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The Role of PiuA, an Outer Membrane Iron Receptor, in the Pathogenesis of Pseudomonas Aeruginosa

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THE ROLE OF PIUA, AN OUTER MEMBRANE IRON RECEPTOR, IN THE PATHOGENESIS OF *PSEUDOMONAS AERUGINOSA*

by

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Introduction

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a Gram negative, rod-shaped bacterium found ubiquitously in the environment. The organism inhabits soil, streambeds and other water environments, subsurface sediments and plants (Hancock & Brinkman 2002; Stapleton et al. 2000). It is known to have the ability to use many carbon sources for nutrition, such as jet fuel, oil and multiple intermediates from the tricarboxylic acid (TCA) cycle. It seems these are preferred over the more common glucose as a carbon source (Li & Lu 2007; Stapleton et al. 2000). *P. aeruginosa* infects multiple different organisms, ranging from insects to humans (Hancock & Brinkman 2000).

P. aeruginosa has a large genome (PA01, the strain commonly used as a wild type in studies, has 5,570 genes or about 6.3 Mbp of DNA), which allows it to persist on a wide variety of surfaces on the human host, including the eyes, ears, burns, wounds, and the respiratory tract (Davies & Bilton 2009; Hancock & Brinkman 2002; Mahajan-Miklos 1999; Winstanley & Fothergill 2008; Wood 1976). It can also persist in anaerobic conditions using NO_3^-, NO_2^-, or arginine for substrate-level phosphorylation, or by using Fe^{3+} as a terminal electron acceptor, making it an ideal pathogen associated with various environments and diseases within the host (Hassett et al. 2009; Shroll & Straatsma 2002).

Pseudomonas aeruginosa and Disease

Using the abilities from the large genome mentioned above, *P. aeruginosa* can proliferate in many areas, making it the causative agent of some of the most prevalent opportunistic diseases in humans. Though little risk to those with normal immune systems, patients with neutropenia, Human Immunodeficiency Virus, Cystic Fibrosis (CF), cancer, severe burns, and those receiving immunomodulatory therapies are at increased risk of developing severe *P. aeruginosa* infections. Patients hospitalized with a ventilator are also at an increased risk of ventilator-associated pneumonia (VAP) (Sadikot et al. 2005; Grgurich et al. 2012). *P. aeruginosa* is one of the most prominent ventilator-associated pathogens, with high mortality when compared with other pathogens of the same niche (Chaste & Fagon 2002; Madiha and Ostrosky-Zeichner 2012). The longer a patient stays in the hospital, the higher their risk for a nosocomial infection from *P. aeruginosa*. Of the 2 million annual hospital-acquired infections, 10% are caused by *P. aeruginosa* (Hancock & Brinkman 2002; Gaynes and Edwards 2005).

Many of these infections are related to interruptions to the innate barriers important to immunity. Burn patients and those with corneal abrasions are examples of open sores that become a route of entry for the bacteria, leading to further invasion and ongoing infection. Cystic Fibrosis is an example of a barrier disturbance that can result in a chronic *P. aeruginosa* infection, in that it affects the innate barrier to infection.

P. aeruginosa is the major cause of chronic lung infections in individuals with Cystic Fibrosis (CF). It is the predominant reason of most morbidity and mortality associated with the disease, leading to inflammation, lung function decline, and

ultimately death (Gomez & Prince 2007; Heijerman 2005; Winstanley et al. 2009). Of all CF patients, approximately 61% test positive for this pathogen in sputum samples, and over 90% will eventually become chronically infected (Govan et al. 1987; Govan & Deretic 1996; Mearns 1980). More recent estimates are up to 80% of adults with CF are affected with *P. aeruginosa* (Ciofu et al. 2013). The ability of *P. aeruginosa* to cause recurrent infection and inflammation leads to widespread effects in the CF population.

Cystic Fibrosis Pathogenesis

Cystic Fibrosis is the most common inheritable disease found in Caucasians, still proving to be lethal despite ongoing research and much improved supportive therapy (Deretic et al. 1994; :Lamont et al. 2009). Apporximately 1 in 2,500 live births in Caucasian populations worldwide result in CF, with 1 in 25 Caucasians being carriers of an allele (Govan & Deretic 1996). CF is a disease that affects many organs, starting before birth and only ending with the expiration of the patient. Before modern medical management of CF, most complications originated in the GI tract, from intestinal blockage and malnutrition, resulting many times in death before the age of one year (Pier 2000). In modern times, the organs most affected by CF are the lungs, with respiratory complications resulting in much of the morbidity of the CF patient throughout life and causing most of the mortality associated with the condition (Deretic et al. 1996; Heijerman 2005; Lamont et al. 2009).

The effects of CF are generally located within epithelial cells of the organs affected. The sweat glands, secretory ducts of the pancreas, gastrointestinal and lung epithelia are among those affected (Boucher 2002). Organs such as the vas deferens,

intestines and pancreas are affected before birth, which can affect functionality such as reproduction later in life (Heijerman 2005).

CF is characterized by a mutation in the gene that encodes a transmembrane protein known as the cystic fibrosis transmembrane conductance regulator (CFTR), resulting in a loss or alteration of function for the protein. CFTR functions in the transport of chloride ions across the plasma membrane and regulation of other ion channels on the epithelial surface, such as the outwardly rectifying chloride channel and the sodium channel, as well as regulating HCO^{3-} and potentially glutathione transport (Pier 2000; Ratjen 2009). Symptoms include excessive ion loss during sweating, and secretion of overly viscous mucous in ducts and airway surfaces. This can lead to defects in the function of these glands, or an increase in ion loss that negatively influences muscle and cardiac function that may result in cardiovascular collapse (Govan & Deretic 1996).

One of the major problems encountered in CF, and usually the one causing morbidity and mortality, is the effect this altered secretion has on the lungs. The defective chloride transport at the apical surface of airway epithelia results in liquid depletion among the airway mucous. This loss of liquid causes the mucous to become sticky and thick, resulting in accumulation to levels where it cannot be easily removed as in normal conditions. Glands secreting this mucous become inflamed and swell. The ability of ciliary hairs on the surface of the respiratory epithelia to wave and move foreign substances along with mucous is also impaired. This results in frequent coughing, difficulty in breathing, and frequent lung infections. Recurrent infections and inflammation, usually bacterial, and disruption of the aforementioned innate immune

response contribute to decline of pulmonary function over the lifetime of the CF patient (Boucher 2002; Gomez & Prince 2007; Govan & Deretic 1996; Ratjen 2009; Lamont et al. 2009). The obstruction of the airway results in a cycle with infection and inflammation, as depicted in Figure A.

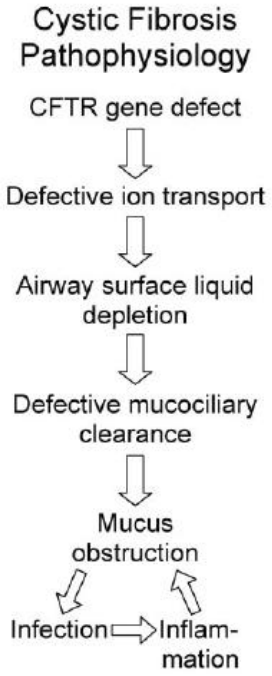

Figure A. Cascade of pathophysiology in cystic fibrosis lung disease (Ratjen 2009).

CF and the Innate Immune Response

CF disease has been mostly associated with problems in the ability of the patient to clear their lungs of the highly viscous mucous buildup. It has been proposed that the salt concentration is high enough on the apical surface of the epithelial tissue to inhibit the function of certain salt-sensitive antimicrobial peptides (defensins) that normally defend against bacterial invasion (Pier 2002). Studies indicated that *P. aeruginosa* added

to epithelium containing surface fluid from normal individuals are killed at a higher rate than when added to epithelia with CF surface fluid. The surface airway fluid collected from CF individuals showed a higher amount of salt than that of unaffected individuals (Smith et al. 1996). Knowles et al. (1997), however, found that the salt concentration on the apical surface was not significantly different than that of healthy persons. They suggest the discrepancies may result from evaporative water loss during sample collection and processing, but have no way of determining the exact cause of these differences in results. Either way, it is evident that bacterial killing is deficient in the airways of CF patients.

Mucin is another molecule that is deficient, both in quantity and quality, in CF patients. In non-diseased individuals, mucin is present in concentrations high enough to bind most foreign invaders, which then are cleared by mucociliary action. Mucin is present in lower-than-normal quantities in the CF lung, with some mucins being degraded in CF individuals (Pier 2002; Ratjen 2009).

Chronic airway inflammation is another key aspect of the innate immune response in CF. Some studies show that inflammation is not present before the airway has been exposed to an infectious agent (Berger 2002). Other studies have shown that inflammatory signs are present in CF infants at as little as 4 weeks old, with markers elevated for IL-8, neutrophil elastase, and neutrophil count from apparently uninfected CF infants (Khan et al. 1995). At some point upon exposure to a pathogen, a hyperinflammatory response begins to occur that feeds into the cycle of infection and obstruction shown in Figure 1 (Ratjen 2009).

CFTR may also act as an uptake protein for pathogens. It has the ability to mediate the uptake of *P. aeruginosa* into lung epithelial cells (Pier et al. 1996). Asialo-GM1 may be another moiety on the apical surface that promotes binding of *P. aeruginosa* to the cell surface (Imundo et al. 1995).

Cytokine excretion seems to be excessive in CF patients along with other defects mentioned above. Increased levels of pro-inflammatory interleukin-8 (IL-8) and neutrophil responses occur in those with CF, far above what is shown in a healthy individual, when measured in a bronchoalveolar lavage fluid. Neutrophils are attracted to the site of infection constantly, at a high rate, even in the absence of detectable infectious agents (Khan et al. 1995). Anti-inflammatory cytokine IL-10 secretion is lower than normal, which, in turn with an excess of GM-CSF, promotes longer survival and activity of neutrophils in the tissues (Chmiel & Davis 2003).

Neutrophils are the most abundant human white blood cell and are a major contributor to innate immunity against infection. Too few neutrophils may leave a person unable to withstand infection, while too many have been associated with morbidity and mortality as well. They are the first cells recruited to inflammatory sites and adhere to damaged areas. They are then stimulated to release inflammatory substances such as reactive oxygen species and proteases. (Sarantis and Grinstein 2012; Zhou et al. 2012).

