Licor), primary antibody was added at a 1:5,000 dilution for one hour to overnight at 4°C. Following membrane washing, Odyssey fluorescent antibody (goat anti-rabbit IRDye® 800CW) was diluted to a working concentration of 1:20,000 according to manufacturer protocol. Immunoblots were analyzed by Odyssey Imaging-system by Licor-Biosciences.

7.10.1 Protein staining

OMV or protein samples run on 15% SDS-PAGE were stained with Sypro Ruby Stain (Molecular Probes). The gel was fixed in a solution of 50% methanol and 7% acetic acid for two 15 min fixation wash steps. The gel was stained with Ruby Stain according to the manufacturer protocol. Following staining, the gel was washed in a solution of 10% methanol and 7% acetic acid. Gels were rinsed in deionized water and exposed on a UV light box so that the fluorescent dye could be visualized and captured via digital camera.

LPS samples run on 15% SDS-PAGE were visualized by silver stain (BioRad). The gel was fixed in a solution of 50% methanol, 10% acetic acid, 10% fixative enhancer, and 30% deionized water for 20 min with shaking. The gel was then rinsed in deionized water 2x10 min. The gel was stained and developed according to the manufacturer protocol until the bands had developed to the desired amount. Following staining, gel development was stopped by washing the gel in a 5% acetic acid solution. Gels were rinsed in deionized water and visualized.

7.11 Electron microscopy

In advance, 400 mesh copper grids with carbon films deposited on them (Electron Microscopy Sciences, #CF400-cu) were cleaned via glow discharge for 1.5 min on a Harrick Plasma Cleaner (PDC-32G). Samples were prepared by applying 10 μl to the grid and incubated 2 min, grids were then washed with 5 drops of 1% aqueous uranyl acetate (Electron Microscopy Sciences). The last drop was left to incubate on the grid for 1.5 min before being wicked off by torn filter paper. Grids were left to dry for 5 min before being viewed on a Tecnai 12 by FEI with a 1024x1024 Gatan Multi-Scan Camera model 794.

7.12 Statistical analysis

All experimental data was performed with a minimum of three biological replicates. Statistical significance of data was determined by using a Student's T-test. Raw data was combined across experimental trials. The averages for the untreated and

treated cultures were compared to determine relative fold change. For experiments

using the lipophilic dye FM4-64, the fluorescence of the probe alone in PBS was

subtracted from all raw data. Immunoblot densitometry values consist of the integrated

intensity of the gel band or area highlighted as determined by the Licor Odyssey

software. Densitometry of SDS-PAGE bands/lanes were performed by ImageJ. Error

bars are representative of standard error.

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Biography

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Publications

Chutkan, H., **MacDonald, I**., Manning, A., and Kuehn, M.J. (2013). Quantitative and qualitative preparations of bacterial outer membrane vesicles. Methods in molecular biology *966*, 259-272.

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