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Examining the Role of Specific Virulence Mechanisms During Pseudomonas Aeruginosa Infection in a Zebrafish Model of Cystic Fibrosis

Ryan T. Phennicie

EXAMINING THE ROLE OF SPECIFIC VIRULENCE MECHANISMS DURING PSEUDOMONAS AERUGINOSA INFECTION IN A ZEBRAFISH MODEL OF CYSTIC FIBROSIS

By Ryan T. Phennicie

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Advisory Committee:

Carol H. Kim, Professor of Microbiology, Advisor

John T. Singer, Professor of Microbiology

Paul J. Millard, Associate Professor of Chemical and Biological Engineering

THESIS ACCEPTANCE STATEMENT

On behalf of the Graduate Committee for Ryan T. Phennicie I affirm that this manuscript is the final and accepted thesis. Signatures of all committee members are on file with the Graduate School at the University of Maine, 42 Stodder Hall, Orono, Maine.

_____ _____

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Thesis Advisor: Dr. Carol H. Kim

An Abstract of the Thesis Presented
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Cystic fibrosis (CF) is the most common lethal hereditary disease. CF is caused by recessive mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene and is associated with multi-organ defects resulting from improper ion transport across epithelial membranes. Chronic lung infection by the environmentally ubiquitous opportunistic human pathogen *Pseudomonas aeruginosa* and the subsequent hyperinflammation that occurs as the host immune system combats the bacterium cause substantial morbidity and mortality in CF. Despite numerous studies that have sought to elucidate the role of CFTR in the innate immune response, the links between CFTR, innate immunity, and *P. aeruginosa* infection remain unclear. The present work highlights the zebrafish as a powerful model organism for human infectious disease. Zebrafish embryos with reduced expression of the *cftr* gene (Cftr morphants) exhibited reduced respiratory burst response and directed neutrophil migration, supporting a connection between *cftr* and the innate immune response. Cftr morphants were also found to display a

significant iron deficiency (ID) compared to control embryos, a symptom commonly diagnosed in CF patients. Cftr morphants were infected with *P. aeruginosa* or other bacterial species that are commonly associated with infections in CF patients, including *Burkholderia cenocepacia*, *Haemophilus influenzae*, and *Staphylococcus aureus*. Intriguingly, the bacterial burden of *P. aeruginosa* was found to be significantly higher in zebrafish Cftr morphants than in controls, a phenomenon that was not observed with any of the other bacterial species examined. The bacterial burden in Cftr morphants infected with a *P. aeruginosa* LasR mutant, a quorum sensing (QS)-deficient strain, was comparable to that in control fish indicating that the regulation of virulence factors through QS is required for enhancement of infection in the absence of Cftr. Cftr morphants were then challenged with *P. aeruginosa* mutants defective in the expression of QS regulated virulence factors. A mutant defective in the production of Exotoxin A (ETA) resulted in similar bacterial clearance in both the Cftr morphant and control embryos. The reduction in directed neutrophil migration to a *P. aeruginosa* infection was also restored when zebrafish embryos were challenged with the ETA mutant. Taken together, these data point towards a possible explanation for the specificity between *P. aeruginosa* and CFTR. The zebrafish system provides a multitude of advantages for studying the unique pathophysiology resulting from defective expression of CFTR, investigating the pathogenesis of *P. aeruginosa* and elucidating the role that the innate system plays in the host response to acute bacterial infections commonly associated with cystic fibrosis.

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INTRODUCTION

1.1. Cystic fibrosis

1.1.1. History

Cystic fibrosis (CF) is recognized as the most common lethal hereditary disease among Caucasian individuals (23). Approximately 40,000 people in the United States, and an additional 40,000 throughout the world, have CF, and an estimated 10 million Americans carry a mutation in the gene responsible for the disease.

The syndrome was first characterized in 1938 when Dr. Dorothy Andersen described cystic fibrosis of the pancreas and for the first time distinguished CF from celiac disease and other gastrointestinal diseases thought to be at fault (23). It was not until 1949, however, that a simple observation by a perceptive pediatrician at a New York children's hospital revealed one symptom of the disease that, not only is still widely used as a diagnostic, but for the first time pointed researchers toward the cause of the collection of symptoms associated with this newly discovered disorder. By identifying a subset of patients suffering from severe heat stroke during a heat wave that summer, he speculated that these patients were dehydrating because of a higher than normal loss of salt. He concluded that children with what we now know as CF had abnormally high, as much as three to five times higher, salt in their sweat than normal children (115). Over the next decade, researchers concluded that the high salt in the sweat of CF patients was a result of a failure to reabsorb NaCl in the sweat duct prior to secretion. The diagnoses at infancy of sweat

concentrations higher than 60 mM remains to this day as a confirmative diagnosis of CF. In 1989, the defective gene responsible for cystic fibrosis was cloned and identified as the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) (63). *CFTR* encodes an ATP dependant cAMP regulated chloride ion channel that is expressed by virtually all epithelial cells and is responsible for chloride conductance and ultimately the regulation of NaCl secretion from the cells (28).

Since 1989, nearly 2000 different mutations in CTFR have been identified (126). About one half of all CF cases, however, result from the deletion of a phenylalanine residue from amino acid position 508 (ΔF508) (105). Among the thousands of mutations that have been identified, those that have been characterized have been grouped into five classes of mutations, based on the result of the mutation. The ΔF508 mutation is considered a class II mutation and results in a mis-folding of the protein and subsequent degradation at the endoplasmic reticulum. Other mutations may result in a protein that is either poorly regulated or completely dysfunctional, a protein that is expressed at lower levels than normal, or a transcript that simply cannot be translated. Regardless of the specific mutation, people carrying two defective alleles express a classic group of respiratory and gastrointestinal symptoms.

1.1.2. Pathophysiology of cystic fibrosis

1.1.2.1. Digestive System

CFTR is highly expressed in the epithelial cells lining the intestine and is responsible for hydrating the mucus by secreting water into the intestine.

Approximately 10-15% of infants with CF will die soon after birth due to a bowel obstruction called meconium ileus, and obstruction of the small intestine is a recurring problem throughout the lives of most people with CF (115). Among the myriad of symptoms associated with CF, progressive pancreatic failure over the course of the patient's life is a grave concern for CF researchers. CFTR is expressed in the cells lining the pancreatic ducts and is responsible for hydrating the mucus that aids the secretion of pancreatic fluid and enzymes into the small intestine. As a result of impaction of these ducts by viscid dehydrated mucus, malnutrition is common and specific dietary regimens and vitamin supplements are necessary. The pancreatic symptoms experienced by CF patients are progressive and failure of the organ over the course of the patient's life ultimately results in reduced levels of insulin and glucagon in circulation and an almost total inability to absorb carbohydrates and fat soluble vitamins (57).

1.1.2.2. Iron deficiency in cystic fibrosis

Iron deficiency (ID) has long been associated with the general malnutrition caused by the reduced pancreatic function and poor nutrient uptake in cystic fibrosis (6). Many of the nutritional deficiencies linked to CF have been overcome with innovative and aggressive therapies over the past few decades. Iron, however, remains a major concern and the deficiency experienced by a large percentage of CF patients that are not responsive to these therapies has led researchers to believe that the ID experienced in CF may in fact be multifaceted and not as simple as once believed (121). The clinical data available varies greatly depending on the age and

location of the subjects studied, but it is clear that 30 – 60% of CF patients are in fact ID, depending on the age of the individual (1, 34, 41, 46, 109, 110, 121, 153). A study by von Drygalski and colleagues found a high prevalence of anemia in CF patients that increased with age. This led the team to conclude that as the health of the individual declined over the course of his or her life, as a result of respiratory failure and diminishing pancreatic function, that the incidence of anemia and, in more than 60% of the cases, a diagnosed iron deficiency, became more prevalent (153). A study of 71 CF patients at Seacroft Hospital in Leeds, UK, showed that 62% of the patients age 16 to 28 were functionally iron deficient, twenty-three percent of the ID patients were also diagnosed as anemic (109). A group led by Dr. David Reid, in Melbourne found that of 30 patients from 21 – 36 years old, 72% were diagnosed as ID determined by both transferrin saturation, a standard clinical measurement for ID, and by a serum iron assay (121). Unfortunately, as is often the case with clinical data, retrospective studies are often incomplete making the data difficult to interpret. There is a growing pool of clinical data that suggests sputum from cystic fibrosis patients contains abnormally high levels of iron (46, 119, 136, 137). While many of these studies effectively showed a significantly higher concentration of iron in the CF lung, the data have been a matter of contention among some researchers who insist that the techniques used to measure iron may in fact be an inaccurate measure of the total available iron in the lung (64) and the limitations of clinical reports which cannot provide a suitable control group may bias the results of the studies. Retrospective studies of patient charts and prospective studies have both

established a significant iron deficiency and anemia in CF patients, including studies that found higher iron levels in sputum samples. Recently, Gifford and colleagues reported a prospective study of 39 CF patients at Dartmouth-Hitchcock Medical Center who were chronically infected with *P. aeruginosa* (46). In this study, the authors found a significant correlation between the ID state of the patient and the clinical worsening of lung function. Contrary to the conclusions of Reid, *et al*. (120), Gifford and colleagues found no significant link between the iron state in the CF lung and the ID state in the patient overall, supporting the notion that the increased iron in the lung may be associated with the presence of the bacteria (46).

1.1.2.3 Respiratory system

By far the most severe symptoms associated with cystic fibrosis, indeed those that are responsible for the majority of the morbidity and mortality in CF, are those of the respiratory tract. Many pathology studies have shown that the CF lung looks relatively normal at birth. Soon after, however, the defect in CFTR has dire consequences on the hydration of the mucus at the surface of the respiratory epithelium (80). This hydration defect results in an airway surface liquid (ASL) that is abnormally thick and viscid and devoid of salts. Eventually, impacted mucus blocks the alveolar ducts resulting in decreased pulmonary function and perpetuating the dehydration of the ASL (10). The advent of modern therapeutic drugs such as mucus thinners and bronchodilators has reduced some of the respiratory distress of CF patients by clearing their airway and removing the impacted mucus (117). Respiratory problems persist however, and ultimately 80 –

90% percent of CF patients will succumb to respiratory failure resulting from tissue destruction brought on by an importunate, yet ineffective, inflammatory response to chronic bacterial infections of the lung (80). For decades, it was accepted that successive bacterial infections of CF children were simply the result of thick mucus lining the respiratory tract and providing an excellent growth medium for the bacteria. Recently, however, certain trends have been emerging in the timeline of these infections and the particular pathogens that predominate. There is now a great deal of evidence that suggests that there may be a more specific relationship between the molecular impacts of a defective chloride channel and the predominant pathogens found in the majority of CF patients.

1.1.3. Bacterial infections of the CF Lung

Bacterial infections are common and usually result in chronic persistent colonization as a consequence of the inability of the host's immune system to properly eradicate these infections. A wide variety of pathogens have been recovered from the lungs of CF patients, and the severity of the infections and indeed the pathogen itself are often closely correlated to the age of the patient. Typically, infections in early childhood are caused by *Staphylococcus aureus* and *Haemophilus influenzae* and often recur throughout childhood and adolescence and are usually easily treated with antibiotics (10, 80). Infections by the bacterium *Stenotrophomonas maltophilia* have been increasing in prevalence over the past few decades, most frequently infecting CF patients in the teen years. These infections, however, are poorly studied and their impact on clinical outcome is still unclear

(23). Recently, infections by the *Burkholderia cepacia* complex of opportunistic pathogens have become a great concern to clinicians with CF patients as studies have shown that these infections result in extremely poor clinical outcomes and a dramatic increase in mortality (72). Lung transplant recipients that were positive for *B. cenocepacia* (*B. cepacia* genomovar III) have a dramatically higher mortality rate after transplantation, prompting many centers to remove culture positive individuals from transplant lists (26). Despite the wide array of bacterial pathogens, as well as viral and fungal pathogens, which will not be discussed here, one species of bacteria is by far the most common and most difficult to eradicate once infection has been established. *Pseudomonas aeruginosa* has been recognized as the most important CF pathogen since the 1960s and today is responsible for chronic respiratory infections in at least 80% of CF patients.

1.1.4. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen of plants and animals (156). The capacity of this bacterium to metabolize a wide array of carbon sources and its ability to grow at a range of temperatures means that *P. aeruginosa* can be found in nearly all environments. Although most people come into contact with this ubiquitous microbe practically every day, infections in otherwise healthy individuals are nearly unheard of. Underlying conditions that result in a compromise of the host's immune defenses are nearly always present when an infection with *P. aeruginosa* is established (156). *P. aeruginosa* is responsible for approximately 10% of all nosocomial infections, making it the fourth

most commonly isolated pathogen in hospital settings (143). *P. aeruginosa* expresses multiple drug efflux pumps and enzymes targeted at many common antibiotics. This coupled with the fact that *P. aeruginosa* has one of the most impermeable outer membranes, roughly 100 times more impermeable than *E. coli* gives this pathogen a high level of intrinsic antibiotic resistance and makes infections very difficult to treat (139). The almost uniquely high resistance to many of our modern antibiotic treatments and the myriad of virulence factors encoded in its genome make *P. aeruginosa* a major concern for patients in intensive care units, particularly those with implanted devices such as catheters.

1.1.4.1. *P. aeruginosa* infections in CF

Although many pathogens are responsible for infections in CF patients and various species of bacteria, virus and fungi are all problematic for these individuals, infection by *P. aeruginosa* ultimately predominates and acute pneumonia rapidly evolves into a chronic, persistent infection of the airway that is nearly impossible to eradicate (49). After nearly 60 years of research, the most puzzling aspect of cystic fibrosis, one that has yet to be sufficiently elucidated, is why this particular population of people is sensitive to infection by this particular microbe. *P. aeruginosa* possesses a vast collection of virulence factors that allow it to infect a broad host range. These virulence factors have been studied in many model systems ranging from *in vitro* work, to infection studies in plants and various animals.

1.1.4.2. Virulence factors of *P. aeruginosa*

1.1.4.2.1. Type III Secretion System

The Type III Secretion System (T3SS) of Gram-negative bacteria facilitates the direct transfer of toxins into the cytoplasm of eukaryotic cells (14, 147). *P. aeruginosa* encodes four exotoxins that have been shown to be delivered via the T3SS apparatus (14). Studies have documented the significance of the T3SS of *P. aeruginosa*, and the exoenzymes delivered through it, in the pathogenesis of infection of humans, plants, insects, nematodes and most recently infection of zebrafish embryos (7, 12, 147).

Table 1.1. Virulence factors of *P. aeruginosa*

ORF (or Operon)	Gene(s)	Description	Classification / Function	Regulation
PA14_18380-18580	*alg844KEGXL*	Alginate	Adhesion	Muc
PA14_19100-19110	*rhlAB*	Rhamnolipid	Biofilm	RhlR, MvfR
		Lipopolysaccharide	Endotoxin	
PA14_48060	*aprA*	Alkaline Metalloprotease	Type I Secreted Toxin - Complement Degradation, Hydrolysis of Fibrin	LasR
PA14_40290	*lasA*	Elastase LasA	Type II Secreted Toxin - Serine Protease	LasR
PA14_16250	*lasB*	Elastase LasB	Type II Secreted Toxin - Protease	LasR
PA14_49560	*toxA*	Exotoxin A	Type II Secreted Toxin - ADP-ribosyltransferase	PvdS, LasR, ToxR
PA14_09900	*prpIL*	Pretease IV	Type II Secreted Toxin - Serine Protease	PvdS
PA14_00300	*plcB*	Phospholipase C	Type II Secreted Toxin - Hemolytic Phospholipase	LasR
PA14_36330-36310	*hcnABC*	Hydrogen Cyanide	Cytochrome C Inhibitor	MvfR
PA14_25195	*exoS*	Exoenzyme S	Type III Secreted Toxin - ADP-ribosyltransferase and GTPase activating	ExsA
PA14_49560	*exoT*	Exoenzyme T	Type III Secreted Toxin - ADP-ribosyltransferase and GTPase activating	ExsA
PA14_51530	*exoU*	Exoenzyme U	Type III Secreted Toxin - Phospholipase	ExsA
PA14_36345	*exoY*	Exoenzyme Y	Type III Secreted Toxin - Adenylate cyclase	ExsA
PA14_34870	*chiC*	Chitinase	Glycosyl hydrolase	MvfR
PA14_09270-09320	*pchDCBAEF*	Pyochelin	Siderophore	PvdS
PA14_33650	*pvdD*	Pyoverdine	Siderophore	PvdS, LasR
PA14_09490	*phzM*	Pyocyanin	Redox-active Phenazine Pigment	LasR, MvfR

1.1.4.2.2. Quorum sensing

Since the first quorum sensing (QS) system was described in 1979, we have learned a great deal about how important cell-to-cell communication is to bacterial survival (93). The LuxR regulatory network that controls genes required for

bioluminescence in the endosymbiotic bacterium *Vibrio fischeri* is by far the most thoroughly characterized QS system of any bacterial species. Since the identification of the *V. fischeri* LuxR system, similar QS networks have been identified in many other species including *P. aeruginosa* and *B. cenocepacia* (71, 155). Unfortunately, while the QS network of *V. fischeri* controls systems that are beneficial for the survival of its host, the QS system of *P. aeruginosa* and other bacterial species is responsible for the regulation of genes that permit invasion and pathogenicity in their host (116). Many of the virulence factors *P. aeruginosa* releases during infection are primarily regulated through the LasR QS network (Figure 1.1), the *P. aeruginosa* homologue of the LuxR system in *V. fischeri*. The sophisticated regulatory network that ultimately results in the transcription of numerous virulence factors begins with the production and secretion of two signal molecules, 3-oxo-C12-Homoserine Lactone (HSL) and C4-HSL (37). When the concentration of these molecules builds to a sufficient threshold, a result of cell density, the Las and Rhl transcriptional regulators are activated and begin to induce the expression of multiple downstream factors, and are ultimately responsible for the transition into biofilm growth in *P. aeruginosa* (102).

Figure 1.1 outlines the hierarchical QS gene regulation pathway of *P. aeruginosa*. While the LasR/RhlR QS system of *P. aeruginosa* is the best characterized of any pathogen, recent studies showing RhlR regulation of factors once thought to be under strict control of LasR have revealed surprising new evidence that the pathway may in fact be more complex than once thought (27).

Despite the controversy that exists over the regulation hierarchy in the LasR/RhlR QS system, it is clear that this pathway, and the downstream products of it, is immensely important in *P. aeruginosa* infections in CF.

Researchers have successfully isolated the Alkaline Metalloprotease, Elastases and Exotoxin A, along with the HSL signals that induced their expression, from the sputum of CF patients chronically infected with *P. aeruginosa* (37, 138). The LasR/RhlR QS system and the factors it regulates are ultimately responsible for and required for the transition from free-swimming planktonic bacteria to a complex multi-cellular community called a biofilm (24). Paradoxically, a recent study has shown that during chronic infections in the CF lung later in the course of infection, inactivating mutations in LasR are common because growth conditions are actually selective for LasR mutants (54). The study revealed that among *P. aeruginosa* isolates from 166 CF patients, 31% of the isolates were LasR mutants. The authors stated that the expression of downstream effectors and the ability of the mutants to grow as biofilms remained intact despite the inactivation of LasR, leading them to speculate that regulation of these genes has been "uncoupled" from the LasR pathway.