Neutrophil elastase is responsible for excess tissue destruction, and cleavage of IgG. This compromises macrophage opsonophagocytosis, since they use antibody to bind to target cells. Elastase also cleaves complement receptor 1 (CR1) and complement receptor type 3 (C3bi), both parts of the complement activation system, thus

compromising yet another system used in innate immunity for phagocytosis of bacteria (Chmiel & Davis 2003).

Collagen and elastin are proteins that are exposed after small breaks in the lung epithelium caused by excessive neutrophil response. Antiprotease activity, normally produced by lung cells to stop the destructive action of neutrophil elastase, is overwhelmed by the elevated neutrophil response, which allows for even more tissue destruction by elastase (Chmiel & Davis 2003).

Reactive oxygen species are present in large amounts from this elevated immune response. CFTR, as mentioned earlier, is speculated to transport glutathione, which aids in neutralizing these reactive oxygen species compounds, thus reducing damage done to the surrounding tissues. Bronchiectasis (an outpunching of the airway wall seen in many lung diseases) eventually occurs due to the tissue damage, leading to fluid pooling and a perfect place for bacterial survival. Along with the phenomenon of biofilms, which will be introduced later, the infection becomes impossible to clear over time. Taken together, constant tissue damage combined with lowered helpful responses to this tissue damage result in massive damage to the lungs long term and decline of lung function (Chmiel & Davis 2003).

Genetics of Cystic Fibrosis

Normally located on the apical surface of an epithelial cell, CFTR in CF epithelia may be present on the surface but not functional, or not present at all on the apical surface, depending on the particular mutation (Boucher 2002). The 250 kb long gene

encoding CFTR is located on the long arm of chromosome 7 and contains 27 exons coding for 1,480 amino acids (Govan & Deretic 1996).

Over 1,800 different mutations have been described in the CFTR gene, though most are very rare (Flume and Devanter 2012). The most common mutation, a loss of three nucleotides resulting in a loss of phenylalanine at position 508 (Δ508), is found in 66% of patients worldwide (Ratjen 2009). There are 6 classes of mutations that have been identified within the CFTR protein, depending on the functional consequences on CFTR in the cell. Class I are stop mutations that assure that CFTR is not synthesized within the cell whatsoever. Class II are inadequately processed CFTR proteins that do not get trafficked to their apical membrane sites; this is the most common and includes the ΔF508 mutation. These proteins do have a chloride transporting capability, though it is reduced. Class III are inadequately regulated CFTR proteins that reach the membrane, but do not properly regulate the chloride channel due to a reduced probability of channel opening. Class IV occurs when CFTR protein is made and trafficked to the membrane correctly, but abnormally disrupts conductance, not allowing chloride to flow correctly. Class V is when the CFTR protein is only partially produced; and class VI, where CFTR protein is degraded early, not allowing it to function adequately or for enough time (Ratjen 2009).

Classes I, II, and III are more common, and usually result in insufficient pancreatic function, whereas classes IV, V and VI are less common and usually result in sufficient function of the pancreas. Lung disease occurs in all classes, but varies in severity. Recently, more progress has been made concerning prognostic determination based on which CFTR mutation is present. New treatment approaches are being put forth

to address specific defects in the CFTR dysfunction. PTC 124 is a derivative of aminoglycoside that may help in class I mutations by increasing read-through to help finish transcribing the CFTR gene. Drugs to increase processing and reduce cellular ability to degrade CFTR in class II mutations may help many patients, while drugs that aid in opening the channels normally may aid in patients with class II and III mutations (Ratjen 2009).

The Ecology of the CF Lung

Virtually all patients with CF are or will eventually become infected with some species of microbes. The 4 species of bacteria studied the most in CF infections are *Staphylococcus aureus*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, and *Burkholderia cepacia*. Which species, as well as the prevalence of these species, are present in the lung changes over time. *S. aureus* is typically found in young patients, with the prevalence of those infected with *P. aeruginosa* going up with age (Figure B). Infections with Respiratory Syncytial Virus are an example of another species directly leading to increased likelihood of *P. aeruginosa* infection. Eventual infection with *P. aeruginosa* coincides with a poor prognosis and a, sometimes steep, decline in lung function. Early and aggressive antibiotic therapy has been shown to be helpful in slowing chronic colonization with *P. aeruginosa* and *S. aureus*, although not enough information exists to determine if this should become the standard of care in CF patients (Harrison 2007; Antunovic et al 2012).

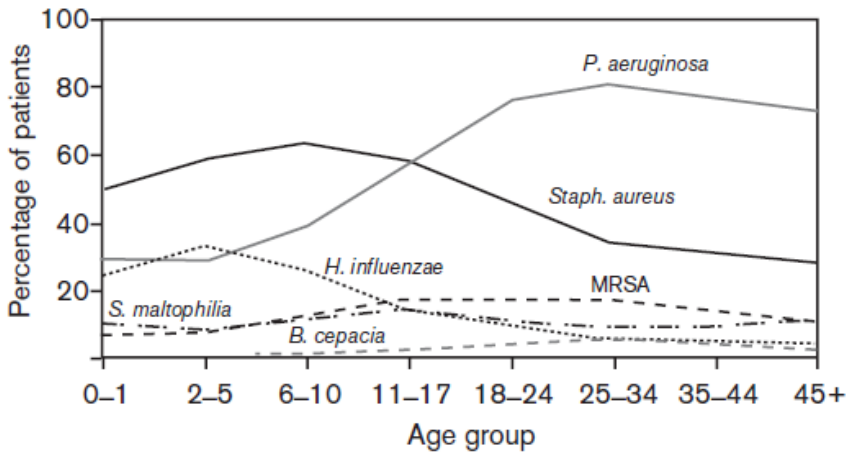

Figure B. Prevalence of selected respiratory pathogens by age group in CF patients (Harrison 2007).

The research that has been done on coinfections with multiple species in CF is scattered. It is not easily put together into meaningful patterns of what is present in individuals and when it is present. Most previous studies do not report individual coinfection status, or report only on bacterial pathogens. Some take samples from multiple areas within the respiratory tract, using different techniques such as lavage and sputum collection. This makes compiling the data in order to determine useful patterns in infection status to be difficult or impossible (Harrison 2007).

This is in contrast to the improvements being made in reporting of species found within CF patients due to improved detection methods and improved life expectancy with the CF population. At this time, over 40% of the CF population is over 18, with a projected average life expectancy at almost 40 years (Antunovic et al. 2012; Flume and Devanter 2012). As expected, as these CF populations have aged, their individual lung ecologies have changed. Figure C shows the current knowledge of species found in CF airways and their prevalence.

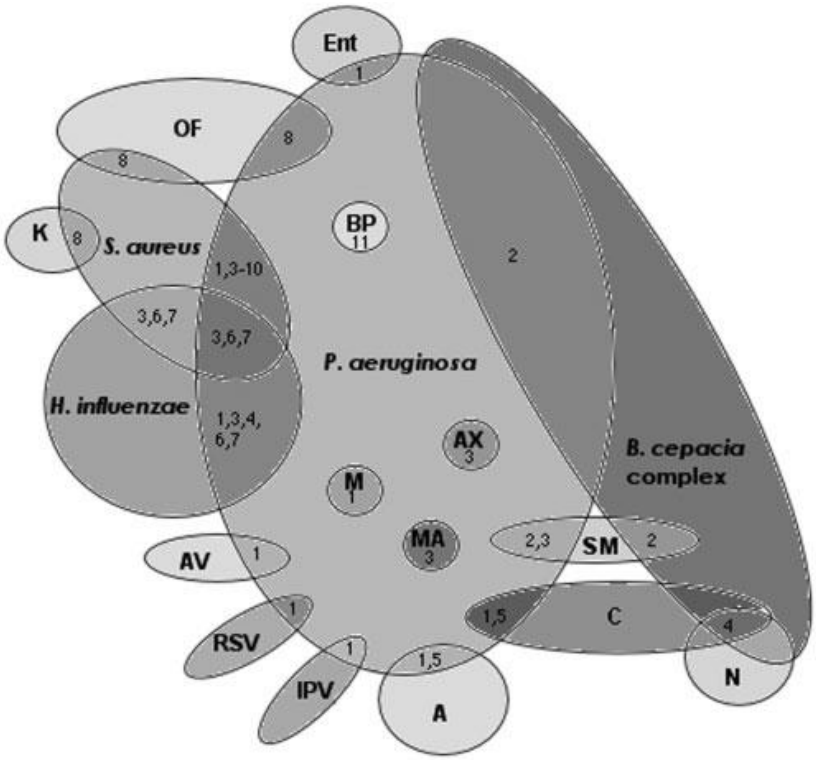

Figure C. Venn diagram showing reported coinfections of the CF airways. (NB: coinfection does not necessarily imply direct interaction between species.) A, *Aspergillus spp*.; AV, adenovirus; AX, A. xylosoxidans; BP, bacteriophage; C, *Candida spp*.; Ent, enterobacteria; IPV, influenza and/or parainfluenza virus; K, *Klebsiella spp*.; M, mycoplasma; MA, *Mycobacterium abscessus*; N, *Neisseria spp*.; OF, oropharyngeal flora; RSV, respiratory syncytial virus; SM, *S. maltophilia* (Harrison 2007).

A significant portion of the CF patient community is infected with more than one pathogen. Just how large a portion is a subject of disagreement between different investigators and papers. Some claim 31% of patients harbor multiple strains of bacteria (Anzaudo et al. 2005), while other authors say most patients are infected (Wahab et al. 2004). The different regions (upper/lower and different lobes) of the respiratory tract have shown to vary in species present (Armstrong et al. 1996, Smith et al. 1998).

Interactions between species will differ based on the pathogens present. These differences in pathogen populations have an influence on progress of disease and decline of lung function (Harrison 2007).

The interactions occurring in the CF lung can take the form of synergism or antagonism. An infection with particular pathogens may change the environment so as to hamper other pathogens from invading, such as *P. aeruginosa* alginate production create a hypoxic environment detrimental to some species (Worlitzsch et al. 2002). Community interactions may also work to modulate the environment to aid in other pathogens invasion. For instance, respiratory syncytial virus infection may facilitate infection by *P. aeruginosa* (Peterson et al. 1981).

Evolution also plays a role in changing the conditions of infection in the CF lung over time. As pathogens are introduced into the lung environment, genetic rearrangements including nonsense, sense and frameshift mutations contribute to changes in phenotype over time. Some changes, such as the conversion to mucoidy, are associated with *P. aeruginosa* chronically infecting CF patients. These changes can aid in immune evasion as well as other survival advantages. Antibiotic treatment also contributes significantly to pathogen evolution over time. Multidrug efflux pumps are one major resistance development contribution (Harrison 2007).

