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Is approved by the final examining committee:

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ROLE OF A PUTATIVE BACTERIAL LIPOPROTEIN IN *PSEUDOMONAS AERUGINOSA*-MEDIATED CYTOTOXICITY TOWARD AIRWAY CELLS.

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LIST OF ABBREVIATIONS

CF	Cystic Fibrosis
°C	Degrees Celsius
CFBE	Cystic Fibrosis Bronchial Epithelial
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
ΔF508	Deletion of Phenylalanine at Position 508
FBS	Fetal Bovine Serum
HCl	Hydrogen Chloride
HRP	Horseradish Peroxidase
LB	Luria-Bertani or Lysogeny Broth
LDH	Lactate Dehydrogenase
LPS	Lipopolysaccharide
CO2	Carbon Dioxide
MEM	Minimal Essential Medium
$MgSO_4$	Magnesium Sulfate
PA14	*Pseudomonas aeruginosa* Strain 14
PCR	Polymerase Chain Reaction
PBS	Phosphate-Buffered Saline
pH	Potential Hydrogen
YPD	Yeast Peptone Dextrose

ABSTRACT

Akhand, Saeed S. M.S., Purdue University, August 2014. Role of a putative bacterial lipoprotein in *Pseudomonas aeruginosa*-mediated cytotoxicity towards airway cells. Major Professor: Gregory G. Anderson.

The patients with Cystic Fibrosis (CF), an inherent genetic disorder, suffer from chronic bacterial infection in the lung. In CF, modification of epithelial cells leads to alteration of the lung environment, such as inhibition of ciliary bacterial clearance and accumulation of thickened mucus in the airways. Exploiting these conditions, opportunistic pathogens like *Pseudomonas aeruginosa* cause lifelong persistent infection in the CF lung by forming into antibiotic-resistant aggregated communities called biofilms. Airway infections as well as inflammation are the two major presentations of CF lung disease. *P. aeruginosa* strains isolated from CF lungs often contain mutations in the *mucA* gene, and this mutation results in higher level expression of bacterial polysaccharides and toxic lipoproteins. In a previous work, we have found a putative lipoprotein gene (*PA4326*) which is overexpressed in antibiotic-induced biofilm formed on cultured CF-derived airway cells. In the current work, we speculated that this particular putative lipoprotein affects cellular cytotoxicity and immune-stimulation in the epithelial cells. We found that mutation of this gene (Δ*PA4326*) results in reduced airway cell killing without affecting other common virulence factors.

Moreover, we observed that this gene was able to stimulate secretion of the pro-inflammatory cytokine IL-8 from host cells. Interestingly, we also found that Δ*PA4326* mutant strains produced less pyocyanin exotoxin compared to the wild type. Furthermore, our results suggest that *PA4326* regulates expression of the pyocyanin biosynthesis gene *phzM*, leading to the reduced pyocyanin phenotype. Overall, these findings implicate *PA4326* as a virulence factor in *Pseudomonas aeruginosa*. In the future, understating the molecular interplay between the epithelial cells and putative lipoproteins like PA4326 may lead to development of novel anti-inflammatory therapies that would lessen the suffering of CF patients.

CHAPTER 1 INTRODUCTION

1.1 Cystic Fibrosis

Cystic fibrosis (CF) is the most common inherited genetic disorder of Caucasians. One in 3500 children are born with CF in United States (Rohlfs et al. 2011). At present, the average life expectancy of an individual with CF is 41.1 years (CFF 2012). Although advanced lung disease is the main reason for most CF related deaths, CF also affects other organs such as the pancreas, liver, and intestine (Quinton 2007; Hardin 2004; O'Malley 2009; Makker et al. 2009). The common signs and symptom of CF are shortness of breath, salty tasting skin, accumulation of thick mucus in the airways, poor growth, and infertility.

This deadly genetic disease is the result of mutations in a gene that encodes for the cystic fibrosis transmembrane conductance regulator (CFTR). Though there are around 1500 mutations that lead to altered CFTR function, deletion of phenylalanine at the 508^{th} position of the protein (ΔF508) is the most common one (Bobadilla et al. 2002). Primarily, CFTR functions as a chloride ion channel and is located in epithelial cells throughout the body. Loss of function of CFTR results in dramatic changes in the CF airway lining and leads to chronic bacterial infection and excessive inflammation (Luciani et al. 2010; Pier 2002). These complications eventually cause the loss of lung

function and ultimate death in patients with CF. Currently, there is no cure for CF. Lack of appropriate animal models of the disease makes it difficult to study the disease *in vivo*. Moreover, the available antibiotics are unable to eradicate the persistent bacterial infection in the CF lung due to the rapid development of drug resistance.

1.2 Inflammation in the CF Lung

Airway inflammation greatly influences the morbidity and mortality in CF patients. Though eosinophils, lymphocytes, and monocytes may be involved and play roles, excessive influx of neutrophils is the hallmark of airway inflammation in CF (Collins et al. 2012; Tan et al. 2011; Sorio et al. 2011; Rosenfeld et al. 2001). Neutrophil elastase (NE), found to be elevated in CF airways early in life, damages structural tissue components and is associated with lung function decline (Sagel et al. 2012). Others mediators such as IL–17, IL-8, IL-6, IL-1β are also detected in increased levels in CF (Armstrong et al. 2005; Tan et al. 2011). Over-activated NF-κB in the epithelial cells enhances the synthesis of these chemokines and pro-inflammatory cytokines in the CF airway (Kube et al. 2001; Venkatakrishnan et al. 2000; DiMango et al. 1998; Barnes & Karin 1997). Unfortunately, compared to normal individuals, airway secretions from CF patients are deficient in the immunoregulatory cytokine IL-10, which results in prolonged neutrophil survival and NF-κB activation (Bonfield et al. 1999; Bonfield et al. 1995; Moore et al. 2001; Osika et al. 1999; Lentsch et al. 1997; Schottelius et al. 1999). This lack of balance between pro-inflammatory and anti-inflammatory cytokines, along with chronic microbial infection, plays a vital role in lung inflammation in CF disease.

1.3 Microbial Infection in the CF Lung

Along with *CFTR* mutation, chronic pulmonary infection is the life-threatening clinical feature that ultimately results in death in CF patients. Chronic bacterial infection and concomitant airway inflammation account for 80 to 95% of patients' respiratory failure in CF (Lyczak et al. 2002). The exact mechanism of how altered CFTR leads to wide-spread bacterial infection is still an enigma. However, several hypotheses have been experimentally proposed, such as I) decreased ion transport results in decreased ciliary clearance of bacteria (Matsui et al. 1998), II) altered salt content in airway surfaces inactivates antimicrobial defensins and negatively affects the neutrophil-mediated bacteria killing (Smith et al. 1996), III) increased level of asialyated glycoprotein in airway surface acts as a receptor for *P. aeruginosa* (Saiman & Prince 1993), and IV) the intrinsic hyper inflammatory environment in the airway hinders the appropriate clearance of microbes (Konstan 1998). Regardless of the mechanism, it is obvious that microorganisms are capable of taking the full advantage of altered airway tissue and gradually damage the pulmonary function by causing chronic persistent infection (Hauser et al. 2011).

The bacterial species that are usually found and play a role in CF pathogenesis are *Haemophilus influenzae, Staphylococcus aureus, P. aeruginosa, Burkholderia cepacia, Stenotrophomonas maltophilia, Achromobacter xylosoxidans (Ciofu et al. 2013)*. In addition to bacteria, fungi (*e.g. Aspergillus* species) and viruses are found to infect the CF lung (Wat et al. 2008; Jubin et al. 2010). *S. aureus* is the most prevalent infecting microbe in early ages. However, with age, *P. aeruginosa* becomes more prevalent, and by

late twenties, *P. aeruginosa* is found in approximately 80% of the patients (CFF patient registry 2012), and it is believed to be associated with the worst outcomes in CF patients. As the disease progresses, the infecting organisms undergo rapid adaptation to the changing lung environment to evade the host immune response. Constant oxidative stress and antibiotic treatment induce mutation in resistance genes and even induce establishment of the recalcitrant biofilm lifestyle of the infecting bacteria (Hoffman et al. 2005; Kaplan et al. 2012; Kaplan 2011).

1.4 *Pseudomonas aeruginosa*

The bacterium *P. aeruginosa* belongs to the Gammaproteobacteria class and is a pathogen of plants and animals, including humans (BH 1996). Generally, this Gram-negative bacterium is classified as an aerobic organism but it has the ability to grow in partial or even in total oxygen-depletion conditions (Alvarez-Ortega & Harwood 2007; Schobert & Jahn 2010; Eschbach et al. 2004). Due to its ability to adapt to diverse environments, this ubiquitous organism can be found in water, soil, skin, and even in medical equipment, including catheters (Trautmann et al. 2005; Frimmersdorf et al. 2010). Its large genome size (6.4 million base pairs encoding approximately 5500 genes) likely supports the versatility of the microorganism (Stover et al. 2000).

While generally harmless in healthy hosts, this opportunistic organism causes medical complications in immunocompromised individuals such as severe pneumonia in CF patients, septic shock in neutropenic patients, urinary tract infection, gastrointestinal infection in premature infants, and skin/soft tissue infection in burn and wound patients

(Lyczak et al. 2000). Moreover, it has been estimated that this bacterium accounts for 10% of hospital-acquired infections in adults as well as in children (Raymond 2006).

P. aeruginosa produces a myriad of virulence factors such as pili, adhesins, exoenzyme S, exotoxin A, phospholipase C, various proteases, siderophores, and alginate that enable it to cause both acute and chronic infections (Ben et al. 2011). This often-called 'Superbug' is hard to eradicate because of its high level intrinsic resistance to diverse antibiotics (Breidenstein et al. 2011). Low permeability of the outer membrane, mutations affecting resistance genes, and development of adaptive resistance to antimicrobials have made this bacterium a formidable one to tackle (Dötsch et al. 2009; Alvarez-Ortega et al. 2010; Fajardo et al. 2008; Nicas & Hancock 1983).

In addition to these numerous virulence traits, *P aeruginosa* can develop into biofilms, which provides the bacteria with another level of protection against current antibiotic regiments (Costerton et al. 2011). Biofilms are defined as bacterial communities, attached to living or abiotic surfaces, that are surrounded by a matrix of exopolysaccharides, DNA, and proteins (Hall-Stoodley et al. 2004). Many bacteria use a biofilm mode of growth as a survival strategy because it provides protection against antibacterial agents (Jefferson 2004). It is believed that the lack of treatment efficacy in CF is due to the formation of antibiotic resistance biofilm in the CF Lung (Moreau-Marquis, Stanton, et al. 2008).

1.5 Pyocyanin Toxin

Among a wide variety of virulence factors, pyocyanin (PYO) is one of the most important ones in *P. aeruginosa* (B Rada & Leto 2013). This heterocyclic, nitrogen containing blue pigment is produced from chorismic acid and secreted during stationary phase of the cultures (Nadal Jimenez et al. 2012). This redox-active compound can freely move across the biological membrane and is capable of accepting and donating electrons (Rada et al. 2008). PYO increases the bioavailability of iron, required for biofilm formation, by creating a redox potential gradient around the biofilm surfaces (Koley et al. 2011; Moreau-Marquis, Bomberger, et al. 2008). PYO can also affect the colony morphology and function as a signaling molecule in quorum sensing networks in *P aeruginosa* (Dietrich et al. 2006; Dietrich et al. 2008).

In various host organisms, PYO has been found to be essential for the full virulence of *P. aeruginosa*. In CF, it has been reported to be involved with the lung function degradation, disease severity, and dominance of *P. aeruginosa* infection (Hunter et al. 2012; Wilson et al. 1988). Moreover, PYO can also cause oxidative stress and pro-inflammatory stimulation in airway epithelial cells that are characteristic of chronic *P. aeruginosa* airway infection (Schwarzer et al. 2008; O'Malley et al. 2003). In addition to that, PYO has also been reported to induce mucin hyper-secretion from the airway epithelium, which is another prominent feature of CF lung disease (Rada et al. 2011).

1.6 Hypothesis

One of the commonly found mutations in CF *P aeruginosa* isolates (in the *mucA* gene) results in constitutive activity of the AlgU sigma factor, which eventually leads to high level production of biofilm matrix polysaccharide (Boucher et al. 1997; Pulcrano et al. 2012). It is thought that prolonged exposure to inflammatory pressures induces mutation of *mucA*. It has been reported that this inflammation-stimulated biofilm formation of *P aeruginosa* due to mutation in the *mucA* gene correlated with over-expression of lipoprotein genes (Firoved et al. 2004).

P aeruginosa encodes around 175 predicted lipoproteins which accounts for ~3.2% of the whole genome (Remans et al. 2010) and *Pseudomonas* Genome Database classifies 60% of these lipoproteins as hypothetical proteins (Winsor et al. 2009; Remans et al. 2010). Lipoproteins have been found to play important role in the pathogenesis of many bacterial infections (Kovacs-Simon et al. 2011). Several antigen presenting cells can recognize lipoproteins via Toll-like receptors results in activation of pro-inflammatory cytokines (Liang et al. 2005). Studies have shown that *P. aeruginosa* lipoproteins, in particular, induce toxic effects on the airway epithelium (Firoved et al. 2004).