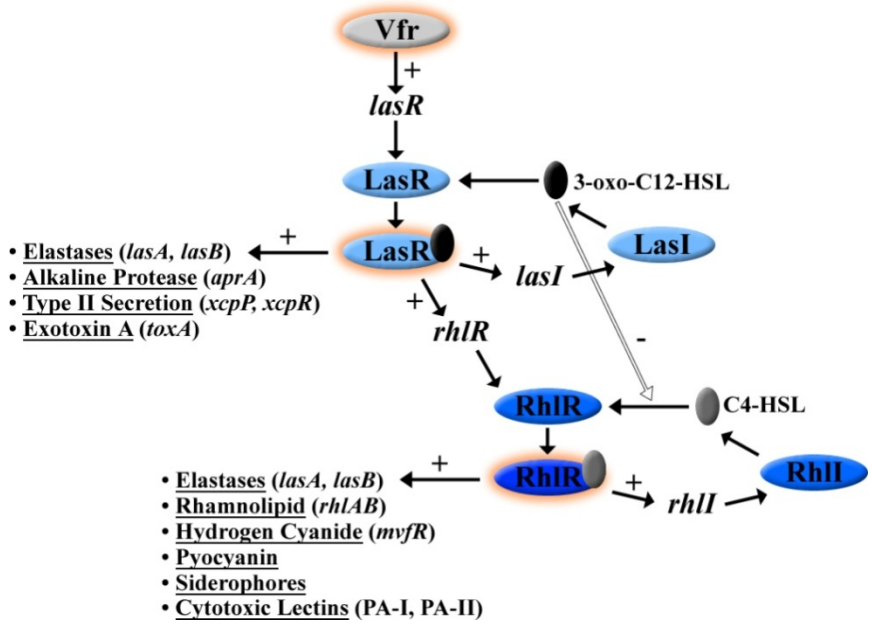

Figure 1.1. The LasR Quorum Sensing System of *P. aeruginosa.* Adapted from: E. C. Pesci, J. P. Pearson, P. C. Seed and B H. Iglewski. 1997. Regulation of *las* and *rhl* Quorum Sensing in *Pseudomonas aeruginosa.* Journal of Bacteriology 179(10) 3127–3132

1.1.4.2.3. Iron regulated virulence factors

1.1.4.2.3.1. Exotoxin A

Exotoxin A (ETA) of *P. aeruginosa* is an ADP-ribosyl transferase that is highly toxic to eukaryotic cells and considered the most toxic virulence factor produced by the bacterium (56, 77). First identified in 1966 as the *P. aeruginosa* lethal toxin, Lui found that the exoproducts produced during cell growth were lethal to mice and caused severe skin lesions on rabbits (77). Ten years later, Iglewski and Kabat described the mechanism of ETAs toxicity and showed that the protein toxin is an ADP-ribosylase and functions to irreversibly modify elongation factor-2 (EF-2) of eukaryotic cells halting protein synthesis and inducing apoptosis (56). ETA

expression is controlled by iron availability and enhanced by the LasR QS system (21, 39, 43, 44). The toxin has been found to reduce the number of circulating leukocytes after IP injection in mice and rabbits and was found to impair the phagocytic ability of macrophages and neutrophils by disrupting the rearrangement of the actin cytoskeleton. Studies have shown ETA to be an essential factor for maximum virulence in a rat chronic lung infection model (158). Later studies using a guinea pig model of acute pneumonia indicated that ETA may in fact be less essential than the elastases of *P. aeruginosa* during acute infection (4). The authors speculate that ETA may offer some protection from *P. aeruginosa* pneumonia by stimulating the host defenses, though this notion has yet to be further elucidated. ETA, the Elastase (LasB) and Serine Protease (LasA) of *P. aeruginosa* have all been shown to be important for infection in mammals, and all three have been found in the sputum of CF patients with chronic *P. aeruginosa* infection (37, 138). The presence of ETA and other virulence factors in the CF lung points to their importance during these chronic lung infections, but their function and their significance early in the course of infection has yet to be sufficiently elucidated.

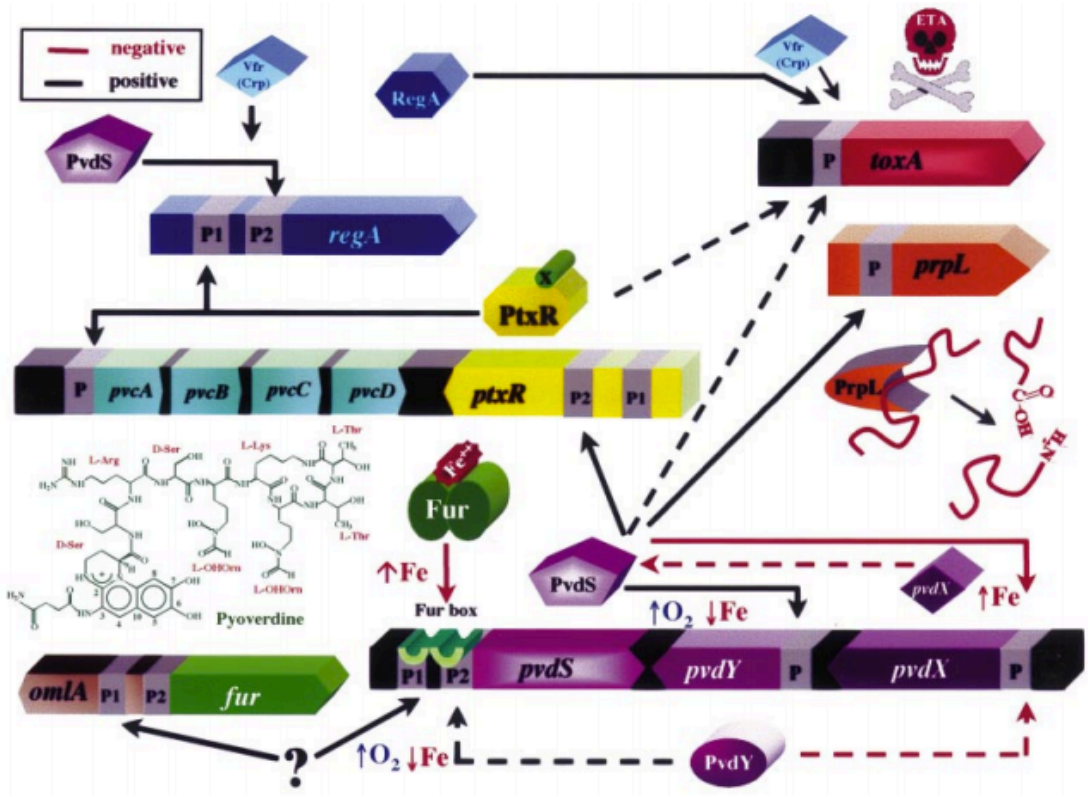

Figure 1.2. Iron Regulated Virulence Network of *P. aeruginosa*. Adapted from: Vasil, M.L. and Ochsner, U.A. 1999. The response of *Pseudomonas aeruginosa* to iron: genetics, biochemistry and virulence. Molecular Microbiology 34(3):399-413

1.1.4.2.3.2. Siderophores

When iron is scarce, *P. aeruginosa* deploys two potent iron chelating siderophores, pyoverdine and pyochelin (116). These siderophores scavenge free iron from the environment and are so efficient that they are capable of sequestering iron from the iron binding proteins, such as transferrins, of their host during infection and delivering the iron to the bacterium through cell surface receptors. Pyoverdine and its specific receptor also serve a gene regulation function. When unbound pyoverdine interacts with the receptor, the sigma factor PvdS is up-

regulated. PvdS, in turn, induces the expression of pyoverdine and the highly cytotoxic Exotoxin A and Serine Protease IV (68). The regulation of these virulence genes through iron availability is yet another example of how *P. aeruginosa* is extremely well adapted to acquiring essential nutrients from its environment by any means necessary.

1.1.4.2.4. Elastases and Proteases

The Elastase LasB, the Serine Protease LasA and the Alkaline Metalloprotease all play a crucial role in pathogenesis and have been studied for their contribution to *P. aeruginosa* infections for over 40 years (90). Although each of these toxins have unique targets and their activity against tissues varies from host to host, all three of these important virulence factors are regulated through the Las and Rhl QS systems in *P. aeruginosa* (102). The destructive impact of elastase has been well described in both acute and chronic lung infection models demonstrating that the elastases and proteases are effective weapons in the arsenal of *P. aeruginosa* (4, 158). It is because of these studies that little surprise is drawn from the finding that elastases and proteases are expressed in the CF lung during chronic infections with *P. aeruginosa* (59, 138).

1.1.4.2.5. Biofilms

Over the past few decades, advances in methodologies and technologies in the field of bacteriology have lead to a change in the understanding of bacterial communities. The dogma of the day was that bacteria are single cell, free-living organisms that rely little on the assistance of others to thrive. The ability to observe

how bacteria live and grow and communicate *in situ* has provided new insights into the bacterial biofilm model showing instead that these unicellular organisms can, and often do, form highly organized and deliberately structured biological communities (19). *P. aeruginosa* is arguably the most well studied biofilm former and the phenotypic changes associated with biofilm growth have been recognized as a major contribution to the persistence of hospital acquired *P. aeruginosa* infections resulting from implantation devices including catheters and breathing tubes (19). The high level of intrinsic antibiotic resistance possessed by *P. aeruginosa* is further enhanced by numerous physiological changes that occur when the bacterium transitions into biofilm growth (33). Recently, developments in molecular biology techniques have enabled researchers to study the mechanisms by which many bacterial species are able to communicate and cooperate to form complex biofilm communities (18). Advances in our understanding of the regulatory networks responsible for triggering the switch from planktonic to biofilm growth have been rapid and studies have revealed that the quorum sensing networks of *P. aeruginosa* that are responsible for this transition are also tightly intertwined with the expression of many virulence factors, providing insights into the contribution of biofilms to pathogenicity in *P. aeruginosa* (24, 25). It is now estimated that over half of all bacterial infections involve biofilms (111). It came as little surprise to CF researchers when Dr. Joseph Lam isolated mucoid microcolonies of *P. aeruginosa* in the sputum of chronically infected CF patients, a discovery that would ultimately lead to the conclusion that the chronic, impossible to eradicate, *P. aeruginosa*

infections in the CF lung are caused by bacteria growing as biofilms with frequent exacerbations (67). Many advances have been made in our understanding of the contribution of biofilms to the pathogenicity of *P. aeruginosa* in the CF lung (3, 154). This model of infection is now widely accepted and the bulk of the current research is directed at dispersing these biofilms by means of new antibiotics, combining antibiotic therapies with enzymes and iron chelators and by exposing biofilms to high doses of iron salts (89, 91, 135).

1.1.5. Current model systems

P. aeruginosa infection has been studied in an assortment of animal models ranging from insects and worms to fish and mammals in an attempt to tease apart the specific mechanisms of its unique virulence and resistance to host defense and antimicrobial therapies (66). These studies, however, have failed thus far to sufficiently explain the specific relationship between this opportunistic pathogen and mutations in the chloride channel CFTR. The favorite tools of researchers studying *P. aeruginosa* infections are mouse models, 19 of which have been established since 1992, and cell culture techniques that utilize both primary and immortalized epithelial cell lines (66). Cell culture techniques have provided a wealth of information about how the loss of ion transport and membrane conductance affects the epithelial cells, but the scope of these experiments has been limited by the capacities of the *in vitro* model itself. Maintaining the epithelial differentiation in the immortalized CF bronchial epithelial lines has proven

problematic and a number of factors related to culture technique have been shown to bias the results of many experiments.

The wide array of available mouse models of CF can replicate many of the clinical symptoms of CF observed in humans. Inflammation, thick mucus secretions, poor nutrient uptake and reduced expression of Nitric Oxide Synthase have all been modeled in mice with mutated or knocked out CFTR. The most difficult symptom to replicate, though, are the respiratory tract infections seen in humans with CF, making most of the CFTR knockout mouse lines a very poor model to study the pathogenesis of *P. aeruginosa* during infections of the lung. Interestingly, the most promising model of the CF lung comes in the form of a mouse line with normal CFTR expression but an over expression of the epithelial sodium channel, closely mimicking the reduced salt secretion into and hydration of the airway surface liquid. This model, unfortunately, fails to recapitulate the majority of symptoms of CF and may not actually be sufficient to model the CF disease (66). Schroeder and colleagues have reported by far the most successful report of *P. aeruginosa* infection of a CF mouse model when they demonstrated reduced clearance of *P. aeruginosa* within 5 hours of inoculation using a CFTRΔF508 mouse line (129). The authors claim that the mechanism for reduced clearance involves epithelial cell ingestion of the bacteria using CFTR as a membrane bound pattern recognition receptor. A great deal of controversy has been raised over the experimental techniques and overall conclusions of this work, but the reduced bacterial clearance may indeed make this

study one of the most successful replications of the bacterial infections associated with CF using a murine model.

1.2. Cystic fibrosis and the innate immune system

1.2.1. Overview

The genetic basis for the disease cystic fibrosis is a defect in the chloride ion channel CFTR. This defective gene results in chronic persistent bacterial infections and a resultant hyperinflammation of the respiratory tract, a product of a failure of multiple facets of the immune response. Despite the resulting immunompromised condition associated with CF, CFTR is not traditionally considered a component of the immune response. The exact mechanisms of the susceptibility to bacterial infection in CF have been studied for decades and have provided insights into how this chloride channel may in fact contribute to an immune response (58).

1.2.2. Innate immune response to bacterial pathogens

1.2.2.1. Pattern recognition receptors

A primary line of defense against invading pathogens is activation of an innate immune response through pattern recognition receptors (PRRs) present on most cells. The Toll gene in drosophila was originally characterized for its role in dorso-ventral patterning and was later implicated in defense against fungal infection in these insects (70). Since this discovery, the family of Toll-Like Receptors (TLRs) has been expanded and well characterized. Upon recognition of their respective pathogen associated molecular patterns (PAMPS) that are distinctive to

many classes of bacterial, fungal and viral pathogens, a signal transduction cascade is initiated and ultimately results in an activation of the appropriate immune response to combat the class of pathogen identified. The innate immune response to bacterial pathogens, including *P. aeruginosa*, have been traced back to several TLRs and the particular PAMPs they recognize have been well characterized (62). Many TLRs have been identified as important in bacterial recognition in the CF lung. TLRs 2, 4, 5, 6 and 9, which recognize triacyl lipopeptides, LPS, flagellin, diacyl lipopeptides and bacterial specific CpG motifs, respectively, all play an important role in bacterial recognition and clearance in the CF lung (81).

A controversial proposal that is still debated among CF researchers originated in a 1996 study by Pier, and colleagues (107). Pier's group showed that a major function of CFTR in the respiratory tract was its activity as a PRR with specificity for the O-side chain of the LPS of *P. aeruginosa*. They showed that direct interaction between CFTR and *P. aeruginosa* resulted in an ingestion of the bacteria by the epithelial cell and ultimately death and sloughing of the infected cell. These findings were refined and this group later identified the specific extracellular region of CFTR involved in the interaction by blocking the region with monoclonal antibodies directed against those residues (106). These findings have been met with a great deal of skepticism and difficulties repeating the experimental conditions described in the publications have led many to reject the hypothesis altogether.

1.2.2.2. Cytokines

The CF lung is characterized by a chronic hyperinflammation defined in part by an excess recruitment of neutrophils into the organ and the subsequent release of cytotoxic granules from these cells resulting in an endless feedback loop of inflammation that is ultimately unable to control the infections that prompted their initial recruitment (58, 81). A debate has been raised among researchers as to whether this massive and importunate neutrophil response occurs independently of or as a result of bacterial infection. The accepted notion that the CF lung is physiologically normal at birth and begins to deteriorate in infancy has been challenged by a handful of clinical reports showing inflammation with no discernable infection, though these have largely been dismissed as evidence of subclinical infections in children makes diagnosis difficult (35, 127). Efforts to explain the unrelenting neutrophil recruitment into the CF airway has led researchers to identify dramatic differences in the regulation of cytokine expression of CF epithelial cells compared to normal cells. Several studies have shown that airway epithelial cells of both primary and immortalized lines express abnormally high levels of the pro-inflammatory cytokines IL-8, IL-6, IL-1β and TNFα, coupled with a decrease in the expression of the anti-inflammatory cytokine IL-10 (5, 120, 140). Several hypotheses exist to describe the excessive production of pro-inflammatory cytokines by the CF cells, among the more widely accepted is that the most common mutation in CF results in an improper folding of the protein resulting

in sequestration at the golgi for destruction (124). This process is thought to explain the increased cell stress that occurs that may ultimately result in inflammation.

1.2.2.3. Phagocyte response

The commonly accepted dogma of hyperinflammation in the CF airway has led many researchers and clinicians to accept the idea of rapid neutrophil infiltration into the lung in response to bacterial pathogens that they will ultimately struggle to eradicate. These notions have been called into question by researchers who have found that isolated neutrophils from CF patients display altered patterns of directed chemotaxis compared to neutrophils from healthy individuals and that these alterations may correlate with the clinical condition of the patients (131). This report trails a study that indicated that CF neutrophils display a significant desensitization to pro-inflammatory cytokines based largely on their over-exposure to these cytokines in CF patients (17).

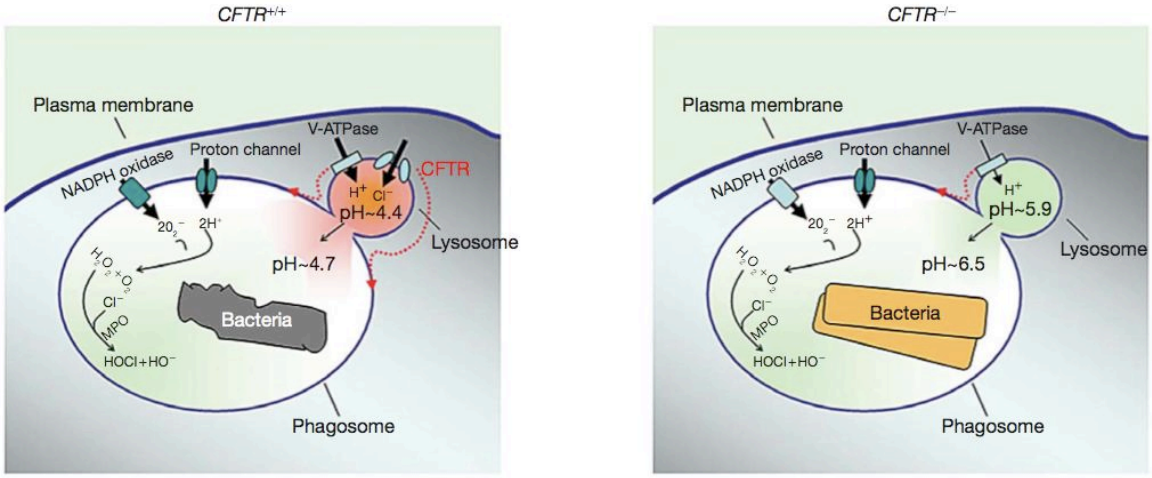

Figure 1.3. Model of the Chlorination Defect in the Lysozome of CF Macrophages. Adapted from Di, et al. 2006. CFTR regulates phagosome acidification in macrophages and alters bactericidal activity. *Nature Cell Biology* 8(9):933-944

Recently, a novel function for CFTR has been described in macrophages, neutrophils and eosinophils (30, 99, 130). CFTR expression has been identified at very low levels in macrophages, neutrophils and eosinophils isolated from healthy individuals and the intracellular localization of the protein is confined to the membrane of the phagolysosome of these cells. The function of the ion channel here is multifaceted and its contributions are thought to include supplying Cl^- for the production of HOCl, a major player in bacterial killing, the establishment of a H+ gradient across the membrane, facilitating the activity of the NADPH Oxidase, and finally lowering the pH of the lysosomal compartment to aid in elimination of phagocytosed pathogens and debris. These recently identified functions of CFTR represent a defined mechanism to explain how loss of this ion channel can significantly alter immune response by reducing the ability of innate immune cells to produce reactive oxygen species to eradicate invading pathogens.

1.3. Zebrafish as a model organism

1.3.1. Overview

The zebrafish has received a great deal of attention as an ideal model for studying embryonic development and many human diseases. In fact, many of the advantages that make the zebrafish ideal for studying development also make this model well suited for studying infectious disease (74). The relatively low cost and ease of maintenance make the zebrafish a cheaper and easier alternative to the more popular mouse models. The optically clear embryos spawned by the zebrafish,

coupled with development external to the mother make the zebrafish an ideal model for studying embryonic development and also offer many advantages for infection studies (84). Because the embryos are optically transparent, they are amenable to microscopic observations of the infection processes in a live animal, something that is very difficult to do with mice. Much progress has been made in understanding the cellular mechanisms by which the zebrafish defends itself from pathogens. An ongoing effort to describe the hematopoietic origins of zebrafish immune cells has lead to the discovery that those origins are very similar to mammalian lymphocytes (20). With the continuing effort to fully sequence the genome and the push to characterize the predicted genes found through this process, much has been learned about the zebrafish and the functional similarities many of its genes share with humans. The ongoing development of genetic tools for the zebrafish has yielded many new ways to study inflammatory processes and host–pathogen responses in a way that could not be replicated *in vitro* and simply could not be performed in murine models (61).

