A SURVEY ON THE PHENOTYPIC VARIABILITY

OF *PSEUDOMONAS AERUGINOSA*

IN CYSTIC FIBROSIS

By

ELIZABETH GRACE O. PASCUAL

Bachelor of Science in Biochemistry

Oklahoma State University

Stillwater, OK

2013

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
May 2015

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Thesis Approved:

Dr. Erika Lutter

Thesis Adviser

Dr. Noha Youssef

Dr. Marianna Patrauchan

Dr. Rita Miller

ACKNOWLEDGEMENTS

The last two years have been guided by the principle of *ad astra per aspera*—it is difficult to reach for the stars. This road has been difficult and now the stars are within my grasp. To receive graduate education is a blessing in itself and is a journey one does not undertake alone. There are hundreds of people who have shaped me into who I am today, both as a professional and as a friend, and I am sorry I do not have the space to acknowledge them all but a particular few.

First, to my mentor and advisor, Dr. Erika Lutter, I thank you for your guidance and wisdom. You have pushed me beyond my conceived notion of my limitations and taught me that research, while essential to progression, does not have to be boring.

Second, to my committee—Dr. Marianna Patrauchan, Dr. Noha Youssef, and Dr. Rita Miller—thank you for your honesty, critical analysis, and guidance on the finer points of presentation and becoming a researcher.

Third, to Mrs. Connie Budd and Dr. Moses Vijayakumar, thank you for teaching me so much. My first year would have been vastly different without either of you.

To my classmates, labmates, and students, who have celebrated every success, mourned every failure, and made me laugh every day, thank you for your beautiful friendship.

To my roommates and closest friends, thank you for grounding me. In my darkest hours, you remind me of the light of the future and give me hope that everything will be all right. Your courage and strength in the face of certain failure and defeat has been, and always will be, an inspiration.

To my parents and my brother—you are the reason I am here. Your support and encouragement to chase after my dreams and ambitions is a priceless gift I will always treasure. Thank you for always lending me an ear and for being the best family a girl could ask for.

And finally, to you who lives in a world where being selfish is much easier than being kind: I leave you with *audeamus* and challenge you to begin some thing new. Dare to attempt the impossible, the improbable, and the unthinkable. Dare to change—hopefully for the better—and dare to dream of a bright future.

Maraming salamat sa inyong lahat!

Acknowledgements reflect the views of the author and are not endorsed by committee members or Oklahoma State University.

Name: ELIZABETH GRACE O. PASCUAL

Date of Degree: MAY 2015

Title of Study: A SURVEY OF THE PHENOTYPIC VARIABILITY OF *PSEUDOMONAS AERUGINOSA* IN CYSTIC FIBROSIS

Major Field: MICROBIOLOGY AND MOLECULAR GENETICS

Abstract: Cystic fibrosis (CF) is one of the most common autosomal recessive genetic disorders, resulting in faulty chloride ion channels in the lungs. As a result, the lungs are severely compromised with viscous secretions leading to chronic and repeated infections, and the colonization of a diverse microbial community. *Pseudomonas aeruginosa* is one of the primary pathogens in the CF lung and prior research has demonstrated a high degree of phenotypic heterogeneity among adult isolates in comparison to control strains. This study has two aims: First, the creation of an open repository of sputum and isolates collected from the Oklahoma Cystic Fibrosis clinic in Oklahoma City, OK to serve as a resource for research groups to use in polymicrobial studies. Second, the assay for a number of virulence factors using a subset of *P. aeruginosa* CF isolates recovered from two patients in each age category: children (under 13), adolescents (13-18), and adults (over 18). The collected repository includes sputum samples from 57 patients demonstrating a wide range of recoverable growth and a broad diversity of observed colony morphologies on nutrient and selective media. Thirty isolates per patient were assayed for surface motility (swim, swarm, and twitch) and production of hydrogen cyanide, biosurfactant, casein proteases, elastases, siderophores, and fluorescent pigments. *P. aeruginosa* isolates from most patients showed a high degree of consistency in producing hydrogen cyanide, fluorescent pigments, casein proteases, elastases, siderophores, and biosurfactant. There was, however, variability seen in the different surface motilities of isolates between the different age groups. These results provide insights to the variability of *P. aeruginosa* between age groups and confirm differences between *P. aeruginosa* isolated from newly infected CF patients and those chronically infected for many years. Further characterization and deeper understanding of the polymicrobial community in the CF lung are required to improve clinical diagnosis and treatment.