Siderophore production decreases over time during chronic *P. aeruginosa* infections. This may be due to environmental changes in the host, or differences in the strain founding the initial infection (De Voss et al. 2001).

Artificially implanting various microbial communities into the lungs to aid in defending against bacterial invasion has been suggested as an option in CF. Even without looking toward treatment options, it is important to understand that microbial ecology plays a large role in determining progression and prognosis in CF (Harrison 2007).

Colonization of the CF Lung

In the past, most infants with CF died due to infection with *Staphylococcus aureus*. Since the advent and regular use of antibiotics, mortality due to infections with pathogens such as *S. aureus* and *Haemophilus influenzae* are less common. Anti-pseudomonal drugs have resulted in progress in the morbidity and mortality of CF patients, though rarely does therapy result in total eradication of infection. The patients generally survive into adulthood, eventually becoming colonized with *P. aeruginosa* and dying from the infection and associated decline in lung function (Govan and Deretic 1996; Flume and Devanter 2012).

Infants born with CF show no initial colonization with normally CF-associated pathogens, but do exhibit signs of immune pathology, such as elevated levels of IL-8 and neutrophil elastase, suggesting neutrophilic inflammation. Even though signs of inflammation are present shortly after birth, there is no corresponding obvious lung pathology when infection is not present (Govan and Deretic 1996; Heijerman 2005).

There are factors present that contribute to the colonization of the lungs by pathogens in CF patients. Defective chloride ion transport may lead to excessive NaCl in the liquid at the airway surface. This may cause certain airway defense factors, such as defensins, to become impaired (Heijerman 2005, Smith et al. 1996). Others have failed

to verify that salt content is elevated (Pier 2000). Another factor that may play into infection is the dehydration seen in the surface liquid in CF patients that may impair clearance of microbes from the airway and leave the lungs open for infection (Matsui 1998). Both of these contributing factors would seem to leave the lungs of a CF patient prone to infection, but not specifically for *P. aeruginosa* infection as argued by Pier (2000), since these defenses are part of innate immunity.

Adherence to airway epithelial cells seems to be a major factor in *P. aeruginosa*'s ability to invade the lungs of CF patients. This will be covered in more depth in a later chapter.

Internalization of *P. aeruginosa* by airway epithelial cells may be partly responsible for clearing of the bacteria from the airway of healthy individuals. *Escherichia coli* is cleared from bladder infections in this manner (Mulvey et al. 1998). CF patients would not be able to clear these cells because of the viscous nature of the airway surface liquid. Internalization in cells with the ΔF508 allele should be decreased compared with normal cells. CFTR is the receptor that binds to *P. aeruginosa* cells in order to internalize them, and CFTR expression in CF is decreased compared to wild-type CFTR (Pier 1997).

Mouse models of multiple CFTR mutations have shown this reduced internalization of *P. aeruginosa* (Delaney et al. 1995; Tang et al. 1995; Zeiher et al. 1995; Zhou et al. 1994). These mutations correspond to mutations seen in human CF, though do not have the same pathology. Inhibition of bacterial ingestion by epithelial cells has been shown to increase the bacterial burden in the lungs. This means epithelial

internalization of bacteria could be an innate defense against lung infection. These factors leave the airway open for habitation of *P. aeruginosa* by not immediately killing or removing the pathogen (Pier 2000).

Factors Leading from Colonization to an Acute Infection

Several virulence factors produced by *P. aeruginosa*, both secreted and cell associated, contribute to its ability to infect susceptible populations. Exotoxin A disrupts protein synthesis and results in cell death, causing local tissue damage and aiding in the initial bacterial invasion (Woods and Iglewski 1983). ExoU, ExoT, ExoS and ExoY have been found to be released by *P. aeruginosa* and injected by the Type III secretion system into the cytoplasm of host cells. ExoY is an adenylate cyclase and ExoU is a cytotoxin that directly lyses cells through phospholipase A_2 activity. Exoenzyme S and exoenzyme T are responsible for directly killing cells and allowing *P. aeruginosa* to disseminate (Nicas et al. 1985; Nicas, Bradley, Lochner and Iglewski 1985; Howell et al. 2013), though this has been disputed recently (Shaver and Hauser 2004).

The hemolysins phospholipase C and rhamnolipid together break down lipids. Rhamnolipid solubilizes pulmonary surfactant to allow phospholipase C access to break down the surfactant. This is possibly responsible for lung collapse (atelectasis) seen in *P. aeruginosa* lung infections (Liu 1974). Rhamnolipid also inhibits ciliary function to make it further impossible for mucociliary transport to clear the airways of infected individuals (Read 1992).

Proteases such as LasA elastase, LasB elastase, and alkaline protease all contribute to infections *by P. aeruginosa*, especially acute infections (Seed et al. 1995;

Clark et al. 2011). The protein elastin is an essential component in the lung, allowing for expansion and contraction integral to lung function and integrity. Elastin is also a major component of blood vessels, which need to expand and contract with the flow of blood (Deldon and Iglewski 1998). LasA, a serine protease, nicks elastin enough to allow more degradation by LasB, a zinc metalloprotease, and alkaline protease, as well as neutrophil elastase (Galloway 1991). LasB also degrades fibrin and collagen (Heck et al. 1968), as well as inhibiting local immune function such as IgA, IgG, and lysozyme. LasB also degrades protease inhibitors such as alpha-1 proteinase inhibitor and bronchial mucus proteinase inhibitor. This makes LasB a major virulence factor that synergistically works with other proteases LasA, alkaline protease and neutrophil elastase (Deldon and Iglewski 1998; Clark et al. 2011).

Pyocyanin is a major virulence factor in *P. aeruginosa* infections. Pyocyanin slows ciliary action as well as aiding in killing cells by oxidative processes (Kanthakumar 1993). Pyocyanin generates reactive oxygen species through a process termed redox cycling. This has been shown to inactivate α_1 protease inhibitor, which may contribute to lung tissue damage by hindering the host's ability to downregulate serine protease activity, such as elastase (Britigan et al. 1999). Pyocyanin has also been shown to decrease catalase activity in cells, further limiting the host's ability to mediate damage from reactive oxygen species (O'Malley et al. 2003). Ciliary beat of ciliated respiratory epithelium was shown to be decreased upon contact with pyocyanin (Kanthakumar et al. 1993). Without pyocyanin, the ability of *P. aeruginosa* to cause disease in cystic fibrosis is markedly decreased (Lau et al. 2004).

Hydrogen cyanide is produced by *P. aeruginosa* for competitive killing of other species, such as *Caenorhabditis elegans*. Cyanide also binds to terminal oxidases of respiratory enzymes resulting in inhibition of aerobic respiration in affected cells (Lenney and Gilchrist 2011).

Lectin A, an outer membrane protein of *P. aeruginosa*, causes cytotoxicity by decreasing growth rates of respiratory epithelial cells. It has also been shown to increase permeability to *P. aeruginosa* exotoxin A, thus increasing effects seen from that virulence factor. Lectin B may aid in adherence to epithelial cells, as well as biofilm structure and formation. Lectin B is also another factor decreasing beat frequency of respiratory cilia (Tielker et al. 2005).

Quorum Sensing

The synthesis of alginate may occur in many strains of *P. aeruginosa*, but not to the degree of overexpression found in mucoid strains. In order for overexpression to occur and begin the conversion to mucoidy, which is a hallmark of chronic lung colonization, the bacteria must undergo genetic alterations to produce virulence factors. Alterations occur due to a phenomenon termed quorum sensing. Quorum sensing is the ability of the bacteria to communicate with each other to bring about widespread changes in transcription based on their density (De Kievit and Iglewski, 2000).

The process of quorum sensing depends on a molecule, called an autoinducer (AHL), produced by the bacteria that will bind to a transcriptional activator known as R protein and induce gene expression when at a high enough density (De Kievit and Iglewski 2000; Pesci et. al. 1999)

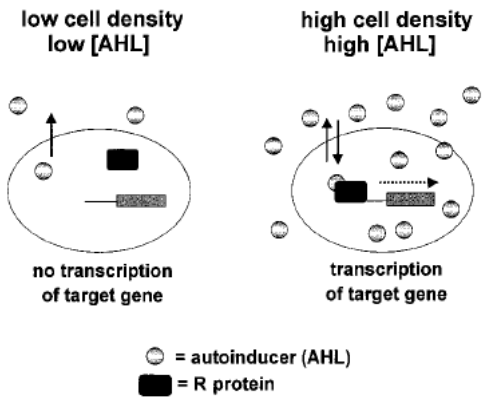

Figure D. Quorum sensing in Gram-negative organisms (De Kievit and Iglewski 2000).

P. aeruginosa has two signaling systems; *las* and *rhl*. These systems together regulate the expression of multiple virulence factors important in establishing and maintaining chronic infection. The LasI autoinducer synthase is responsible for producing the acylated homoserine lactone N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C_{12}-HSL). When at sufficient quantities, the transcriptional activator LasR will function with the AHL to express virulence genes including *lasA*, *lasB*, *aprA*, and *toxA*. This will also induce expression of more *lasI*, which creates a positive feedback loop. The LasR-3-oxo-C_{12}-HSL complex also controls expression of *rsaL*, which inhibits *lasI* expression (De Kievit and Iglewski 2000; Pesci et. al. 1999; Smith and Iglewski 2003).

The second signaling system, *rhl*, is controlled by the transcriptional activator RhlR , which is activated by the RhlI autoinducer synthase-produced N-butyryl-L-homoserine lactone (C_4-HSL). The expression of pyocyanin, cyanide, *lasB*, *aprA*, and *rhlAB*, required for rhamnolipid production, is dependent on this RhlR-C_4-HSL complex (De Kievit and Iglewski 2000; Smith and Iglewski 2003).

The components of these two systems are very specific in their interactions. Even with the similarities in the AHLs and RhlR and LasR, neither AHL seems to significantly activate the opposite transcriptional activator. The products of both systems are generally generated by one system only. In addition, the *las* system positively regulates *rhlR* and *rhlI*. 3-oxo-C_{12}-HSL can also compete with C_4-HSL for RhlR binding, in order to inhibit the *rhl* system. This means the *las* system is the dominant system in the hierarchical quorum sensing system of *P. aeruginosa*. This suggests a separation between the two systems, though they are linked together (De Kievit and Iglewski 2000).

A third autoinducer molecule very different from the other two has been reported by Pesci et al. (1999). This molecule, 2-heptyl-3-hydroxy-4-quinolone (PQS), induces *lasB* expression. It is under the control of the las system, but needs RhlR for activation. Much is still unknown about PQS, but the fact that 3 autoinducers have been found along with the other regulatory components of the quorum sensing system suggests tight control of expression (De Kievit and Iglewski 2000; Pesci et. al. 1999).