1.3.2. Zebrafish innate immunity

1.3.2.1. TLRs and Cytokines

It has been shown that the innate immune system is well conserved among all animals, from insects to vertebrates, and is a crucial first line of defense against invading microorganisms. This conservation of both structure and function holds true when comparing the zebrafish and human innate immune system (146). Pathogen recognition and subsequent immune response, including the release of

pro-inflammatory cytokines and interferon, through TLRs occurs in a similar manner in zebrafish and humans and although some of the intermediates are different, the end result is very much the same. Twenty-four TLR genes have been identified in zebrafish, many more than in mammals, and characterization of their expression and function has been ongoing. The conservation of downstream expression of pro-inflammatory cytokines between zebrafish and human has been well documented (146). Numerous studies in the zebrafish have demonstrated cytokine responses to bacterial pathogens. In 2005, Pressley and colleagues demonstrated that TNFα and IL-1β were found to be up-regulated upon infection with the natural fish pathogen *Edwardsiella tarda* (113). Researchers have begun to explore the specific functions of some of these pro-inflammatory cytokines, such as the neutrophil recruiting Interleukin 8, to compare them to the more thoroughly described mammalian orthologs (97).

1.3.2.2. Zebrafish phagocytes

Many aspects of the cell-mediated branch of the innate immune system of zebrafish have been elucidated through studies challenging the fish with many viral and bacterial pathogens. The lineage of zebrafish neutrophils has recently been traced back to the same myeloid lineage as zebrafish macrophages (69). Through histological staining techniques and fluorescently labeled antibodies, Le Guyader and collages were able to show that neutrophils of zebrafish behave much differently than mammalian neutrophils. Unlike in mammals, where macrophages are often found in tissue and neutrophils are more frequently found circulating in blood,

zebrafish neutrophils typically reside in the tissues and exhibit rapid chemotaxis to wound or infection sites (69). They were also able to show that although migration to infection with *E. coli*, zebrafish neutrophils were rarely found to engulf these bacteria, leading them to speculate that the neutrophils and macrophages of zebrafish respond differently depending on the pathogen. Since this study, many reports have been published showing the varying degree of participation neutrophils play in combating infections with different pathogens (7, 69, 112, 152). The introduction of the Tg(*mpx*:GFP) transgenic line of zebrafish in which a green fluorescent protein is expressed under the neutrophil specific myeloperoxidase (MPX) promoter has provided new insights into how neutrophils migrate to and combat different pathogens in a live animal model (123). This transgenic line has been utilized in studies attempting to elucidate neutrophil locomotion and how these cells are recruited to sites of infection and tissue injury (122).

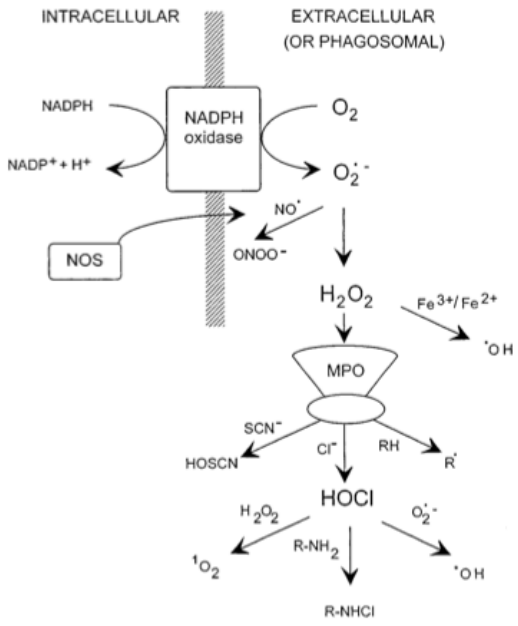

Figure 1.4. Production of Reactive Oxygen Species Following the Respiratory Burst. Adapted from Hampton, et al. 1998. Inside the Neutrophil Phagosome: Oxidants, Myeloperoxidase, and Bacterial Killing. *Blood* 92(9):3007-3017

A crucial contribution of macrophages and neutrophils during the immune response to pathogens is the respiratory burst response and the resulting production of reactive oxygen species (ROS) (Figure 1.4). Many assays are available to measure the production of reactive oxygen species in isolated neutrophils and methods have been reported for neutrophils of both fish and mammals (8, 50).A novel technique for performing whole embryo measurements of respiratory burst has been reported in which exposure of zebrafish embryos to toxicants has been shown to hamper the ability of zebrafish neutrophils to produce ROS (53, 92). This assay was adapted from one reported to detect immune affects of toxicant exposure in neutrophils extracted from rainbow trout (8).In the whole embryo assay,fish are

exposed to the non-fluorescent, reduced 2',7'-dichlorodihydrofluorescein diacetate and phorbol 12-myristate 13-acetate (PMA) is used to activate protein kinase C and synthetically induce a respiratory burst response. The ROS produced during this burst are then quantified by measuring the fluorescence produced by the de-acetylated dichlorodihydrofluorescein. The availability of this assay is a significant advantage of the zebrafish model as it is a relatively inexpensive, technically easy and quantitatively precise method of measuring the overall immune health of the animal after exposure to a toxicant or genetic manipulation. The assay is particularly useful in studies involving host – pathogen interactions in CF because of the critical role neutrophils play in combating infections in the CF lung.

1.3.3. Zebrafish and infectious disease

1.3.3.1. Bacterial infection models

Recently, the zebrafish has gained popularity among researchers as a powerful tool for elucidating host–pathogen interactions *in vivo*. In the past 10 years alone, the number of bacterial pathogens that have been described in zebrafish has risen from only a handful to nearly 30 different organisms (listed in Table 1.2.) (61). This rise in popularity as a model host has come on the heels of studies describing the structural and functional homology of the zebrafish immune system to that of mammals (20, 122). Recently, two separate groups of researchers have described the pathogenesis of *P. aeruginosa* in the zebrafish (7, 12). Both groups described the importance of the phagocytes of the zebrafish embryo in the clearance of a *P. aeruginosa* infection and showed that several virulence factors of *P. aeruginosa*

contribute to the bacterium's lethality during a disseminated infection in these embryos.

Table 1.2. Zebrafish bacterial infection models

Bacteria	Infection method	Embryos / Adults	Reference
Aeromonas hydrophila	Injection, Static Immersion	Embryo, Adult	Rawls, 2004; Rawls, 2006; Rodriguez, 2008
Aeromonas salmonicid	Static Immersion	Adult	Lin, 2007
Aeromonas veronii	Static Immersion	Embryo, Adult	Bates, 2006
Bacillus subtilus	Injection	Embryo	Herbomel, 1999
Burkholderia cenocepacia	Injection	Embryo	Deng, 2009; Vergunst, 2010
Edwardsiella ictaluria	Injection	Adult	Petrie-Hanson, 2007
Edwardsiella tarda	Injection, Static Immersion	Embryo, Adult	Phelan, 2005; Nayak, 2007; Pressley, 2005
Escherichia coli MG1655	Static Immersion	Embryo	Rawls, 2006; Rawls, 2007
Escherichia coli O157:H7	Static Immersion	Embryo	Szabady, 2009
Flavobacterium columnare	Injection, Static Immersion	Adult	Moyer, 2007
Flavobacterium johnsoniae	Injection, Static Immersion	Adult	Moyer, 2007
Francisella spp.	Injection	Adult	Vojtech, 2009
Leptospira interrogansa	Injection	Embryo	Davis, 2009
Listeria monocytogenes	Injection	Adult	Menudier, 1996; Levraud, 2009
Listeria spp.	Injection	Embryo, Adult	Menudier, 1996
Listonella anguillaruma	Injection, Static Immersion	Adult	Rojo, 2007
Mycobacterium haemophiluma	Injection	Adult	Whipps, 2007
Mycobacterium marinum	Injection, Static Immersion	Embryo, Adult	Davis, 2002; Meijer, 2005; Gao, 2006; Clay, 2007; Harrif, 2007; Clay, 2008; Davis, 2009; Volkman, 2010
Mycobacterium peregrinum	Static Immersion	Adult	Harrif, 2007
Pseudomonas aeruginosa	Injection, Static Immersion	Embryo	Rawls, 2004; 2006; 2007; Brannon, 2009; Clatworthy, 2009; Llamas, 2009; Vasil, 2009; Singer, 2010; Phennicie, 2010
Pseudomonas fluorescens	Static Immersion	Embryo, Adult	Bates, 2006
Salmonella arizonae	Injection	Embryo	Davis, 2002
Salmonella typhimurium	Injection	Embryo	van der Sar, 2003; van der Sar, 2006; Stockhammer, 2009
Staphylococcus aureus	Injection	Adult	Lin, 2007; Prajsnar, 2008
Streptococcus iniae	Injection	Adult	Neely, 2002; Miller, 2005; Lowe, 2007
Streptococcus pyogenes	Injection	Adult	Neely, 2002; Bates, 2005; Cho, 2005; Montanez, 2005; Kizy, 2009
Vibrio anguillarum	Static Immersion	Embryo	O'Toole, 2004

CHAPTER 2

SPECIFIC RESISTANCE TO *PSEUDOMONAS AERUGINOSA* INFECTION IN ZEBRAFISH IS MEDIATED BY THE CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR

2.1. Abstract

Cystic fibrosis (CF) is a genetic disease caused by recessive mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene and is associated with prevalent and chronic *Pseudomonas aeruginosa* lung infections. Despite numerous studies that have sought to elucidate the role of CFTR in the innate immune response, the links between CFTR, innate immunity, and *P. aeruginosa* infection remain unclear. The present work highlights the zebrafish as a powerful model organism for human infectious disease, particularly infection by *P. aeruginosa*. Zebrafish embryos with reduced expression of the *cftr* gene (Cftr morphants) exhibited reduced respiratory burst response and directed neutrophil migration, supporting a connection between *cftr* and the innate immune response. Cftr morphants were infected with *P. aeruginosa* or other bacterial species that are commonly associated with infections in CF patients, including *Burkholderia cenocepacia*, *Haemophilus influenzae*, and *Staphylococcus aureus*. Intriguingly, the bacterial burden of *P. aeruginosa* was found to be significantly higher in zebrafish Cftr morphants than in controls, but this phenomenon was not observed with the other bacterial species. Bacterial burden in Cftr morphants infected with a *P.*

aeruginosa ΔLasR mutant, a quorum sensing-deficient strain, was comparable to that in control fish indicating that the regulation of virulence factors through LasR is required for enhancement of infection in the absence of Cftr. The zebrafish system provides a multitude of advantages for studying the pathogenesis of *P. aeruginosa* and for understanding the role that innate immune cells such as neutrophils play in the host response to acute bacterial infections commonly associated with cystic fibrosis.

2.2. Introduction

Cystic fibrosis (CF) is a multi-organ genetic disorder characterized by chronic pulmonary infections as well as gastrointestinal and reproductive abnormalities. In 1989 it was discovered that CF results from mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene (63), which encodes a chloride ion channel present in most epithelial cells. Today, 80–95% of CF patients succumb to respiratory failure resulting from chronic lung infections that begin in early childhood and that are caused primarily by *P. aeruginosa* (16, 80). Chronic infection with the bacterium leads to hyperinflammation characterized by excessive recruitment of neutrophils to the airways, and subsequent release of oxidants and enzymes from activated neutrophils, which damages the lung tissue over time (80).

Pseudomonas aeruginosa is a flagellated Gram-negative opportunistic pathogen of plants and animals and is found naturally in soil and water. As a nosocomial pathogen, it is the third most commonly isolated organism, primarily

leading to urinary tract or wound infections in immunocompromised patients (47). Due to its production of alginate, the low permeability of its outer membrane and the presence of multidrug efflux pumps, *P. aeruginosa* exhibits a high degree of intrinsic antibiotic resistance (125), making infections by this bacterium a serious concern to hospital-bound patients. Myriad virulence factors carried by this bacterium afford *P. aeruginosa* the ability to infect a broad range of species (12). The Type III Secretion System enables the direct transfer of exotoxins from *P. aeruginosa* into the cytoplasm of eukaryotic cells and has been shown to be required for full virulence in zebrafish (7, 12) and directly linked to tissue destruction and infection of the mouse lung (40). LasR, a transcriptional regulator of quorum sensing, controls the expression of numerous virulence factors, including elastases, proteases, pyocyanin and hydrogen cyanide, and facilitates the transition into biofilm growth in *P. aeruginosa* (12, 31).

Among the most puzzling questions in cystic fibrosis research is why patients are susceptible to *P. aeruginosa* infection in particular. In non-CF patients, the most common respiratory bacterial pathogen is *Streptococcus pneumoniae* (114) while *P. aeruginosa* is rarely isolated (79). Studies that identified CFTR as a pattern recognition receptor for *P. aeruginosa* have linked CFTR deficiency to reduced bacterial clearance early in the course of infection in CFTR knockout mice (38, 48, 65, 129).

Recently, the zebrafish has become recognized as a useful model for the study of infectious disease (22, 52, 75, 84-86, 94, 95, 103, 113, 123, 149, 160) and

the pathogenesis of *P. aeruginosa* has been described (7, 12). Zebrafish embryos are particularly amenable to microscopic visualization, as they are optically clear, thus allowing real-time analysis of a bacterial infection. By far the most important advantage is the fact that for the first 30 days post fertilization zebrafish rely solely on the innate immune system for host defense against pathogens (20). After approximately 4 weeks zebrafish gain a functional adaptive immune system, at which time the interaction between innate and adaptive immunity may be examined. Invertebrates such as *C. elegans* and *Drosophila* have no adaptive immune system but possess only a very simple innate immune system and one primitive cell type, the hemocyte, which is thought to carry out some of the functions of both macrophages and neutrophils in evolutionarily higher species (78). The use of morpholino antisense oligonucleotide (MO) technology in zebrafish embryos has facilitated the functional characterization of many zebrafish genes, including those involved in innate immunity (2, 7, 150).

In the present study, the role of Cftr in the zebrafish innate immune response to acute infection with *P. aeruginosa* was evaluated through targeted knockdown of Cftr. Knockdown of Cftr perturbs multiple innate immune responses in the zebrafish embryo. Dampened respiratory burst response and reduced neutrophil migration both indicate that *cftr* plays a vital role in the host response to infection. Of greatest interest was the finding that *P. aeruginosa* bacterial burden was significantly higher in Cftr morphants than control embryos at 8 hours post infection (hpi), which parallels similar studies performed in mice and human

bronchial epithelial cells. The reduced ability to clear the infection was observed only when morphants were challenged with *P. aeruginosa* and not with other bacterial pathogens, including those commonly isolated from CF sputum. Infection with a *P. aeruginosa* mutant defective in the LasR transcriptional regulator did not result in an increase in bacterial burden in the morphants compared to the controls. The zebrafish embryo is a promising new model for the study of the host response to acute infections by *P. aeruginosa* and the effect of *cftr* on innate immunity and bacterial clearance.

2.3. Materials and Methods

2.3.1. Zebrafish care and maintenance.

Zebrafish were housed at the University of Maine Zebrafish Facility in recirculating systems (Aquatic Habitats, Apopka, FL). Water temperature was maintained at 28°C with a total system flow rate of 150 L/min. Zebrafish were maintained in accordance with the Institutional Animal Care and Use Committee standards. Adult zebrafish (>3 months old) were used for breeding embryos which were reared in Petri dishes containing 60 mL of embryo rearing medium (ERM) (0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 4.2 mM $NaHCO_3$). Strains used were Wild Type AB and the transgenic AB strain Tg(*mpx*:GFP)^{i114} expressing enhanced green fluorescent protein (EGFP) driven by the neutrophil specific myeloperoxidase promoter (123), kindly provided by Dr. Stephen Renshaw (University of Sheffield, Sheffield, UK). Until 24 hours post

fertilization (hpf), ERM was supplemented with 0.3 mg of methylene blue/L to prevent fungal growth. ERM was changed daily.

2.3.2. Detection of *cftr* expression in zebrafish tissues.

Zebrafish tissues were dissected into Trizol (Invitrogen, Carlsbad, CA) and total RNA was purified. Lymphoid and myeloid cells isolated from zebrafish kidneys by flow cytometry (145, 161) and stored as frozen pellets were generously provided by Dr. David Traver (University of California, San Deigo, La Jolla, CA) and resuspended in Trizol for RNA purification. Total RNA from tissues (2 μg) and sorted cells (1 μg) was reverse transcribed (SuperScript™ III Reverse Transcriptase: Invitrogen) and subjected to thermal cycling with gene-specific primers and TITANIUM™ Taq DNA polymerase (Clontech, Mountain View, CA). Expression of *cftr* was detected using nested primers. The initial *cftr* PCR was completed using the "*cftr* qPCR OUTSIDE FOR" primer (CTGTTTACAGAGATGCTGATCTTTAC), the "*cftr* qPCR OUTSIDE REV" primer (ACTGATATTGTCGTAGGCCTCCAAAC), and 30 cycles with an annealing temperature of 60^0C. The nested CFTR PCR was completed using the "*cftr* qPCR FWD (b1-t7)" primer (TGACGCACCATTCACTCATCTGGA), the "*cftr* qPCR REV (b1-t8)" primer (AGCTCAGGAAAGGTGCCGTAGAAA) and 30 cycles with an annealing temperature of 63^0C. PCR conditions and primer sequences for detecting myeloperoxidase (*mpx*), *TCRα* and β-*actin* expression were described previously (162). PCR products were analyzed by gel (2% agarose) electrophoresis.

2.3.3. Bacterial strains and growth conditions.

Wild-type non-mucoid *P. aeruginosa* strain PA14, PA14 ΔLasR and PA14 ΔT3SS were generously provided by Dr. George A. O'Toole and Dr. Deborah A. Hogan (Dartmouth Medical School, Hanover, NH). Strain PA14(p67T1) was constructed recently (134) and carries a constitutive plasmid-encoded RFP variant of d-Tomato that was engineered by Shaner *et al*. (132). *P. aeruginosa*CF lung isolate, designated SMC573 was provided by Dr. Joe Schwartzman (Dartmouth-Hitchcock Medical Center, Lebanon, NH). *P. aeruginosas*trains were grown on cetrimide selective agar (Accumedia, Lansing MI). PA14(p67T1)cultures were supplemented with 750 μg of ampicillin/ml. *Edwardsiella tarda* was grown on *Edwardsiella ictaluri* medium (EIM) (133). *Burkholderia cenocepacia* clinical isolate strain J2315 was cultured on *Burkholderia cepacia* selective agar (BCSA) (51). *Staphylococcus aureus* laboratory strain MZ100 (provided by George O'Toole) was grown on tryptic soy agar supplemented with 5% sheep blood (Northeast Laboratories, Waterville, ME). *Haemophilus influenza* type b strain EAGAN, a gift from Dr. Brian Ackerley (University of Massachusetts Medical School, Worcester, MA), was grown on chocolate agar supplemented with 16,000 units of bacitracin per liter (BD Bioscience, Sparks, MD). *E. coli* XL-10 (Stratagene, Cedar Creek, TX) was grown on sorbitol Mackonkey Agar (BD Bioscience, Sparks, MD). Prior to infection experiments, liquid cultures were grown for 12 h at 37°C with shaking (250 rpm) in L-broth(USB, Cleveland, OH), except *H. influenzae*, which was grown in brain heart infusion broth supplemented with 10 mg of Hematin (Sigma-Aldrich) and 10 mg of

NAD per liter (MP Biomedical, Solon, OH), harvested by centrifugation and washed twice in Dulbecco's phosphate buffered saline (dPBS). Cell density was determined spectrophotometrically and adjusted to 10^7 CFU/ml in dPBS.