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CHAPTER I

INTRODUCTION

Cystic fibrosis (CF) is an autosomal disorder affecting approximately 30,000 individuals in the United States [22]. It results from mutations in the cystic fibrosis transmembrane regulator (CFTR) gene [22]. Although CF was first observed in infants with pancreatic problems, it is widely studied for its contributions to pulmonary decline [22]. The most notable feature of pulmonary CF is the production of viscous mucus in the lungs allowing for the retention of a rich polymicrobial community that would otherwise be expelled [85].

The polymicrobial community in the CF lung has presented a new direction for CF research. In childhood, the dominant pathogens are *Staphylococcus aureus* and *Haemophilus influenzae*. As a patient ages and becomes infected with *Pseudomonas aeruginosa*, the lung maintains the same microbial load but *P. aeruginosa* dominates the lung. The degree of cooperativity between *P. aeruginosa* and other CF pathogens such as *Burkholderia cepacia* complex is thought to enhance the virulence of the dominant pathogen [12], and places emphasis on the need to understand interspecies interactions in detail.

Due to its genetic flexibility, *P. aeruginosa* clones demonstrate a high degree of phenotypic variance even within the same patient [107]. Among the phenotypes studied by Workentine et al. [107], many are virulence factors contributing to the pathogenicity of *P. aeruginosa* such as protease and siderophore production, and swarm, swim, and twitch motility. Most research is focused on adult patients and provides little insight to the early years of infection in children and adolescents. Moreover, obtaining sputum from children and adolescents presents another challenge for CF research. Parents and guardians of young patients are often uncomfortable with external studies and prefer to limit their children's participation. Because children and adolescents do not readily produce sputum as much as adults do, it is easier to collect throat swab cultures from them, but this may not be an accurate representation of infections in the lower airways. With this being said, a combination of challenges presents a serious gap in heterogeneity studies.

This study has two aims in order to address these gaps. First, the creation of a repository of CF sputum and isolates from all ages will address the challenge of obtaining suitable samples for research use. By setting up an open access library of samples, this fosters collaborations with interested research groups to further enhance our current knowledge and understanding of the CF lung and associated infections. Second, surveying *P. aeruginosa* strains collected from patients at the Oklahoma Cystic Fibrosis clinic will address gaps in heterogeneity studies. We hypothesize that *P. aeruginosa* strains recovered from cystic fibrosis sputa from children (under 13), adolescents (13-18), and adults (over 18) will exhibit differences in virulence factor production between the three age groups. Additionally, heterogeneity is also expected between isoaltes obtained from the same patient.

CHAPTER II

REVIEW OF LITERATURE

I. Cystic Fibrosis

Cystic fibrosis (CF) is one of the most common autosomal recessive disorders, affecting approximately 30, 000 individuals in the United States (reviewed in [22]). Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) negatively affect the epithelial lining of the lungs [22] and allow for the retention of a diverse microbial community that would otherwise be expelled [85]. Cystic fibrosis, also referred to as mucoviscidosis, first gained attention as a pancreatic ailment resulting in lesions and malnutrition. At the time, the understanding of the disease was that it was a collection of related clinical states. However, this dogma changed when the work of Dorothy Andersen was published in 1938 [67], which provided a genetic understanding of what was previously considered to be a collection of disease states to a single disease with many clinical manifestations and effects [3]. Further work led to the establishment of a few key observations common to CF individuals. The sweat of CF patients contains abnormally

high concentrations of sodium (Na^+), chloride (Cl^-), and potassium (K^+) ions [30, 67]. Those patients who reach adulthood are also sterile [95].

While the alimentary effects of CF have been studied, much research focuses on the respiratory manifestation of CF, particularly on the repeated bacterial infections of the lungs. These infections are typically prevented in a healthy individual through a number of innate host defense mechanisms. In CF, mutations in the CFTR gene result in malfunctioning chloride (Cl^-) ion transporters in epithelial cells, leading to viscous mucus secretions and accounts for the increased levels of ions in the sweat. The most common mutation in the CFTR gene is a ΔF508 frameshift mutation that encodes isoleucine instead of phenylalanine (reviewed in [67]). The defective protein is accumulated in the endoplasmic reticulum [39, 67] and does not traffic to the Golgi [67, 106], an action necessary for membrane expression. As such, the ΔF508 mutation results in low Cl^- transport across the epithelial membrane and contribute to abnormal epithelial fluid secretions [67].