Adhesion of *P. aeruginosa* to Airway Epithelial Cells

Outer membrane proteins that aid in bacterial adherence to epithelial cells also aid in infection. Pili have been implicated in the adherence of *P. aeruginosa* (Chi et al. 1991). Flagella, and exoproducts such as elastase, alkaline protease and phospholipase C, have also been shown to aid in adherence to epithelial cells (Saiman et. al 1990; Azghani et al. 1992).

Pili expression was found to aid in adhesion and virulence of *P. aeruginosa*. Virulence in non-piliated strains was found to be less than PA01, including the ability to

cause pneumonia and disseminate to other organs, and rate of mortality. This indicates pili are important in adherence and infection (Tang et al. 1995; Bucior et al. 2012). RpoN, a regulatory protein involved in the expression of pili, also plays a role in adherence to lung epithelia (Chi et al. 1991).

Upon incubation with *P. aeruginosa*, human alveolar epithelial cells in culture (A549 cell line) internalize the bacteria, as determined by transmission electron microscopy. Lysosomal-mediated killing by the A549 cells was not observed for this population of bacteria, as internalized bacteria were viable after recovery hours later. This could play a role in sheltering the bacteria from host defenses during chronic infections (Chi et al. 1991).

Another binding factor in the airway is respiratory mucins. Bacteria have been shown to bind to mucins present in the respiratory mucosa, independent of pilin production. This is increased upon iron limitation (Scharfman et al. 1996). OprF, an outer membrane protein seen in *Pseudomonas* sp., has been shown to aid in adherence to A549 cells as well. OprF-deficient *P. aeruginosa* strains adhere less well to A549 cells. Pre-incubation with purified OprF or monoclonal antibody for OprF decrease bacterial-cell adherence as well, showing a specific interaction event (Azghani et al. 2002).

Flagella may be another important factor in *P. aeruginosa* binding to airway epithelium. Flagellar knockouts showed less ability to adhere to bovine tracheal cells. This may be due to a decreased ability to associate with the epithelial cells because of less chemotaxis, or because pilin and flagella are both regulated by RpoN (Saiman et al. 1990). This is corroborated by a study showing flagellated strains of *P. aeruginosa* were

mostly responsible for initially infecting CF patients (Luzar et al. 1985). The importance of flagella has been verified more recently as well (Feldman et al. 1998; Bucior et al. 2012). All of these factors together result in *P. aeruginosa* being well-suited for invasion of CF lungs. The many factors present help to establish an infectious process in immunocompromised patients, and indeed, infection and colonization correlate highly with the ability to adhere to these cell types (Woods et al. 1980).

Mucoid *P. aeruginosa* Causes Chronic Infection

An acute infection does not always result in a chronic infection in those with CF. Intermittent infections will only result in sporadic colonization that does not result in significant decline in lung function (Kerem et al. 1990). Chronic colonization may not occur for up to 5.5 years after introduction (Govan and Deretic 1996). Once the *P. aeruginosa* has been cultured for 6 months continuously and the patient develops an increased antibody response to precipitins, it can be considered a chronic infection (Johansen and Hoiby 1992).

Chronic infection by *P. aeruginosa* coincides with conversion of the bacterium to a mucoid state. The time from initial colonization can vary drastically, anywhere from immediate detection of mucoidy to 5 or more years (Govan and Deretic 1996). It has been demonstrated that random mutations causing the conversion to mucoidy occur at a low rate, so it is thought to be a significant advantage for the pathogen to convert to this state (Martin et al. 1993).

Mucoid strains of *P. aeruginosa* show an appearance like that of mucous, with a thick, slimy coating on a nutrient agar plate. Alginate production is the reason for this telltale appearance. Antibodies to alginate are detectable in all sputum from chronic *P. aeruginosa* CF infections and are associated with declining lung function (Pederson et al. 1990). Alginate has been shown to be involved in many aspects of chronic infection in CF patients; in particular in three main areas: protective barrier against opsonization and phagocytosis, immunomodulation against host defenses, and biofilm aspects of infection such as adhesion and protection from antibiotic attack (Govan and Deretic 1996; Pritt et al. 2007). Table 1 shows many proposed roles of alginate in *P. aeruginosa* infection in the CF lung.

Alginate is an exopolysaccharide that is secreted from mucoid *P. aeruginosa* cells that enables the pathogen to persist in a structured matrix that gives it multiple advantages over wild-type free living bacteria. In addition to the properties already mentioned, alginate scavenges reactive oxygen intermediates and hypochlorite, which greatly impair the ability of the immune system to destroy the infection (Deretic et al. 1994; Govan and Deretic 1996; Pritt et al. 2007).

Alginate, combined with LPS or other *P. aeruginosa* virulence factors, is associated with an increase in immunoglobulins *in vivo*. It also elicits pro-inflammatory cytokine release such as IL-1, TNF-α and IL-8. These contribute to continuous inflammation and neutrophil accumulation in the airways, thus resulting in the decline of lung function common to CF patients (Govan and Deretic 1996).

Aforementioned alginate properties demonstrate selective pressures for *P. aeruginosa* to convert to the mucoid state upon infection in the CF patient. These changes are a large part of what make it possible for the bacterium to survive for such long periods of time in the CF host (Govan and Deretic 1996).

Conversion to Mucoidy

The alginate biosynthetic cluster is responsible for producing alginate. The cluster is regulated by the *algD* promoter, which is regulated by several transcription factors. Initiation of *algD* transcription is dependent upon AlgU, and alternative sigma factor. Once activated, *P. aeruginosa* converts to mucoidy and begins the chronic colonization of the CF host. Alg R and AlgB also effect transcription of *algD*. Inactivation of *algR* stops *algD* transcription and mucoidy conversion. AlgB activity is not known at this time (Deretic et. al. 1994; Govan and Deretic 1996).

Alginate synthesis can be affected by environmental factors, many of which are present in the CF lung, such as dehydration, abnormal osmolarity and nutrient deprivation. Mutations have been attributed to the changes that occur in mucoid phenotypes of *P. aeruginosa* (Deretic et. al. 1994; Govan and Deretic 1996).

Mutations that cause a mucoid condition to develop lead to changes over time in the genetics of *P. aeruginosa*. Mutations within *mucA* and *mucB* can cause conversion to mucoidy over time. Mutations in *mucA* and *mucB* deprive the anti-sigma factor of *algU* and allow for overproduction of alginate (Deretic et. al 1994).

There is also an AlgU-independent pathway for alginate production. Whereas AlgU-dependent alginate production relies on AlgU for induction of *algD* expression, another sigma factor, RpoN, can also participate in conversion to mucoidy. The two pathways are not independent as they regulate each other as well. This leaves another pathway that mutation may affect mucoid conversion (Boucher et. al. 2000).

Significance of Iron in the Metabolism of Bacteria

Iron is an essential nutrient for bacteria to thrive in their environment. It participates in multiple roles that are essential for their survival, such as respiration, nitrogen fixation, and gene regulation. Iron must be incorporated into proteins in order to perform most of its functions. Most iron available in the lungs is in the ferric form, which is an extremely insoluble form, at 10^{-18} M at a pH of 7.0 (Andrews et al. 2003).

Bacteria must be able to pick up iron from the environment and utilize it in order to perform the various metabolic functions associated with iron, thus they need a high affinity method for obtaining iron. In the case of *P. aeruginosa*, the lungs of human hosts generally contain very little iron, which necessitates iron scavenging. The CF host, in contrast with the non-diseased human, possesses lungs which are higher in iron content. Iron is still not freely available, however; so the bacterium still must scavenge for iron (Andrews et al. 2003; Cornelis and Matthijs 2002; Lamont et al. 2009).

Iron Acquisition by *Pseudomonas aeruginosa*

P. aeruginosa acquires much of its iron through siderophore-mediated processes. Siderophores are high affinity iron chelators that are excreted by bacteria to bind to iron in the environment. These siderophores form ferric-siderophore complexes, which the bacteria take up using specific outer membrane receptors. Periplasmic binding proteins move this complex to ATP-binding cassette (ABC) transporters that move the complex into the cell. The complex then dissociates to form free iron for use by various metabolic systems (Figure E). This process is common to Gram negative organisms, and is distinct from Gram positives (Andrews et al. 2003).

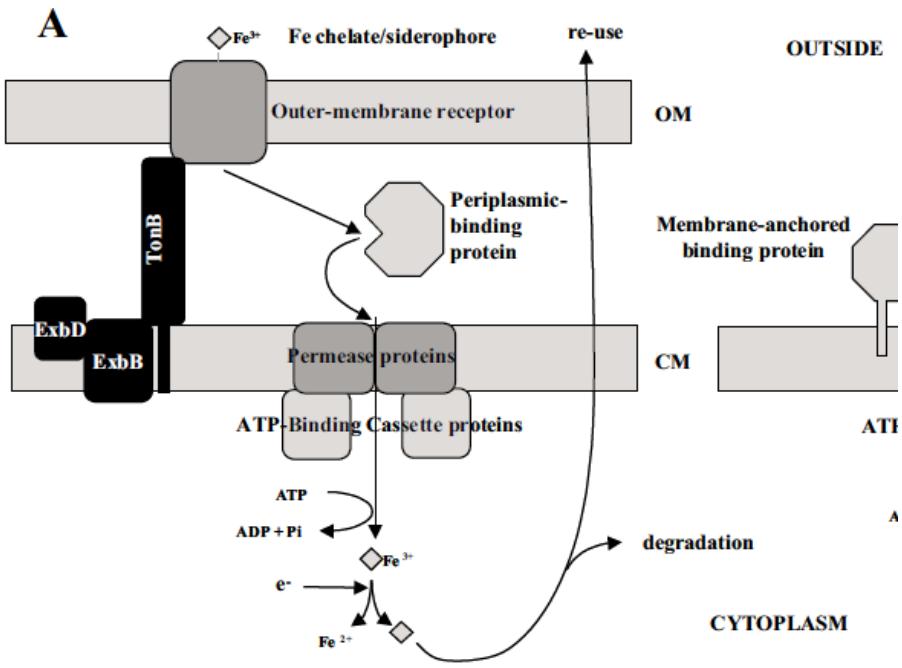

Figure E. Siderophore-mediated iron uptake by Gram negative bacteria (Andrews et al. 2003)

The Siderophores of *P. aeruginosa*

Siderophores have been observed in use by many bacterial species, as well as fungal and plant species (Andrews et al. 2003). *P. aeruginosa* possesses multiple siderophores, of which the main one is pyoverdine (Figure F). There are 3 separate species of pyoverdine in this bacterium, and each binds to a different receptor. There is evidence, however; that species may be able to cross-react with receptors other than theirs. Production of these siderophores is of great importance to the ability of *P. aeruginosa* to establish and maintain an infection Cornelis and Matthijs 2002).