2.3.4. Morpholino knockdown.

Morpholino antisense oligonucleotides (MOs) against zebrafish Cftr (GenBank accession no. NM_001044883) were synthesized by Gene Tools (Philomath, OR), reconstituted, and stored in Danieau's buffer. CFTR I1E2 MO (5'-CCACCTGTAAATATTCAGAGCAGAT-3') anneals to the intron 1/exon 2 boundary of the transcript and results in the deletion of exon 2. CFTR Trans MO (5'-CATCCTCCACAGGTGATCTCTGCAT-3') anneals to the AUG start site and blocks translation. Unless otherwise noted, Cftr knockdown was achieved by co-injecting 1.25 ng of each CFTR MO per embryo. Two and one half nanograms of Standard Control Morpholino (Gene Tools, Philomath, OR) was injected per control embryo. Prior to injection, MO solutions were supplemented with 0.01% phenol red to permit visualization. Zebrafish embryos at the 1–2 cell stage were disinfected by rinsing with Perosan (Zep Manufacturing, Atlanta, GA) for 1 minute, then microinjected with 5 nL of MO solution in the yolk, using an MPPI-2 pressure injection system (Applied Scientific Instrumentation, Eugene, OR). To determine whether co-injection of both CFTR morpholinos would yield the same effect on bacterial burden as would each injected separately, embryos were injected with either CFTR MO or both MOs simultaneously, and subsequently infected. Each CFTR MO was capable of exerting an independent effect on the bacterial burden (data not

shown). However, the two MOs injected together appeared to exert a synergistic effect, despite the fact that the dose of total MO (2.5 ng per fish) remained constant in all groups. For rescue experiments, wild type zebrafish *cftr* was cloned into pCS2^{+}(148), linearized, transcribed into capped RNA by SP6 mMessage mMachine (Ambion) and purified by LiCl precipitation. Approximately 100 pg of *cftr* RNA in 1x Danieau buffer containing 0.01% phenol red was simultaneously injected at the 1–2 cell stage with the 2.5 ng of I1E2 MO.

2.3.5. Infection by microinjection.

Zebrafish embryos (48 hpf) were manually dechorionated and anesthetized by immersion in Tris-buffered tricaine methane sulfonate (200 μg/mL) (Western Chemicals, Inc., Ferndale, WA). Unless otherwise noted, approximately 5 nl of bacterial suspension was microinjected into the duct of Cuvier as described by Clatworthy *et al.* (12) using the injection system described above. Inocula of 50 CFU per fish were injected into embryos for infection experiments with *P. aeruginosa, E. coli, E. tarda, B. cenocepacia, S. aureus* and *H. influenzae.* Statistical significance of mortality was determined by Log-Rank Test and by the Wilcoxon Test.

2.3.6. Determination of bacterial burden.

Infected embryos were incubated at 28°C for 8 h prior to re-isolation. Twenty fish in triplicate treatments were thoroughly washed in sterile dPBS, then homogenized in 1 ml of L broth. Ten-fold serial dilutions were performed in L broth and each dilution was plated in duplicate onto the appropriate selection medium. Plates were incubated at 37°C for 24 h, then colonies were counted and

corresponding titers were calculated. Bacterial burden ± standard error of the mean is expressed as CFUs per ml of embryo homogenate. Error bars represent the standard errors of the mean between replicates. Data represent three independent experiments. Statistical significance was determined by the Student's t-test.

2.3.7. Respiratory burst assay.

Respiratory burst assays were performed as described previously (53), with the following modifications. Twelve embryos per treatment were induced with phorbol 12-myristate 13-acetate (PMA), while 12 remained uninduced. Fluorescence was measured using a Synergy2 plate reader (Biotek, Winooski, VT) every 2 min 32 sec for a total of 95 measurements. Excitation / emission wavelengths were 485 nm / 528 nm, respectively. The 95th measurement was used to determine the fold induction value. Error bars represent standard errors of the mean. Data represent three independent experiments. Statistical significance was determined by Two Factor ANOVA.

2.3.8. Neutrophil migration assay.

Fifty bacterial cells were introduced into the hindbrain ventricle of 10−15 Tg(*mpx*:GFP) zebrafish embryos at 48 hpf by microinjection. Embryos were imaged by epi-fluorescence microscopy at 3 hpi and the total number of neutrophils within the ventricle was counted. Data shown was collected in three independent experiments for each pathogen. 42 Cftr morphant and 42 control embryos were infected with PA14; 35 were infected with *E. coli* and 30 were PBS injected controls. Statistical significance was determined by the Student's t-test.

2.3.9. Fluorescence microscopy.

Imaging was performed with an Olympus IX-81 inverted epi-fluorescence microscope equipped with a Hamamatsu ORCA-ER CCD camera and IPLab software (BD Biosciences, Rockville, MD). Confocal microscopy was performed using an Olympus IX-81 equipped with an Olympus FV-1000 laser scanning confocal using the Olympus FV-10 software package. Objective lenses 4x/0.16 NA, 10×/0.4 NA, 40x/0.9 NA and 100× oil immersion/1.4 NA were used. Prior to imaging, fish were anesthetized by immersion in Tris-buffered tricaine methane sulfonate (200 μg/ml). Images represent overlays of wide-field differential interference contrast (DIC) and fluorescence images unless otherwise noted. Optical filters providing excitation / emission at 543 nm / 610 nm and 488 nm / 510 nm were used for the detection of RFP and EGFP, respectively.

2.4.Results

2.4.1. Assessment of *P. aeruginosa* infection by microinjection.

P. aeruginosa infection by microinjection has been described (7, 12). Injection into the duct of Cuvier (12) resulted in the dissemination of bacteria into the bloodstream and proved to be the most effective method of infection. The LD_{50} of PA14 was determined to be approximately 50 CFU / embryo (Fig. 1a), resulting in 50% mortality by 48 hpi, significantly higher than PBS-injected controls. Bacteria initially colonized the yolk and spread to other regions in the fish, including the vasculature and tissues of the heart and tail, within a few hours post infection. In

addition, bacteria were readily engulfed by professional phagocytes circulating in the bloodstream. Neutrophils of the Tg(*mpx*:GFP)^{i114} zebrafish containing phagocytosed PA14(p67T1) were abundant (Fig. 1b) and were observed primarily near the site of injection, the duct of Cuvier along the anterior yolk.

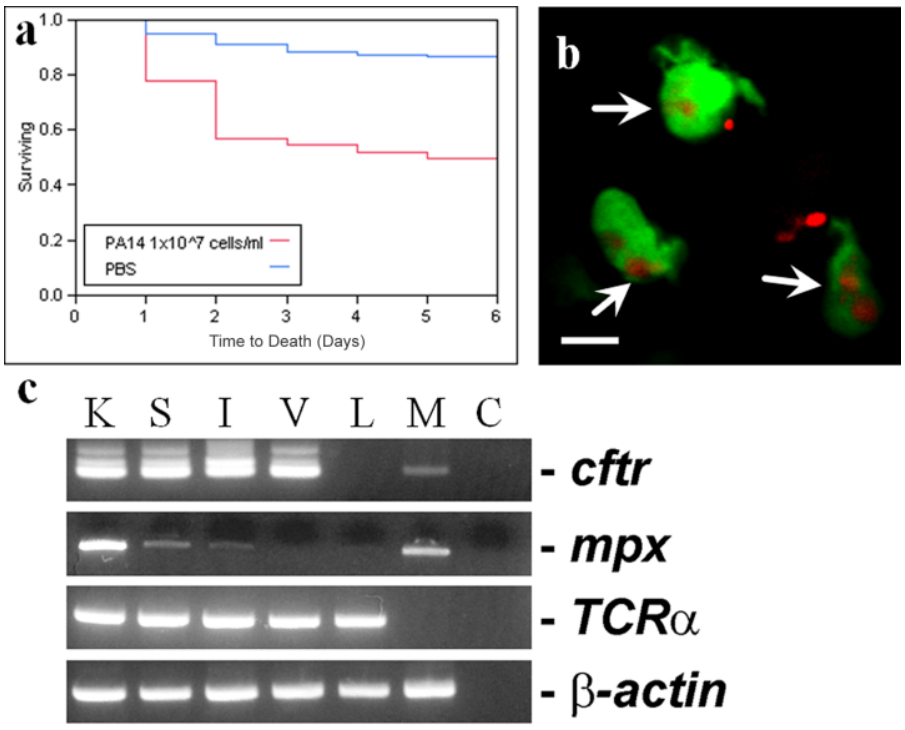

Figure 2.1. Zebrafish Are Infected by Microinjected *P. aeruginosa*.50 CFUs of PA14 were injected into the duct of Cuvier of 48 hpf fish. (a) Kaplan-Meier survival curve showing approximately 50% mortality in PA14 infected embryos by 48 hpi in wild type AB zebrafish. This mortality is significantly higher than mock injected controls by Log-Rank Test and by Wilcoxon ($p < 0.0001$). (b) Red fluorescent bacteria (white arrows) phagocytosed by green fluorescent neutrophils in the Tg(*mpx*:GFP)^{i114} transgenic zebrafish embryo. Scale bars represent 10 um. (c) PCR amplification of 200 bp fragment of *cftr* from zebrafish tissues. K: Kidney, S: Spleen, I: Intestine, V: Liver, L: Lymphocytes, M: Myeloid cells, C: No template control.

2.4.2. *cftr* Expression in the zebrafish.

cftr has been cloned and characterized in several fish species (9) including the zebrafish. Zebrafish *cftr* was expressed in the liver, kidney, spleen and intestine (Fig. 1c). *cftr* transcripts were detected in cells of the myeloid lineage that includes macrophages and neutrophils. Expression of *cftr* was not detected in the lymphocyte population. T-cell receptor α (*TCRα*) and *mpx,* which are expressed in zebrafish T lymphocytes and neutrophils, respectively, were used as controls to ensure the purity of cDNA derived from lymphocytes and myeloid cells. Expression levels of *β-actin* were consistent in all tissues examined.

2.4.3. Respiratory burst response in Cftr morphants.

It has been proposed that the lack of functional CFTR has deleterious effects on the production of reactive oxygen species (ROS) by macrophages and neutrophils resulting in reduced bactericidal activity (30, 32, 98). To determine whether this occurs in the zebrafish, a whole-embryo respiratory burst assay was employed (53, 92). Cftr morphants were generated as described above and no phenotypic differences between morphants and controls were observed. The respiratory burst response was significantly dampened (1.8-fold lower) in Cftr morphant embryos compared with controls between 48 and 56 hpf (Fig. 2).

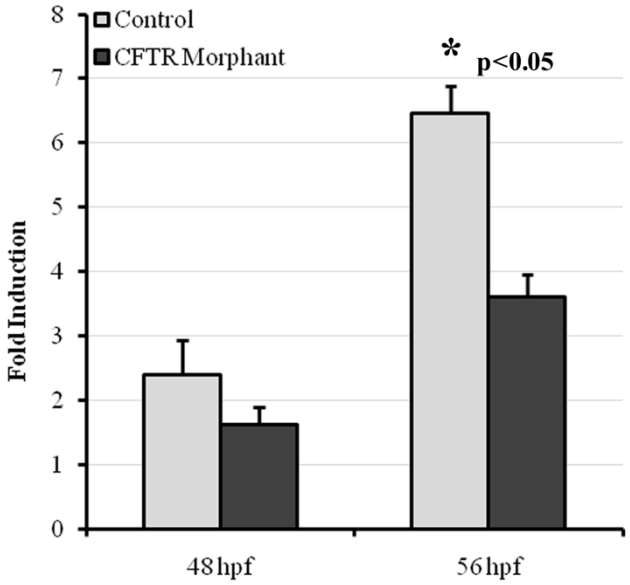

Figure 2.2. Dampened Respiratory Burst Response in Cftr Morphant Embryos. At 48 and 56 hpf respiratory burst is significantly dampened in morphant embryos versus controls, indicating a decrease in production of ROS in morphants. Results are representative of three independent experiments. Error bars represent standard errors of the mean. Statistical significance was determined by Two Factor ANOVA.

2.4.4. Delayed neutrophil migration in Cftr morphants.

With the finding that Cftr knockdown disrupts overall immune health by reducing ROS production, directed chemotaxis of neutrophils to a localized infection was assessed in the zebrafish. At 48 hpf, PA14(p67T1) was injected into the hindbrain ventricle (HBV), as described by Davis *et al*(22), of morphant and control Tg(*mpx*:GFP)^{i114} transgenic embryos. Neutrophils began to arrive at the site of infection at 90 minutes post infection (mpi) with peak migration at 180 mpi. Neutrophils in the HBV were enumerated at 180 mpi (Fig. 3a). The average number of neutrophils in the HBV of Cftr morphants (9 cells) was significantly lower than in

infected controls (15 cells) (Fig. 3b). Upon infection with avirulent *E. coli*, an average of 5 neutrophils arrived at the HBV at 180 mpi and there was no significant difference between the morphants and controls (Fig. 3b). The perturbation in migration behavior of neutrophils in response to a *P. aeruginosa* infection may account for the reduced clearance of the bacteria in the Cftr morphants discussed below.

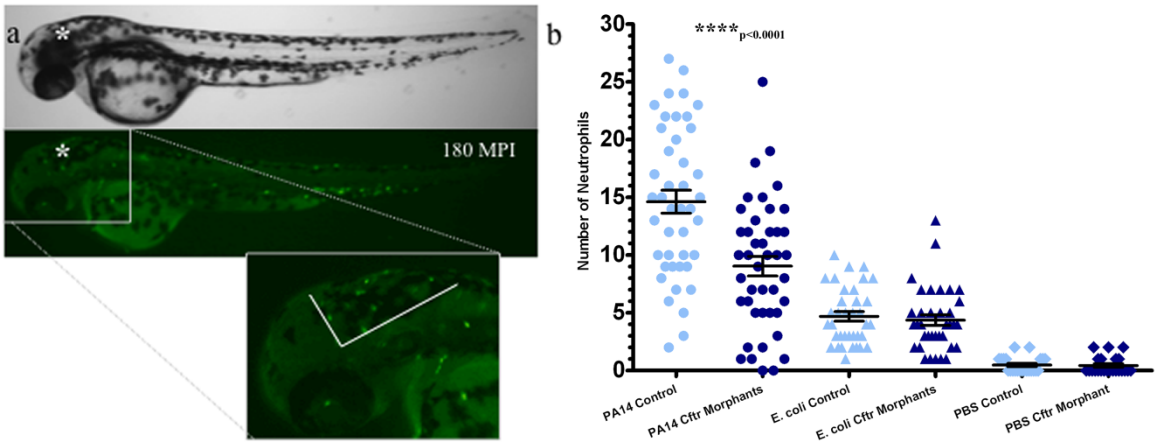

Figure 2.3. Neutrophil Migration is Impaired in Cftr Morphants. (a) Neutrophil migration to the hindbrain ventricle (*) of Tg(*mpx*:GFP)^{i114} zebrafish embryos at 48 hpf and site of infection was monitored for 8 hours post infection. Total numbers of neutrophils within the boundary of the hindbrain ventricle (white lines) were counted at 180 mpi. (b) Significantly fewer neutrophils were observed in the Cftr morphants than Control infected embryos when challenged with 50 CFUs of PA14(p67T1) (n=42), but no difference in neutrophil migration was observed when infected with 50 CFUs of *E. coli* XL-10 (n=35) or injected with 5 nl sterile PBS (n=30). Error bars represent standard errors of the mean, statistical significance was determined by the Student's t-test.

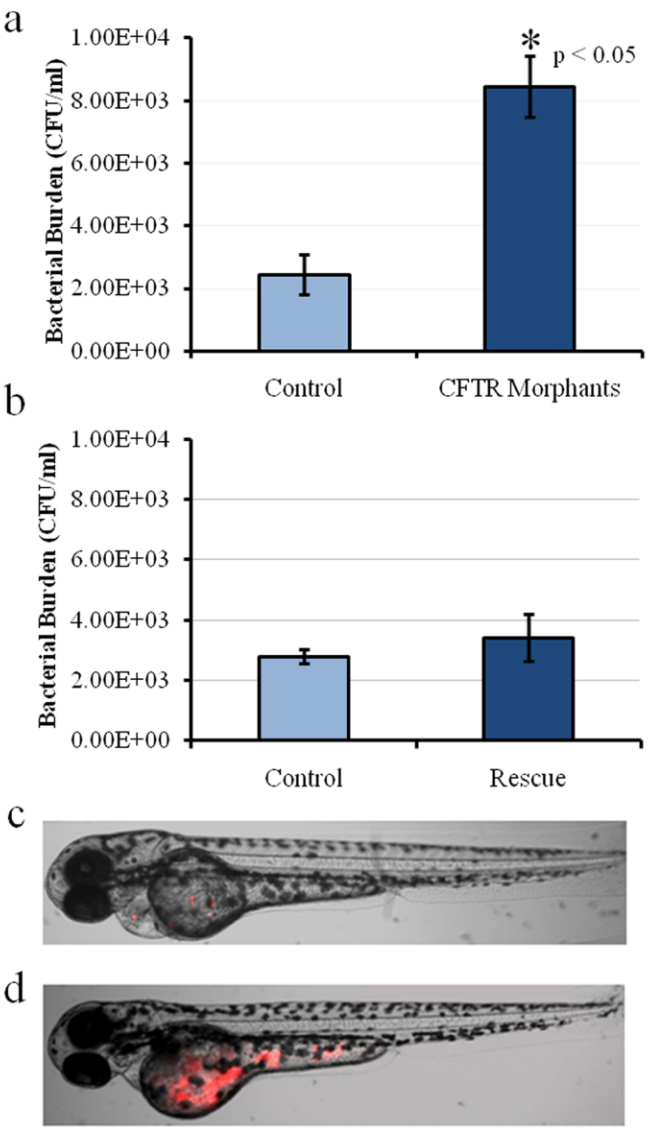

Figure 2.4. Bacterial Clearance Is Impaired at 8 Hours Post Infection in Cftr Morphants. (a) Cftr morphant embryos infected with 50 CFUs of PA14 at 48 hpf exhibit a 3.47-fold greater bacterial burden than control infected embryos at 8 hpi. (b) No difference in bacterial burden was observed when the Cftr knockdown was rescued. (c-d) Results were visually confirmed using fish infected with PA14(p67T1). Fluorescence of Cftr morphants (d) was observably greater than that of control fish (c). Results are representative of three independent experiments. Error bars represent standard errors of the mean and statistical significance was determined by the Student's t-test.

2.4.5. Bacterial burden in Cftr morphants.

One of the most significant observations in CF models is that mutated or deficient CFTR results in increased host colonization (15), and an inability of the host to clear *P. aeruginosa* infection (48, 129). This observation, however, has not been obtained consistently and appears to depend on experimental design. One aim of this study was to determine if a Cftr knockdown would affect the ability of the zebrafish host to clear a *P. aeruginosa* infection. At 48 hpf, morphant and control embryos were microinjected with 50 CFUs of PA14 into the duct of Cuvier. After 8 h the morphant embryos exhibited a bacterial burden that was significantly greater than that in control embryos, 8.45 ± 0.98 x 10^3 CFU/ml, versus 2.45 ± 0.65 x 10^3 CFU/ml, respectively (Fig. 4a). Interestingly, the significantly higher bacterial burden early in the course of infection did not result in higher mortality in the Cftr morphants compared to the controls (data not shown). A rescue experiment was performed, wherein co-injection of the CFTR I1E2 morpholino with the full length transcript of zebrafish *cftr* confirmed that the effect of morpholino knockdown was specific. Infection of the rescued Cftr morphants with PA14 resulted in no significant difference in bacterial burden when compared with the controls, 3.43 ± 0.76 x 10^3 CFU/ml versus 2.80 ± 0.5 x 10^3 CFU/ml, respectively (Fig. 4b).

To visually confirm the increased bacterial burden, morphant embryos were challenged with PA14(p67T1). At 8 hpi, 20 fish were selected at random from the control and morphant groups and examined by fluorescence microscopy. Greater fluorescence signal intensity was observed in the Cftr morphants compared to

controls, and this fluorescence was most apparent within the yolk (Figs. 4c and 4d). We have previously shown that RFP-expressing and wild-type strains of *P. aeruginosa*are equally virulent, and that the production of RFP has no effect on bacterial burden(134).

2.4.6. Increased bacterial burden is unique to *P. aeruginosa*.

Cftr morphant embryos have a 3.5 fold higher number of PA14 cells after 8 h of infection than do control embryos. To determine if this finding was unique to PA14, the bacterial burden assay was repeated with a CF clinical isolate of *P. aeruginosa* SMC573 and strains of *H. influenza*, *B. cenocepacia* and *S. aureus,* all of which are commonly isolated from CF patients, as well as the fish pathogen *E. tarda* and an avirulent strain of *E. coli*. All strains of *P. aeruginosa* examined showed significant differences in bacterial burden between the Cftr morphants and controls (Fig. 5) and no difference was observed with the other bacterial species.