The classification of CF as an infectious disease stems from repeated invasion of the normally sterile lungs by opportunistic pathogens such as *Staphylococcus aureus*, *Haemophilus influenzae,* and *Pseudomonas aeruginosa* [67]. This repeated invasion is partially due to poor action of the mucocilliary escalator in the trachea, which normally moves mucus secretions from the lungs up to the throat to be expelled or swallowed and destroyed in the stomach [67]. In CF, poor Cl^- ion transport across the epithelial cell membranes thickens the mucus, creating plugs that serve as a breeding ground for pathogens, and the thickened mucus cannot be easily moved by the villi of the mucocilliary escalator [67].

One of the clinical symptoms associated with CF is the intermittent occurrence of pulmonary exacerbations. An exacerbation is loosely defined as a period of acute worsening of symptoms and is associated with a decrease in lung function [41]. Exacerbations are usually

treated with antibiotic therapies to eradicate pathogens that may be causing pulmonary decline through host-pathogen responses such as inflammation.

The relationship between airway inflammation and infection has been the subject of several studies. In a normal immune response, inflammation serves to quickly bring neutrophils and macrophages to the site of infection to eradicate pathogens (reviewed in [35]). Inflammation is normally self-limiting [35], however, in CF, the airways experience chronic inflammation due to the recognition of whole pathogens such as respiratory viruses or pathogen-associated molecular patterns (PAMPs), which are components shed by pathogens such as flagellum and lipopolysaccharide (reviewed in [75]). PAMPs bind to receptors on the epithelial cells to stimulate an inflammatory response [75].

It has been suggested that exaggerated inflammation in response to early infections with pathogens such as *S. aureus* causes initial tissue damage [67]. However, it has also been suggested that lung damage may also occur before chronic pathogenic infections occur in the lungs due to the increased number of neutrophils and the elevated amounts of neutrophil elastase, α_1–antiprotease, and interlukin-8 (IL-8) present in bronchoalveolar lavage (BAL) fluid taken from infants with CF [55]. In this study, seven of the sixteen infants did not culture any CF pathogens from the BAL fluid but still had increased numbers of neutrophils and levels of IL-8 [55].

In a comparative study of bronchial epithelial cells from CF and non-CF individuals, Bonfield, et al. measured the levels of secreted anti-inflammatory cytokine IL-10, and pro-inflammatory cytokines IL-8 and IL-6 [11]. Their study revealed that non-CF epithelial cells secrete IL-10 but no pro-inflammatory cytokines [11]. On the other hand, CF epithelial cells secrete pro-inflammatory cytokines but not IL-10 [11]. This difference between non-CF and CF epithelial cell cytokine production may be due to continuous stimuli from pathogens or PAMPs

present in the lungs. Furthermore, another study demonstrated that *P. aeruginosa* stimulates the release of pro-inflammatory cytokine IL-8 by bronchial epithelial cells [32]. An elevated level of activated NF-κB in epithelial cells also contributes to the continuous inflammatory response [31].

This failure to regulate pro-inflammatory signaling by epithelial cells has led to the use of anti-inflammatory therapies such as ibuprofen to improve patient symptoms [57]. However, the link between chronic airway inflammation, microbial infection, and CF is still not well understood despite years of research. How do pathogens continue to thrive in the lungs despite the constant recruitment of neutrophils by IL-8? One study suggests that the elevated Cl^- concentration in the CF lung may inhibit neutrophil phagocytosis [100], indicating that the abnormal secretions in the CF lung may play a role in pathogen persistence and ineffective killing by neutrophils. As such, chronic inflammation in CF is marked by the presence of neutrophils and pro-inflammatory cytokines in the sputum [89]. During periods of pulmonary exacerbations, inflammation increases in the lungs, but whether this heightened response is due to increased epithelial cell recognition of PAMPs remains under debate.

I. a. Major Pathogens of Cystic Fibrosis

Pseudomonas aeruginosa is one of the primary pathogens in the CF lung and prior research has demonstrated a high phenotypic variability among adult isolates in comparison to control strains [107]. Other major pathogens include *Haemophilus influenzae, Burkholderia cepacia* complex (BCC) [12] and *Staphylococcus aureus* [69]. In childhood, the dominant pathogens are *S. aureus* and *H. influenzae* [67]. As a patient ages and becomes infected with *P. aeruginosa,* the lungs maintain the same microbial load but *P. aeruginosa* or *B. cepacia* complex dominates the lung, replacing less virulent microorganisms frequently found in the oral cavity with more severe pathogens [28]. Infection with *B. cepacia* complex is often of high concern, as

patients may succumb to "cepacia syndrome," a fatal condition brought about by rapid onset of necrotizing pneumonia [40] or septicemia [112].