Previously, in a microarray study, we observed that tobramycin treatment of *P. aeruginosa* biofilm on cystic fibrosis-derived human bronchial epithelial cells (CFBE cells) cultured human CF-derived airway cells (CFBE cells) led to overexpression of the gene *PA4326* (Anderson et al. 2008). This gene is predicted to encode a lipoprotein (Winsor et al. 2011). In our current study, we explored the role of this putative lipoprotein PA4326 in *P aeruginosa*-mediated cytotoxicity towards airway cells. We

found that mutation of *PA4326* resulted in decreased killing of cultured airway cells and diminished secretion of IL-8 from these cells. These phenotypes correlated with decreased production of pyocyanin. These results suggest that disruption of PA4326 can dramatically affect the virulence and immmuno-stimulatory properties of *P. aeruginosa*.

CHAPTER 2 METHODS AND MATERIALS

2.1 Bacterial Strains and Plasmid

In Table 1 and Table 2, all the bacterial strains and plasmids used in this study are provided. Unless otherwise noted in the text, for all the experiments *P. aeruginosa* strains were cultured in LB media supplemented with suitable antibiotics at 37^0C overnight and yeast was grown in YPD (Yeast Peptone Dextrose).

2.2 Gene Manipulations

2.2.1 Deletions of Gene

Utilizing an allelic replacement method as described previously by Shanks *et al.* 2006, we constructed the isogenic deletion mutant strains (Shanks et al. 2006). In short, 4326Lfor/4326Lrev and 4326Rfor/4326Rrev primers (Table 3) were used in PCR reaction to amplify ~1,000 bp flanking both sides of the *PA4326*. The amplified PCR product and an EcoRI cut pMQ30 plasmid vector were transformed in *S. cerevisiae* IncSc1 (Shanks et al. 2006). pMQ30 is a suicide vector for *P. aeruginosa*, and the PCR primers contained regions of homology with this plasmid such that after transformation,

S. cerevisiae directed recombination of the PCR fragments into the vector. The resultant deletion plasmid was electroporated into Escherichia coli S17-1. Conjugations were performed with transformant S17-1 and *P. aeruginosa* PA14 (Kuchma et al. 2005). LB plates with 5x gentamycin (1000x stock: 10 mg/ml) and 1x nalidixic acid (1000x stock: 20 mg/ml) were used to select the successful conjugants. During conjugation, by a single-crossover recombination step, the deletion constructs were joined at the gene locus of the chromosome. The strains were plated on selective LB plates (10% sucrose) to yield the strains with successful loss of *sacB* gene from the suicide vectors as a result of successful deletion of insert. Finally, using PCR with 4326for/4326rev primers (Table 3), the isogenic deletion mutant strains were validated.

2.2.2 Gene Complementation

A pair of primers 4326Comfor/4326Comrev was used to PCR amplify the gene of interest as well as ~500 bp upstream of the start codon; this region likely carries the natural promoter for *PA4326*. Then, an EcoR1-cut PCR fragment was ligated with EcoR1 digested vector plasmid pMQ72 (Shanks et al. 2006) and transformed into *E. coli* DH5α. The isolated complementation plasmid pSSA004 was transformed onto Δ*PA4326* using electroporation.

2.3 Growth Curve

Cultures were grown overnight at 37^0C in LB media, diluted 1:100 into fresh medium and sampled in triplicate in 96–well microtiter plates. Growth was measured by absorbance at 600nm in a spectrophotometer every 30 min for 12 hours. Samples were kept at 37°C throughout the assay.

2.4 Tissue Culture

Cystic fibrosis derived bronchial epithelial (CFBE) cells were used for this study (Cozens et al. 1994). Minimal essential medium (MEM 1x with 10% FBS, 50U/mL penicillin, and 50µg/mL streptomycin) was used for feeding the CFBE cells every 2-3 days, and incubated at 37^0C in 5% CO_2. The cells were harvested at 37^0C in 5% CO_2 with growth media. Confluent monolayers of CFBE cells were grown in 24-well plates prior to the experiments (Anderson et al. 2008). Aseptic technique was maintained throughout the CFBE cell culture procedure.

2.5 Cytotoxicity Assays

For cytotoxicity assays, CFBE cell monolayers in 24-well plates were inoculated with $1.2x10^7$ CFU/mL in 0.5ml MEM/well without FBS, penicillin, streptomycin, and phenol red and incubated at 37°C for 1 hour. Then, the medium was removed and replaced with fresh medium containing 0.4% arginine, whereupon the co-culture was

incubated at 37°C for an additional 5 hours. Cytotoxicity was measured as a percent release of lactate dehydrogenase (LDH), using the CytoTox96 kit (Promega, Madison, WI) as directed.

2.6 Flow Cytometry

Bacteria-induced apoptosis was analyzed using a flow cytometry method. In brief, CFBE cells were incubated with bacteria at 37 ^{0}C for 3.5 hours, and then trypsinized for 15 minutes. Cells were collected, centrifuged in tubes, and resuspended in 200µl of 1x Binding Buffer (5x Binding Buffer: 50 mM HEPES, 700 mM NaCl, 12.5 mM CaCl2, pH 7.4). After that, PE-conjugated anti-human antibody D34 monoclonal antibody, annexin V-FITC, and 7 aminoactinomycinD (7AAD) were added to the tubes. After incubating for 15 min in the dark at room temperature, the cells were analyzed in a BD FACSCalibur cell analyzer. This method detects the viable cells (negative for both annexin V and 7AAD staining), early apoptotic cells (positive for annexin V but no 7AAD uptake), and late apoptotic/necrotic cells (binding with annexin V as well as positive for 7AAD uptake as a result of cytoplasmic disruption) in a sample.

2.7 Enzyme-Linked Immunosorbent Assay (ELISA) for IL-8

To measure IL-8 levels, we infected CFBE cells with *P. aeruginosa* for 3.5 hours to allow biofilm formation, as previously described (Anderson et al. 2008). The

supernatant was then collected and IL-8 was quantified using a Human IL-8 CytoSet™ ELISA kit (Invitrogen, Camarillo, CA) following the manufacturer's protocol.

2.8 RT-PCR Analysis

For determining the level of *phzM* transcripts, we grew our bacterial isolates 24h at 37°C in LB media. We harvested RNA, synthesized cDNA, and performed semiquantitative RT-PCR according to described methods (Mah et al. 2003; Kuchma et al. 2005), using primer pair phzMforRT/phzMrevRT (Table 3) at 56°C annealing temperature. We utilized *fbp* (fructose bisphosphatase gene) as a normalization control because, irrespective of growth condition, we found the same transcript level for *fbp* in several lab datasets (Anderson et al. 2008).

2.9 Pyocyanin Assay

Pyocyanin was measured with chloroform extraction methods (Essar et al. 1990). To enhance pyocyanin production, bacterial strains were grown overnight in King A medium at 37^0C (KING et al. 1954). To determine the level of secreted pyocyanin, 500 µl of chloroform was mixed with 1ml sample of the culture supernatant, and the lower organic layer was extracted. 500 µl of 200mM tris-HCl (pH 8.0) was added and the organic layer, rich in pyocyanin, was collected. The level of pyocyanin was determined by multiplying the A_{376} of the separated lysate with a factor of 56. To quantify the pyocyanin within the cells, pelleted cells from 250ml of overnight culture were washed

with diH_2O. Afterwards, the washed cells were diluted with fresh media and mixed with 500 µl of chloroform before proceeding with quantification as above.

2.10 *Staphylococcus aureus* Killing Assays

Overnight-grown bacterial cultures of wild type and mutant ΔPA4326 were spotted (3 µl) onto freshly swabbed LB plates with *Staphylococcus aureus* MZ100. After 20-24 h of incubation at 37°C, the zones of inhibition were observed for each of the cultures.

2.11 Bioassay of C_4-HSL

Reporter strains *P. aeruginosa* PAO-JP2 containing plasmid pECP61.5 was used for this assay. pECP61.5, which functions as a reporter of C_4-HSL, contains an *rhlA-lacZ* fusion along with the *rhlR* gene under strong promoter *tac* (P_{tac})(Pearson et al. 1997). In brief, overnight grown *P. aeruginosa* PAO-JP2/pECP61.5 strains were subcultured in 5ml PTSB (5.0% peptone, 0.25% TSB & pH 7.0) media for 2 hours with added test supernatants. Finally, the β-galactosidase activity was measured according to previously described protocol (Miller 1972).

2.12 Biofilm Formation

The crystal violet assays (O'Toole & Kolter 1998) were performed to assess the bacterial biofilm formation on polyvinyl chloride (PVC) plastic microtiter plates. Minimal M63 media supplemented with 0.4% arginine was used as biofilm media.

2.13 Motility Assays

Bacterial swimming motility was determined by pipetting 2 µl of bacterial culture into LB 0.3% agar plates. After incubation for 20 to 24 hours at 37°C the swimming zones were measured. Bacterial swarming ability was measured following a previously described method, using M5 medium plates with phosphate buffer, 1 M magnesium sulfate, 1M calcium chloride, and 20% casamino acids, with 0.4% agar (Tremblay & Deziel 2008). At first, 2 µl of bacterial culture were dropped onto the surface of the plates, and then the plates were incubated at 37°C overnight. After that, the swarming plates were incubated for 48h at room temperature.

2.14 Statistical Analyses

'Mean ± standard deviation' calculation was used to present the results. Student's *t*-tests were calculated to find out statistically significant variations among the mean results. A P-value < 0.05 was considered statistically significant.

CHAPTER 3 RESULTS

3.1 *PA4326* encodes a likely lipoprotein

PA4326 is annotated on the *Pseudomonas* Genome Database (Winsor et al. 2011) as a putative lipoprotein. We confirmed that the protein hypothetically encoded by this gene contains a putative type II export signal, commonly found in lipoproteins, and this protein has been predicted to be lipoprotein by LipoP server (Fig. 1).

3.2 *PA4326* is involved in cytotoxicity

Interestingly, we found that isogenic deletion mutation of *PA4326* results in reduced cell killing of CFBE monolayers compared to wild type (Fig. 2). Additionally, we observed that the Δ*PA4326* mutant is less cytotoxic toward CFBE epithelial cells than the wild type, as measured by release of lactate dehydrogenase (LDH) from the airway cells (Fig. 3). Next, we complemented the cytotoxic phenotype of the Δ*PA4326* strain with plasmid p*4326* and measured the cytotoxicity. A shown in Figure 4, wild type strain PA14 with empty control vector pMQ72 showed more cytotoxicity than the mutant strain Δ*PA4326* pMQ72. We also found that the complemented Δ*PA4326* p4326 strain exhibited partially restored cytotoxic effects on the CFBE cells compared with the mutant

with empty vector (ΔPA4326 pMQ72).To rule out the possibility of any growth defect of the Δ*PA4326* gene, we performed bacterial growth curves in LB broth. We found that Δ*PA4326* grew similarly as the wild type strain PA14 (Fig. 5).

3.3 *PA4326* mediates cell death patterns

Because the *PA4326* gene was found to be involved in cellular cytotoxicity in the co-culture system, we intended to investigate lipoprotein-mediated cell death patterns of the CFBE monolayer. To understand the cell killing (apoptosis and necrosis), we used flow cytometry to measure fluorescent intensity of CFBE cells stained with anti-CD34, annexin V, and 7 aminoactinomycin D (7-AAD) (Abrahamsen et al. 2002). This method detects the viable cells (negative for both annexin V and 7AAD staining), early apoptotic cells (positive for annexin V but no 7AAD uptake), and late apoptotic/necrotic cells (binding with annexin V as well as positive for 7AAD uptake as a result of cytoplasmic disruption) in a sample. After 3.5 hours of incubation with wild type and Δ*PA4326* (Fig. 6), the percent of early apoptotic cells was same for both the wild type (53.48%) and mutant (53.73%) infected cells. However, the percent of nonviable late necrotic/apoptotic cells was higher for wild type stimulated cells (38.75%) compared to Δ*PA4326* stimulated cells (29.26%). This data suggests that the PA4326 putative lipoprotein is involved in active cell killing, which is coherent with our earlier results and supports our hypothesis on its role in cellular tissue destruction.

3.4 *PA4326* stimulates IL–8 production.

It has been reported that purified lipoproteins stimulate cytokine secretion from epithelial cells (Firoved et al. 2004). This result led us to wonder whether the putative lipoprotein PA4326 can also stimulate CFBE cells to express more pro-inflammatory cytokines. In a broader picture, we hypothesize that lipoproteins play a definite role in epithelial inflammation in the CF lung, and that PA4326 is an important lipoprotein in that process. We detected the release of the cytokine IL–8 from CFBE cell monolayers 3.5 hours post-inoculation with different strains, using an IL-8 ELISA kit (CytoSet^{TM}, Invitrogen). We observed 204.55 ± 39.73 pg/ml IL-8 in the supernatants CFBE cells inoculated with the wild type PA14 pMQ72 at this time point. In contrast, Δ*PA4326* pMQ72 mutants induced only 162.814 ± 2.4 pg/ml IL-8 (Figure 7). The CFBE cells that were inoculated with Δ*PA4326* p*4326* restored wild type-stimulated IL-8 levels (199.93 ± 29.79 pg/ml). This observation that the *PA4326* mutant stimulates a decreased amount of proinflammatory cytokines than wild type supports the hypothesis that this factor participates, in part, in the inflammatory reaction in the epithelial cells of CF patient lungs.