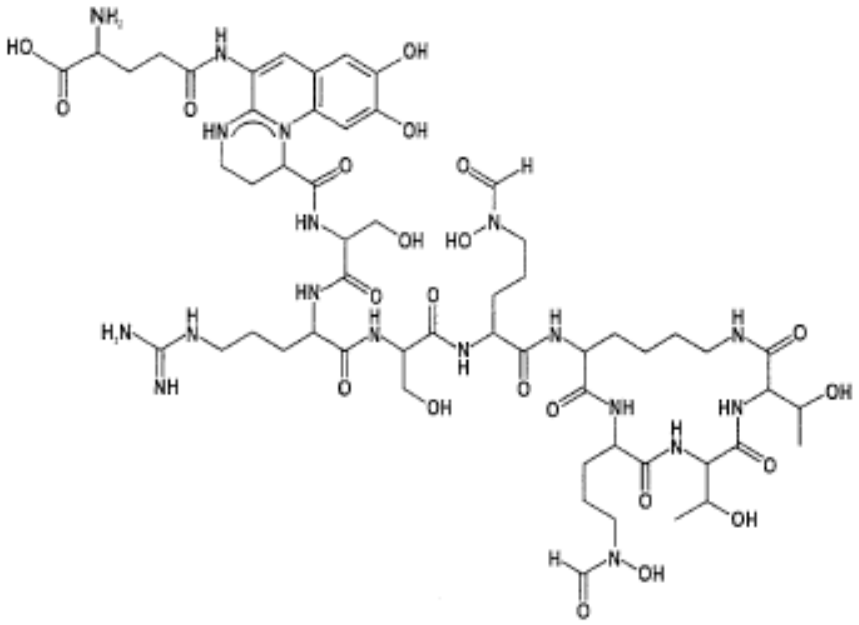

Figure F. Chemical structure of PA01 pyoverdine (Cornelis and Matthijs 2002).

Another siderophore produced by *P. aeruginosa* is termed pyochelin. This molecule is a derivative of sialic acid and has a lower affinity than pyoverdine (Figure G). It is possible that pyochelin scavenges for metals other than iron as well. In the presence of pyocyanin, ferripyochelin forms reactive oxygen species that play a role is

tissue damage. *P. aeruginosa* also has the ability to use other species' siderophores to scavenge iron, and can also use heme as an iron source, making it an extremely versatile organism (Cornelis and Matthijs 2002).

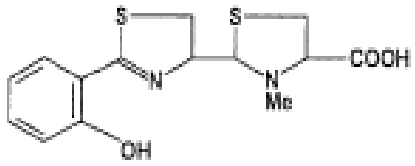

Figure G. Chemical structure of Pyochelin (Cornelis and Matthijs 2002)

Transport of Ferri-siderophore Complexes into the Cell

Iron-siderophore complexes must bind to outer membrane proteins before entering the cell, as described earlier. The pyoverdines have a receptor for each species in *P. aeruginosa*. The three species (Types I, II and III) each bind to receptors termed FpvAI, FpvAII and FpvAIII (Lamont et al. 2009). These ferri-siderophore receptors are present on the membrane normally only during iron starvation (Andrews et al. 2003).

The TonB-ExbB-ExbD complex is needed in order to supply energy for the process of ferri-siderophore uptake (Figure E). Through direct contact between TonB and the OM receptor, energy derived from the chemical gradient at the membrane is delivered to the OM protein to allow for transport. ExbB-ExbD use the chemical gradient to change the confirmation of TonB to an active form, which then conducts the energy to the OM protein (Andrews et al. 2003).

Once in the periplasmic space, the complex must still cross the inner membrane to get into the cytoplasm of the cell. A periplasmic binding protein binds to the ferri-

siderophore one at a time, through an unknown mechanism, to shuttle the complex to an ABC permease at the inner membrane. There are only 4 types of ABC permeases in *P. aeruginosa*, which is in contrast to the larger number of OM proteins (Andrews et al. 2003)

Once inside the cell, the iron is reduced to ferrous iron, for which the siderophore has low affinity, and is thus released as free iron in the cytoplasm. The siderophores are then either degraded or exocytosed to scavenge more iron (Andrews et al. 2003).

Genetics of Iron Regulation

Iron-dependent genes are regulated by the Fur repressor in bacteria. Fur binds iron in the cell, which represses iron uptake protein gene expression. The opposite is true as well; that without iron, Fur will not bind to the Fur box and repress its genes. Fur represses PvdS , which is involved in pyoverdine synthesis, as well as many other genes, including several extracellular factors (Andrews et al. 2003; Cornelis and Matthijs 2002; Vasil and Ochsner 1999). Iron regulated genes play a role in the pathogenesis of *P. aeruginosa*. Genes such as *toxA* and *prpL*, that encode exotoxin A and extracellular protease respectively, are induced by iron (Vasil and Ochsner 1999).

PA4514 is the open reading frame assigned to *piuA*, encoding PiuA, which is a putative TonB-dependent ferrisiderophore receptor on the outer membrane of *P. aeruginosa*. Using a SELEX technique to screen potential DNA fragments for Fur binding, piuA was found to be under Fur regulation. PiuA is upregulated during iron restriction, indicating it is an important gene for survival in a low-iron environment (Ocshner and Vasil 1996; Vasil and Ochsner 1999). At this time, not much else is known

about PiuA, such as its siderophore specificity or its role in virulence (Cornelis and Bodillis 2009).

Role of Iron in the Virulence of *P. aeruginosa*

As mentioned above, some studies show that exotoxin A production is increased under conditions of iron restriction. Exotoxin A, various proteases, elastase and hemagglutinin were all shown to increase in multiple strains of *P. aeruginosa* grown in low-iron conditions (Bjorn et al. 1979; Vasil and Ochsner 1999). Growth of the bacterium in low iron media has been shown to be inhibited (Bjorn et al. 1979). Adhesion to respiratory epithelial cells is enhanced in iron-limited growth conditions, allowing infection in harsh conditions (Scharfman et al. 1996).

A pyocyanin-related compound (phenazine-1 carboxylic acid) has been shown to aid in the scavenging of iron in low iron environments during *P. aeruginosa* infection (Wang et al. 2011). These factors together may aid in bacterial survival by making essential nutrients more available during infection. The virulence of the bacterium increases by 100 fold after being passaged under iron restricted conditions (Forsberg and Bullen 1972).

Significance of Research

Exactly which iron receptors on the outer membrane of *P. aeruginosa* are important, and to what extent, are not known at this time. PA4514 is homologous for the *piuA* gene, which encodes an outer membrane iron uptake receptor protein. Goals for this study include determining how inactivation of this protein affects the pathogenesis of *P. aeruginosa*.

We hypothesize that inactivation of the *piuA* gene and iron depletion will result in a lower ability of a knockout strain of *P. aeruginosa* to grow. This harsh environmental change will, in turn, result in increased virulence factor production and adherence to A549 cells. Overall, we expect to see an increased ability to kill respiratory epithelial tissue.

We assayed for the ability of a strain of *P. aeruginosa* containing no *piuA* gene to grow in iron depleted media, adhere to lung epithelia, produce the major virulence factor pyocyanin and destroy lung epithelial cells to investigate the role of this protein in causing disease.

Long term goals for studies on the properties of PiuA, as well as other outer membrane iron receptors, are to determine which receptors on the outer membrane of *P. aeruginosa* are important in circumventing host responses and establishing infection, maintaining infection, causing tissue destruction, and long-term survival in the host.

Understanding the factors, both on the bacterial side and host side of this infectious process, will lead to better understanding the interplay between host and pathogen during long-term infection in those with cystic fibrosis, as well as those who acquire other *P. aeruginosa* infections. This may aid in development of novel antimicrobial drugs, as well as provide potential vaccine targets for future development.

Materials and Methods

Bacterial Strains. The *P. aeruginosa* strain PW8599 (ΔPiuA) was obtained from the University of Washington two-allele library. The strain was constructed using a transposon mutagenesis process. The strain was confirmed by PCR using flanking primers for the PA4514 gene with a primer complementary to the transposon from the University of Washington (5'-GGGTAACGCCAGGGTTTTCC -3') using Amptaq Gold (Applied Biosystems) directly from a colony grown overnight on LB agar. Reactions were run at 95° for 10 minutes, followed by 30 cycles of 95° (30 seconds), 55° (30 seconds), 72° (2 minutes), and a final elongation of 72° for 7 minutes. Sequences for flanking primers: 4514 Forward (5'-CATAGCGCCAGTAGGACGGG – 3') and 4514 Reverse (5'-GGGTATCACCTTCGGCG -3'). The wild type strain used was the PA01 wild-type strain (B. Holloway, Monash University, Australia).

Culture Conditions. The strains of *P. aeruginosa* (PA01 and PW8599) were grown overnight in Luria Broth (LB) with and with 400 µM 2,2' dipyridyl (LBD), to chelate iron (Fisher Scientific, Pittsburgh, PA, U.S.A.). The PA01 and PW8599 strains were washed three times with LB and LBD , respectively, and diluted to a concentration of 10^5 cells/ mL (OD_{600} = 0.07) using a BioRad SmartSpec Plus Spectrophotometer (BioRad, Hercules, CA, U.S.A.).

For determination of pyocyanin concentrations, PA01 and PW8599 were grown in LB and LBD for 24 hours while shaking. The cultures were 1/10 of the total volume of the containing vessel to ensure proper aeration.

For adhesion assays and cytotoxicity studies, overnight cultures of PA01 and PW8599 were grown in LB and LBD. Bacteria were diluted to an OD_{600} of 0.07 (10^5 cells/ mL). Human alveolar type II like epithelial carcinoma (A549) cells were grown in RPMI medium supplemented with 10% Fetal Bovine Serum (FBS). 96-well tissue culture plates were seeded with 2.0 x 10^4 cells and allowed to grow to confluence in a 5% CO_2 incubator at 37°C.