I. b. Polymicrobial infections in the CF lung

It has been well appreciated that CF individuals possess a unique microbiome among each other and that their polymicrobial communities are also different from those of non-CF individuals [28, 37]. This presents a challenge for therapeutic strategies and places identification of the polymicrobial community members a high priority for effective diagnosis and care.Current research is focused on understanding the polymicrobial community present in the CF lung. Traditional sputum studies were conducted under aerobic conditions and indicated that a select group of microorganisms colonized the CF lung in a particular order, beginning with *H. influenzae* and *S. aureus* and ending with *P. aeruginosa* and *B. cepacia* complex dominant infections [37, 85]. However, other opportunistic pathogens such as non-tuberculosis *Mycobacterium, Stenotrophomonas maltophilia,* and *Achromobacter xylosoxidans* have been cultured with increased frequency in recent years [37].

However, aerobic-based studies are misrepresentative of the true nature of the CF lung microbiome. The levels of oxygen present in the lungs fluctuate as mucus accumulates in the airways and are cleared by expectoration. Therefore, it is possible that anaerobic microorganisms may colonize the lungs as well. Both facultative and obligate anaerobes have been cultured from sputum in addition to the traditional aerobic pathogens [28, 37, 87]. As respiratory viral infections are also common in CF patients, other researchers began to address the question of what other microorganisms are present in the sputum. When fungi were cultured from sputum, the need for a more sensitive and high throughput method of detection became a priority when it became apparent the CF community was more complex than previously thought.

With the advent of next-generation sequencing, the study of CF microbiome became more feasible. Using culture-independent techniques, Delhaes, et al. identified several members of the polymicrobial community, including opportunistic fungal pathogens such as *Aspergillus fumigatis,* yeasts such as *Candida albicans*, anaerobic bacteria, and traditional CF pathogens such as *P. aeruginosa* [28]. In another microbiome study conducted by Fodor, et al. [37], the impact of exacerbations and antibiotic treatment on the microbial community was assessed. They showed that, at the end of exacerbations *Pseudomonas* and *Burkholderia* populations did not significantly or consistently decrease when compared to the onset of exacerbation or during clinically stable periods [37]. Moreover, antibiotic treatment affected a small number of less abundant microorganisms such as *Neisseria* and *Pasteurella,* reducing their numbers or clearing them entirely from the lungs [37]. However, treatment during exacerbations with high doses of antibiotics is usually associated with temporary improvement of clinical status [37], as shown by changes in community structure after antibiotic treatment as opposed to during periods of stability and the beginning of exacerbation periods [37]. The use of culture-independent techniques for rapid identification of all members of the complex and highly diverse community present in the lower airways might prove useful in prescribing a personalized and effective drug treatment plan [28].

Other studies focused on assessing the active rather than the total community in CF lungs. This technique ensures that the active members of the community are targeted with personalized antibiotics even if they are present in low numbers. Metabolically active community is investigated by assessing the levels of the RNA rather than the DNA. Metabolic activity measured by Reverse Transcription Terminal Restriction Fragment Length Polymorphism (RT-T-RFLP) in comparison to Terminal Restriction Fragment Length Polymorphism (T-RFLP) showed that microorganisms present in low numbers, such as *B. cepacia* complex, were metabolically active [87].

I. c. Polymicrobial interactions in the CF lung

Inter- and intra-species interactions play a major role in determining the overall effect that the polymicrobial community will have on the clinical status of the host. In particular, the interactions between *S. aureus*, *B. cenocepacia*, and *P. aeruginosa* are of particular interest owing to their prevalence in the CF lung and their impact on mortality. It has been observed that the CF lung contains a lower microbial diversity in comparison to the microbial communities present on the skin or in the gut [28]. However, this reduced diversity in fungal and bacterial populations was associated with poorer clinical status and decreased lung function [28]. Therefore, the select group of pathogens colonizing the CF lung interacts with each other using a variety of signaling mechanisms and synergistic interactions.