3.5 Effects on pyocyanin production

P. aeruginosa produces a greenish blue pigment toxin called pyocyanin. This exotoxin of *P. aerguinosa* has redox active properties, can be inhibitory against competing microorganisms, and has been reported to cause damage in lung epithelial

cells during CF infection (Price-Whelan et al. 2007; Bal Rada & Leto 2013). In our Δ*PA4326* strain, we observed that the cultures were less bluish green than the wild type growth culture (Fig. 8). This phenotype could be reversed by the complementation plasmid (p*4326*). This phenotypic difference made us wonder how PA4326 lipoprotein is affecting the production/secretion of the toxin pigment.

According to the *Pseudomonas* Genome Database, around 80% of the *P. aeruginosa* lipoproteins are outer membrane proteins playing essential structural roles (Winsor et al. 2011; Remans et al. 2010). Since, as a zwitterion, pyocyanin can move across the cell easily, we speculated that the modification of the bacterial membrane due to the deletion of *PA4326* might reduce the secretion of the toxin in the bacterial growth media. To test this hypothesis, we measured the pyocyanin quantitatively both in supernatant of the bacterial culture and in whole cell lysates that combine the intracellular compartment with the supernatant.

In both of the cases, we found more pyocyanin in wild type than our mutant Δ*PA4326* strain. The wild type strain secreted 58.73% more pyocyanin than Δ*PA4326* in the culture supernatant (Fig. 9A). We also found a similar result for whole cell pyocyanin levels (Fig. 9B), indicating that PA4326 might affect pyocyanin synthesis rather than pyocyanin secretion.

3.6 Effects on the expression of *phzM*

To explore further the effects of Δ*PA4326* on pyocyanin synthesis, we examined if the deletion of *PA4326* affects expression of *phzM*, which is required for phenazine

modification in the pyocyanin biosynthesis pathway (Mavrodi et al. 2001). PhzM is required to convert PCA to 5-methylphenazine-1-carboxylic acid betaine, which in turn is converted to pyocyanin (Mavrodi et al. 2001). qPCR showed that deletion mutation of *PA4326* results in lower transcript level of the *phzM* gene (Figure 10). The level of *phzM* transcripts were 72.3% lower in Δ*PA4326* than the wild type strain. These results indicated that lower level expression of the *phzM* gene may account for the reduced pyocyanin phenotype of Δ*PA4326.*

3.7 Effects on quorum sensing molecules

2-alkyl-4-quinolone (PQS) is a signaling molecule of *P. aeruginosa* that enhances the expression of *rhlI* and *rhlR,* which are essential for synthesis of the quorum sensing molecule C_4-HSL (McKnight et al. 2000). C_4-HSL induces the expression of the *phzA1* gene locus required for the conversion of chorismic acid to the pyocyanin precursor molecule (Whiteley et al. 1999). Thus, we tested the idea that PQS signaling was interrupted in Δ*PA4326,* resulting in low levels of C_4-HSL. To answer this question, we determined the level of C_4-HSL in the culture supernatant quantitatively using a cross-feeding bioassay (Pearson et al. 1997). In Figure 11, we found both the wild type and the mutant strains were capable of producing considerable amount of C4-HSL signal molecule.

To test whether PQS signaling was affected in Δ*PA4326,* we took advantage of the fact that *P. aeruginosa* requires PQS and related compound 4-quinolone to kill *S. aureus* and several other Gram-positive bacteria (Anon n.d.; Déziel et al. 2004). We

spotted 3 µl of overnight cultures of wild type, Δ*PA4326*, and complemented strains onto agar plates freshly swabbed with *S. aureus*, and then incubated these plates for 20–24 hours at 37^0C. In the co-culture plate, the zone of inhibition represents cell killing, and hence the active PQS molecule. We observed that the zones of inhibition were the same for all of the spotted strains, indicating that PQS levels were unaffected in Δ*PA4326* (Fig. 12).

3.8 Effects on other virulence factors

To further investigate the effects of *PA4326* deletion on other virulence factors, we measured biofilm formation. Biofilm assays were performed at various time points, and we found no statistical difference at any time point in biofilm development in either of the strains (Figure 13).

We also compared motility of wild type and ΔPA4326. Swarming motility was identical between the strains (Fig. 14A). On the other hand, swimming motility was slightly reduced in the Δ*PA4326* strain, compared to wild type (Fig. 14B).

CHAPTER 4 DISCUSSION

In this research study, we demonstrated that putative lipoprotein-encoding gene *PA4326* is involved in cellular cytotoxicity and regulates bacterial pigment toxin production. Various studies have reported the role of lipoproteins as virulence factors in *P. aeruginosa*, particularly influencing host inflammation. Here, we reported a previously unstudied putative lipoprotein gene *PA4326*, which we found to be involved in cellular cytotoxicity and inflammatory reaction. Interestingly, we found that *PA4326* also affected pyocyanin regulation via an unknown mechanism. Overall, this study supports our hypothesis that *P. aeruginosa* lipoproteins cause CF airway tissue destruction and remodeling as a downstream effect of immune stimulation.

Excessive inflammation in lung tissue is one of the hallmark presentations of CF patients (Rowe et al. 2005; Govan & Deretic 1996). Increased IL-8 levels are common in the CF lung during bacterial chronic infection, ultimately leading to neutrophil infiltration and elaboration of inflammatory molecules (Courtney et al. 2004). Here, we found that putative lipoprotein PA4326 (Fig. 1) can stimulate IL-8 secretion, which implied its role in overall bacterially-mediated inflammation in the CF lung (Fig. 7). Previously, Firoved *et al.* has reported that purified *P. aeruginosa* lipoproteins induced IL-8 production in primary human respiratory epithelial cells and our finding supports their observation (Firoved et al. 2004). Given the fact that expression of lipoproteins is

greatly enhanced upon contact with epithelial cells, this observation of cytokine induction by *P. aeruginosa* lipoproteins like PA4326 indicates significant clinical implications (Frisk et al. 2004; Chugani & Greenberg 2007). Although it has been suggested that TLR2 may play a role in these activities (Firoved et al. 2004), the mechanism how epithelial cells respond to lipoprotein PA4326 is still unclear.

Various effector molecules of *P. aeruginosa* have been implicated to play a role in bacterially-mediated induction of apoptosis during infection (Hauser & Engel 1999; Shafikhani et al. 2008; Jia et al. 2006). Bacterial lipoprotein mediated cell activation and apoptosis have also been reported (Aliprantis 1999). Here, we made a similar observation with our putative lipoprotein *PA4326* gene (Fig. 6). Δ*PA4326* induced CFBE cells undergo less apoptotic/necrotic death compared to the wild type induced cell, which indicates the involvement of *PA4326* in active bacterially-mediated cell killing. It is important to note that the presented flow cytometry data are representative of a single experiment, requiring more experimentation to conclude anything regarding cell death pattern. However, this preliminary observation restated the significance of PA4326 in *P. aeruginosa* virulence. In the future, following the cell deaths (apoptosis/necrosis) over time would tell us a broader picture of lipoprotein-mediated cell death dynamics.

Our results also suggest a possible regulatory function of *PA4326* in bacterial toxin production which may be involved, in part, in cell killing. Our initial observation of reduced CFBE cell destruction (Fig. 2) with mutant Δ*PA4326* was followed by quantitative measurement of the cellular cytotoxicity. We found significant reduction in cytotoxicity measured by LDH release with our mutant compared to wild type (Fig. 3), and this effect was partially reversed by the complementation vector (Fig. 4). The partial

complementation could be the result of lower expression of vector plasmid in the mutant strain due to the growth conditions and media, and does not undermine the effects of the *PA4326* gene in virulence. The bacterial growth curve ruled out the possibility of defective growth of the mutant playing any role in this reduced cytotoxic phenotype (Fig. 5). Generally, strains that have higher cytotoxicity *in vitro* are also found to have higher virulence in animal models of pneumonia (Schulert et al. 2003; Sawa et al. 1998). As a result, our phenotypic observation *in vitro* suggested a role of PA4326 and possibly other lipoproteins in the pathogenesis of pneumonia caused by *P. aeruginosa*. Moreover, considering our previous observation that tobramycin prevented bacterial killing of CFBE epithelial cells (Anderson et al. 2008), we hypothesize that antibiotic-driven selective pressure on bacteria may inhibit effector proteins involved in cell killing; as a result bacteria overexpress lipoproteins like PA4326 in an effort to compensate for reduced cell killing (Anderson et al. 2010; Yahr & Wolfgang 2006). More in-depth experimental approaches are required to elucidate the mechanism of the lipoprotein-mediated CFBE cell killing.

We also made an interesting observation that our mutant strains produced less of the blue redox-active phenazine compound pyocyanin in growth media than wild type (Fig. 8). It was a very significant observation considering that pyocyanin is an important virulence factor for *P. aeruginosa* pathogenesis and has been shown to have inhibitory effects on cell respiration, ciliary function, and cell growth in mammalian cells (Wilson et al. 1987; Gee W Lau et al. 2004). Moreover, the characteristic bluish hue of CF sputum is the result of high level (~100 µM) pyocyanin accumulation (Wilson et al. 1988), and pyocyanin has also been reported to induce oxidative stress and apoptosis in neutrophils

(Usher et al. 2002; Muller 2002). We believe that the reduced CFBE cell destructions with Δ*PA4326*, as well as cytokine and apopotosis effects were the result, in part, of this reduced pyocyanin phenotype.

The question how *PA4326* is affecting exotoxin pyocyanin is still not clear. Initially, we thought that *PA4326* might affect the membrane transportation of pyocyanin, as most *P. aeruginosa* lipoproteins (80%) are outer membrane proteins and play important structural role (Remans et al. 2010). However, we found deficiency in production of pyocyanin both in culture supernatant and in whole cell lysate indicating that *PA4326* might be involved in pyocyanin synthesis rather than in secretion (Fig. 9).This observation was not surprising as plenty of other *P. aeruginosa* toxic lipoproteins have been implicated in regulatory roles (Remans et al. 2010; Gee W. Lau et al. 2004). Moreover, this observation also demands investigation of the cellular localization of the putative lipoprotein PA4326, to determine whether it, in fact, resides in the outer membrane.

To further elucidate the relationships between *PA4326* and pyocyanin, we also showed that deletion of *PA4326* resulted in low level expression of *phzM* transcripts (Fig. 10). *phzM*, encoding a SAM-dependent methyltransferase, is a crucial gene which converts the phenazine intermediate to 5-methylphenazine-1-carboxylic acid betaine which in turn is converted to pyocyanin (Mavrodi et al. 2001). Our finding suggested that one probable reason for the reduced pyocyanin phenotype was insufficient expression of *phzM* gene. However, at this point, we do not know whether *PA4326* affects the *phzM* expression directly or indirectly. We examined the possible effects of *PA4326* on quorum sensing signaling molecule C_4-HSL, because C_4-HSL and its receptor RhlR have a

positive regulatory effects in the expression of *phzA1* gene locus (Whiteley et al. 1999). However, we did not observe any statistically significant variation in C_4-HSL level among strains (Fig. 11), which implied that the level of C_4-HSL was not involved in pyocyanin deficiency in Δ*PA4326* strains. Moreover, we also showed qualitatively (Fig. 12) that in Δ*PA4326* strains, PQS (2-alkal-4-quinolone) signaling, which promotes the expression of *rhlI* and *rhlR* required for C_4-HSL synthesis (McKnight et al. 2000), was likewise unaffected.

In a recent paper, it has been reported that phenazines control colony size and biofilm thickness in *P. aeruginosa* strain PA14 (Ramos et al. 2010). Phenazines have also been implicated to control gene expression and community behavior, as well as thickness of *Pseudomonas chlororaphis* biofilms (Dietrich et al. 2008; Maddula et al. 2008; Ramos et al. 2010). Moreover, Das *et al.* have found that pyocyanin promotes extracellular DNA release, which is required to maintain biofilm integrity in *P aeruginosa* (Das & Manefield 2012). However, in our biofilm assay in PVC with our pyocyanin defective Δ*PA4326* strains, we did not find any significant variations in biofilm levels among different strains at various time points (Fig. 13). This discrepancy in observations between biofilm systems may be due to the differences in experimental approaches used in previous studies. Similar to Ramos *et al.*, we observed that pyocyanin might not influence the surface attachment (early biofilm) of the PA14 strain, which data contrast with observations with *P. chlororaphis* (Ramos et al. 2010; Maddula et al. 2008). However, Ramos *et al.* also reported that pyocyanin might influence the swarming motility, whereas we failed to find similar effects (Fig. 14) with our Δ*PA4326* strain (Ramos et al. 2010). This previous study used a double *phz1/2* mutant, which was

completely deficient in any phenazine production (Dietrich et al. 2006). Further studies using co-culture CFBE biofilm assay will give a more clinically-significant picture of the relationship, if any, between *PA4326*, pyocyanin, and biofilm formation (Anderson et al. 2008).

In this study, we presented the role of a previously unstudied putative lipoprotein PA4326 in *P. aeruginosa*-mediated cytotoxicity towards airway cells. In the future, in-depth analysis on how CFBE cells respond to with bacterial lipoproteins, like PA4326, will facilitate better understanding of this dynamic host/virulence interplay. Nonetheless, our current research has shown the importance of PA4326 in bacterial virulence and may lead toward novel anti-inflammatory therapies against lipoproteins like PA4326 which would lessen the suffering of the patients with CF.

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REFERENCES

REFERENCES

Abrahamsen, J.F. et al., 2002. Flow cytometric measurement of apoptosis and necrosis in cryopreserved PBPC concentrates from patients with malignant diseases. *Bone Marrow Transplantation*, 29, pp.165–171.