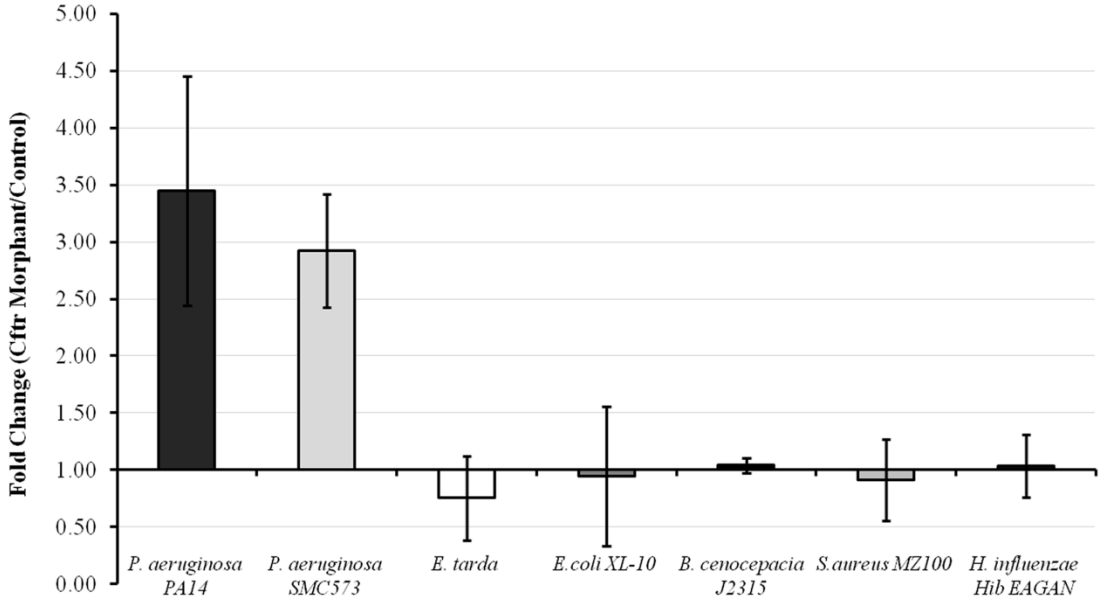

Figure 2.5. Increased Bacterial Burden in Cftr Morphants Is Specific for *P. aeruginosa.* Infection of Cftr morphants with PA14 shows a statistically significant 3.47-fold change in bacterial burden over control. *P. aerigoinosa* clinical isolate SCM573 shows a similar 2.9-fold higher bacterial burden in morphants compared to controls. Infection of morphants with *Edwardsiella tarda, Escherichia coli, Burkholderia cenocepacia, Staphylococus aureus* and *Haemophilus influenzae* shows no significant change in bacterial load over controls. Results are representative of three independent experiments. Error bars represent standard errors of the mean.

2.4.7. LasR quorum sensing regulator but not Type III Secretion is required for increased virulence in Cftr morphants.

The Type III Secretion System (T3SS) and LasR have been previously shown to be important virulence determinants in *P. aeruginosa* infection in zebrafish (7, 12). The connection between these systems and the reduced clearance of *P. aeruginosa* in the Cftr morphants was examined. Infection with PA14 ΔT3SS in Cftr morphants resulted in a 4-fold increase in the bacterial burden, similar to that

observed with wild type PA14, compared to control embryos (Fig. 6), though the total number of bacteria recovered was significantly higher than with wild type PA14. Upon infection with PA14 ΔLasR, bacterial burden in the Cftr morphants was similar to that of the controls (Fig. 6) and the number of bacteria recovered at 8 hpi was consistent with that of wild type PA14, approximately 2 x 10^3 CFU/ml, suggesting that components of the LasR system, not T3SS, may be responsible for the reduced bacterial clearance in morphants.

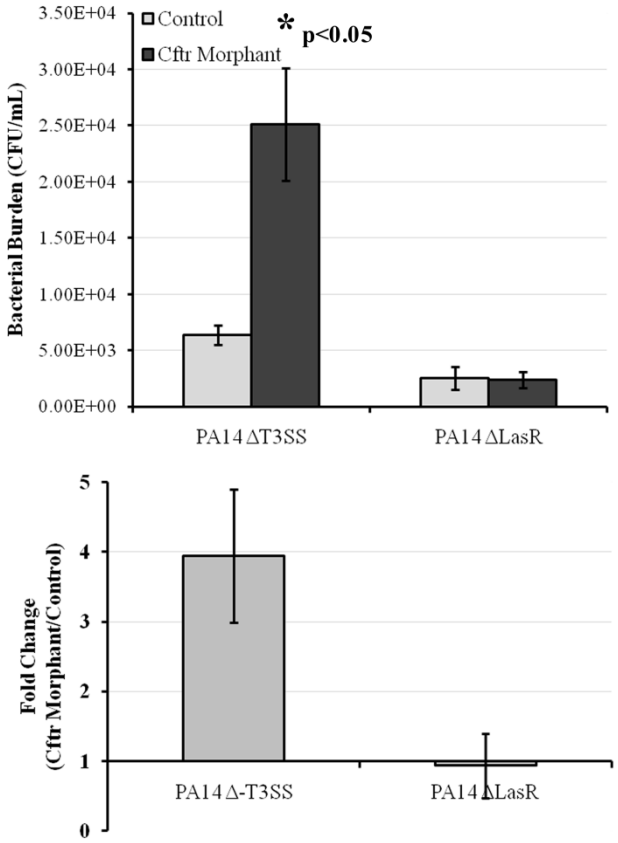

Figure 2.6.LasR contributes to the Reduced Bacterial Clearance of *P. aeruginosa* in Cftr Morphant Zebrafish Embryos**.** 48 hour post fertilization morphant and control embryos were challenged with 50 CFUs of PA14 mutants defective in either T3SS or the LasR transcriptional regulator and incubated 8 hours. Morphant embryos

infected with the T3SS mutant exhibit a 4.5-fold greater bacterial burden than controls. No difference in bacterial burden was observed upon infection with PA14 defective in LasR. Results are representative of three independent experiments. Error bars represent standard errors of the mean, statistical significance determined by Student's t-test.

2.5. Discussion

In this zebrafish study, Cftr knockdown during acute *P. aeruginosa* infection resulted in a greater bacterial burden, observed at 8 hpi. The trend of reduced early clearance of *P. aeruginosa* has been demonstrated previously in mice (129), where differences between CFTR knockout and control mice were only observed a few hours post infection. This study, however, has generated controversy (11). Our findings were not strain-specific and were noted in morphants infected with PA14, the constitutive RFP-expressing PA14(p67T1) and a CF clinical isolate SMC573. Rescue experiments, in which the knockdown of Cftr was complemented with full length transcripts of the gene, showed no measureable difference in bacterial burden compared to controls, confirming that the findings are a direct result of Cftr knockdown.

P. aeruginosa is only one of the several species of bacteria commonly isolated from sputum of CF patients. *S. aureus*and *H. influenzae* are typically associated with early childhood infections in CF, usually before *P. aeruginosa* becomes prevalent (16, 45, 60, 87, 151), and infections with *B. cepacia* complex are often associated with a very poor clinical outcome (128). We determined the effect of Cftr knockdown on bacterial clearance by the host. The bacteria observed included *E.*

tarda, a natural fish pathogen that has been previously described in the zebrafish (113), an avirulent strain of *E. coli*, XL-10, and *S. aureus, H. influenzae* and *B. cenocepacia,* which are common CF lung pathogens. Clearance was examined under identical infection conditions and no significant difference in bacterial burden between Cftr morphants and controls was observed at 8 hpi, providing evidence of Cftr-mediated specific resistance to *P. aeruginosa*. These findings indicate that although Cftr is required for the initial innate immune response to *P. aeruginosa*, it is not required for response to other bacterial pathogens, including other common respiratory pathogens and those that naturally infect the zebrafish.

Two mechanisms of virulence most commonly associated with *P. aeruginosa* pathogenesis were examined as possible contributors to the impaired bacterial clearance observed in the Cftr morphants. The T3SS plays a significant role in the progression of infection, and *P. aeruginosa* mutants defective in T3SS have been shown previously to have an impaired ability to infect zebrafish embryos (7, 12). Infection with PA14 ΔT3SS resulted in the same trend as wild type PA14, indicating that even though T3SS is an important virulence determinant in *P. aeruginosa,* it does not influence impaired clearance in the morphants.

The LasR transcriptional regulator is key to quorum sensing in *P. aeruginosa* and regulates the expression of multiple virulence factors. According to one retrospective study, in which clinical isolates of *P. aeruginosa* were genotypically categorized, approximately 30% of the isolates obtained from cystic fibrosis patients were LasR mutants (54). Hoffman and colleagues speculated that during

chronic infection there is selective pressure for LasR mutants in the CF lung because virulence factors required for acute infection become disadvantageous for maintaining chronic infection. In addition, a recent study by this group determined that the mechanism for this selection lies in the availability of specific amino acids found in the CF airway, which offer a metabolic advantage to the LasR mutants (55). Low oxygen tension and high levels of nitrates in the lung favor utilization of nitrogen species over oxygen for metabolism by the bacteria. The authors show that denitrification by *P. aeruginosa* results in an accumulation of NO·, and exogenous sources of NO· overwhelm the detoxification processes by the bacterium. This makes *P. aeruginosa* LasR mutants highly susceptible to reactive nitrogen species (RNS) but confers a resistance to oxidative stress (55). We found no difference in bacterial burden between morphants and controls challenged with PA14 ΔLasR, identifying one or more components of this complex system as critical to the enhanced virulence of the bacterium in CF. It is probable that although loss of LasR is key to survival during chronic infections, the transcription of virulence factors downstream from LasR is critical for the initial infection in CF. These data support the conclusions drawn from studies in the mouse CF model, which identified CFTR as a pattern recognition receptor for *P. aeruginosa* and show reduced early bacterial clearance in CFTR-/- mice (129), but offer an alternative explanation for the specificity observed between CFTR and *P. aeruginosa* early in the course of infection. Because only the PA14 LasR mutant was examined in this study, further

investigation with mutants of other strain backgrounds is required to confirm the role of LasR during infection.

Respiratory burst is a key host defense mechanism against invading microorganisms. *In vitro* studies have recently revealed that CFTR is required for acidification of the phagosomal compartment of professional phagocytes, such as macrophages and neutrophils, and may shed light on the nature of the involvement of CFTR in ROS production and bacterial killing (30, 32, 98). This phagosomal chlorination defect is thought to result in impaired function of the NADPH oxidase and peroxidases due in part to an insufficient H^+ gradient between the lumen of the phagosome and the cytosol. The respiratory burst response of many organisms serves as a good indicator of overall innate immune health (53), and zebrafish are an ideal model for studying the dynamics of this response because whole-embryo measurements of respiratory burst can be made *in vivo* (53). Respiratory burst levels in Cftr morphants were significantly lower than controls at 48 and 56 hpf. The 56 hpf time point corresponds to an 8 hour infection of 48 hpf embryos, the developmental stage in which the bacterial burden was greater in morphant embryos. These data suggest that Cftr knockdown in zebrafish embryos leads to an impaired innate immune response characterized in part by a significant reduction in the production of ROS. Because there was marked reduction of ROS production in morphant fish at these time points and *cftr* expression was demonstrated in zebrafish myeloid cells, the data could explain the impaired ability to clear the infection. It has been shown that *P. aeruginosa* LasR mutants are much more

susceptible to ROS than LasR competent strains (55). The increased sensitivity of the bacteria could explain why the morphant embryos are as proficient as the control embryos at clearing the LasR mutants, despite the reduced production of ROS overall. Further investigation is required to determine the underlying mechanism for this observation and the potential role of RNS in bacterial clearance of the *P. aeruginosa* LasR mutant.

The major cause of morbidity and mortality in CF has been attributed to destruction of the lung epithelia by a massive and importunate neutrophylic inflammation (66). Proteases and oxygen radicals released by neutrophils in an attempt to eradicate bacterial infection cause damage to the epithelial surface (35). One question that remains heavily debated is whether the inflammation occurs independently of bacterial infection or as a result of it. Observations of neutrophil inflammation in CF infants, where no pathogens were detected, have been challenged by evidence showing colonization of the lower respiratory tract at subclinical levels before presentation of symptoms (35). An *in vitro* study has shown a significant decrease in directed chemotaxis between neutrophils isolated from healthy individuals and from acutely infected CF patients (131). These studies, however, were unable to sufficiently answer the complex questions surrounding neutrophil recruitment to an infection. An advantage of the optically clear zebrafish embryo is that assessment of infection can be made *in vivo* using fluorescently-labeled pathogens and established transgenic zebrafish lines. This makes the zebrafish an ideal model for the study of phagocyte-pathogen interactions and the

kinetics of bacterial clearance *in vivo*, which is not easily conducted in mice. Zebrafish neutrophils demonstrate strong chemotaxis toward wound and infection sites (69) and actively engulf invading pathogens. A transgenic line of zebrafish expressing GFP in neutrophils, Tg(*mpx*:GFP)^{i114} , under the control of the myeloperoxidase promoter, has been characterized (123). Changes in neutrophil behavior in embryos were assessed with imaging studies performed in Tg(*mpx*:GFP)^{i114} embryos infected with PA14(p67T1). These experiments showed that a localized infection in a discrete location could be established resulting in a vigorous neutrophil migration by 3 hpi. Significantly fewer neutrophils were observed in response to the infection of Cftr morphant embryos indicating an impaired response to pathogens. *E. coli* XL-10 was used as a control and no difference in the number of neutrophils between morphants and controls was observed at 3 hpi. Fewer neutrophils migrated to the HBV because the overall neutrophil response to *E. coli* is less robust in zebrafish (69). This indicates some specificity in the neutrophil response or signaling to *P. aeruginosa*. Dampened neutrophil migration, coupled with the LasR-dependent enhancement of infection in the Cftr morphants, offers an explanation as to why the CF lung is so easily colonized by *P. aeruginosa*, an organism that rarely causes respiratory tract infections in healthy individuals. It is possible that the slower, less robust neutrophil response may afford the bacteria more time to establish sufficient density to undergo phenotypic changes associated with LasR quorum sensing, rendering the bacteria more resistant to host defenses and allowing infection to persist.

The zebrafish ortholog of *cftr* (Subclass C, member 7) was identified in 2003 by Thomas *et al*.(142). We have found 78% conserved amino acid sequence similarity between zebrafish and human with the highest degree of identity in both nucleotide binding domains. Not only is CFTR conserved among species, but the outcome of *P. aeruginosa* infection in response to Cftr knockdown in zebrafish is similar to that observed in other model organisms. The zebrafish is advantageous for larger studies, in which hundreds to thousands of individuals may be studied in a single experiment. As an added benefit, the lack of an adaptive immune response in embryos for the first 30 days renders the zebrafish an ideal model for studying innate immunity, which is the critical defense mechanism in human lung infections (32) and may provide the key to understanding the basis for impaired bacterial clearance in the CF lung. The zebrafish has recently emerged as a valuable model for studying host-pathogen interactions and innate immunity (7, 12, 22, 52, 75, 84-86, 94, 95, 103, 113, 123, 149, 160) and the findings presented here underscore the significant advantages of the zebrafish embryo model and offer an intriguing new system for the study of *P. aeruginosa* pathogenesis and the host innate immune response in the context of CF.

CHAPTER 3

THE IRON REGULATED VIRULENCE NETWORK OF *PSEUDOMONAS AERUGINOSA* PERMITS RAPID AND ENHANCED COLONIZATION IN A ZEBRAFISH MODEL OF CYSTIC FIBROSIS

3.1. Abstract

Cystic fibrosis (CF) is the most common lethal hereditary disease and is caused by recessive mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene. CF is associated with multi-organ defects resulting from improper ion transport across epithelial membranes. Chronic lung infection by the environmentally ubiquitous opportunistic human pathogen *Pseudomonas aeruginosa* and the subsequent hyperinflammation that occurs as the host immune system combats the bacterium are responsible for the bulk of the morbidity and mortality in CF. Despite numerous studies that have sought to elucidate the role of CFTR in the innate immune response, the links between CFTR, innate immunity, and *P. aeruginosa* infection remain unclear. Our previous work has demonstrated that zebrafish embryos with reduced expression of the *cftr* gene (Cftr morphants) exhibited reduced respiratory burst response, directed neutrophil migration and had a reduced ability to clear *P. aeruginosa* infections, a phenomenon that was unique to this pathogen and was caused by virulence factors regulated through quorum sensing (QS). The present work highlights our ability to use the zebrafish to model and elucidate mechanisms of infection by replicating multiple facets of the CF

condition in humans. In this study, Cftr morphants were infected with *P. aeruginosa* mutants defective in the expression of QS regulated virulence factors. A mutant defective in the production of Exotoxin A (ETA) resulted in similar bacterial clearance in both the Cftr morphant and control embryos. The reduction in directed neutrophil migration to a *P. aeruginosa* infection was also restored when challenged with the ETA mutant. Cftr morphants were found to display a statistically significant iron deficiency (ID) compared to control embryos, a symptom commonly diagnosed in humans with CF that is believed to be a result of deficiencies in pancreatic function and poor nutrient uptake. Taken together, these data point towards a possible explanation for the specificity between *P. aeruginosa* and CF. The zebrafish system provides a multitude of advantages for studying the unique pathophysiology resulting from defective expression of *cftr*, the pathogenesis of *P. aeruginosa* and elucidating the role that the CF innate immune system plays in response to acute bacterial infections.

3.2. Introduction

Cystic fibrosis (CF) is a lethal hereditary multi-organ disorder caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), a chloride ion channel expressed in most epithelial cells in the body (28). Thick mucus produced by all mucosal surfaces, a symptom of poor ion transport across these membranes, is responsible for many of the clinical manifestations of this disease. Poor pancreatic function and thick mucus blocking the intestinal tract results in

poor uptake of nutrients, vitamins and minerals. Iron deficiency (ID), and often ID associated anemia, is common in CF patients and has been attributed to poor pancreatic function and loss of iron into the airway caused by excessive infection and inflammation (46). The bulk of morbidity and mortality among people with CF results from chronic persistent bacterial infections of the respiratory tract caused primarily by the opportunistic pathogen *Pseudomonas aeruginosa*. *P. aeruginosa* is a common cause of nosocomial infections and is particularly problematic for ICU patients with implanted devices such as breathing tubes and catheters (143). Despite the frequent isolation of this bacterium in immunocompromised patients, infections by *P. aeruginosa*, particularly infections of the respiratory tract, are extremely rare in otherwise healthy individuals (79). Scientists and clinicians have sought to elucidate the factors that make CF patients particularly vulnerable to chronic infections by *P. aeruginosa,* but decades of research have failed to fully explain the mechanisms underlying this unique susceptibility.

P. aeruginosa is an environmentally ubiquitous Gram-negative bacterium that is found in soil and water. This environmental microbe is also a common opportunistic pathogen in both plants and animals (79). Because of the diverse environments in which *P. aeruginosa* thrives and the highly recombinant and promiscuous nature of the bacterium, a wide array of potent toxins and other virulence factors are encoded within its genome. Cell-to-cell communication, or QS, has been implicated in the gene regulatory networks of many bacterial species (155), and it was not surprising that the regulation of virulence genes by *P.*

aeruginosa was traced back to these QS networks (102). *P. aeruginosa* possesses two QS controlled transcriptional regulators, LasR and RhlR. These regulators and the specific inducers that activate them have been implicated in the expression of multiple genes associated with pathogenicity and toxicity during infection in plants and animals (See Figure 1.2 and Table 1.1) (102).

Recent studies have highlighted the zebrafish (*Danio rerio*) as a powerful model for studying host–pathogen interactions during bacterial and viral infections and the pathogenicity of *P. aeruginosa* in the zebrafish embryo has been described (22, 52, 75, 84-86, 94, 95, 103, 113, 123, 149, 160). Our previous study established the zebrafish embryo as a useful model for studying the unique relationship between the CFTR and bacterial infections (104). We found that embryos with reduced expression of Cftr (Cftr morphants) had an enhanced susceptibility to infection by *P. aeruginosa*. Cftr morphants had a less robust neutrophil response to *P. aeruginosa*, and the bacterial clearance defect in the Cftr morphants was the result of virulence factors regulated through the LasR Quorum Sensing (QS) system.