An important feature of polymicrobial communities is community surveillance, which is defined as the ability of one pathogen to sense and respond to stimuli from another pathogen [58]. For example, upon exposure to N-acetylglucosamine (GlcNAc), a component of peptidoglycan, *P. aeruginosa* increases the production of *Pseudomonas* quinolone signal (PQS)-regulated virulence factors [58]. Gram-positive bacteria that shed high amounts of GlcNAc are co-cultured with *P. aeruginosa,* who in turn express higher than wild type amounts of pyocyanin. Further work showed that GlcNAc induces *pqsA* transcription, the first gene in the synthesis of PQS, and thereby induces pyocyanin and elastase production [58]. In a *Drosophila melanogaster* infection model, the commensal Gram-positives in the crop served as the primary source for peptidoglycan. This suggests that the production of elastase induced by the transcription of *pqsA* [58] allows for the lysis of Gram positives not only to harvest nutrients from the lysed cell, but also to reduce competition [7]. The increased production of PQS-mediated toxins is an effect of community surveillance and not the result of mutational overproduction of pyocyanin and elastase.

Interactions between *P. aeruginosa* and the dimorphic yeast *Candida albicans* are dependent on secreted signals from both species. *N*-acyl-homoserine lactone (AHL) quorum-sensing signals from *P. aeruginosa* are able to influence *C. albicans* morphology, allowing it to switch from yeast to filamentous morphologies depending on the amount of AHL signals present in co-cultures [70]. Farnesol, a secreted *C. albicans* virulence factor that represses the switch from yeast to filamentous morphologies, is capable of reducing swarming motility in *P. aeruginosa* [70]. These results imply that bacterial-fungal interactions may play a greater role in the polymicrobial community of the CF lung and contribute to the persistence of chronic polymicrobial infections.

A long established tenant of CF chronic infections is the necessity for biofilm formation. Biofilms in the lung contribute to antimicrobial resistance and poor clearance despite chemical therapy. Co-infections of *P. aeruginosa* and *B. cenocepacia* in a planktonic culture demonstrate that while *P. aeruginosa* inhibits *B. cenocepacia* growth, biofilm formation is positively affected by *B. cenocepacia* [12]. When grown alone, *P. aeruginosa* forms flat biofilms but in mixed cultures, it forms filamentous biofilms[12]. Biomass also increases in co-culture [12].

Perhaps the most curious aspect of the CF lung is the obvious dominance by *P. aeruginosa* in the lung over the presence of other microorganisms. Bragonzi et al. show that co-infections of *P. aeruginosa* and *B. cenocepacia* increase host inflammation response [12]. In synergistic biofilms, virulence factor production also increases [12]. In a study conducted by Sibley, et. al, polymicrobial infections and community interactions were studied in *Drosophila melanogaster,* an infection model that is often used to study CF infections. Oral microbes, which are either avirulent or beneficial to the fly, demonstrate synergy and enhancement of pathogenicity of the crop polymicrobial community [96]. Two oral strains, which are phenotypically identical in a natural-route *Drosophila* infection model, are viewed as two separate and different infections by the co-infecting *P. aeruginosa* and by the host's innate

immune system [96]. This result suggests a level of complexity to polymicrobial infections and forms the basic framework for further studies in bacterial interactions.

II. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative rod ubiquitous in soil and water environments. It is an opportunistic pathogen of nosocomial concern and is frequently found to colonize burn wounds [66]. In the past thirty years, *P. aeruginosa* has gained attention as a bacterium capable of bioremediation and oil recovery due to its production of surface-active chemical compounds called biosurfactants [44, 111]. *P. aeruginosa* is highly motile by both flagellum [94] and Type IV pili [14]. It is considered an obligate aerobe, although some strains demonstrate growth in facultative anaerobic conditions using nitrate or nitrite as an alternate electron acceptor [19].

The production of a wide variety of virulence factors produced by the bacterium marks it as a pathogen of nosocomial concern. It is capable of forming biofilms and an intricate hierarchy of quorum sensing systems controls many of its cellular processes. *P. aeruginosa* is known to produce colored pigments which act as signaling molecules, redox cascade inhibitors, and siderophores [61, 84]. It also produces a large number of proteases that contribute to tissue destruction and evasion of host immune response. Anti-pseudomonal drugs such as ciprofloxacin are often prescribed to treat infection but with limited success, as it is highly antimicrobial resistant due to the formation of biofilms and the acquisition of antimicrobial resistance plasmids.