Aliprantis, A.O., 1999. Cell Activation and Apoptosis by Bacterial Lipoproteins Through Toll-like Receptor-2. *Science*, 285(5428), pp.736–739. Available at: http://www.sciencemag.org/cgi/doi/10.1126/science.285.5428.736 [Accessed May 23, 2014].

Alvarez-Ortega, C. et al., 2010. Genetic determinants involved in the susceptibility of Pseudomonas aeruginosa to beta-lactam antibiotics. *Antimicrobial Agents and Chemotherapy*, 54, pp.4159–4167.

Alvarez-Ortega, C. & Harwood, C.S., 2007. Responses of Pseudomonas aeruginosa to low oxygen indicate that growth in the cystic fibrosis lung is by aerobic respiration. *Molecular Microbiology*, 65, pp.153–165.

Anderson, G.G. et al., 2008. In vitro analysis of tobramycin-treated Pseudomonas aeruginosa biofilms on cystic fibrosis-derived airway epithelial cells. *Infection and Immunity*, 76, pp.1423–1433.

Anderson, G.G. et al., 2010. The Pseudomonas aeruginosa magnesium transporter MgtE inhibits transcription of the type III secretion system. *Infection and Immunity*, 78, pp.1239–1249.

Anon, Staphylococcus aureus Serves as an Iron Source for Pseudomonas aeruginosa during In Vivo Coculture. Available at: http://jb.asm.org/content/187/2/554.full.pdf [Accessed March 6, 2014].

Armstrong, D.S. et al., 2005. Lower airway inflammation in infants with cystic fibrosis detected by newborn screening. *Pediatric Pulmonology*, 40, pp.500–510.

Barnes, P.J. & Karin, M., 1997. Nuclear factor- k B--a pivotal transcription factor in chronic inflammatory diseases. *New England Journal of Medicine*, 336, pp.1066–1071.

Ben et al., 2011. Virulence factors in Pseudomonas aeruginosa: mechanisms and modes of regulation. *Annales de Biologie Clinique*, 69(4), pp.393-403.

BH, I., 1996. *Pseudomonas. In: Baron's Medical microbiology (Baron S et al., eds.)* 4th ed., Univ of Texas Medical Branch.

Bobadilla, J.L. et al., 2002. Cystic fibrosis: A worldwide analysis of CFTR mutations - Correlation with incidence data and application to screening. *Human Mutation*, 19, pp.575–606.

Bonfield, T.L. et al., 1995. Inflammatory cytokines in cystic fibrosis lungs. *American Journal of Respiratory and Critical Care Medicine*, 152, pp.2111–2118.

Bonfield, T.L., Konstan, M.W. & Berger, M., 1999. Altered respiratory epithelial cell cytokine production in cystic fibrosis. *The Journal of Allergy and Clinical Immunology*, 104, pp.72–78.

Boucher, J.C. et al., 1997. Mucoid Pseudomonas aeruginosa in cystic fibrosis: characterization of muc mutations in clinical isolates and analysis of clearance in a mouse model of respiratory infection. *Infection and Immunity*, 65, pp.3838–3846.

Breidenstein, E.B.M., de la Fuente-Núñez, C. & Hancock, R.E.W., 2011. Pseudomonas aeruginosa: all roads lead to resistance. *Trends in Microbiology*, 19(8), pp.419–26. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21664819 [Accessed June 6, 2014].

CFF, 2012. *Cystic Fibrosis Foundation - Patient Registry Annual Data Report*,

Chugani, S. & Greenberg, E.P., 2007. The influence of human respiratory epithelia on Pseudomonas aeruginosa gene expression. *Microbial Pathogenesis*, 42, pp.29–35.

Ciofu, O., Hansen, C.R. & Høiby, N., 2013. Respiratory bacterial infections in cystic fibrosis. *Current Opinion in Pulmonary Medicine*, 19, pp.251–8.

Collins, J. et al., 2012. Allergic bronchopulmonary aspergillosis treated successfully for one year with omalizumab. *Journal of Asthma and Allergy*, 5, pp.65–70.

Costerton, A.J.W., Stewart, P.S. & Greenberg, E.P., 2011. Bacterial Bioflims : Persistent Common Cause of Persistent Infection. *Science*, 284, pp.1318–1322.

Courtney, J.M., Ennis, M. & Elborn, J.S., 2004. Cytokines and inflammatory mediators in cystic fibrosis. *Journal of Cystic Fibrosis : Official Journal of the European Cystic Fibrosis Society*, 3(4), pp.223–31. Available at: http://www.sciencedirect.com/science/article/pii/S1569199304001705 [Accessed February 16, 2014].

Cozens, A.L. et al., 1994. CFTR expression and chloride secretion in polarized immortal human bronchial epithelial cells. *American Journal of Respiratory Cell and Molecular Biology*, 10, pp.38–47.

Das, T. & Manefield, M., 2012. Pyocyanin Promotes Extracellular DNA Release in Pseudomonas aeruginosa. *PLoS ONE*, 7.

Déziel, E. et al., 2004. Analysis of Pseudomonas aeruginosa 4-hydroxy-2-alkylquinolines (HAQs) reveals a role for 4-hydroxy-2-heptylquinoline in cell-to-cell communication. *Proceedings of the National Academy of Sciences of the United States of America*, 101, pp.1339–1344.

Dietrich, L.E.P. et al., 2008. Redox-active antibiotics control gene expression and community behavior in divergent bacteria. *Science (New York, N.Y.)*, 321, pp.1203–1206.

Dietrich, L.E.P. et al., 2006. The phenazine pyocyanin is a terminal signalling factor in the quorum sensing network of Pseudomonas aeruginosa. *Molecular Microbiology*, 61(5), pp.1308–21. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16879411 [Accessed February 14, 2014].

DiMango, E. et al., 1998. Activation of NF-kappaB by adherent Pseudomonas aeruginosa in normal and cystic fibrosis respiratory epithelial cells. *The Journal of Clinical Investigation*, 101, pp.2598–2605.

Dötsch, A. et al., 2009. Genomewide identification of genetic determinants of antimicrobial drug resistance in Pseudomonas aeruginosa. *Antimicrobial Agents and Chemotherapy*, 53, pp.2522–2531.

Eschbach, M. et al., 2004. Long-term anaerobic survival of the opportunistic pathogen Pseudomonas aeruginosa via pyruvate fermentation. *Journal of Bacteriology*, 186, pp.4596–4604.

Essar, D.W., Eberly, L. & Crawford, I.P., 1990. Evolutionary differences in chromosomal locations of four early genes of the tryptophan pathway in fluorescent pseudomonads: DNA sequences and characterization of Pseudomonas putida trpE and trpGDC. *Journal of Bacteriology*, 172, pp.867–883.

Fajardo, A. et al., 2008. The neglected intrinsic resistome of bacterial pathogens. *PLoS ONE*, 3(2), e1619.

Firoved, A.M., Ornatowski, W. & Deretic, V., 2004. Microarray analysis reveals induction of lipoprotein genes in mucoid Pseudomonas aeruginosa: implications for inflammation in cystic fibrosis. *Infection and Immunity*, 72, pp.5012–5018.

Frimmersdorf, E. et al., 2010. How Pseudomonas aeruginosa adapts to various environments: A metabolomic approach. *Environmental Microbiology*, 12, pp.1734–1747.

Frisk, A. et al., 2004. Transcriptome Analysis of Pseudomonas aeruginosa after Interaction with Human Airway Epithelial Cells. *Infect. Immun.*, 72, pp.5433–5438.

Govan, J.R. & Deretic, V., 1996. Microbial pathogenesis in cystic fibrosis: mucoid Pseudomonas aeruginosa and Burkholderia cepacia. *Microbiological Reviews*, 60, pp.539–574.

Hall-Stoodley, L., Costerton, J.W. & Stoodley, P., 2004. Bacterial biofilms: from the natural environment to infectious diseases. *Nature Reviews. Microbiology*, 2, pp.95–108.

Hardin, D.S., 2004. GH improves growth and clinical status in children with cystic fibrosis -- a review of published studies. *European Journal of Endocrinology*, 151, pp.S81–S85.

Hauser, A.R. et al., 2011. Clinical significance of microbial infection and adaptation in cystic fibrosis. *Clinical Microbiology Reviews*, 24(1), pp.29–70. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3021203&tool=pmcentrez&rendertype=abstract [Accessed June 4, 2014].

Hauser, A.R. & Engel, J.N., 1999. Pseudomonas aeruginosa induces type-III-secretion-mediated apoptosis of macrophages and epithelial cells. *Infection and Immunity*, 67, pp.5530–5537.

Hoffman, L.R. et al., 2005. Aminoglycoside antibiotics induce bacterial biofilm formation. *Nature*, 436, pp.1171–1175.

Hunter, R.C. et al., 2012. Phenazine Content in the Cystic Fibrosis Respiratory Tract Negatively Correlates with Lung Function and Microbial Complexity. *American Journal of Respiratory Cell and Molecular Biology*, 47, pp.738-745.

Jefferson, K.K., 2004. What drives bacteria to produce a biofilm? *FEMS Microbiology Letters*, 236, pp.163–173.

Jia, J. et al., 2006. Expression of Pseudomonas aeruginosa toxin ExoS effectively induces apoptosis in host cells. *Infection and Immunity*, 74(12), pp.6557–70. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1698105&tool=pmcentrez&rendertype=abstract [Accessed June 1, 2014].

Jubin, V. et al., 2010. Risk factors for aspergillus colonization and allergic bronchopulmonary aspergillosis in children with cystic fibrosis. *Pediatric Pulmonology*, 45, pp.764–771.

Kaplan, J.B., 2011. Antibiotic-induced biofilm formation. *The International Journal of Artificial Organs*, 34, pp.737–751.

Kaplan, J.B. et al., 2012. Low Levels of -Lactam Antibiotics Induce Extracellular DNA Release and Biofilm Formation in Staphylococcus aureus. *mBio*, 3, pp.e00198–12–e00198–12.

King, E.O., Ward, M.K. & Raney, D.E., 1954. Two simple media for the demonstration of pyocyanin and fluorescin. *The Journal of Laboratory and Clinical Medicine*, 44, pp.301–307.

Koley, D. et al., 2011. Discovery of a biofilm electrocline using real-time 3D metabolite analysis. *Proceedings of the National Academy of Sciences*, 108, pp.19996–20001.

Konstan, M.W., 1998. Therapies aimed at airway inflammation in cystic fibrosis. *Clinics in Chest Medicine*, 19, pp.505–513.

Kovacs-Simon, A., Titball, R.W. & Michell, S.L., 2011. Lipoproteins of bacterial pathogens. *Infection and Immunity*, 79, pp.548–561.

Kube, D. et al., 2001. Proinflammatory cytokine responses to P. aeruginosa infection in phuman airway epithelial cell lines. *Am J Physiol Lung Cell Mol Physiol*, 280, pp.L493–502.

Kuchma, S.L., Connolly, J.P. & O'Toole, G.A., 2005. A three-component regulatory system regulates biofilm maturation and type III secretion in Pseudomonas aeruginosa. *Journal of Bacteriology*, 187, pp.1441–1454.

Lau, G.W. et al., 2004. Pseudomonas aeruginosa pyocyanin is critical for lung infection in mice. *Infection and Immunity*, 72(7), pp.4275–8. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=427412&tool=pmcentrez&rendertype=abstract [Accessed June 5, 2014].

Lau, G.W. et al., 2004. The role of pyocyanin in Pseudomonas aeruginosa infection. *Trends in Molecular Medicine*, 10(12), pp.599–606. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15567330 [Accessed June 1, 2014].

Lentsch, A.B. et al., 1997. In vivo suppression of NF-kappa B and preservation of I kappa B alpha by interleukin-10 and interleukin-13. *The Journal of Clinical Investigation*, 100, pp.2443–2448.

Liang, M.D. et al., 2005. Bacterial peptidoglycan-associated lipoprotein: a naturally occurring toll-like receptor 2 agonist that is shed into serum and has synergy with lipopolysaccharide. *The Journal of Infectious Diseases*, 191, pp.939–948.

Luciani, A. et al., 2010. Defective CFTR induces aggresome formation and lung inflammation in cystic fibrosis through ROS-mediated autophagy inhibition. *Nature Cell Biology*, 12, pp.863–875.

Lyczak, J.B., Cannon, C.L. & Pier, G.B., 2000. Establishment of Pseudomonas aeruginosa infection: Lessons from a versatile opportunist. *Microbes and Infection*, 2, pp.1051–1060.

Lyczak, J.B., Cannon, C.L. & Pier, G.B., 2002. Lung infections associated with cystic fibrosis. *Clinical Microbiology Reviews*, 15, pp.194–222.

Maddula, V.S.R.K., Pierson, E.A. & Pierson, L.S., 2008. Altering the ratio of phenazines in Pseudomonas chlororaphis (aureofaciens) strain 30-84: effects on biofilm formation and pathogen inhibition. *Journal of Bacteriology*, 190, pp.2759–2766.

Mah, T.-F. et al., 2003. A genetic basis for Pseudomonas aeruginosa biofilm antibiotic resistance. *Nature*, 426, pp.306–310.

Makker, K., Agarwal, A. & Sharma, R., 2009. Oxidative stress & male infertility. *The Indian Journal of Medical Research*, 129, pp.357–367.

Matsui, H. et al., 1998. Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease. *Cell*, 95, pp.1005–1015.

Mavrodi, D. V et al., 2001. Functional analysis of genes for biosynthesis of pyocyanin and phenazine-1-carboxamide from Pseudomonas aeruginosa PAO1. *Journal of Bacteriology*, 183(21), pp.6454–65. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=100142&tool=pmcentrez&rendertype=abstract [Accessed February 18, 2014].