In the present study, we sought identify which QS regulated virulence factors were directly responsible for the reduced ability of the morphants to clear infections caused by *P. aeruginosa* and to attempt to elucidate the underlying mechanisms that are responsible for this unique susceptibility. We performed infection studies using *P. aeruginosa* PA14 transposon insertion mutants defective in the expression of virulence factors that have been (i) identified as important for full virulence during infections, (ii) highlighted for their involvement in the pathogenicity of *P. aeruginosa*

in the CF lung, and (iii) controlled by either the LasR or RhlR QS system of *P. aeruginosa*. We have identified *P. aeruginosa* Exotoxin A (ETA) as being responsible for the reduced clearance observed in the Cftr morphant zebrafish embryos and have found that neutrophil migration to this mutant is restored to levels equal to the control embryos. The lethal effects of *P. aeruginosa* ETA on the host during infection have been well characterized (101, 159) and the mechanisms by which the toxin is regulated have been thoroughly described (43, 44). The present study highlights the advantages of the zebrafish embryo for infectious disease research. Large scale studies involving multiple strains of bacteria and bacterial mutants with varying pathogenicity are technically easy and relatively inexpensive to perform in zebrafish embryos and a myriad of reports are available demonstrating that the immune response of zebrafish embryos to bacterial pathogens closely mirrors experiments performed in murine models (146). The data presented here provide insight into a potential molecular mechanism that results in the susceptibility of CF patients to infection by *P. aeruginosa* and identifies a new avenue for future research in this field.

3.3. Materials and methods

3.3.1. Zebrafish care and maintenance

Zebrafish were housed at the University of Maine Zebrafish Facility in a recirculating system (Aquatic Habitats, Apopka, FL). Water temperature was maintained at 28°C with a total system flow rate of 150 L/min. Zebrafish were

maintained in accordance with the Institutional Animal Care and Use Committee standards. Adult zebrafish (>3 months old) were used for breeding embryos which were reared in Petri dishes containing 60 mL of Egg water (60 µg Instant Ocean (Spectrum Brands)/ml). Strains used were Wild Type AB and the transgenic AB strain Tg(*mpx*:GFP)^{i114} expressing enhanced green fluorescent protein (EGFP) driven by the neutrophil specific myeloperoxidase promoter (123), kindly provided by Dr. Stephen Renshaw (University of Sheffield, Sheffield, UK). Until 24 hours post fertilization (hpf), Egg water was supplemented with 0.3 mg of methylene blue/L to prevent fungal growth. Egg water was changed daily.

3.3.2. Morpholino knockdown

Morpholino antisense oligonucleotides (MOs) against zebrafish Cftr (GenBank accession no. NM_001044883) were synthesized by Gene Tools (Philomath, OR), reconstituted, and stored in Danieau's buffer. CFTR I1E2 MO (5'-CCACCTGTAAATATTCAGAGCAGAT-3') anneals to the intron 1/exon 2 boundary of the transcript and results in the deletion of exon 2. CFTR Trans MO (5'-CATCCTCCACAGGTGATCTCTGCAT-3') anneals to the AUG start site and blocks translation. Cftr knockdown was achieved by co-injecting 1.25 ng of each CFTR MO per embryo. Two and one half nanograms of Standard Control Morpholino (Gene Tools, Philomath, OR) was injected per control embryo. Prior to injection, MO solutions were supplemented with 0.01% phenol red to permit visualization. Zebrafish embryos at the 1–2 cell stage were disinfected by rinsing with Perosan (Zep Manufacturing, Atlanta, GA) for 1 minute, then microinjected with 5 nL of MO

solution in the yolk, using an MPPI-2 pressure injection system (Applied Scientific Instrumentation, Eugene, OR).

3.3.3. Bacterial strains and growth conditions

Wild-type non-mucoid *P. aeruginosa* strain PA14and mariner transposon PA14 mutants of *lasA, lasB, hplS, mvfR, rhlA,phzM* and *toxA* (described by Liberati, 2005) were generously provided by Dr. George A. O'Toole (Dartmouth Medical School, Hanover, NH). *P. aeruginosa* strains were grown on cetrimide selective agar (Accumedia, Lansing MI). Mariner cultures were supplemented with 30 μg of gentamicin/ml. Prior to infection experiments, liquid cultures were grown for 12 h at 37°C with shaking (250 rpm) in L-broth (USB, Cleveland, OH), harvested by centrifugation and washed twice in Dulbecco's phosphate buffered saline (dPBS). Cell density was determined spectrophotometrically and adjusted to 10^7 CFU/ml in dPBS.

3.3.4. Identification of transposon insertion site in PA14 mutants.

The location of the transposon insertion was identified as previously described(73). Briefly, overnight cultures of each mutant were grown in L-Broth with 30 μg of gentamicin/mland lysed at 95°C for 15 min. Cell debris was pelleted by centrifugation and 5 μl of supernatant were used as template for a two round PCR reaction with a transposon specific primer and an arbitrary primer as described. PCR products from the second round were gel extracted using the MiniElute Gel Extraction kit (Qiagen), ligated into pGEM-T easy (Promega) and sequenced by the University of Maine DNA Sequencing Facility (Orono, ME).

Sequencing results were blasted against the PA14 genome using the Pseudomonas Genome Database(157). In three mutants, PA14ΩAprA, PA14ΩPlcB and PA14ΩPvdD, fragments could not be amplified through the arbitrary PCR method. For confirmation that the two step method was not biased against these genomic regions, the entire ORF was amplified through standard PCR methods and the fragment was sequenced and compared to wild type PA14.

3.3.5. Infection by microinjection

At 48 hpf, zebrafish embryos were manually dechorionated and anesthetized by immersion in Tris-buffered tricaine methane sulfonate (200 μg/mL) (Western Chemicals, Inc., Ferndale, WA). Unless otherwise noted, approximately 5 nl of bacterial suspension was microinjected into the duct of Cuvier as described by Clatworthy *et al.*(12) using the injection system described above. Inocula of 50 CFU per fish were injected into embryos for infection experiments with all *P. Aeruginosa* mutant strains.

3.3.6. Determination of bacterial burden

Infected embryos were incubated at 28°C for 8 h prior to re-isolation. Twenty fish in triplicate treatments were thoroughly washed in sterile dPBS, then homogenized in 1 ml of L broth. Ten-fold serial dilutions were performed in L broth and each dilution was plated in duplicate onto Cetrimide agar (Accumedia, Lansing MI) for selection of only *P. aeruginosa.* After a 24 h incubation at 37°C, colonies were counted and corresponding titers were calculated. Data is expressed as either bacterial burden ± standard error of the mean per ml of embryo homogenate or the

fold difference in burden of the Cftr morphant embryos relative to control embryos. Error bars represent the standard errors of the mean between replicates. Data are representative of three independent experiments. Statistical significance was determined by the Student's t-test.

3.3.7. Neutrophil migration assay

Fifty bacterial cells were introduced into the hindbrain ventricle of 15–20 Tg(*mpx*:GFP) zebrafish embryos at 48 hpf by microinjection. Embryos were imaged by epi-fluorescence microscopy at 3 hpi and the total number of neutrophils within the ventricle was counted. Data shown were collected in three independent experiments and pooled together. Statistical significance was determined by the Student's t-test.

3.3.8. Fluorescence microscopy

For all microscopy experiments, an Olympus IX-81 inverted epi-fluorescence microscope equipped with a Hamamatsu ORCA-ER CCD camera and Olympus CellSens Dimension Software. Objective lenses 4x/0.16 NA, 10×/0.4 NA and20x/0.75 NA were used. Prior to imaging, fish were anesthetized by immersion in Tris-buffered tricaine methane sulfonate (200 μg/ml). Optical filters providing excitation / emission at 543 nm / 610 nm and 488 nm / 510 nm were used for the detection of AlexaFlour594 and EGFP, respectively.

3.3.9. Ferrozine based iron assay

A ferrozine based assay wasperformed as previously described by Riemer, *et al.,* 2004, with slight modification. In brief, one zebrafish embryo was euthanized in

a lethal dose of tricaine methane sulfonate, transferred to a 0.7 ml microcentrifuge tube and lysed for 1 hour at 60°C in 100 ul of 50 mM NaOH. Bound iron was released using freshly prepared acidic potassium permanganate at 60°C for two hours. Total iron was detected using the iron-detection reagent as described, transferring the assay to a clear 96-well plate and reading the absorbance of each well at 550 nm using a Synergy2 plate reader (Biotek, Winooski, VT) 30 minutes after adding the detection reagent. For experiments with de-yolked embryos, the protocol was the same except that each embryo was manually de-yolked using fine forceps immediately before NaOH lysis.

3.3.10. *in situ* whole mount TUNEL assay

After an 8 h infection with wild type PA14, Cftr morphant and control embryos were fixed overnight in 4% paraformaldehyde in PBS at 4°C. After fixation, embryos were transferred through a methanol gradient and stored at -20°C in 100% methanol until TUNEL assays were performed. Measurements of apoptosis were achieved using the Click-it TUNEL Alexa Fluor 594 Assay Kit (Invitrogen, Carlsbad, CA) with slight modification. In brief, the TdT reaction was extended to 2 hours, blocking steps were extended to 40 minutes and the recommended BSA blocking buffer was substituted with Roche Western Blocking buffer (Roche 1921673, Roche, Nutley, NJ) as previously described (13).

3.4. Results

3.4.1. Identification of transposon insertion sites in PA14 mutants from the mariner transposon library.

PA14 Mariner transposon mutants, a gift from Dr. George A. O'Toole, were characterized as described in the methods. Transposon insertion was confirmed by a 2 step PCR reaction and insertion of the transposon was confirmed in all but 3 of the genes selected for this study (Table 3.1.). The transposon was not identified in 3 of the mutants and this was confirmed by amplifying and sequencing the entire ORF. The 3 mutants which did not appear to contain the transposon in the correct gene were not used for the remainder of the study.

Table 3.1. Mariner transposon mutants used for this study.

Gene	Product	ORF	Tn
aprA	Alkaline Mettaloprotease	PA14_48060	N
lasA	Serine Protease LasA	PA14_40290	Y
lasB	Protease LasB	PA14_16250	Y
hplS	Type II Secretion apparatus	PA14_29500	Y
mvfR	Multiple Virulence Factor Regulator	PA14_51340	Y
rhlA	Rhamnolipid	PA14_19100	Y
phzM	Pyocyanin	PA14_09490	Y
toxA	Exotoxin A	PA14_49560	Y
pvdD	Pyoverdine	PA14_33650	N
plcB	Phospholipase C	PA14_00300	N

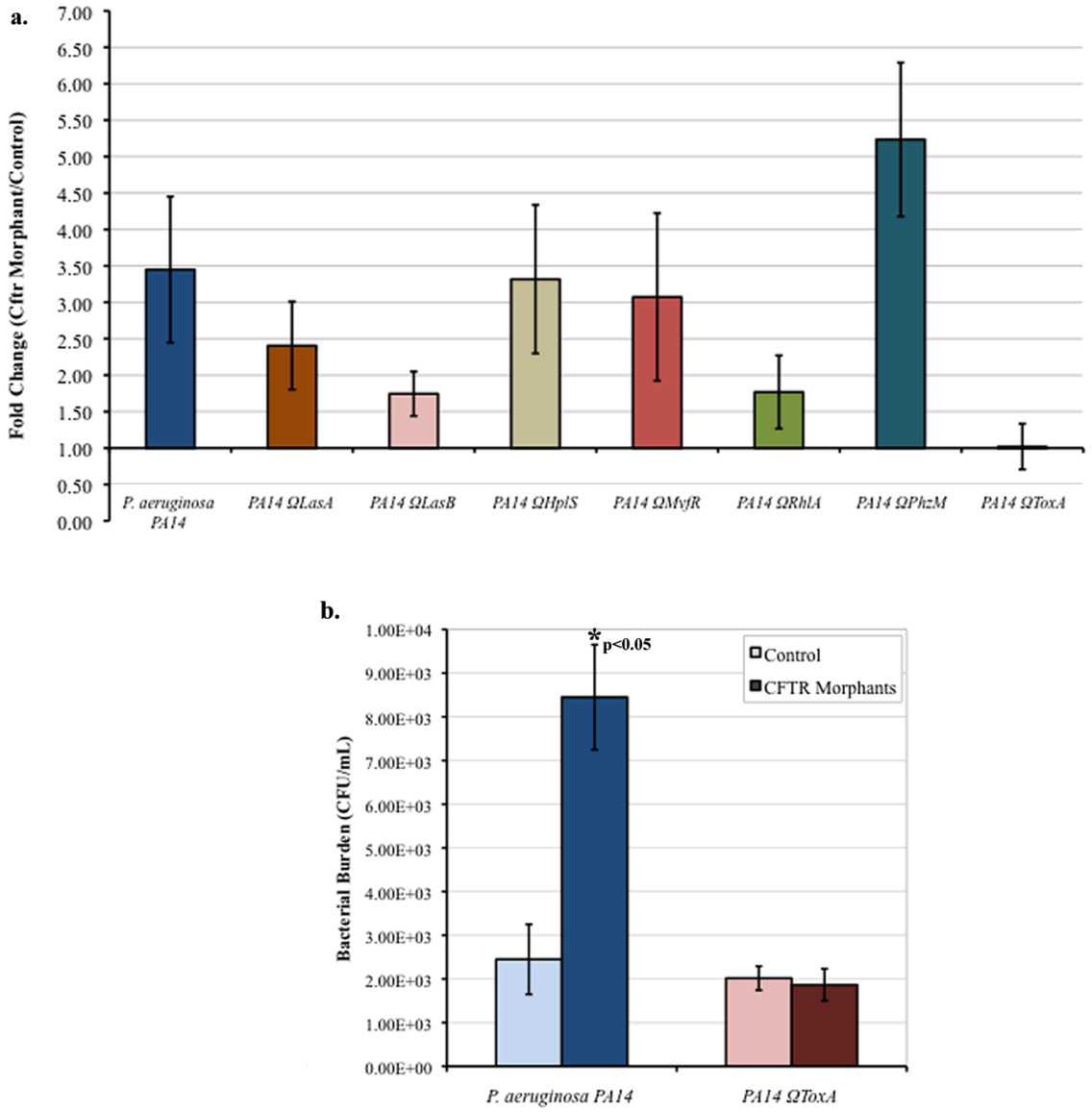

Figure 3.1. Bacterial Burden of Cftr Morphants and Controls Challenged with Mutants of *P. aeruginosa*. (a)Bacterial burden assays were performed as described using seven *P. aeruginosa* mutants. All but one showed a statistically significantly higher burden in Cftr morphant compared to control embryos.(b) Challenge with the *P. aeruginosa* Exotoxin A mutant PA14 ΩToxA resulted in similar burden levels in control and Cftr morphant embryos. Results are representative of three independent experiments. Error bars represent standard errors of the mean, statistical significance determined by Student's t-test.

3.4.2. Reduced bacterial clearance in Cftr morphants is ablated when challenged with a PA14 ToxA mutant.

Our previous study demonstrated that Cftr morphant embryos have a reduced ability to clear *P. aeruginosa* infection compared to control embryos. This clearance defect was found to be a result of factors regulated by the LasR quorum sensing system of this bacterium. Cftr morphant embryos challenged with PA14 transposon insertion mutants of *lasA, lasB, hplS, mvfR, rhlA* and *phzM* were found to have significantly higher bacterial burdens than control embryos infected with those mutants. Upon infection with PA14 ΩToxA, a mutant which cannot produce Exotoxin A, bacterial burden in the Cftr morphants was similar to that of the controls (Figure 3.1.a) and the number of bacteria recovered at 8 hpi was consistent with that of wild type PA14, approximately 2 x 10^3 CFU/ml (Figure 3.1.b).

3.4.3. Neutrophil migration in Cftr morphants is restored upon infection with a PA14 ToxA mutant.

An unexpected result from our previous study was the finding that directed chemotaxis of neutrophils to a localized infection with *P. aeruginosa* was dramatically reduced in the Cftr morphant embryos. Because the bacterial clearance defect in the Cftr morphants was restored when embryos were challenged with an ETA mutant of PA14, and ETA has previously been implicated in altering leukocyte activity and function (76, 108), we next examined neutrophil migration to the PA14 ETA mutant. At 48 hpf, 50 CFUs of PA14 ΩToxA were injected into the hindbrain ventricle (HBV), as described previously, of morphant and control Tg(*mpx*:GFP)^{i114}

transgenic embryos. Neutrophils in the HBV were enumerated at 180 minutes post infection (mpi) (Figure 3.2). The average number of neutrophils in the HBV of Cftr morphants was found to be the same as in infected controls (15 cells), and similar to the number shown previously in control fish infected with WT PA14. The reduction in migration behavior of neutrophils in response to a *P. aeruginosa* infection appears to be a result of ETA production by the bacterium.

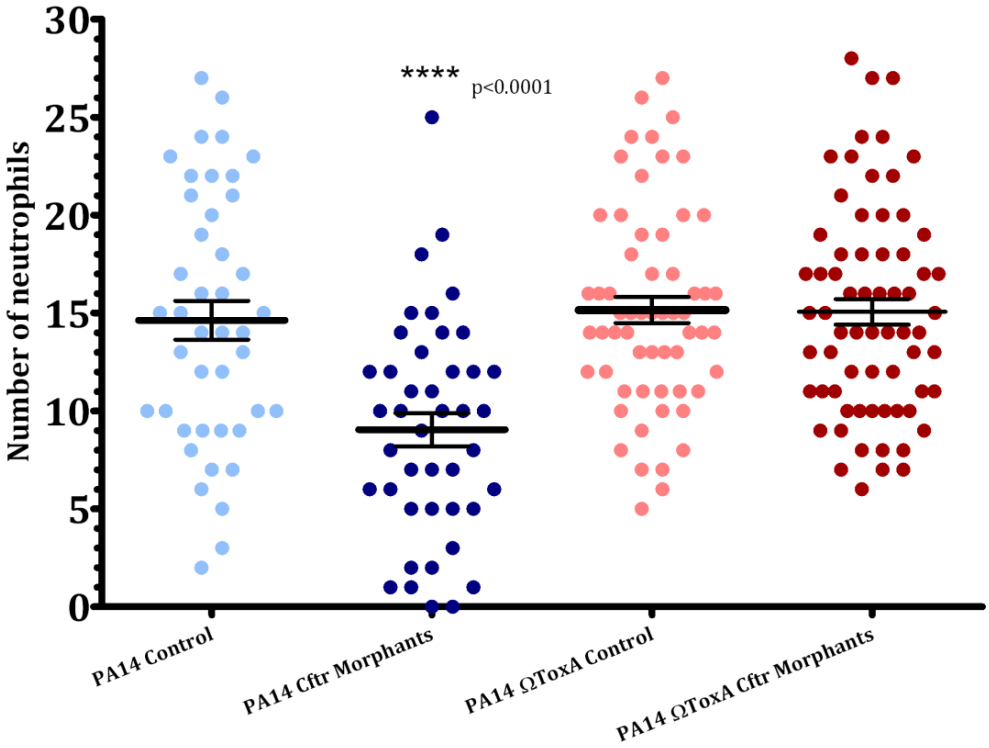

Figure 3.2. Neutrophil Migration in the Cftr Morphants is Restored When Infected with PA14 ΩToxA. Significantly fewer neutrophils were observed in the Cftr morphants than Control infected embryos when challenged with 50 CFUs of PA14(p67T1) (n=42), but no difference in neutrophil migration was observed when infected with 50 CFUs of PA14 ΩToxA (n=65). Data was collected in three independent experiments and pooled. Error bars represent standard errors of the mean, statistical significance was determined by the Student's t-test.