II. a. Quorum Sensing Systems

Perhaps the most unique quality of *P. aeruginosa* is its ability to easily adapt to a wide range of environments. This is accomplished through fine-tuning of its phenotypic expression using quorum sensing. This adaptive ability is often termed "genetic plasticity" by some researchers but this term is misleading since the genome remains relatively constant from isolate to isolate.

P. aeruginosa has three known quorum sensing systems that contribute to the production of many virulence factors and interspecies interactions. The first two quorum sensing systems are categorized as *N*-acyl homoserine lactone (AHL) signal systems. These AHLs bind to LuxR-type intracellular protein receptors. A related quorum sensing system was first identified in *Vibrio* species. The first of these AHL systems is the *las* system, a complete quorum sensing system that uses 3-oxo-dodecanoyl-homoserine lactone (3-oxo-C12-HSL) as an autoinducer. 3-oxo-C12-HSL binds to LasR, a cognate transcriptional regulator. The binding complex controls the expression of over 300 genes. Furthermore, the *las* system controls the activation of the *rhl* system, but this is dependent on nutrient availability [45]. The second AHL system is the *rhl* system. Its autoinducer is *N*-butanoyl L-homoserine lactone (C4-HSL), which binds to RhlI, which is also a cognate transcriptional regulator. The binding complex also controls the expression of 300 genes [45].

The third quorum sensing system is the *Pseudomonas* quinolone signal (PQS), which acts as one of the primary quorum sensing signals in *P. aeruginosa*. The autoinducer, 2-heptyl-3-hydroxy-4-quinolone, binds to the cognate receptor, PqsR, to regulate the production of PQS, elastase, rhamnolipids, and pyocyanin [38]. The *pqsABCDE* operon produces two products, 2-alkyl-4-quinolones (AQs) and 2-heptyl-4-quinolone (HHQ) [29]. AQs have been detected in the supernatant with PQS [29]. AQs also play a role in cell-to-cell communication with other

bacteria, a function essential in the CF lung where a diverse microbial community exists. PqsH, a monooxygenase whose gene is not part of the *pqsABCDE* operon, aerobically converts HHQ to PQS [38]. Furthermore, HHQ binds to PqsR and induces *pqsA* transcription [108]. Research suggests that the binding of HHQ, despite its lower binding affinity to PqsR in comparison to PQS, is important in anaerobic conditions [92]. However, the significance of this accumulation of HHQ in anaerobic environments has yet to be studied.

AQs have been also shown to be vital for interspecies interactions. While PQS is only produced by *P. aeruginosa*, HHQ is produced by *P. putida* and *Burkholderia* species produce methylated AQs [105]. A study shows that a *pqsA* mutant of *P. aeruginosa* is unable to lyse *S. aureus*, suggesting that *S. aureus* lysis involves AQs or genes regulated by AQs [68]. Heptylquinoline-N-oxidase (HQNO) is an AQ that inhibits respiration and the uptake of aminoglycosides. The production of HQNO by *P. aeruginosa* confers protection for *S. aureus* and results in the selection of small colony variant *S. aureus* [50, 101].

II.b. Denitrification

Denitrification is described as anaerobic respiration using nitrate (NO_3^-) or nitrite (NO_2^-) as a terminal electron acceptor instead of oxygen. NO_3^- or NO_2^- is reduced to gaseous N-oxides and N_2 in this process [102]. Chen et al. [19] demonstrated that *P. aeruginosa* is capable of denitrification, an ability advantageous in the low oxygen environment of the CF lung and for bacterium deep within a biofilm in contrast to those at the surface. The regulation of denitrification is controlled by levels of N-oxides and oxygen. These two factors also mediate the switch between aerobic respiration and denitrification through the CRP/FNR protein family [102].

In addition to regulation by environmental levels of N-oxides and oxygen, denitrification is also regulated by the quorum sensing molecules of *P. aeruginosa* [102]. Studies conducted by Toyofuku et al. [102] show that the AHL quorum sensing systems, *las* and *rhl*, repress denitrification. The third quorum sensing system, PQS, affects denitrification by acting on the enzyme activity of NO_3^- reductase (NAR), NO_2^- (NIR), NO reductase (NOR), and N_2O reductase (NOS) through iron chelation. Hoffmann, et al. [51] proposed that nutrient availability induced a metabolic shift in *P. aeruginosa*, creating mutant *lasR* genes. This shift confers resistance to multiple antibiotics and a heavy reliance on denitrification to gain energy along with some aerobic respiration [51].