McKnight, S.L., Iglewski, B.H. & Pesci, E.C., 2000. The Pseudomonas quinolone signal regulates rhl quorum sensing in Pseudomonas aeruginosa. *Journal of Bacteriology*, 182, pp.2702–2708.

Miller, J.H., 1972. Experiments in molecular genetics. *Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY*.

Moore, K.W. et al., 2001. Interleukin-10 and the interleukin-10 receptor. *Annual Review of Immunology*, 19, pp.683–765.

Moreau-Marquis, S., Bomberger, J.M., et al., 2008. The DeltaF508-CFTR mutation results in increased biofilm formation by Pseudomonas aeruginosa by increasing iron availability. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, 295, pp.L25–L37.

Moreau-Marquis, S., Stanton, B.A. & O'Toole, G.A., 2008. Pseudomonas aeruginosa biofilm formation in the cystic fibrosis airway. *Pulmonary Pharmacology & Therapeutics*, 21(4), pp.595–9. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2542406&tool=pmcentrez&rendertype=abstract [Accessed May 27, 2014].

Muller, M., 2002. Pyocyanin induces oxidative stress in human endothelial cells and modulates the glutathione redox cycle. *Free Radical Biology & Medicine*, 33, pp.1527–1533.

Nadal Jimenez, P. et al., 2012. The Multiple Signaling Systems Regulating Virulence in Pseudomonas aeruginosa. *Microbiology and Molecular Biology Reviews*, 76, pp.46–65.

Nicas, T.I. & Hancock, R.E., 1983. Pseudomonas aeruginosa outer membrane permeability: isolation of a porin protein F-deficient mutant. *Journal of Bacteriology*, 153, pp.281–285.

O'Malley, C.A., 2009. Infection control in cystic fibrosis: cohorting, cross-contamination, and the respiratory therapist. *Respiratory Care*, 54, pp.641–657.

O'Malley, Y.Q. et al., 2003. The Pseudomonas secretory product pyocyanin inhibits catalase activity in human lung epithelial cells. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, 285, pp.L1077–L1086.

O'Toole, G.A. & Kolter, R., 1998. Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development. *Molecular Microbiology*, 30, pp.295–304.

Osika, E. et al., 1999. Distinct sputum cytokine profiles in cystic fibrosis and other chronic inflammatory airway disease. *The European Respiratory Journal*, 14, pp.339–46. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10515411 [Accessed February 14, 2014].

Pearson, J.P., Pesci, E.C. & Iglewski, B.H., 1997. Roles of Pseudomonas aeruginosa las and rhl quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. *Journal of Bacteriology*, 179, pp.5756–5767.

Pier, G.B., 2002. CFTR mutations and host susceptibility to Pseudomonas aeruginosa lung infection. *Current Opinion in Microbiology*, 5, pp.81–86.

Price-Whelan, A., Dietrich, L.E.P. & Newman, D.K., 2007. Pyocyanin alters redox homeostasis and carbon flux through central metabolic pathways in Pseudomonas aeruginosa PA14. *Journal of Bacteriology*, 189, pp.6372–6381.

Pulcrano, G. et al., 2012. Different mutations in mucA gene of Pseudomonas aeruginosa mucoid strains in cystic fibrosis patients and their effect on algU gene expression. *The New Microbiologica*, 35, pp.295–305.

Quinton, P.M., 2007. Cystic fibrosis: lessons from the sweat gland. *Physiology (Bethesda, Md.)*, 22, pp.212–225.

Rada, B. et al., 2011. Reactive oxygen species mediate inflammatory cytokine release and EGFR-dependent mucin secretion in airway epithelial cells exposed to Pseudomonas pyocyanin. *Mucosal Immunology*, 4, pp.158–171.

Rada, B. et al., 2008. The Pseudomonas toxin pyocyanin inhibits the dual oxidase-based antimicrobial system as it imposes oxidative stress on airway epithelial cells. *Journal of Immunology (Baltimore, Md. : 1950)*, 181, pp.4883–4893.

Rada, B. & Leto, T., 2013. Pyocyanin effects on respiratory epithelium: relevance in Pseudomonas aeruginosa airway infections. *Trends in Microbiology*, 21(2), pp.73–81. Available at: http://www.sciencedirect.com/science/article/pii/S0966842X12001904 [Accessed June 8, 2014].

Rada, B. & Leto, T.L., 2013. Pyocyanin effects on respiratory epithelium: Relevance in Pseudomonas aeruginosa airway infections. *Trends in Microbiology*, 21, pp.73–81.

Ramos, I. et al., 2010. Phenazines affect biofilm formation by Pseudomonas aeruginosa in similar ways at various scales. *Research in Microbiology*, 161, pp.187–191.

Raymond, J., 2006. Pseudomonas aeruginosa and hospital-acquired infections. *Archives of Pediatrics*, 13 Suppl 1, pp.S2–S4.

Remans, K. et al., 2010. Genome-wide analysis and literature-based survey of lipoproteins in Pseudomonas aeruginosa. *Microbiology*, 156(Pt 9), pp.2597–2607. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20616104 [Accessed February 16, 2014].

Rohlfs, E.M. et al., 2011. Cystic fibrosis carrier testing in an ethnically diverse US population. *Clinical Chemistry*, 57, pp.841–848.

Rosenfeld, M. et al., 2001. Early pulmonary infection, inflammation, and clinical outcomes in infants with cystic fibrosis. *Pediatric Pulmonology*, 32, pp.356–366.

Rowe, S.M., Miller, S. & Sorscher, E.J., 2005. Cystic fibrosis. *The New England Journal of Medicine*, 352, pp.1992–2001.

Sagel, S.D. et al., 2012. Sputum Biomarkers of Inflammation and Lung Function Decline in Children with Cystic Fibrosis. *American Journal of Respiratory and Critical Care Medicine*. 186, pp.857-865.

Saiman, L. & Prince, A., 1993. Pseudomonas aeruginosa pili bind to asialoGM1 which is increased on the surface of cystic fibrosis epithelial cells. *The Journal of Clinical Investigation*, 92, pp.1875–1880.

Sawa, T. et al., 1998. In vitro cellular toxicity predicts Pseudomonas aeruginosa virulence in lung infections. *Infection and Immunity*, 66, pp.3242–3249.

Schobert, M. & Jahn, D., 2010. Anaerobic physiology of Pseudomonas aeruginosa in the cystic fibrosis lung. *International Journal of Medical Microbiology*, 300, pp.549–556.

Schottelius, A.J. et al., 1999. Interleukin-10 signaling blocks inhibitor of kappaB kinase activity and nuclear factor kappaB DNA binding. *The journal of Biological Chemistry*, 274, pp.31868–31874.

Schulert, G.S. et al., 2003. Secretion of the toxin ExoU is a marker for highly virulent Pseudomonas aeruginosa isolates obtained from patients with hospital-acquired pneumonia. *The Journal of Infectious Diseases*, 188, pp.1695–1706.

Schwarzer, C. et al., 2008. Oxidative stress caused by pyocyanin impairs CFTR Cl- transport in human bronchial epithelial cells. *Free Radical Biology and Medicine*, 45, pp.1653–1662.

Shafikhani, S.H., Morales, C. & Engel, J., 2008. The Pseudomonas aeruginosa type III secreted toxin ExoT is necessary and sufficient to induce apoptosis in epithelial cells. *Cellular Microbiology*, 10(4), pp.994–1007. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18053004 [Accessed June 5, 2014].

Shanks, R.M.Q. et al., 2006. Saccharomyces cerevisiae-based molecular tool kit for manipulation of genes from gram-negative bacteria. *Applied and Environmental Microbiology*, 72, pp.5027–5036.

Smith, J.J. et al., 1996. Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid. *Cell*, 85, pp.229–236.

Sorio, C. et al., 2011. Defective CFTR expression and function are detectable in blood monocytes: Development of a new blood test for cystic fibrosis. *PLoS ONE*, 6.

Stover, C.K. et al., 2000. PA01 - Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen. *Nature*, 406, pp.959–64.

Tan, H.-L. et al., 2011. The Th17 pathway in cystic fibrosis lung disease. *American Journal of Respiratory and Critical Care Medicine*, 184, pp.252–258.

Trautmann, M., Lepper, P.M. & Haller, M., 2005. Ecology of Pseudomonas aeruginosa in the intensive care unit and the evolving role of water outlets as a reservoir of the organism. *American Journal of Infection Control*, 33.

Tremblay, J. & D??ziel, E., 2008. Improving the reproducibility of Pseudomonas aeruginosa swarming motility assays. *Journal of Basic Microbiology*, 48, pp.509–515.

Usher, L.R. et al., 2002. Induction of Neutrophil Apoptosis by the Pseudomonas aeruginosa Exotoxin Pyocyanin: A Potential Mechanism of Persistent Infection. *The Journal of Immunology*, 168(4), pp.1861–1868. Available at: http://www.jimmunol.org/cgi/doi/10.4049/jimmunol.168.4.1861 [Accessed June 5, 2014].

Venkatakrishnan, A. et al., 2000. Exaggerated activation of nuclear factor-kappaB and altered IkappaB-beta processing in cystic fibrosis bronchial epithelial cells. *American Journal of Respiratory Cell and Molecular Biology*, 23, pp.396–403.

Wat, D. et al., 2008. The role of respiratory viruses in cystic fibrosis. *Journal of Cystic Fibrosis*, 7, pp.320–328.

Whiteley, M., Lee, K.M. & Greenberg, E.P., 1999. Identification of genes controlled by quorum sensing in Pseudomonas aeruginosa. *Proceedings of the National Academy of Sciences*, 96(24), pp.13904–13909. Available at: http://www.pnas.org/cgi/doi/10.1073/pnas.96.24.13904 [Accessed March 11, 2014].

Wilson, R. et al., 1988. Measurement of Pseudomonas aeruginosa phenazine pigments in sputum and assessment of their contribution to sputum sol toxicity for respiratory epithelium. *Infection and Immunity*, 56, pp.2515–2517.

Wilson, R. et al., 1987. Pyocyanin and 1-hydroxyphenazine produced by Pseudomonas aeruginosa inhibit the beating of human respiratory cilia in vitro. *The Journal of Clinical Investigation*, 79, pp.221–229.

Winsor, G.L. et al., 2009. Pseudomonas Genome Database: Facilitating user-friendly, comprehensive comparisons of microbial genomes. *Nucleic Acids Research*, 37.

Winsor, G.L. et al., 2011. Pseudomonas Genome Database: Improved comparative analysis and population genomics capability for Pseudomonas genomes. *Nucleic Acids Research*, 39.

Yahr, T.L. & Wolfgang, M.C., 2006. Transcriptional regulation of the Pseudomonas aeruginosa type III secretion system. *Molecular Microbiology*, 62, pp.631–640.

TABLES

Table 1: Experimental Organisms

Organisms	Description or Purpose	Source
Pseudomonas aeruginosa PA14	wild type, clinical isolate	Rahme et al.1995
Pseudomonas aeruginosa GGA50	PA14 ΔPA4326	This study
Staphylococcus aureus MZ100	Wild type	Shanks et al., 2005
Agrobacterium tumefaciens NTL4	With two plasmids: *tra* gene and *traI-traZ* fusion	Fuqua & Winans, 1996; Fuqua et al., 1996; Stickler et al., 1998
Pseudomonas aeruginosa PA0-JP2/pECP62.5	PA01 *lasΓ/rhlΓ* with pECP61.5 plasmid	Pearson et al.,1997
S. cerevisiae InvSc1	ura3-52 / ura3-52; creation of plasmids by homologous recombination	Invitrogen

Table 2: Description of plasmids

Plasmid	Description	Source
pSSA004	Complementation of *PA4326* gene	This study
pMQ72	Control expression vector with gentamicin-resistant gene	Shanks et al.
pMQ30	Suicide vector	Shanks et al.