Growth Curve. *P. aeruginosa* strains PA01 and PW8599 were incubated in LB and LBD media at 37 °C overnight. Both PA01 and PW8599 strains were washed three times in LB and LBD media, respectively, followed by a 100-fold dilution in the same media. These dilutions were prepared in a vessel 10 times larger than the total volume to ensure proper aeration. The optical density of each strain was measured at OD_{600} for 18 hours at 30 minute intervals to determine cell density. Three readings every time period were taken for each strain and condition. Each strain and condition was replicated in triplicate and each assay was repeated in triplicate.

Pyocyanin Quantification. The pyocyanin production of *P. aeruginosa* strains PA01 and PW8599 was quantified in LB and LBD culture media. Strains were grown in LB and LBD for 24 hours at 37 °C. The PA01 and PW8599 cultures were centrifuged at 4,500 g for 15 minutes. followed by the extraction of pyocyanin pigment using a chloroform/0.2 M HCl (1:1, v/v) solution. The A_{520} of the pyocyanin pigment was measured using a BioRad SmartSpec Plus (BioRad, Hercules, CA, U.S.A.). This assay was repeated in triplicate.

Adhesion Assay. To assay for the ability of PA01 and PW8599 to adhere to A549 cells (as an *in vitro* analogue of the human lung), PA01 and PW8599 were grown overnight in LB and LBD and washed three times in the respective medium. The A549 cells were prepared as noted in culture conditions above. The *P. aeruginosa* PA01 and PW8599 strains were diluted to an OD_{600} of 0.07, and incubated with the A549 cells for 1 hour at 37°C and 5% CO_2. Manual desorption was carried out four times with RPMI growth medium with no FBS supplementation. The PA01- and PW8599-adhered A549 cells were removed with the addition of trypsin. The cells were diluted serially and plated onto a LB agar medium. The LB agar plates were incubated at 37 °C overnight and colony forming units were examined for each strain and condition.

Cytotoxicity Assay. The *P. aeruginosa* PA01 and PW8599 cytotoxicity to A549 cells was measured at a 100:1 multiplicity of infection in PBS incubated at 37 °C and 5% CO_2 for 1 hour in 12-well plates. The adherent cells were removed with the addition of trypsin and stained with 0.4% Trypan Blue (1:1, Sigma Aldrich). The number of dead (blue) cells and viable (dye excluding) cells per 100 cells was determined using low-power microscopy with a hemocytometer.

Statistical Methods. A paired, two-tailed Student's t-test was used to compare the statistical significance of the *P. aeruginosa* PA01 and PW8599 strains using Prism (GraphPad Software, Inc. LaJolla, CA).

Results

Determination of Growth Capabilities of *Pseudomonas aeruginosa* strains in differing media

The growth properties of the *P. aeruginosa* PA01 and PW8599 strains were determined in LB and LBD media. It is expected that the growth of the *P. aeruginosa* PA01 and PW8599 strains would be diminished in media that is iron deficient for bacterial metabolic processes. The *P. aeruginosa* PA01 strain in LB media significantly increased in growth, compared to the PA01 strain in LBD media ($p = 0.0006$) and the *P. aeruginosa* PW8599 strain in either LB ($p = 0.0008$) or LBD media ($p = 0.0003$), with an increase of 79%, 42% and 83% respectively. The *P. aeruginosa* PW8599 strain in LB media had higher growth compared to the PW8599 strain in LBD media ($p = 0.0002$) with an increase of 129%; however, the PA01 strain in LBD had lower growth that of the PW8599 strain in LB media ($p = 0.0032$) with a yield 79% that of PW8599 in LB. There was no significant difference between the two strains in LBD media (Figure 3).

Pyocyanin Production between strains in differing media

To determine the relative quantities of the virulence factor pyocyanin produced in the *P. aeruginosa* PA01 and PW8599 strains, we measured its production in LB and LBD media. A significant decrease in pyocyanin production was observed for *P. aeruginosa* PA01 strain in the LB medium compared to the LBD medium ($p < 0.0001$) and the *P. aeruginosa* PW8599 strain in LBD medium ($p < 0.0001$), with decreases of 56% and 67% respectively. The *P. aeruginosa* PA01 strain in LBD medium produced significantly more pyocyanin than the *P. aeruginosa* PW8599 strain in LB medium ($p <$

0.0001) with an increase of 91%, but the pyocyanin was significantly less than the PW8599 strain in LBD medium ($p < 0.0001$) with a 25% decreased yeild. The *P. aeruginosa* PW8599 strain in LB medium produced 61% less pyocyanin than the PW8599 strain in LBD medium ($p < 0.0001$). There was no significant difference between the two strains in LB medium (Figure 4).

Comparison of *P. aeruginosa*'s ability to adhere to lung epithelium

To determine the relative abilities of PA01 and PW8599 to adhere to lung epithelial tissue, and thus potential infection and invasion potential, we assayed adhesion using A549 cells. A significant increase in adherence was observed with PA01 in LBD compared to PA01 in LB ($p<0.0001$) and PW8599 in LB ($p=0.0003$), with increases of 94% and 70% respectively. PW8599 in LBD showed a significant increase in adherence compared to PA01 in LB ($p<0.0001$), PA01 in LBD ($p=0.0273$) and PW8599 in LB ($p=0.0002$), with increases of 176%, 43% and 144% respectively. No significant difference was seen between PA01 in LB and PW8599 in LB (Figure 5).

Cytotoxicity of differing strains of *P. aeruginosa*

To determine the cytotoxic abilities of *P. aeruginosa* PA01 and PW8599, we performed a trypan blue cytotoxicity assay using A549 cells. PA01 in LB showed significantly less cytotoxicity than PA01 in LBD ($p=0.0003$), PW8599 in LB ($p=0.0071$), and PW8599 in LBD ($p<0.0001$), with decreases of 36%, 27% and 47% respectively. PW8599 in LBD showed significantly more cytotoxicity than PW8599 in LB ($p=0.0004$) and PA01 in LBD ($p=0.0152$), with increases of 37% and 21% respectively. All strains

and conditions exhibited significantly increased cytotoxicity compared to the control. No significant difference was seen between PW8599 in LB and PA01 in LBD (Figure 6).

Discussion

This study provides evidence that *piuA*, encoding an outer membrane protein iron receptor, is important in the pathogenesis of *Pseudomonas aeruginosa*. As expected, growth was diminished, both under iron restriction and in normal media, compared to the wild type bacterium. This suggests that *P. aeruginosa* harboring a non-functional PiuA iron receptor may grow to a lower density in the host.

Biofilm formation would likely be reduced as well, to a certain extent. This phenomenon of limited iron availability disrupting biofilm formation has been described by Forsberg and Bullen (1972) and Lamont et al. (2009). A link between the pyoverdine and alginate regulatory networks, with AlgQ being a positive regulator of pyoverdine synthesis genes (Ambrosi et al. 2005). Iron limitation also plays a role in upregulating the *fagA-fumC-orfX-sodA* operon, important in alginate production, along with other alginate regulators such as AlgU (Hassette et al. 1997).

The increased production of the major virulence factor, pyocyanin, was observed in iron deficient conditions. The strain harboring the mutation in *piuA* produced more pyocyanin than the wild type *P. aeruginosa* strain in iron deficient conditions. While wild type bacteria did produce more pyocyanin under iron restriction than while in iron replete conditions, the difference was much more pronounced for the knockout strain. Under conditions where iron is not plentiful, this strain would produce more pyocyanin that could increase its virulence on a cell-by-cell basis.

This pyocyanin increase may be an attempt by *P. aeruginosa* to increase the concentration of iron that is available to it. Other phenazines have been shown to aid in iron scavenging by reducing Fe (III) to Fe (II). Biofilm development was shown to be aided by phenazines as well. Pyocyanin itself seems to aid biofilm development through an iron-independent manner (Wang et al. 2011).

We found that adhesion was shown to be affected in the strain possessing no functional *piuA*, as well. The adhesion was not significantly different between the two strains grown in normal media, but once grown in iron deficient media, the knockout strain adhered far better than the wild type strain. The mechanism for this increase is unknown, but may be related to increased exoproduct expression. This increase in exoproducts such as elastase, exotoxin A and other proteases, has been observed under iron limited conditions (Bjorn et al. 1979), and, thus, may play a role in increased bacterial adherence to epithelia. Although, there may be other host factors, such as increased immune response, that might contribute to the increase in bacterial adherence to epithelia (Darling and Evans 2003; Gomez and Prince 2007).

We showed that the ability to kill cells, or cytotoxicity, was increased by knocking out the *piuA* gene. The iron limitation increased this effect as expected. This suggests that responses to a lowered ability to scavenge iron from its environment may increase virulence, thus increasing the ability to destroy respiratory epithelial cells. Those virulence factors may be exotoxin A, which has been shown to have increased expression during iron restriction, pyocyanin, and extracellular proteases (Bjorn et al. 1979).

The strain possessing decreased expression of PiuA outer membrane iron receptor showed increased virulence on a cell by cell basis. However, *in vitro* studies demonstrate this strain to grow to a lower cell density than wild type *P. aeruginosa*. This means that, in an *in vivo* infection scenario, this strain may have a reduced ability to cause disease or kill the host. This situation would be true if the reduced growth overshadowed the increased individual cells' virulence.

Further study with the PW8599 strain of *P. aeruginosa* will reveal more information on the functionality of the PiuA protein and its effect on virulence. Further work to determine differences at the promoter level, such as XylE assays, would validate the concept that this gene is upregulated under iron limited conditions. Methods to determine the virulence using conditions that more closely resemble iron conditions in the human *in vivo* CF situation would yield more information on the activity of the PW8599 strain in the host. Since no media exists that mimics the iron content of the CF lung, further study in the murine model to determine adherence, cytotoxicity, growth and virulence factor production in this knockout is indicated.

The siderophore to which the PiuA receptor binds is currently unknown. Studies to determine which species of siderophore is using this receptor for cellular entry would aid in characterization of this protein and further the knowledge of its behavior. Knowning the identity of this siderophore may also enhance our ability to predict how important this protein is for overall *P. aeruginosa* pathogenesis (Vasil 2007).

Proteins in other species that function in similar ways to PiuA have been characterized to a greater extent. The PiuA in *Streptococcus pneumonia* has been shown

to elicit an opsonophagocytic antibody response in a mouse model (Jomaa et al. 2005). If this is true for PiuA of *P. aeruginosa*, it could open up avenues for treatment of infection; if not in CF, possibly in other forms of infection such as those associated with burns.