II.c. Biosurfactant

P. aeruginosa biosynthesizes biosurfactant molecules called rhamonolipids [44]. A rhamnolipid is a glycolipid and has the general structure of a hydrophobic fatty acid bound to a hydrophilic portion that contains either one or two rhamnose sugars [97] . Research performed by Davey et al. [27] indicates that rhamnolipids are required to maintain biofilm architecture by opening channels between macrocolonies to allow for the distribution of oxygen and nutrients throughout the biofilm.

II.d. Motility

Motility is often considered to be a virulence factor for its role in host colonization and biofilm formation. Three types of motility are studied in depth in this work. The first is swimming, and is defined by Henrichson as a kind of surface translocation produced through flagellar action [48]. Swimming only occurs on a sufficiently thick surface and the cells move

individually and at random in unorganized patterns [48]. In *P. aeruginosa*, swimming is utilized to obtain nutrition, escape toxic substances, and colonize new hosts and environments. Previous studies show that mutations in any of the genes involved in flagellum synthesis causes reduced virulence [94].

The second type of motility observed in this study is twitching motility. It is also a type of surface translocation but is made possible by Type IVa pilli, a type of adhesin (reviewed in [14]). Movement appears intermittent and jerky [48] and is observed in both flagellated and unflagellated bacteria. The Type IVa pilli, as an adhesin, promotes surface attachment and is important in establishing biofilms (reviewed in [14]). Twitching motility also contributes to biofilm architecture development. Mutants are unable to form structurally stable biofilms. With regards to pathogenicity, Type Iva pilli are used in the early or acute stages of *P. aeruginosa* infection but are lost in chronic infections either through mutations or down-regulation.

The third type of motility is swarming. In particular, the swarming motility requires two exoproducts to stimulate swarming—rhamnolipids and 3-(3-hydroxyalkanoyloxy) alkanoic acids which act as wetting agents and chemotactic stimuli [103]. As previously mentioned, rhamnolipids are an integral part in biofilm maintenance and architecture. Swarming is defined as rapid translocation across a semisolid surface [48], and Tremblay and Deziel suggested that swarming behavior is not virulent, but rather, is a method by which a colony quickly controls a maximum amount of space [103].

II. e. Siderophores

In order to thrive in any environment, all living organisms must find a way to meet iron nutrient demands to carry out essential metabolic processes. While most eukaryotes are capable of acquiring iron through diet and humans meet most of their iron nutritional demands through

the recycling of red blood cells by macrophages, bacteria must possess some kind of iron-uptake system to do the same (reviewed in [18]). Upon invasion of a host, bacteria must free iron from different sources such as the heme groups found in hemoglobin or lactoferrin present in mucosal secretions and neutrophils [18]. Caza and Kronstad outlined four microbial iron uptake systems in their review of bacterial and fungal iron acquisition. The first system is the secretion of hemolysins to lyse red blood cells and uptake heme to acquire iron [18]. The second is the uptake of transferrin and lactoferrin [18]. The third system is the secretion of siderophores, low molecular weight chelators capable of binding to environmental iron and taking it into the cell where it is broken down and freed from the transporter ferric complex [13]. Finally, the fourth iron uptake method is the direct uptake of ferrous iron [18].

P. aeruginosa secretes two siderophores, pyoverdine and pyochelin, in iron-limiting conditions. The presence of two siderophore systems in *P. aeruginosa* is interesting, considering that pyoverdine has a much higher affinity for iron than pyochelin does. However, since pyochelin is able to bind to other metal co-factors, this second siderophore most likely plays an additional role in metal homeostasis. Brandel, et al. [13] confirmed that while pyochelin is rarely synthesized, even in clinical isolates, its primary role is to facilitate iron uptake, especially when the primary pyoverdine-based iron uptake system is deficient. Other studies demonstrate that pyoverdine and pyochelin are capable of increasing *P. aeruginosa*'s tolerance to toxic metal ions in the environment [13]. This observation lends further support to the siderophores' probable role in metal homeostasis.