Table 3: Description of primers:

Primer	Sequence (5’ – 3’)
4326L For	aattctgttttatcagaccgcttctgcgttctgatttaatcaccatcgactacctgaatg
4326L Rev	gatacagttcccaacggaggggatttcacggaggaggttc
4326R For	gaacctcctccgtgaaatcccctccgttgggaactgtatc
4326R Rev	ggaattgtgagcggataacaatttcacacaggaaacagcaccagcaggttatgcaggttc
4326F	cgcgacgagcatcgaaatac
4326R	cccttcagtcacgccaatac
4326Comfor	tactgtttctccatacccgttttttgggctagcgaattcaggaggagcgcgctatgcctatgcgaagcttg atc
4326Comrev	ctgtatcaggctgaaaatcttctctcatccgccaaaacagctacaggcgggacggcacgg
5110forA	ttcgccatcaacatgtccaaccag
5110revA	acatcgggttggcttcgtacatcag
phzMRTFor	aaggcaagtcgctggtgaactg
phzMRTRev	tactgcaggatcaactggttgcg

FIGURES

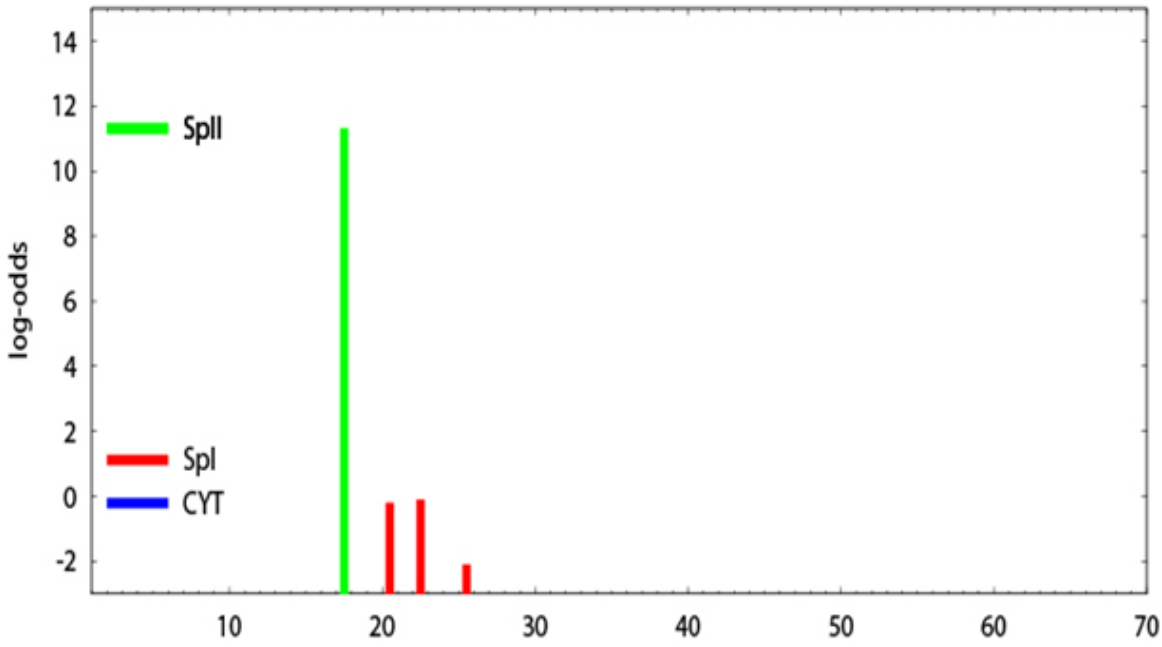

Figure 1: Type II (lipoprotein) export signal predicted by LipoP (cleavage after residue 17). SpI = signal peptide (signal peptidase I) SpII = lipoprotein signal peptide (signal peptidase II) CYT = cytoplasmic.

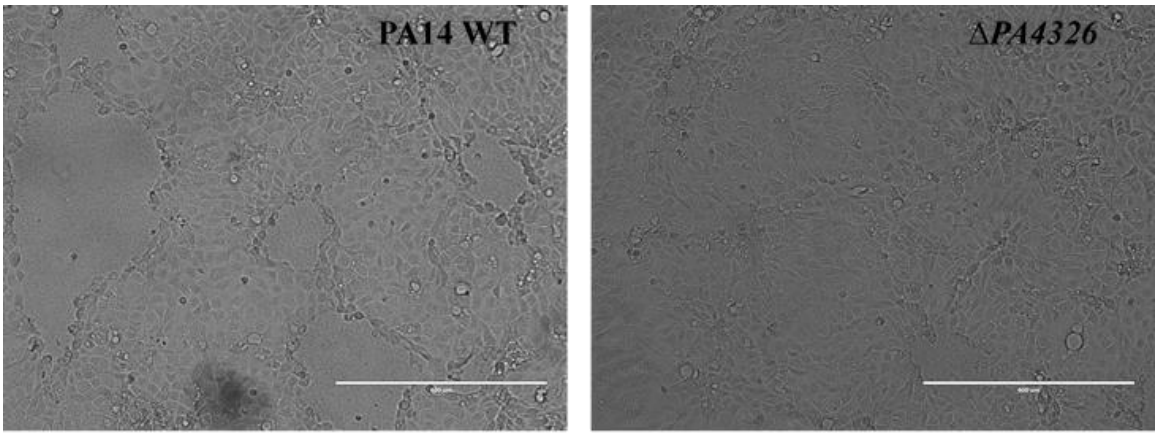

Figure 2: Cell monolayers are killed by *P. aeruginosa* Wild Type (WT) but not killed by the Δ*PA4326* mutant, 8 hours after incubation, cultured on CF-derived bronchial epithelial cells (CFBE cells).

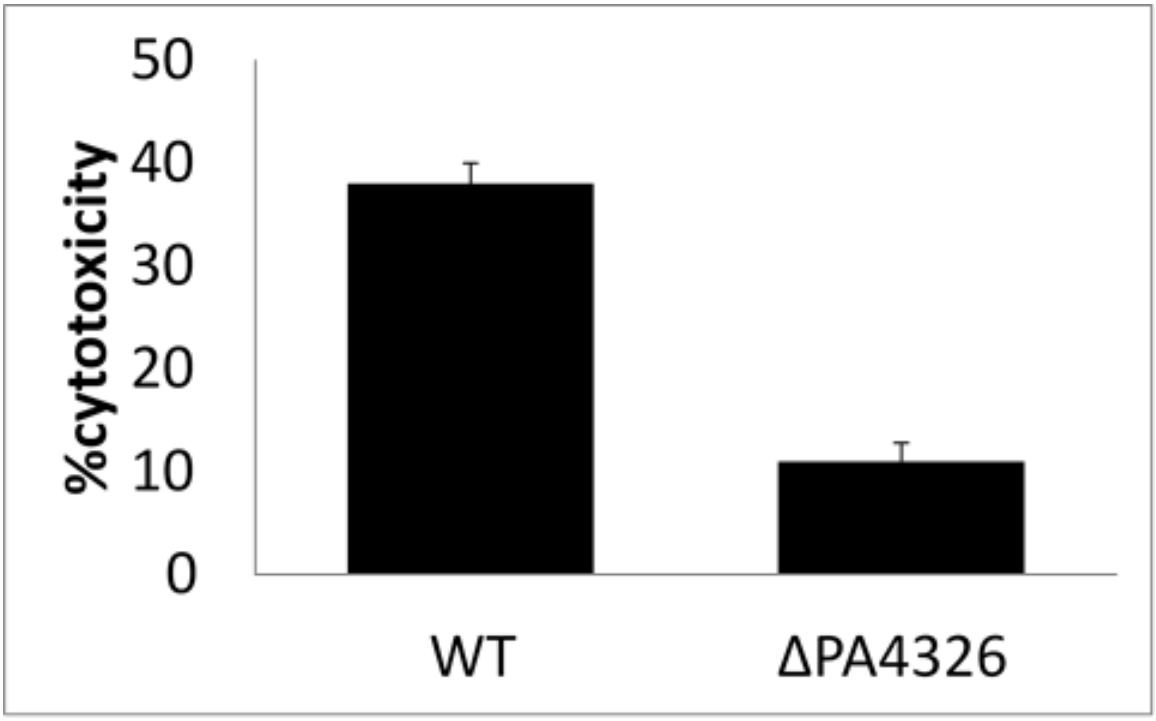

Figure 3: The Δ*PA4326* mutant strain is less toxic to CFBE cells than the wild type (WT), measured by Lactate Dehydrogenase (LDH) release after 6 hours.

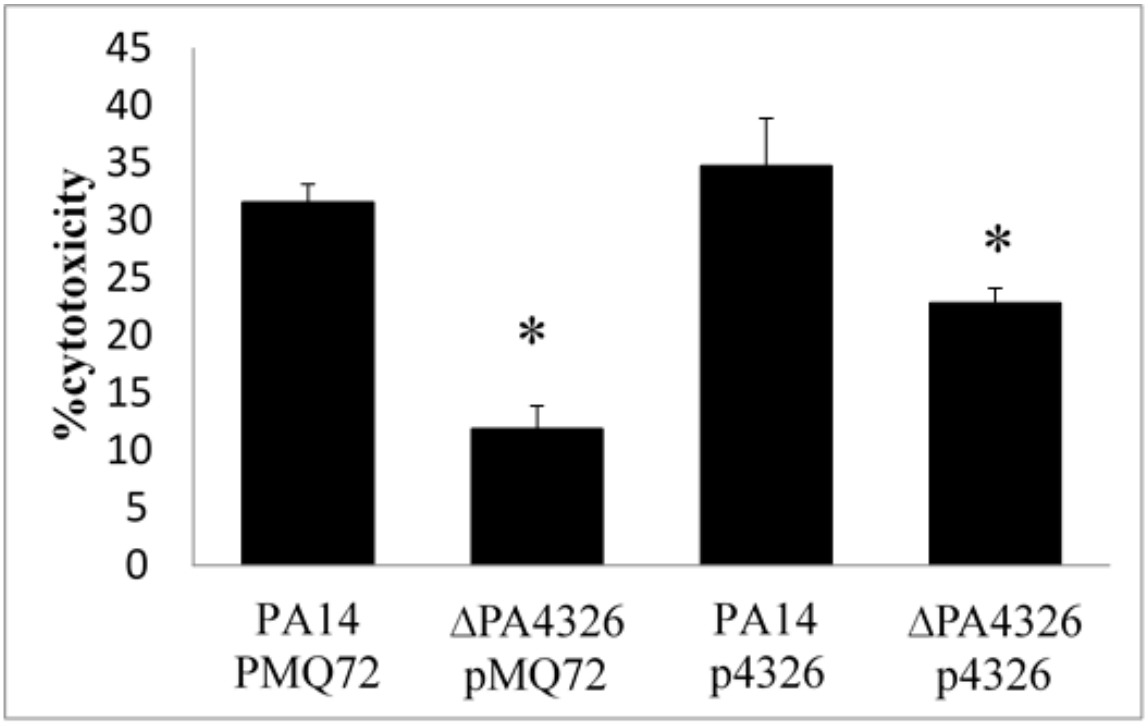

Figure 4: **Cytotoxicity Assay shows that the Δ*PA4326* strain is less cytotoxic.** Δ*PA4326* with complementation plasmid was significantly more cytotoxic than Δ*PA4326* with empty vector. The Y-axis displays percent cytotoxicity, normalized as a percentage of maximum LDH release from CFBE cells treated with Triton® X-100. PA14 is the wild type strain, p*4326* is the *PA4326* complementation plasmid, and pMQ72 is the empty vector. Data are representative of 4 independent experiments, performed in triplicate. *p< 0.05.

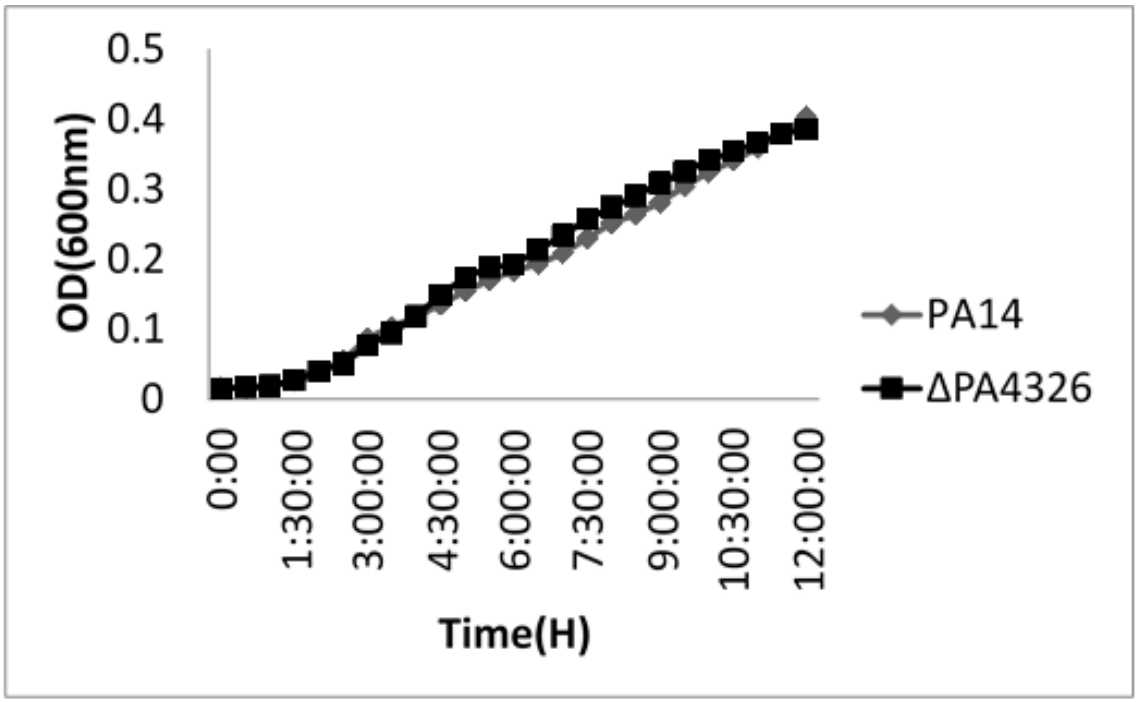

Figure 5: Growth is unaffected by mutation of *PA4326*. Absorbance values were measured at 600 nm in a spectrophotometer held at 37°C. Here, both the wild type and Δ*PA4326* strains showed similar growth patterns. Data are representative of 3 independent experiments performed in triplicate.