Vaccination against *P. aeruginosa* could protect against infection in the future for people at risk for disease, such as individuals with CF. This route may not be ideal, since prevention of infection against *P. aeruginosa* would allow other pathogens to invade the lungs and increase tissue damage. Vaccination may be desirable for other populations at risk, such as burn patients or those with frequent infections caused by *P. aeruginosa*. In *S. pneumoniae*, PiuA has been shown to be a potential vaccine determinant. It was shown to prevent respiratory infection in mice using a mucosal method of innoculation. If, as was shown for *S. pneumoniae*, PiuA is well conserved across many strains of *P. aeruginosa*, vaccination may prevent a multitude of infections (Jomaa et al. 2006).

Further study of this protein in the context of infection and colonization with *P. aeruginosa* is indicated. Multiple routes of investigation present themselves, such as understanding bacterial response to environmental changes or development of vaccines or modulators of this protein. Increased knowledge of the processes that this protein mediate may serve to aid in treatment and prevention *of P. aerigunosa* infections in the future.

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Table 1: Proposed roles for alginate in the pathogenesis of the cystic fibrosis lung (Govan and Deretic 1996).

Function or property	Comments
Inhibition of phagocytosis by neutrophils and macrophages	Nonopsonic
	Opsonic
Hypochlorite scavenging	Acetyl groups important
Quenching of reactive oxygen intermediates	
Interference with effective opsonization	Opsonic antibodies against alginate are protective
Suppression of oxidative burst in neutrophils	In biofilms; free alginate in low doses induces, in high doses inhibits
Suppression of neutrophil chemotaxis	Complement or bacterial products driven chemotaxis
Suppression of lymphocyte functions	Acetyl groups important
Suppression of opsonic antibody production	Immune complex-dependent cytotoxic T lymphocytes kill opsonic antibody-producing hybridomas; high doses of alginate
Mitogen activity and polyclonal B-cell stimulation	Possibly related to hypergammaglobulinemia; associated with clinical deterioration
Correlation of IgG and IgA against alginate and poor lung function	Antibodies also detectable in patients infected with nonmucoid strains only
No activation of alternative complement pathway	
IL-1 and tumor necrosis factor alpha induction	Mannuronate-rich polymer more active
Biofilm formation/microcolony mode of growth in vivo	See Fig. 1
Resistance to antibiotics	Innate and biofilm mode of growth related
Adhesion	Variable; possibly not a major ligand; indirectly as biofilm
Other	Nutrient scavenging; protection from dehydration; viscosity of sols and gelling with Ca^{2+}; interaction with phosphorylcholine

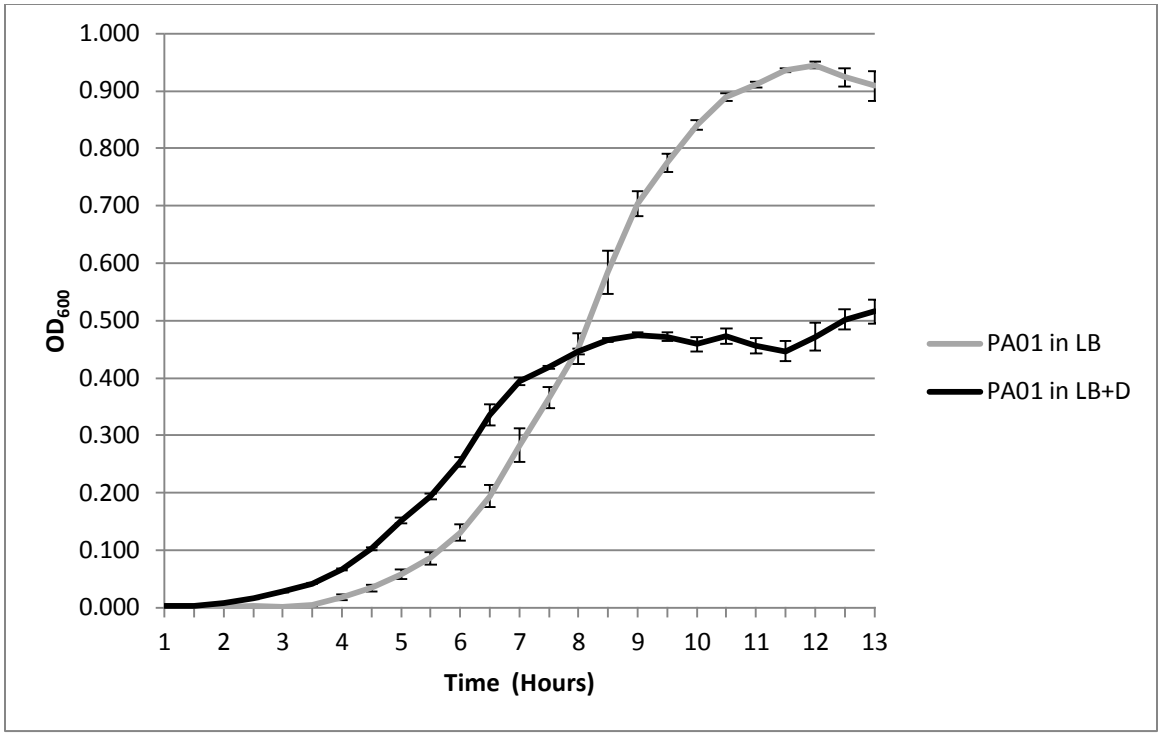

Figure 1. Growth Curves of PA01 in conditions of normal and limited iron showing Standard Deviation. PA01 was grown in media with and without 400 µM 2-2' dipyridyl for 12 hours. Turbidity was assessed with OD_{600} every 30 minutes during this time. Time points indicate sampling periods. N=3, SD.

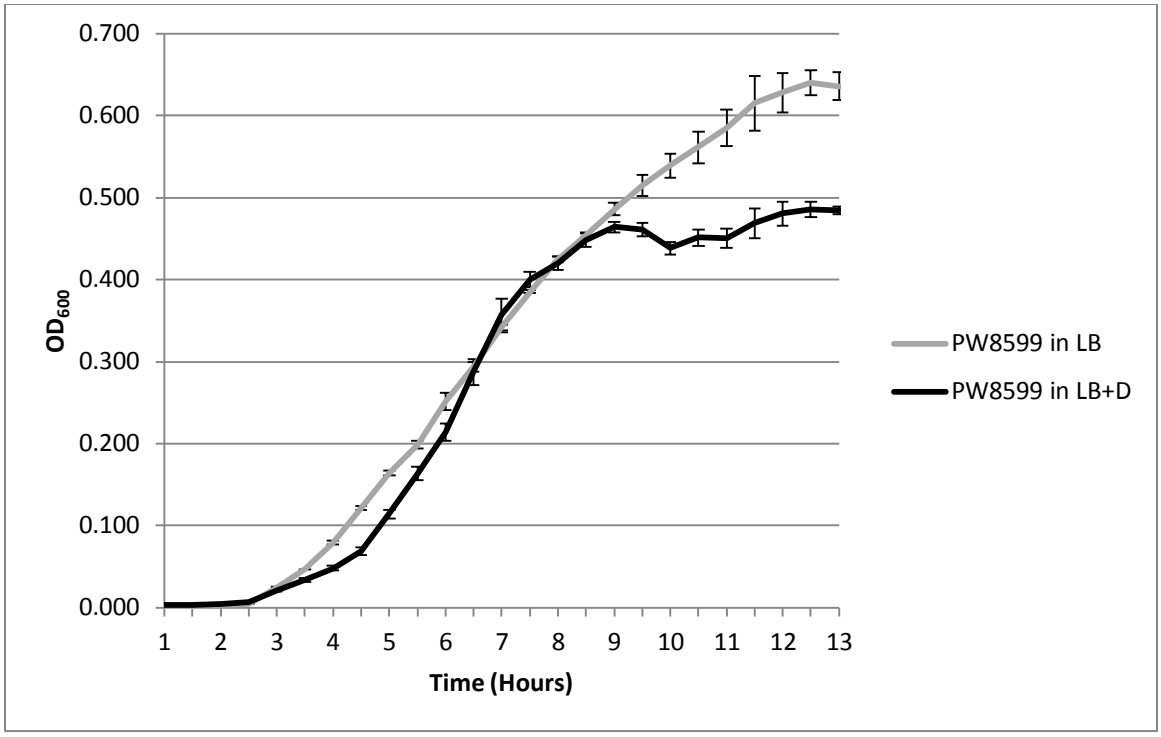

Figure 2. Growth Curves of knockout strain of *P. aeruginosa* in conditions of normal and limited iron. PW8599 was grown in media with and without 400 µM 2-2' dipyridyl for 12 hours. Turbidity was assessed with OD_{600} every 30 minutes during this time. Time points indicate sampling periods. N=3, SD.

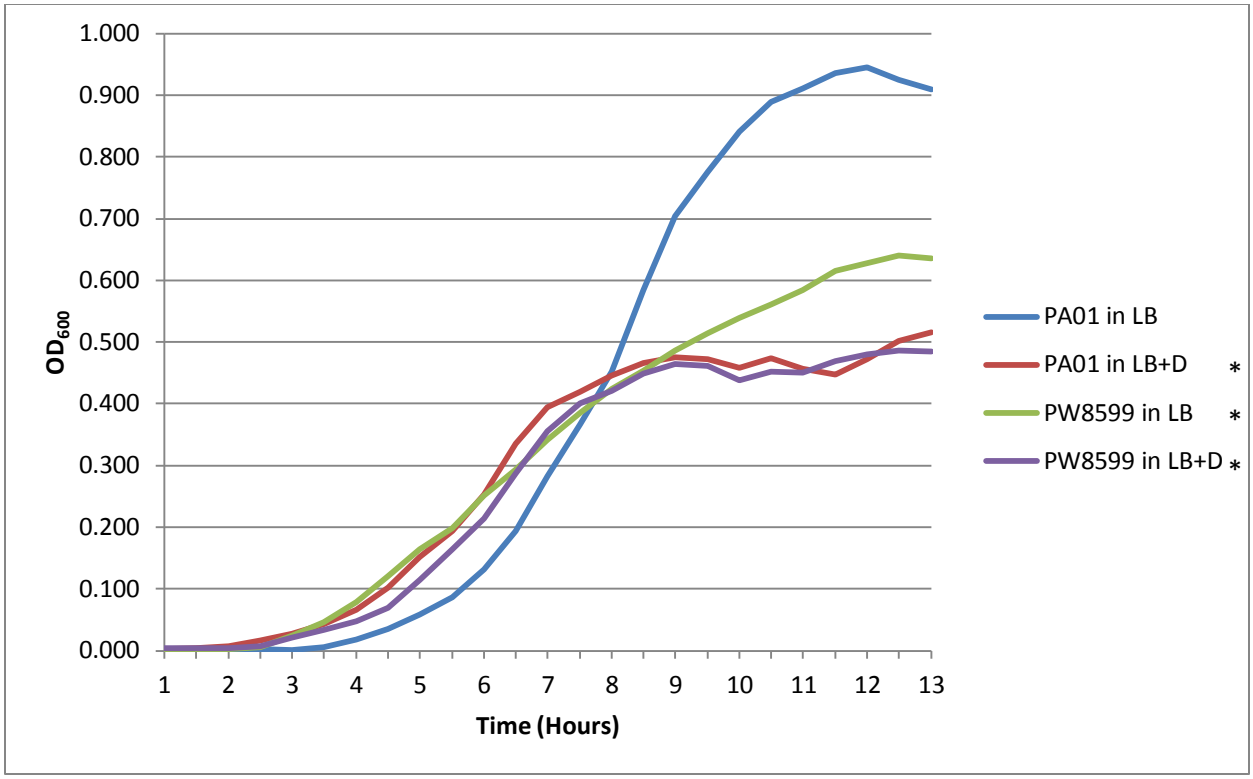

Figure 3. Growth Curves of wild type and ΔpiuA *P. aeruginosa* strains in normal and iron limited conditions. PA01and PW8599 were grown in media with or without 400 µM 2-2' dipyridyl for 12 hours. Turbidity was assessed with OD_{600} every 30 minutes during this time. Time points indicate sampling periods, n=3. Asterisks indicate a significant difference from PA01 in LB ($p<0.01$).