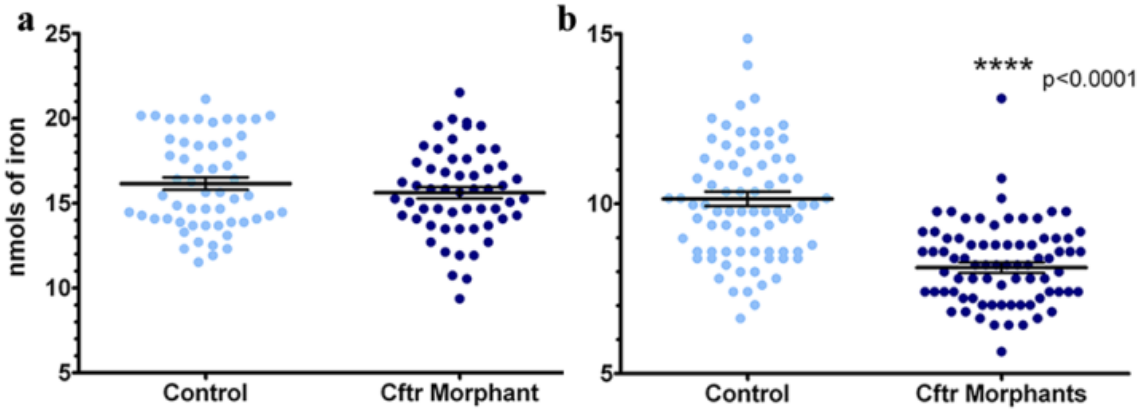

Figure 3.3. Cftr Morphant Zebrafish Embryos Display an Iron Deficiency. Total iron per embryo, represented and nano-moles per embryo, in whole (a) and de-yolked (b) zebrafish embryos at 48 hpf. No significant difference was found in whole fish measurements but significantly less iron was found in the bodies of the Cftr morphant fish after removal of the yolk. Results are shown as the pooled data from three independent experiments. Error bars represent standard errors of the mean, statistical significance was determined by the Student's t-test.

3.4.4. Iron status of Cftr morphant zebrafish embryos.

Iron deficiency is common among CF patients and is thought to be a consequence of chronic inflammation in response to respiratory tract infections and poor dietary uptake of iron resulting from poor pancreatic function. To test the iron status of the Cftr morphant zebrafish embryos, a colorimetric Ferrozine based assay was employed. The total amount of iron was found to be about 16 nmols per embryo and was similar in Cftr morphant and control embryos (Figure 3.3.a). Because the embryos are completely reliant on their yolk for nutrients at 48 hpf, and the morphant and control fish were both spawned from the same wild type parents, we next sought to measure the ability of the embryo to take up iron from its yolk. The

assay was performed on embryos that had been manually de-yolked using fine forceps and Cftr morphant embryos were found to have taken up significantly less iron from their yolk (7.9 ±0.15 nmols) than control embryos (10.26±0.24 nmols) (Figure 3.3.b).

3.4.5. ETA induced apoptosis in Cftr Morphant embryos.

ETA of *P. aeruginosa* has been found to induce apoptosis by inhibiting protein synthesis of eukaryotic cells. To test whether it was possible to visualize this effect *in vivo*, control and Cftr morphant zebrafish embryos were injected with 50 CFUs of wild type PA14 at 48 hpf as described previously. At 8 hpi, embryos were fixed with 4% paraformaldehyde and a whole mount *in situ* fluorescence based TUNEL assay was performed as described in methods. A disseminated infection with a small inoculum does not appear to result in a significant amount of measurable cell death at this early time point in either Cftr morphant or control embryos (data not shown).

3.5. Discussion

Our previous study established the zebrafish embryo as a viable model organism for the study of *in vivo* host–pathogen response in the context of cystic fibrosis (104). Using morpholino antisense oligonucleotides targeted at the zebrafish CFTR ortholog, we were able to effectively knock down the expression of zebrafish Cftr and use these Cftr morphant zebrafish to study the dynamics of early innate immune responses to acute bacterial infections. We found that Cftr morphant

embryos exhibited a dampened overall immune response characterized in part by reduced production of reactive oxygen species (ROS) and a reduction in the directed chemotaxis of zebrafish neutrophils to a *P. aeruginosa* infection. We further demonstrated that Cftr morphant embryos had a reduced ability to clear acute infections caused by *P. aeruginosa*, and not by other respiratory pathogens commonly isolated from CF patients. This reduction in bacterial clearance was determined to be a result of the LasR quorum sensing (QS) system, and the downstream virulence factors that are regulated through LasR.

Numerous virulence factors are transcriptionally regulated by LasR QS (102). It was the goal of this study to attempt to identify which of these virulence factors are particularly important early in the course of acute *P. aeruginosa* infections in our Cftr morphant zebrafish embryos. *P. aeruginosa* strain PA14 mutants from the mariner transposon mutant library (73) were selected based on previous reports of genes that are transcriptionally controlled or otherwise regulated by LasR and RhlR (102). The earliest described transcriptional regulation by LasR and RhlR, indeed the genes they were named after, are the elastases LasA and LasB and rhamnolipid, a biosurfactant essential for biofilm growth in *P. aeruginosa*(96, 144). Infection of Cftr morphant zebrafish embryos with mutants of either LasA, LasB or RhlA resulted in a significantly higher bacterial burden after 8 h of infection than what was observed in control embryos. The difference in bacterial burden in Cftr morphants compared to controls was significantly smaller than what was previously observed with wild type PA14, indicating that virulence in this model is impacted when

expression of these genes is lost, but that these genes are not solely responsible for the reduced bacterial clearance. LasR QS system also controls the expression of components of the Type II general secretion pathway of *P. aeruginosa*. To test for the involvement of this pathway, we selected an *hplS* mutant. Infection with a PA14 HplS mutant resulted in the same reduction in bacterial clearance observed with wild type PA14. The redox active blue-green pigment pyocyanin that is produced during *P. aeruginosa* growth and is indicative of *P. aeruginosa* infections in most clinical settings was tested for its role in virulence in our Cftr morphant zebrafish. Although pyocyanin is regulated by LasR, it is also regulated through another QS system, the Multiple Virulence Factor Regulator (*mvfR*), which controls the expression of pyocyanin, hydrogen cyanide and others (29). Infection with PA14 mutants of either *phzM*, an enzyme essential for the production of pyocyanin, or with a mutant of *mvfR*, QS controlled regulator of pyocyanin production, yielded the same reduced bacterial clearance observed during infections with wild type PA14, indicating that pyocyanin and other genes under control of MvfR are not responsible for the bacterial clearance defect in the Cftr morphants.

Among the PA14 mutants examined in this study, only infection with a PA14 ToxA mutant, a mutant that cannot express Exotoxin A (ETA), resulted in bacterial clearance by Cftr mophant embryos that was equal to that of controls. While ETA expression is not under the control of LasR per se, LasR QS has been shown to enhance the expression of this toxin (44). The iron-starvation sigma factor PvdS is responsible for the regulation of ETA and pyoverdine in *P. aeruginosa*, and the

expression of these genes is dramatically up-regulated during growth in iron-limited environments. ETA is a powerful and lethal toxin and has been recovered from sputum of CF patients chronically infected with *P. aeruginosa* (59). The expression of ETA was also shown to strongly correlate with expression levels of LasR in the lungs these patients (138). By ADP-ribosylating elongation factor 2 and inhibiting protein synthesis, ETA effectively induces the death of any eukaryotic cells that encounter this toxin (56).

ETA has been shown in both mouse and dog models to dramatically reduce the number of circulating leukocytes following ip or iv injection (76) and cytotoxicity has been demonstrated using isolated human macrophages (108). Pollack and Anderson also noted a significant reduction in the phagocytic ability of these isolated human macrophages following a sub-lethal dose of ETA (108). To determine if ETA may be the cause of the reduced neutrophil migration to a *P. aeruginosa* infection in the hindbrain ventricle (HBV) of Cftr morphant zebrafish, we repeated the assay with the PA14 ToxA mutant. Neutrophil migration to infection with the ETA mutant in Cftr morphant embryos was restored to levels similar to control embryos and the numbers of neutrophils were consistent with previous data using wild type PA14 in control embryos. This indicates that ToxA may in fact be responsible for the reduced ability of Cftr morphant embryos to clear an acute infection with *P. aeruginosa*. The finding that bacterial clearance and directed neutrophil chemotaxis were restored in our Cftr morphant embryos when infected with a ToxA mutant was unanticipated because previous studies by Woods, *et al.*

(158), and Blackwood, *et al.* (4), demonstrated that in rats and guinea pigs, ETA played a major role in virulence during chronic, but not acute lung infections. This data is not in contradiction to these previous reports, in fact we saw no significant difference in the infection of control embryos infected with wild type PA14 or PA14ΩToxA. This may be the first report that implicates ETA as a major virulence factor in acute infections by *P. aeruginosa* in the context of CF. While the expression of this toxin has been confirmed in CF clinical isolates of *P. aeruginosa* and the toxin has been directly detected in sputum of chronically infected patients, we were unable to find any reports of the importance of ETA in the initial stages of *P. aeruginosa* infection of the CF lung.

ETA is not the only toxin produced by *P. aeruginosa*. Indeed, ETA is not even the only ADP-ribosylase that is expressed by *P. aeruginosa* (141). Exoenzyme S has also been described as an ADP-ribosylase and pathogenicity during acute infection of a neonatal mouse lung was not reduced by a *P. aeruginosa* mutant defective in Exo-S expression (141). Despite the obvious effects ETA has on neutrophil migration in response to an infection, and the reduced bacterial clearance that seems to be a result of this defect, we sought to identify a possible link between ETA and CF in humans that has not yet been described. It is well known that ETA expression is highly up-regulated during the iron-starvation response of the bacterium (43). The iron status of CF patients has been a matter of great debate over the past decade. A multitude of clinical reports exist showing that anywhere from 30–70% of CF patients are iron deficient (ID) and a large proportion of these

patients suffer from anemia linked to this ID (1, 34, 41, 64, 109, 121, 153). Recent studies, however, have identified extremely high levels of iron in the sputum of CF patients compared to iron levels in healthy people (118, 120, 136, 137). There is a great deal of controversy over whether these findings are indicative of complications of defective expression of CFTR or whether they are a product of chronic persistent bacterial infections and the resultant hyper-inflammation. Recent studies have revealed that the increased iron in the sputum is caused by higher than normal iron secretion by CF epithelial cells, prompting them to begin testing iron chelation therapy to attempt to remove the chronic *P. aeruginosa* infections in these patients (46, 88, 89). Interestingly, the same group has recently reported that the iron in CF sputum strongly correlated with their clinical condition and infection status, and they failed to correlate sputum iron concentrations with serum iron levels, indicating that the iron loss through airway inflammation is not as significant as once believed (46, 121).

Our findings regarding ETA and the clinical data available regarding iron deficiency of CF patients prompted us to investigate if the iron status of our Cftr morphant zebrafish was measurable and if it was altered compared to the control embryos. Because the zebrafish embryo is reliant on its yolk for nutrients at 48 hpf, and the morphant embryos were spawned from the same wild type parents as the control embryos, we found that the total amount of iron in the whole embryo was about 16 nmols per fish. Upon removal of the remainder of the yolk at 48 hpf, we found that the Cftr morphant embryos had taken up significantly less iron from their

yolk than did control embryos. This reduction in the uptake of iron closely replicates the ID that is observed in humans with CF and may explain why ETA plays such a pivotal role in inhibiting bacterial clearance in the morphant embryos.

While high levels of iron present in the CF lung after the establishment of chronic *P. aeruginosa* infection permit the enhancement of biofilm growth and a reduction in the effectiveness of antibiotic therapies and the host immune response, it is plausible that the ID state of the CF patient prior to the infection may induce the up-regulation of ETA and other iron regulated virulence factors that have not previously been implicated in acute infection, enabling the bacterium to establish a foothold in the lung and a transition into a chronic infection through other mechanisms.

CHAPTER 4

DISCUSSION AND FUTURE DIRECTIONS

Researchers have been struggling for decades to elucidate the molecular mechanisms that make cystic fibrosis (CF) patients susceptible to infection by the opportunistic pathogen *P. aeruginosa*, a pathogen that rarely causes infection in healthy individuals (80). Many advances have been made in the characterization of the unique pathophysiology of the CF condition in humans, but these studies have fallen short of determining the specifics of the relationship between this environmental microbe and the chloride channel CFTR in the lungs of CF patients. Researchers have been at a disadvantage in these studies because of a lack of a suitable animal model. Much has been learned in recent years about the growth and adaptation of this bacterium to the conditions in the CF lung through the use of cell culture models, using both primary and immortalized epithelial cell lines, or through studies in murine models. Although CF researchers have developed nearly a dozen CF mouse models with varying mutations in the CFTR gene, researchers must select the appropriate mouse for their work because none of these models have successfully replicated the wide array of symptoms that are caused by defective CFTR in humans. At the most basic level, the mouse models fail to be useful in infection studies because CFTR knockout mice fail to spontaneously develop *P. aeruginosa* infections, as humans do, and it is especially difficult to artificially establish a *P. aeruginosa* infection in these models, particularly acute infections,

because mice have mechanisms in place that protect them from these challenges that are found in human airways.

We have proposed the use of the zebrafish embryo as a model for studying the innate immune response to acute infections with *P. aeruginosa*. A criticism we commonly receive when presenting our work is that the damage that is caused by *P. aeruginosa* in the CF lung is a result of chronic persistent infections, not acute infections. While we do not disagree with researchers attempting to develop strategies to combat chronic *P. aeruginosa* infections that are particularly difficult to treat, we argue that every chronic bacterial infection that has been established begins first as an acute infection, and that it is the responsibility of the innate immune system to combat these acute infections and rapidly eradicate them before the condition worsens. Although our model suffers the disadvantage of not having a structure homologous to the human airway, we have been able to identify many important factors contributing to susceptibility to infection that are impacted by the loss of Cftr expression.

In chapter 2, we discuss the finding that loss of Cftr perturbs the innate immune response in the zebrafish embryo, characterized in part by the reduced ability of the Cftr morphant zebrafish to produce reactive oxygen species, an essential element of bacterial killing by neutrophils. We also identified a reduced neutrophil migration response in the neutrophils of Cftr morphant fish to infection by *P. aeruginosa*. Taken together, these data indicate that loss of Cftr may make the zebrafish embryos more susceptible to bacterial infection. The pivotal finding of this

initial report was that Cftr morphant embryos had a reduced ability to clear infection by *P. aeruginosa*. We further addressed the notion of susceptibility to bacterial infection in general by challenging the Cftr morphant embryos with other bacterial species commonly implicated in respiratory infections in CF. We found that the bacterial clearance defect of the Cftr morphants was unique to *P. aeruginosa* and subsequently identified the LasR quorum sensing (QS) system of *P. aeruginosa*, and the virulence factors that are expressed through it, as being responsible for this reduced ability to clear the infection.

In chapter 3, we attempted to examine each of the virulence factors that are regulated by LasR in *P. aeruginosa* in order to identify which of these systems contribute to pathogenesis in the zebrafish embryos. We performed infection studies using several *P. aeruginosa* mutants from the mariner transposon library developed by Liberati, *et al.* (73) and compared bacterial burdens in the Cftr morphant and control zebrafish. We found that infection with four of the *P. aeruginosa* mutants studied resulted in a reduction in the bacterial clearance defect observed in the Cftr morphant embryos, and infection by one of these mutants resulted in bacterial burdens that were identical in the Cftr morphant and control embryos. When challenged with a *P. aeruginosa* mutant that cannot produce Exotoxin A (ETA), a potent and lethal toxin secreted by the bacterium during infection and as a result of the iron starvation response, Cftr morphant zebrafish embryos were able to clear the infection as efficiently as the control embryos were,

implicating ETA as a crucial component in the susceptibility of CF patients to infection by *P. aeruginosa*.

Although the mechanism of toxicity of ETA has been well described, and the toxin is routinely identified in the sputum of CF patients infected with *P. aeruginosa,* the contributions of this toxin to the initial stages of infection and colonization of the CF lung by *P. aeruginosa* have not been previously identified. In fact, a previous study had ruled out the significance of ETA during acute respiratory tract infections in a guinea pig model, seemingly in contradiction to our hypothesis. Indeed, while ETA is not important during acute infections of normal animals, reports of infection studies with ETA in a CF model do not exist. Thus, we are the first to report that ETA is a critical component in suppressing the immune response of the host during an acute infection using an animal model of CF. When challenged with the ETA mutant of *P. aeruginosa*, we found that the ability of Cftr morphant embryos to clear the infection was restored to the level of the control embryos, and that the neutrophil migration response in the morphants was as robust in the morphants as in the controls.

The ablation of the neutrophil migration defect in the Cftr morphants may in fact result in the restoration of bacterial clearance, indicating that the slowed response of Cftr morphant neutrophils to *P. aeruginosa* may actually be a result of exposure to the bacterial toxin. This effect has been previously described in mouse and dog models, where direct injection of the purified toxin resulted in a reduction in the number of circulating leukocytes and toxic shock in these animals (76). ETA

has also been shown to induce apoptosis in human macrophages and directly inhibit phagocytosis by these cells (108). Previous work has demonstrated that ETA has little effect on phagocytosis and bacterial killing by neutrophils in rabbits (100), but no studies could be found examining inhibition of neutrophil migration by ETA. Our studies of the responses of zebrafish phagocytes have been limited to neutrophils because a true macrophage specific transgenic line has not been available. Recently, a transgenic line of zebrafish, which express a fluorescent protein driven by the macrophage specific mpeg1 promoter, has been described (36). Because ETA has a more dramatic effect on normal macrophages from humans and other animals, future studies of migration and phagocytosis responses may prove to be another critical component of bacterial clearance that is altered in our zebrafish CF model.

The inhibition of neutrophil and possibly macrophage recruitment and killing in the Cftr morphant zebrafish by *P. aeruginosa* ETA identifies a potential mechanism by which the bacterium evades the immune responses of the host in order to establish an infection in the CF lung. The next question we wanted to address was why ETA was significant during these infections. ETA is only one of many virulence factors expressed by *P. aeruginosa* during infections, and indeed, only one of the toxins with ADP-ribosylase activity. Exoenzyme S (ExoS) has been shown to act in the same manner and target the same eukaryotic cell processes as ETA. One of the major findings from our initial report described in chapter 2 was that the Type III Secretion System, the only mechanism by which ExoS is delivered to eykaryotic cells, is not responsible for the reduction in bacterial clearance. The

action of inducing apoptosis and inhibiting phagocytosis and bacterial killing, therefore, is not simply specific to ETA, but could be an effect of enhanced expression of this toxin by the bacteria during infection in the Cftr morphant embryos.

We next focused on the regulatory networks that control ETA, since the expression level of the exoenzyme seemed to be a plausible explanation for the importance of the toxin during acute infection in our Cftr morphants, but the dispensable nature of the toxin during acute infection has been described in other models. Our initial report identified genes regulated through the LasR pathway, including ETA, as targets for future study. While the expression of ETA is highly up-regulated when the LasR QS system is activated, ETA is primarily regulated through the iron-starvation sigma factor PvdS. When the bacterium is growing in environments where iron is limited, including inside hosts during infection, PvdS activates the expression of ETA and the siderophore pyoverdine (PVD). ETA is released into the environment to kill any eukaryotic cells that are nearby, releasing the iron they have stored. PVD expression increases and the siderophore is subsequently released to chelate the iron that was released by the dying cells and return it to the bacterium. This is a highly effective and elegant mechanism of attaining iron in environments where it is very limited. The system is also highly effective in acquiring iron, among other vital nutrients during pathogenesis in plant and animal hosts.

The fact that ETA is primarily regulated by iron availability is, in itself, a significant component of the specificity of these infections. Iron deficiency (ID), and often an ID associated anemia is a common symptom of CF, observed in about 30-70% of CF patients depending on their age and clinical condition (1, 34, 41, 64, 109, 121, 153). We next wanted to determine whether our CF zebrafish model replicated the ID status of humans with CF. Adapting a cull culture based colorometric iron assay, we found that our Cftr morphant zebrafish embryos had significantly less iron than control embryos did. The difference in total iron we found was small, but it is an accurate measure of the total iron the fish were able to absorb from their yolk. Future studies are needed to elucidate the exact mechanisms by which our Cftr morphant zebrafish are ID. A great deal of controversy exists in clinical reports of ID in CF patients. The cause of the ID is generally attributed to (i) a reduced ability of the body to take up iron and other key nutrients because of deteriorating pancreatic function, (ii) a loss of iron through bleeding into the intestines, a common symptom in CF, or (iii) loss of iron through chronic persistent infections of the respiratory tract, and the subsequent inflammation in response (46). It may be possible to use the CF zebrafish model we have established to determine which of these possibilities are the most significant. Studies could be performed after infection to measure the contribution of the inflammation response to iron loss in the zebrafish. Iron measurements could be performed on older fish, after the yolk is completely absorbed, but before the fish begins actively feeding. These experiments may shed light on any loss of iron that occurs simply as an effect of the symptoms of CF.