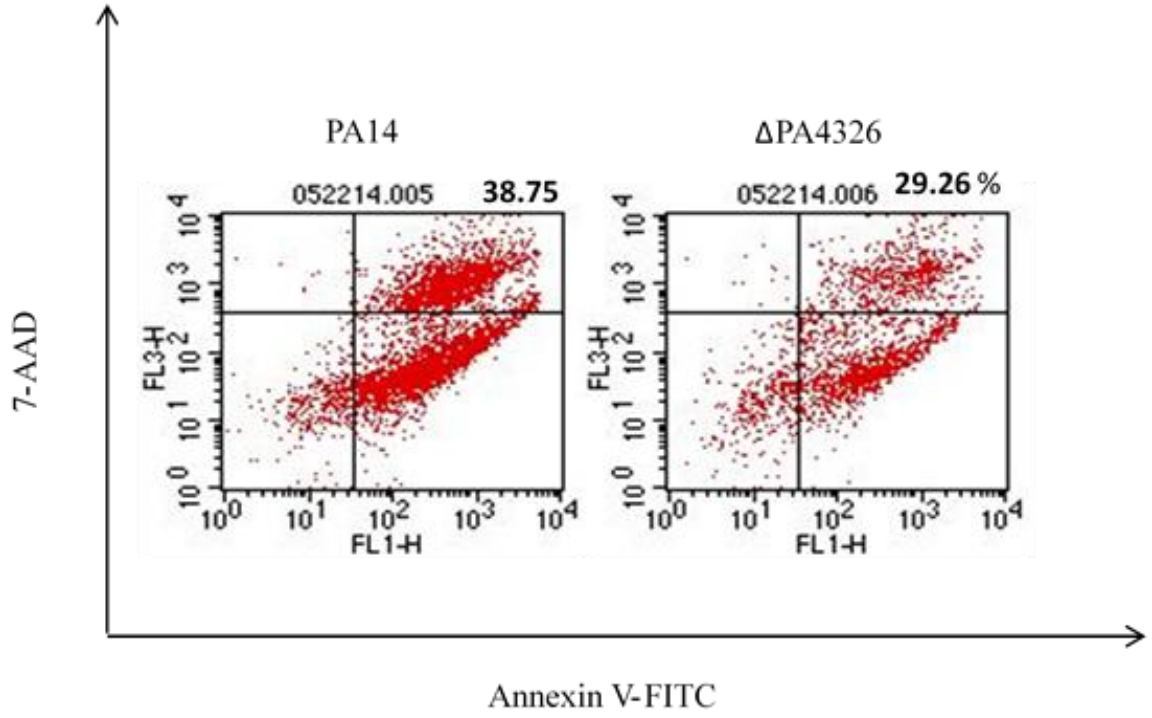

Figure 6: Mutation of *PA4326* reduces apoptosis and necrosis in CFBE cells cultured with bacteria. Three and a half hours post-inoculation with wild type PA14 and Δ*PA3226*, CFBE cells were triple stained with PE-conjugated anti-human antibody D34 monoclonal antibody, annexin V-FITC, and 7 aminoactinomycinD (7AAD). According to the control samples (Not shown in figure), the lower right quadrant was defined as early apoptotic cells, which bindannexin V but still retain their cytoplasmic integrity and exclude 7AAD, whereas the upper right quadrant represents nonviable, late apoptotic/necrotic cells, which are positive for annexin V and actinomycin D staining. Left Panel: 38.75% of the cells inoculated with PA14 wild type were late apoptotic/necrosis cells. Right panel: 29.26% of the stimulated with ΔPA4326 were late apoptotic/necrosis. Data represent a single experiment.

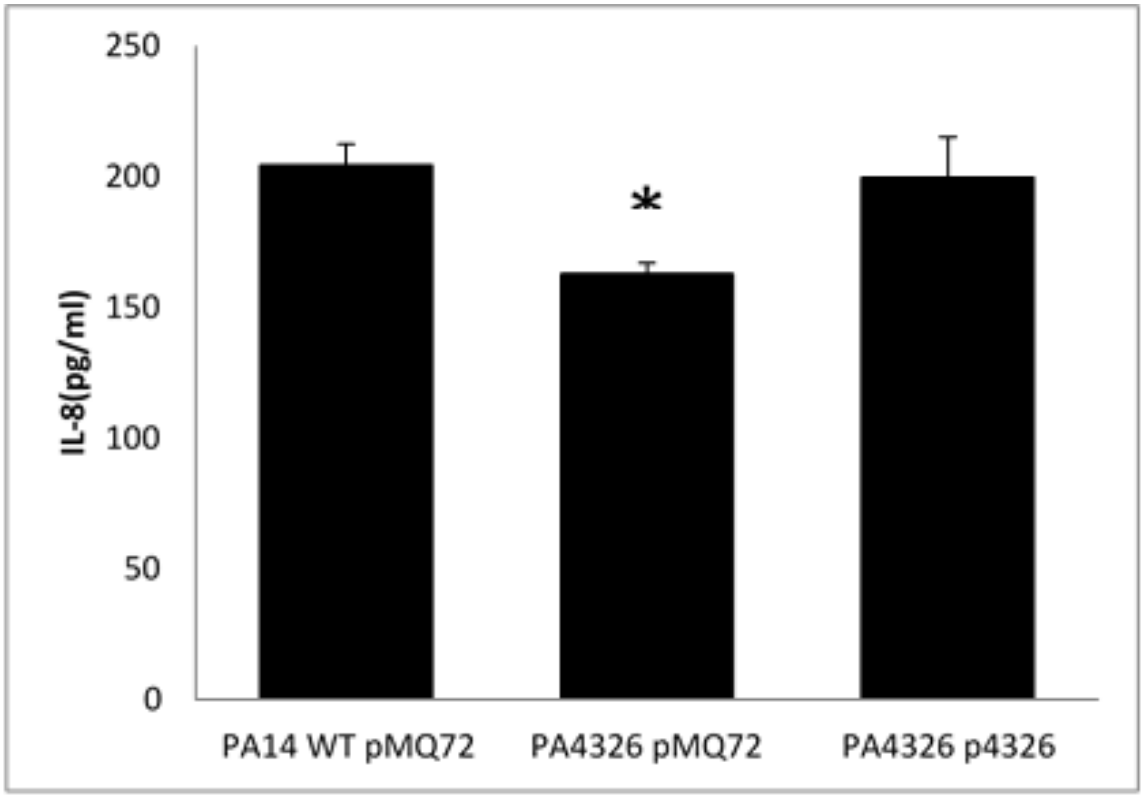

Figure 7: Decreased IL-8 secretion from CFBE cells inoculated with the *PA4326* mutant. IL-8 levels in the supernatant of CFBE monolayers inoculated with wild type ΔPA4326 strains were measured by ELISA (detection Limit 12.5 to 800 pg/ml). p*4326* is the complementation plasmid and pMQ72 is the empty vector. The results are the means of four independent experiments ± the standard deviation. *$p<0.05$.

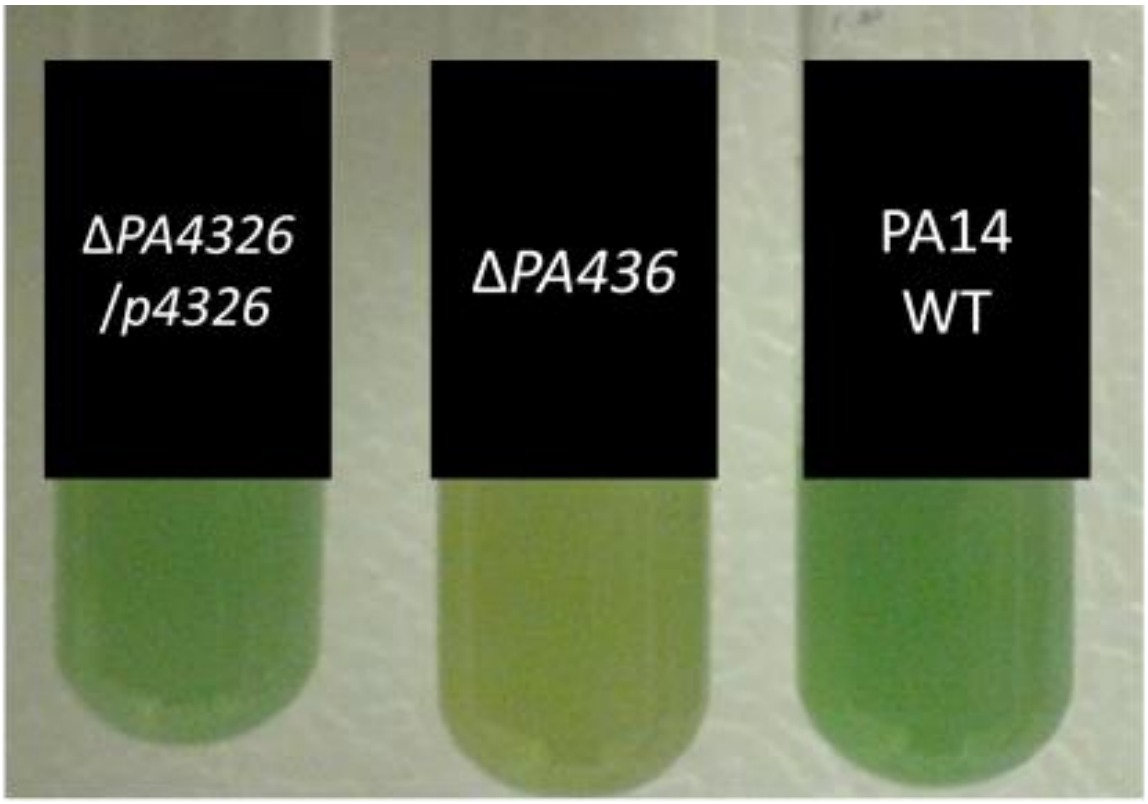

Figure 8: Δ*PA4326* culture is less green than wild type. The bacterial cultures were grown overnight at 37^0 C in LB media. Qualitative observations reveal a lighter color to Δ*PA4326* cultures than wild type. p*4326* is the complementation plasmid.

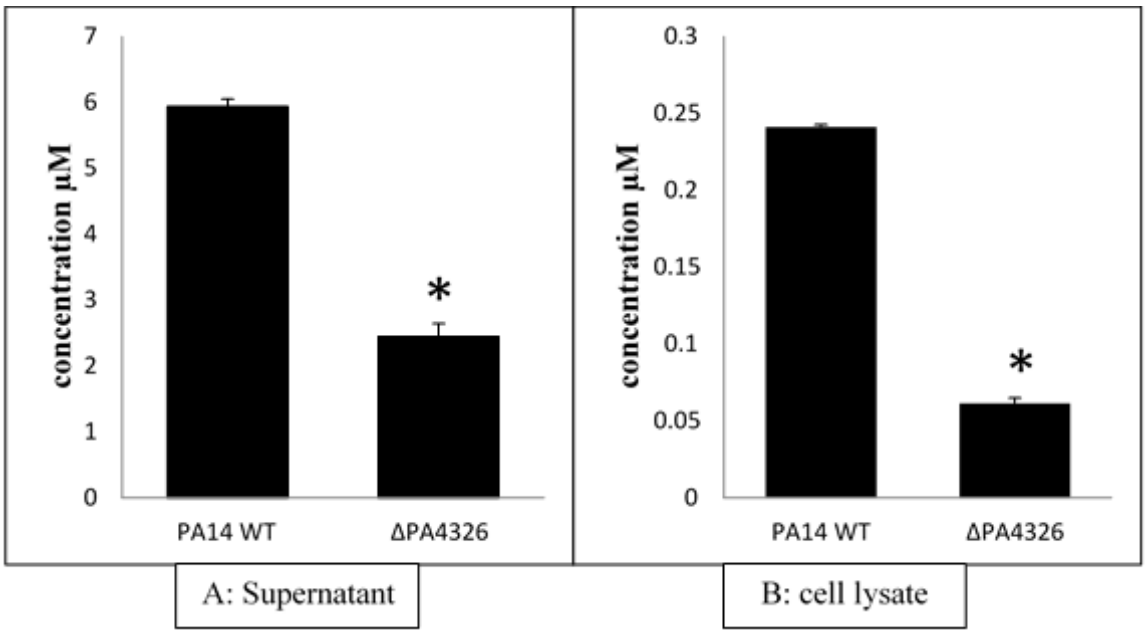

Figure 9: Δ*PA4326* affects pyocyanin levels. The pyocyanin levels of the bacterial strains were determined by spectrophotometry of chloroform extracted bacterial fraction (Essar et al. 1990), as described in Materials and Methods. We found less pyocyanin in A) culture supernatants and B) whole cell lysates of Δ*PA4326* than in these fractions of wild type. Results are representative of three independent experiment performed in triplicate. *$p<0.05$.