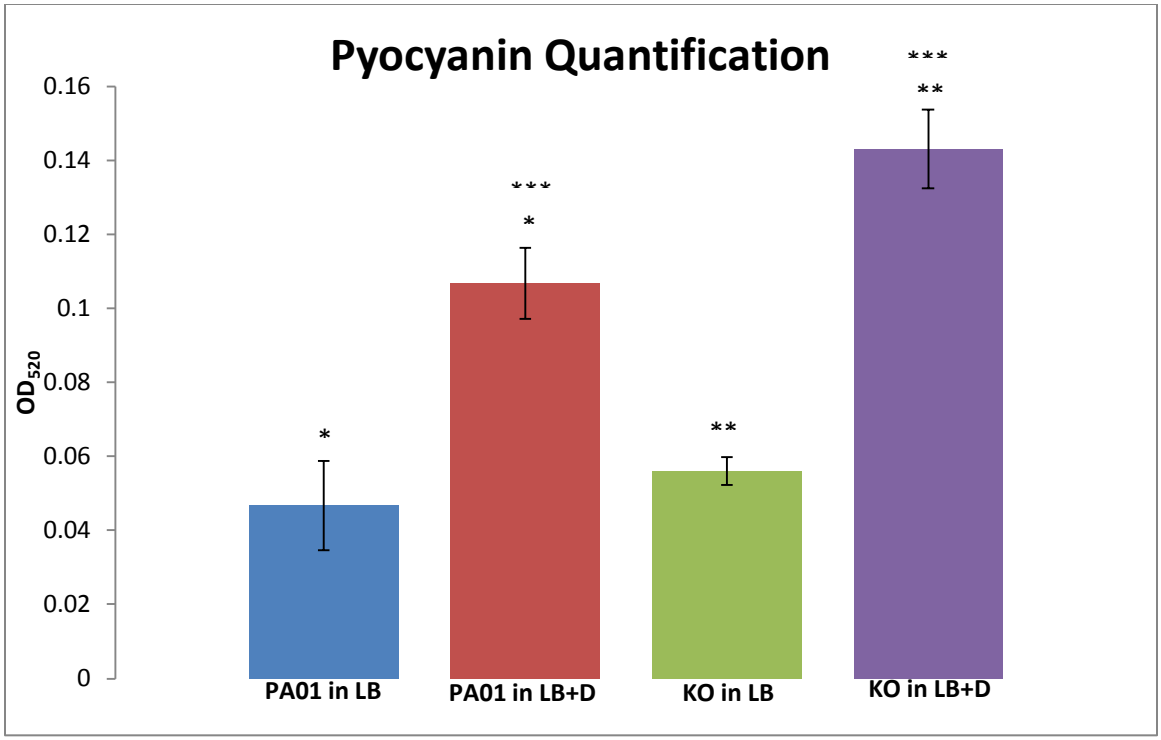

Figure 4. Increased pyocyanin production in *P. aeruginosa* grown in iron limited media. PA01 and PW8599 were grown for 24 hours in LB or LB with 400 µM 2-2' Dipyridyl. Pyocyanin was extracted by chloroform/HCl and analyzed by spectrophotometry. N=3, SD. Data was anazlyzed by student's t-test using Prism. *, **, and *** indicate a significant difference between groups ($p<0.0001$).

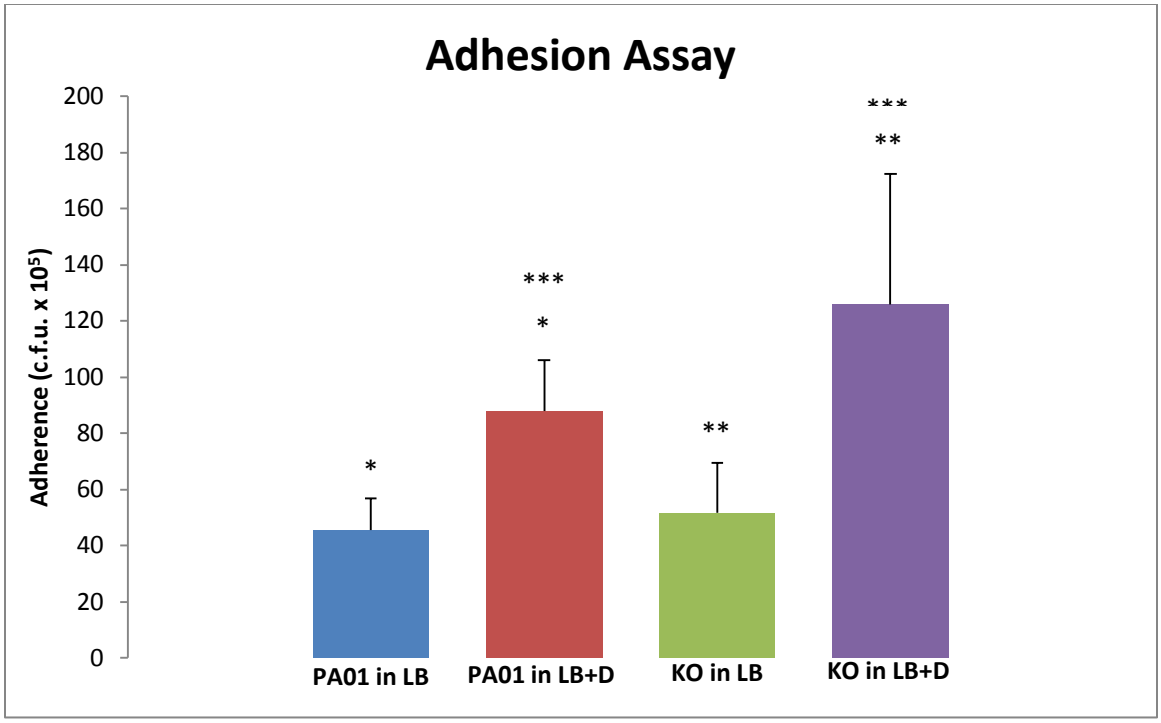

Figure 5. Differing abilities of *P. aeruginosa* strains to adhere to lung epithelium. PA01 and PW8599 were grown in LB with and without 400µM 2-2' Dipyridyl overnight and allowed to adhere to confluent A549 cells for 1 hour. Adhesion was quantified by plating serial dilutions of adhered bacteria overnight on LB plates and counting CFUs. N=3, SD. *, ** and *** indicate significant differences between groups ($p<0.05$).

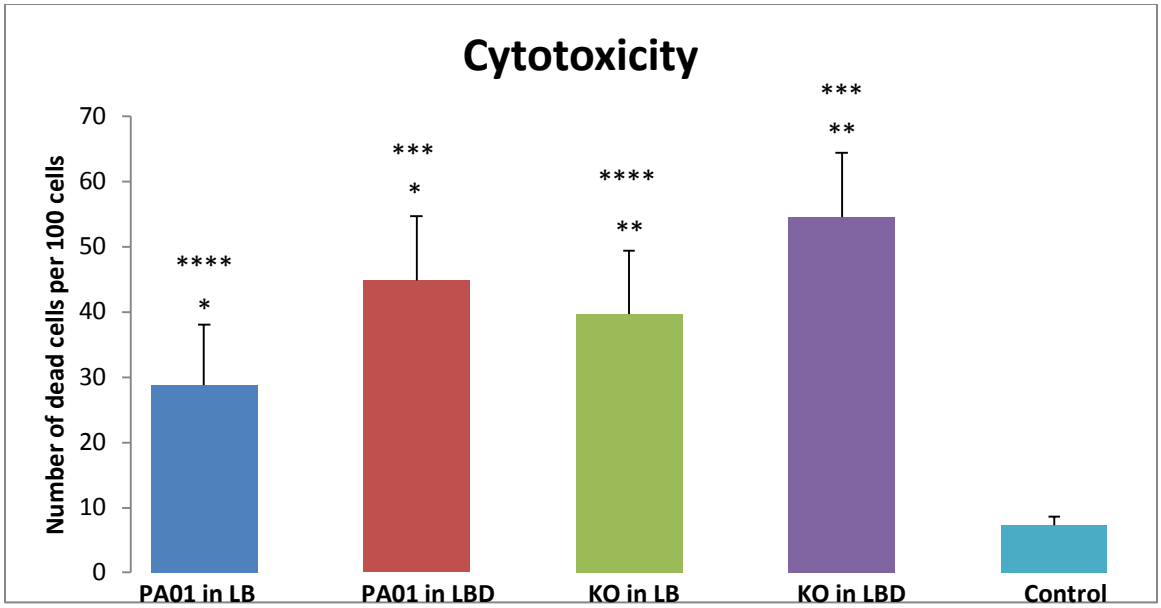

Figure 6. Cytotoxicity of *P. aeruginosa* strains. Bacterial strains were grown in LB with and without 400µM 2-2' Dipyridyl overnight then added to A549 monolayers at an MOI of 100:1 for 1 hour. Cells were removed and stained with Trypan Blue and counted using a hemocytometer. Control contained A549 with no bacteria added. N=3, SD. *, **, *** and ****indicate a significant difference between groups ($p<0.05$).

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