A common technique use to measure DNA fragmentation resulting from apoptotic signaling cascades is the dUTP nick end labeling (TUNEL) assay. The TUNEL assay employs a terminal deoxynucleotidyl transferase that can incorporate fluorescently labeled nucleotides onto fragmented DNA, indicating apoptosis or necrosis. Attempts to utilize a whole mount TUNEL assay to measure ETA induced apoptosis and necrosis were unsuccessful. This was probably a result of the relatively low inoculum size and because the disseminated infection that resulted from directly introducing the bacteria into the bloodstream may cause some cytotoxicity spread throughout the fish, which would be very difficult to measure using this assay. It may be possible to establish a discrete infection, similar to those infections used for the neutrophil migration assays, and measure cytotoxicity in those discrete areas of the fish. If these assays are successful, they are a good indication that our hypothesis is correct. They are not by any means confirmative, however, because as discussed in chapter 1, there are a myriad of virulence factors and toxins produced during infection that could account for this necrosis and apoptosis.

A more confirmative and indeed more quantitative approach would be to measure the expression levels of ETA and other iron regulated virulence factors during infection in the zebrafish by quantitative RT-PCR. Total RNA could be extracted from infected Cftr morphant and control embryos and the relative copy number for the iron regulated *P. aeruginosa* genes could be measured. Other techniques, including whole mount fluorescence-based assays using a probe or

antibody specific for ETA could also potentially be used to measure the expression of the toxin.

One of the major disadvantages of our model is that the knockdown of Cftr using morpholinos (MOs) is only transient and the effects of the MOs are diluted out after the first week of the embryos life. Several methods for developing knockout mutants in zebrafish have become popular, ENU mutagenesis and tilling for mutations has been used but the process is time consuming and technically difficult. A new technique that has come into favor recently is the use of sequence specific zinc finger nucelases (ZFNs) to target and mutate genes in the zebrafish. A novel "open-source" method of engineering ZFNs to target specific genome locations has been developed and the new ZFNs have been successfully used to modify the zebrafish genome (42, 82, 83). With the support of an NIH grant, the Zinc Finger Consortium has recently generated customized ZFNs directed against the zebrafish *cftr* gene. These ZFNs can now be used to induce mutations in the gene of zebrafish and could ultimately enable the creation of a Cftr -/- knockout mutant line. Such a mutant could be extremely useful for studying the dynamics of long-term infections and repeated exposure experiments. Another potential avenue for future studies would be sequential, or co-infections with *P. aeruginosa* and other CF pathogens. Speculation has recently been raised about the advantages of treating childhood CF infections. Some retrospective clinical studies have identified that children treated with anti-staphylococcal antibiotics were more likely to develop infections with *P. aeruginosa* earlier than children not treated with the drugs. Because multiple

infections would be challenging during the short timeframe we can work with the fish now, having a knockout line at our disposal would be particularly useful.

When taken together, our data establish the value of our new CF model and provide new insights into the pathogenesis of *P. aeruginosa* infection in humans with CF. The advantages of this model organism make these infection studies technically easy and relatively inexpensive to perform. Because the zebrafish does not have a respiratory tract that is homologous to mammals, we cannot perform the traditional lung infection experiments. This may not necessarily be a disadvantage here though, as we can investigate the molecular mechanisms of susceptibility to bacterial infections in CF without the added complications of particular anatomical structures. Our findings highlight the advantages of the zebrafish embryo for infection studies and provide exciting avenues for future research.

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APPENDICES

Appendix A

Raw Bacterial Burden Data

Table A.1. Raw plate count data for embryos infected with WT PA14

	Control Morphant			Cftr Morphant		
	Rep 1	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**
Plate 1	11	26	19	84	61	100
Plate 2	22	39	30	61	132	69
Avg. Colonies	16.5	32.5	24.5	72.5	96.5	84.5
Avg. CFU/mL	1.65E+03	3.25E+03	2.45E+03	7.25E+03	9.65E+03	8.45E+03
AVERAGE			2.45E+03			8.45E+03
STD DEV			8.00E+02			1.20E+03
SEM			5.66E+02			8.49E+02

Table A.2. Raw plate count data for embryos infected with WT PA14

	Control Morphant			Rescued Cftr Morphant		
	Rep 1	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**
Plate 1	25	15	23	21	23	26
Plate 2	19	45	33	47	83	26
Avg. Colonies	22	30	28	34	53	26
Avg. CFU/mL	2.20E+03	3.00E+03	2.80E+03	3.40E+03	5.30E+03	2.60E+03
AVERAGE			2.80E+03			3.43E+03
STD DEV			4.32E+02			1.32E+03
SEM			2.16E+02			6.61E+02

Table A.3. Raw plate count data for embryos infected with SMC573

	Control Morphant			Cftr Morphant		
	Rep 1	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**
Plate 1	32	88	35	120	145	125
Plate 2	21	42	5	72	96	125
Avg. Colonies	26.5	65	20	96	120.5	125
Avg. CFU/mL	2.65E+03	6.50E+03	2.00E+03	9.60E+03	1.21E+04	1.25E+04
AVERAGE			3.05E+03			1.09E+04
STD DEV			2.39E+03			1.58E+03
SEM			1.20E+03			7.92E+02

Table A.4. Raw plate count data for embryos infected with *E. tarda*

	Control Morphant			Cftr Morphant		
	Rep 1	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**
Plate 1	26	32	41	5	24	10
Plate 2	19	41	32	16	51	14
Avg. Colonies	22.5	36.5	36.5	10.5	37.5	12
Avg. CFU/mL	2.25E+04	3.65E+04	3.65E+04	1.05E+04	3.75E+04	1.20E+04
AVERAGE			3.18E+04			2.00E+04
STD DEV			8.08E+03			1.52E+04
SEM			4.67E+03			8.76E+03

Table A.5. Raw plate count data for embryos infected with *E. coli* XL-10

	Control Morphant			Cftr Morphant		
	Rep 1	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**
Plate 1	21	12	19	28	19	14
Plate 2	24	12	11	14	19	7
Avg. Colonies	22.5	12	15	21	19	10.5
Avg. CFU/mL	2.25E+03	1.20E+03	1.50E+03	2.10E+03	1.90E+03	1.05E+03
AVERAGE			1.26E+03			1.06E+03
STD DEV			5.41E+02			5.58E+02
SEM			3.12E+02			3.22E+02

Table A.6. Raw plate count data for embryos infected with *B. cenocepacia*

	Control Morphant			Cftr Morphant		
	Rep 1	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**
Plate 1	6	41	24	28	80	52
Plate 2	37	106	16	16	65	37
Avg. Colonies	21.5	73.5	20	22	72.5	44.5
Avg. CFU/mL	2.15E+04	7.35E+04	2.00E+04	2.20E+04	7.25E+04	4.45E+04
AVERAGE			31625			46333.3
STD DEV			28261.8			25299.9
SEM			16317			14606.9

Table A.7. Raw plate count data for embryos infected with *S. aureus*

	Control Morphant			Cftr Morphant		
	Rep 1	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**
Plate 1	28	24	60	31	40	29
Plate 2	32	45	67	34	57	29
Avg. Colonies	30	34.5	63.5	32.5	48.5	29
Avg. CFU/mL	3.00E+04	3.45E+04	6.35E+04	3.25E+04	4.85E+04	2.90E+04
AVERAGE			4.13E+04			3.96E+04
STD DEV			1.51E+04			1.03E+04
SEM			8.73E+03			5.97E+03

Table A.8. Raw plate count data for embryos infected with *H. influenzae*

	Control Morphant			Cftr Morphant		
	Rep 1	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**
Plate 1	138	120	300	101	157	280
Plate 2	139	203	115	120	305	284
Avg. Colonies	138.5	161.5	207.5	110.5	231	282
Avg. CFU/mL	1.39E+06	1.62E+06	2.08E+06	1.11E+06	2.31E+06	2.82E+06
AVERAGE			1.85E+06			2.08E+06
STD DEV			4.25E+05			8.81E+05
SEM			2.46E+05			5.08E+05

Table A.9. Raw plate count data for embryos infected with PA14 ΔT3SS

	Control Morphant			Cftr Morphant		
	Rep 1	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**
Plate 1	8	30	22	70	47	73
Plate 2	23	37	23	59	43	78
Avg. Colonies	15.5	33.5	22.5	64.5	45	75.5
Avg. CFU/mL	1.55E+03	3.35E+03	2.25E+03	6.45E+03	4.50E+03	7.55E+03
AVERAGE			2.38E+03			6.17E+03
STD DEV			9.07E+02			1.54E+03
SEM			5.24E+02			8.92E+02

Table A.10. Raw plate count data for embryos infected with PA14 ΔLasR

	Control Morphant			Cftr Morphant		
	Rep 1	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**
Plate 1	26	6	7	17	15	33
Plate 2	33	16	28	16	24	17
Avg. Colonies	29.5	11	17.5	16.5	19.5	25
Avg. CFU/mL	2.95E+03	1.10E+03	1.75E+03	1.65E+03	1.95E+03	2.50E+03
AVERAGE			2.03E+03			1.93E+03
STD DEV			4.31E+02			9.39E+02
SEM			2.49E+02			5.42E+02

Table A.11. Raw plate count data for embryos infected with PA14 ΩLasA

	Control Morphant			Cftr Morphant		
	Rep 1	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**
Plate 1	62	93	191	271	300	347
Plate 2	63	95	216	315	320	292
Avg. Colonies	62.5	94	203.5	293	310	319.5
Avg. CFU/mL	6.25E+03	9.40E+03	2.04E+04	2.93E+04	3.10E+04	3.20E+04
AVERAGE			1.20E+04			3.08E+04
STD DEV			7.40E+03			1.34E+03
SEM			4.27E+03			7.75E+02

Table A.12. Raw plate count data for embryos infected with PA14 ΩLasB

	Control Morphant			Cftr Morphant		
	Rep 1	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**
Plate 1	28	16	12	60	30	31
Plate 2	36	33	20	40	42	62
Avg. Colonies	32	24.5	16	50	36	46.5
Avg. CFU/mL	3.20E+04	2.45E+04	1.60E+04	5.00E+04	3.60E+04	4.65E+04
AVERAGE			2.42E+04			4.42E+04
STD DEV			8.01E+03			7.29E+03
SEM			4.62E+03			4.21E+03

Table A.13. Raw plate count data for embryos infected with PA14 ΩHplS

	Control Morphant			Cftr Morphant		
	Rep 1	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**
Plate 1	18	70	41	163	107	134
Plate 2	24	55	41	169	150	103
Avg. Colonies	21	62.5	41	166	128.5	118.5
Avg. CFU/mL	2.10E+03	6.25E+03	4.10E+03	1.66E+04	1.29E+04	1.19E+04
AVERAGE			4.15E+03			1.38E+04
STD DEV			2.08E+03			2.50E+03
SEM			1.20E+03			1.45E+03

Table A.14. Raw plate count data for embryos infected with PA14 ΩMvfR

	Control Morphant			Cftr Morphant		
	Rep 1	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**
Plate 1	45	117	75	23	41	14
Plate 2	50	130	58	20	30	18
Avg. Colonies	47.5	123.5	66.5	21.5	35.5	16
Avg. CFU/mL	4.75E+03	1.24E+04	6.65E+03	2.15E+04	3.55E+04	1.60E+04
AVERAGE			7.92E+03			2.43E+04
STD DEV			3.96E+03			1.01E+04
SEM			2.28E+03			5.80E+03

Table A.15. Raw plate count data for embryos infected with PA14 ΩRhlA

	Control Morphant			Cftr Morphant		
	Rep 1	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**
Plate 1	71	52	97	152	115	220
Plate 2	139	52	154	180	125	207
Avg. Colonies	105	52	125.5	166	120	213.5
Avg. CFU/mL	1.05E+04	5.20E+03	1.26E+04	1.66E+04	1.20E+04	2.14E+04
AVERAGE			9.42E+03			1.67E+04
STD DEV			3.79E+03			4.68E+03
SEM			2.19E+03			2.70E+03

Table A.16. Raw plate count data for embryos infected with PA14 ΩPhzM

	Control Morphant			Cftr Morphant		
	Rep 1	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**
Plate 1	6	2	13	55	47	83
Plate 2	13	19	15	43	54	74
Avg. Colonies	9.5	10.5	14	49	50.5	78.5
Avg. CFU/mL	9.50E+02	1.05E+03	1.40E+03	4.90E+03	5.05E+03	7.85E+03
AVERAGE			1.13E+03			5.93E+03
STD DEV			2.36E+02			1.66E+03
SEM			1.36E+02			9.59E+02

Table A.17. Raw plate count data for embryos infected with PA14 ΩToxA

	Control Morphant			Cftr Morphant		
	Rep 1	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**
Plate 1	13	24	21	18	25	11
Plate 2	15	25	23	21	26	11
Avg. Colonies	14	24.5	22	19.5	25.5	11
Avg. CFU/mL	1.40E+03	2.45E+03	2.20E+03	1.95E+03	2.55E+03	1.10E+03
AVERAGE			2.02E+03			1.87E+03
STD DEV			5.48E+02			7.29E+02
SEM			2.74E+02			3.64E+02

Appendix B

Raw Respiratory Burst Assay Data

Table B.1. Raw relative fluorescent units from the 95th read

	48 hpf embryos + H_2DCFDA					
	1	2	3	4	5	6
Control	11037	15994	5698	8119	18729	1035
	1138	9171	5131	5320	1544	11183
Cftr Morphant	11533	1335	13959	3727	802	2212
	5394	4477	4167	2211	1432	449
	56 hpf embryos + H_2DCFDA					
Control	5502	4219	1972	2702	2546	1283
	3493	8027	4109	2727	2506	1556
Cftr Morphant	3520	1705	7413	4065	3157	1833
	18692	3751	5737	2001	3455	858
	48 hpf embryos + H_2DCFDA + PMA					
	7	8	9	10	11	12
Control	19428	13243	4928	5383	8069	6086
	10247	2918	4364	1261	5306	8910
Cftr Morphant	6697	2691	8391	3801	6933	13805
	15851	4875	3912	1035	3173	4240
	56 hpf embryos + H_2DCFDA + PMA					
Control	17425	18535	23882	27220	17463	14396
	29653	25006	27270	24688	20723	15946
Cftr Morphant	17537	20152	12792	17926	17328	22061
	20639	10220	10274	27148	8386	18477

Appendix C

Raw Neutrophil Migration Data

Table C.1. Raw Neutrophil migration numbers per embryo at 3 hpi

WT PA14						E. coli XL-10 Control					
Control Morphant			Cftr Morphant			Control Morphant			Cftr Morphant		
Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
16	9	17	1	12	11	6	5	2	5	11	4
8	10	9	0	16	8	3	18	3	3	7	4
2	20	10	19	8	7	6	3	3	7	3	3
15	18	22	12	6	10	2	6	2	4	2	2
10	19	15	14	14	15	4	7	10	7	6	4
14	23	37	6	10	11	5	4	2	2	3	1
26	14	12	13	18	6	5	8	3	5	5	5
16	24	22	12	5	9	4	5	2		4	1
7	21	22	10	12	7	5	8	3		1	7
12	6	24	3	10	20	8	4	9		13	5
9	27	7	15	5	7			7		19	7
9	23	15	0	25	8			8			8
13	10		10	5				9			4
13	21		14	12				2			4
17			2	25				3			3
5			7	15				2			3
3			2	5				1			1
14			1	1				1			2
											1

Table C.1. Continued

PBS Injected Control						PA14ΩToxA					
Control Morphant			Cftr Morphant			Control Morphant			Cftr Morphant		
Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
1	0	0	0	1	0	16	10	6	11	11	12
0	1	0	0	0	0	8	18	12	20	13	22
1	1	0	1	0	2	11	13	5	24	15	17
1	2	1	2	0	0	20	16	26	14	15	32
0	2	0	0	0	0	14	10	7	23	16	20
0	0	0	0	2	0	14	8	20	14	10	23
0	0	0	1	0	0	16	13	10	28	18	7
0	0	0	0	0	1	12	15	15	18	10	13
0	0	0	0	0	0	15	17	9	19	13	8
1	1	1	2	1		15	20	24	19	13	22
0	0	0	0	0		15	38	16	13	13	16
0			0			14	19	25	10	11	15
						31	15	11	18	9	17
						19	23	23	11	16	8
						24	15	27	7	23	9
						14	23	11	13	20	21
						22	11	13	14	7	10
						17	11	16	8	14	14
						20	14	14	18	17	24
						13		32	10	11	12
								7	16	27	6
								16	14	16	10
								12		9	17

Appendix D

Raw Ferrozine Assay Data

Table D.1. Raw ferrozine assay A_{550} absorbance data (for each embryo)

48 hpf Whole Zebrafish Embryos						48 hpf De-Yolked Zebrafish Embryos					
Control Morphant			Cftr Morphant			Control Morphant			Cftr Morphant		
Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.184	0.169	0.172	0.182	0.180	0.186	0.141	0.152	0.172	0.142	0.139	0.173
0.168	0.169	0.162	0.207	0.184	0.190	0.135	0.147	0.172	0.146	0.140	0.190
0.200	0.169	0.165	0.185	0.187	0.172	0.146	0.198	0.189	0.146	0.139	0.152
0.184	0.177	0.168	0.190	0.162	0.169	0.151	0.158	0.190	0.146	0.133	0.128
0.200	0.17	0.222	0.178	0.184	0.175	0.144	0.152	0.174	0.139	0.141	0.124
0.359	0.198	0.172	0.191	0.159	0.109	0.149	0.139	0.153	0.152	0.142	0.135
0.192	0.188	0.173	0.172	0.181	0.158	0.145	0.138	0.150	0.147	0.140	0.166
0.158	0.199	0.160	0.197	0.172	0.179	0.141	0.148	0.161	0.141	0.142	0.127
0.194	0.199	0.181	0.177	0.197	0.145	0.152	0.147	0.172	0.149	0.133	0.146
0.199	0.188	0.168	0.178	0.199	0.174	0.142	0.149	0.173	0.139	0.135	0.130
0.171	0.2	0.176	0.190	0.171	0.162	0.141	0.137	0.160	0.143	0.137	0.132
0.177	0.191	0.175	0.151	0.167	0.172	0.146	0.145	0.157	0.142	0.146	0.149
0.164		0.199	0.125		0.198	0.179	0.141	0.191	0.144	0.135	0.185
0.167		0.181	0.197		0.170	0.161	0.148	0.238	0.102	0.140	0.145
0.199		0.176	0.166		0.178	0.143	0.140	0.181	0.150	0.130	0.130
0.180		0.167	0.193		0.182	0.133	0.132	0.227	0.155	0.139	0.125
0.193		0.161	0.172		0.166	0.146	0.140	0.146	0.144	0.134	0.162
0.185		0.156	0.187		0.175	0.150	0.141	0.138	0.143	0.144	0.130
0.205		0.171	0.170		0.167	0.154	0.150	0.175	0.147	0.135	0.138
0.280		0.191	0.174		0.178	0.153	0.131	0.162	0.135	0.130	0.143
0.187		0.170	0.183		0.179	0.157	0.147	0.178	0.147	0.138	0.126
0.172		0.160	0.152		0.166	0.141	0.147	0.132	0.142	0.141	0.129
0.192		0.167			0.169	0.144		0.179	0.145		0.130
		0.199			0.158	0.147			0.138		

BIOGRAPHY OF THE AUTHOR

Ryan Terrell Phennicie was born in Woodbridge, VA on August 8th, 1985. He and his three brothers were raised in Haverhill, MA and Ryan graduated from Haverhill High School in 2003. Ryan then attended the University of Maine in Orono and graduated with a B.S. in Microbiology in 2008. He returned in the fall of 2009 and entered the Microbiology graduate program at the University of Maine. Ryan is a candidate for a Master of Science degree in Microbiology at the University of Maine in May 2011.