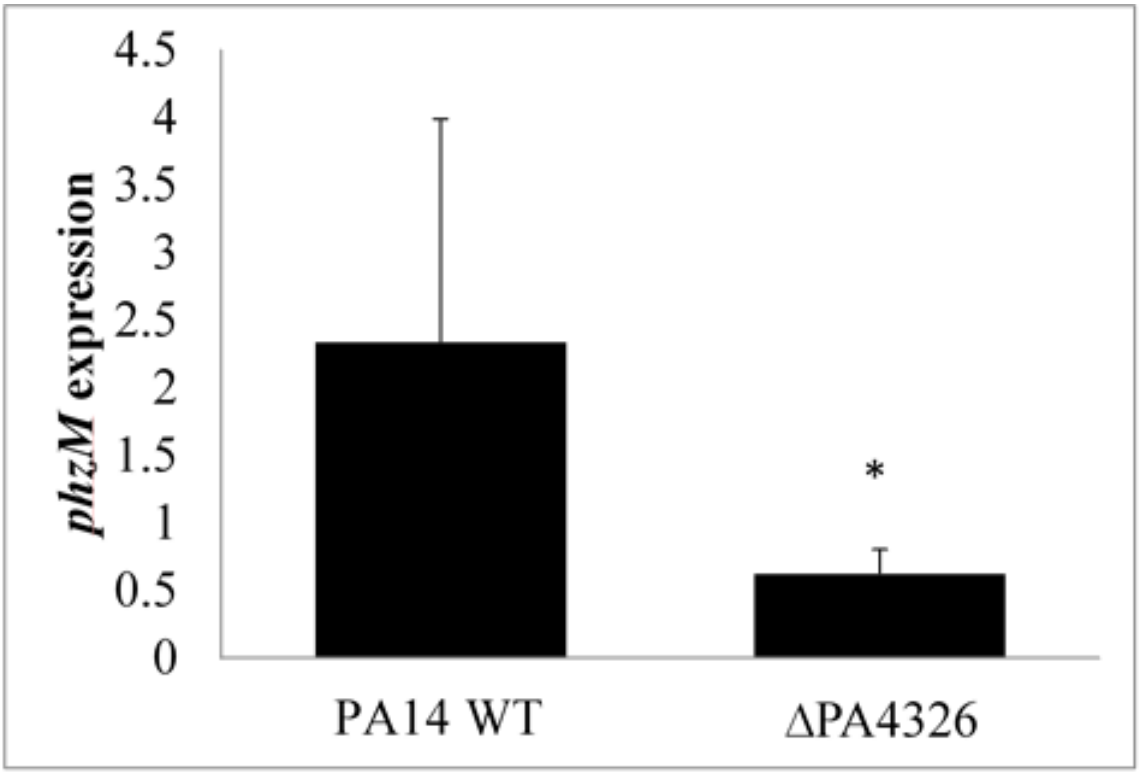

Figure 10: Deletion of *PA4326* results in reduced expression of *phzM*. Transcript level was assessed by qPCR. Data were normalized to control transcript *PA5110*. The presented results are the mean value of four independent experiments performed in triplicate. *p <0.05.

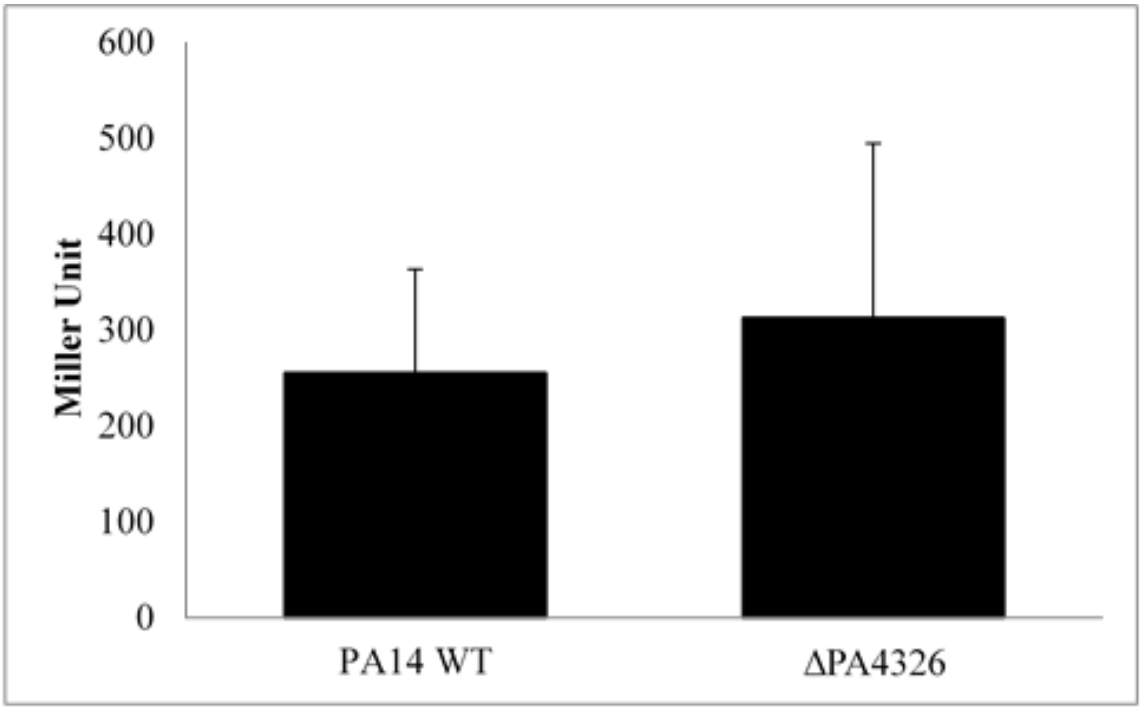

Figure 11: Level of C_4-HSL molecules within the supernatant fraction of the strains (PA14 WT and Δ*PA4326).* As described in methods, the level was quantified as Miller Units of β–galactosidase activity in the cross-feeding activity. No statistically significant differences were observed among the strains. Data are representative of 3 independent experiments.

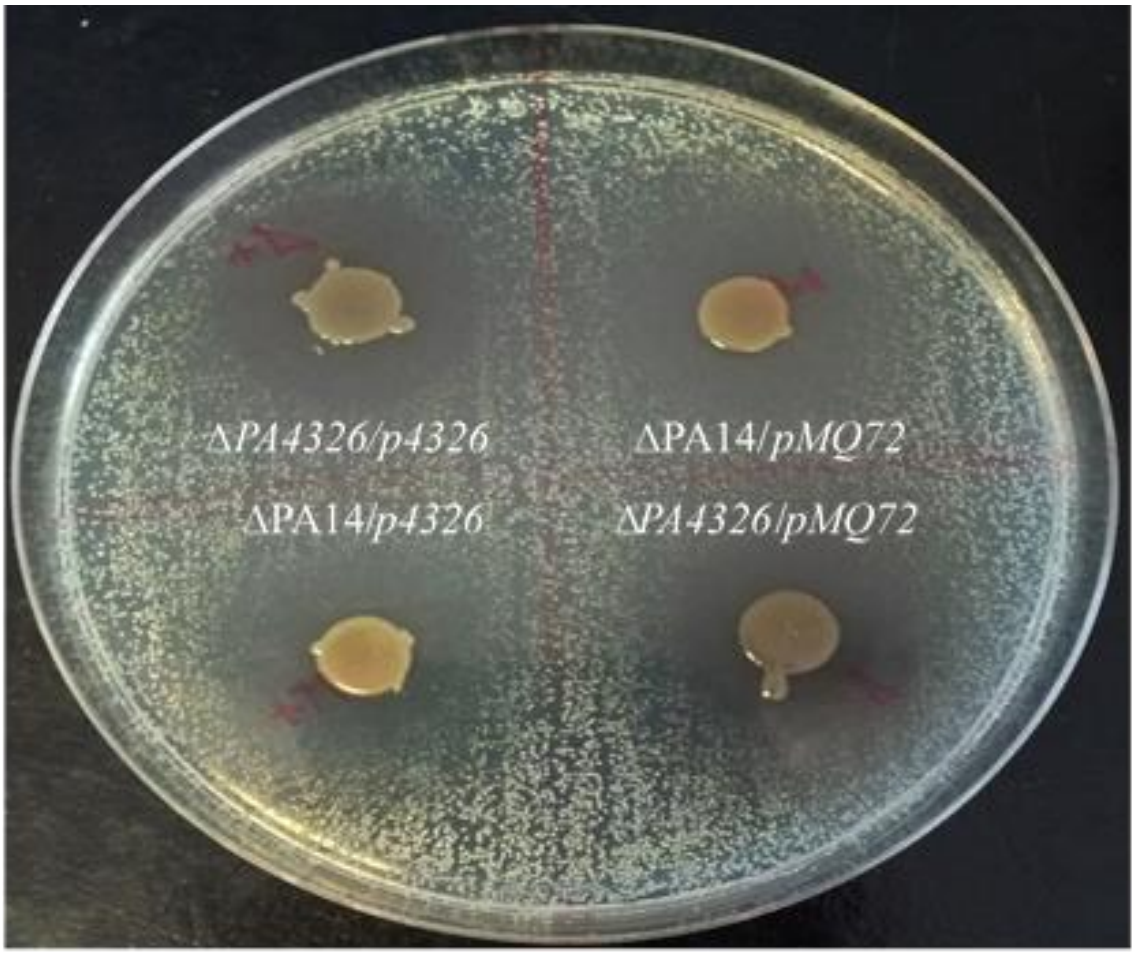

Figure 12: Deletion of *PA4326* does not disrupt PQS levels. The plates were swabbed with *S. aureus* and spotted with four different constructs (Δ*PA4326/p4326,* Δ*PA14/pMQ72,* Δ*PA14/p4326,* and Δ*PA4326/pMQ72*). After incubation overnight at 37^0 C, the zones of inhibition were observed for these different strains. Data are representative of 3 independent experiments.

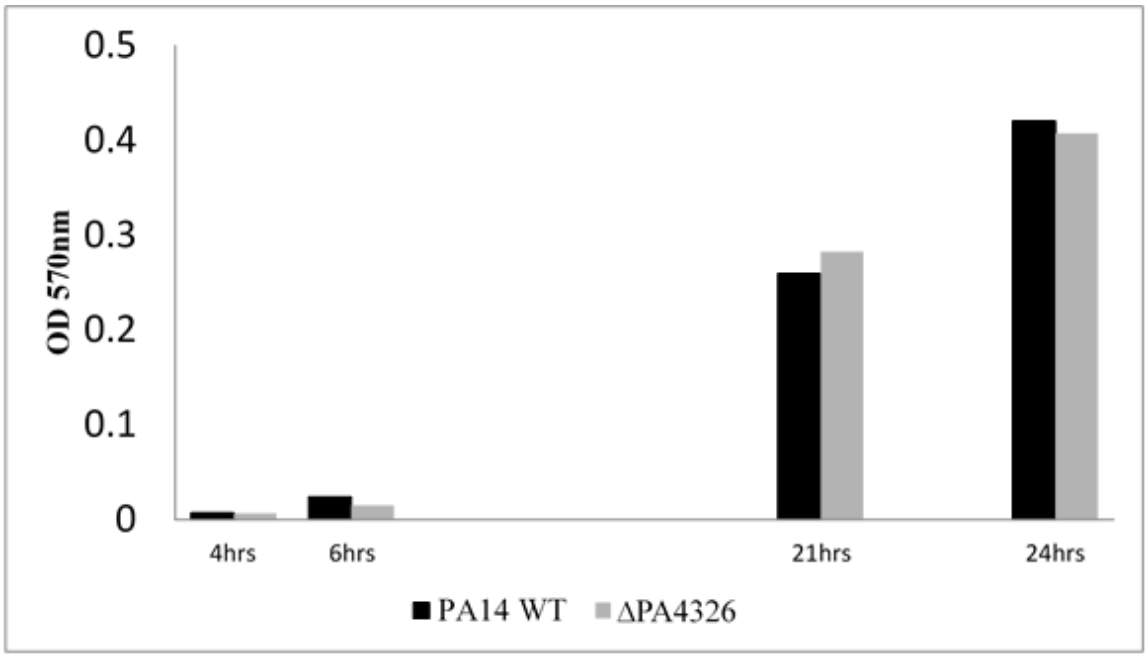

Figure 13: Δ*PA4326* forms comparable biofilm to wild type at various time points. As described in the methods, wild type and Δ*PA4326* were assayed for biofilm formation in minimal M63 media supplemented with 0.4% arginine. No statistically significant differences were observed among the strains.

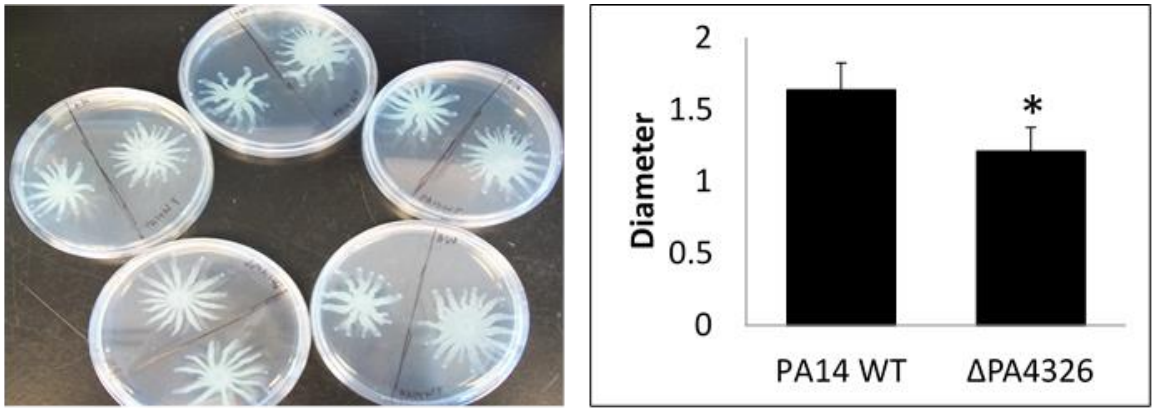

Figure 14: Motility of Δ*PA4326*. A) Swarming motility was identical between wild type and Δ*PA4326* strains. Shown are 5 replicate assays, depicting wild type on the left side of the plate and Δ*PA4326* on the right side. B) Swimming motility was significantly decreased in the Δ*PA4326* strain, compared to wild type. The swimming zones were measured after 16 to 20 hours of incubation at 37^0 C. *p< 0.05.