II. e. i. Pyoverdine

One of the major siderophores in *P. aeruginosa*, pyoverdine (PVD) is also found in other fluorescent pseudomonad species (reviewed in [91]). It is characterized by its green-yellow

pigment that fluoresces blue under UV light [74]. Structurally, pyoverdine consists of a fluorescent chromophore linked to a strain-specific peptide and a side chain bound to the chromophore (reviewed in [91]). A single *pvd* locus in the PAO1 genome contains nearly all the genes for synthesis and uptake [54]. Iron uptake occurs when the apo-PVD is secreted outside the cell and binds to iron. The ferr-PVD complex then binds to the outer membrane receptor FpvA [54]. PAO1 has three types of pyoverdine: PVDI, PVDII, and PVDIII.

With regard to pulmonary infections, pyoverdine is an important asset to a bacterium attempting to colonize an environment with a low availability of free iron. Colonization is further hindered by neutrophil-gelatinase-associated lipocalin (NGAL), an innate defense protein that targets bacterial siderophores in both apo- and ferric forms [79]. However, pyoverdine evades binding to NGAL in both apo- and ferric forms due to its inability to bind to the ligand-pocket of NGAL, allowing *P. aeruginosa* to establish infection within an otherwise hostile environment [79].

In addition to its traditional role as an iron-sequestering molecule, Lamont et. al showed that pyoverdine also acts as a signaling molecule controlling the expression of exotoxin A, PrpL, an exoprotease, and pyoverdine itself in *P. aeruginosa* [61]. Pyoverdine is also thought to have a role in biofilm control, cell-to-cell communication, and virulence factor regulation [54].

II. e. ii. Pyochelin

Pyochelin (PCH) is the other major siderophore of *P. aeruginosa* and it is produced through the condensation of salicylate and cysteine [24]. It is structurally different from pyoverdine, containing two thioazoline and thiozolidine heterocyclic rings [20]. Pyochelin binds to iron in a 2:1 ratio [24] but it is also capable of chelating other biologically significant metals such as copper(II) and zinc(II), suggesting pyochelin may play a specific role in metal

homeostasis [13]. Two gene clusters, *pchDCBA* and *pchEFGHI*, are responsible for biosynthesis but the specifics of pyochelin expression are regulated by cytoplasmic iron concentrations and the binding of chelated iron to cytoplasmic regulator protein PchR [13]. Unlike pyoverdine, pyochelin utilizes FptA for outer membrane transport [13] and FptX for inner membrane translocation [24].

Chelated iron from the environment is transported to the cytoplasm where it activates PchR to induce pyochelin synthesis and activate genes necessary for iron uptake[13]. Immediately after synthesis in a siderosome, pyochelin is exported outside the cell to avoid accidental iron chelation in the cytoplasm [24]. Synthesis is later inhibited when iron(II) in the cytoplasm binds to the Fur protein to stop further iron acquisition in order to maintain iron homeostasis in the bacterium [13].

Unlike pyoverdine, pyochelin is able to fit inside the ligand-binding pocket of NGAL, but it does not bind strongly to NGAL [79]. This suggests that while pyochelin may be recognized by NGAL, the overall host response to *P. aeruginosa* siderophore secretion remains mostly absent due to the bacterium's reliance on pyoverdine to act as the major siderophore contributing to successful infection and biofilm establishment [79].

II. f. Pigments

The pigments of *P. aeruginosa* are easy to identify on solid media and serve secondary roles in signaling, metabolism and virulence. Pyocyanin presents as a blue-green soluble pigment and is discussed below. The role of pyoverdine, a green-yellow, fluorescent pigment, as a siderophore was described in depth previously. Pyomelanin is a red-brown pigment and pyorubin is red in color [109]. The exact functions of pyomelanin and pyorubin are unknown but some researchers believe they play a role in protection from oxidative stress [34].

II.f. i. Pyocyanin

Among the pigments it produces, *P. aeruginosa* is most known for the production of pyocyanin (PCN), a blue-green pigment. It is a redox-active secondary metabolite, a member of the phenazines, and has a low molecular weight. As a zwitterion, it is capable of passing through biological membranes. Pyocyanin interferes with several mammalian cell functions such as cell respiration, prostacyclin release from lung endothelial cells, and IL-2 release, which limits T-lymphocyte growth. It is also able to initiate redox cascade in order to inhibit microbial growth, enabling *P. aeruginosa* to dominate in the CF lung [84]. Furthermore, research suggests that pyocyanin plays a role in epithelial cell dysfunction, altered pulmonary immunity, and proteolytic injury [84].

Pyocyanin synthesis is regulated by an AHL-quorum sensing system using OdDHL autoinducer. Specifically, synthesis is regulated by RhlR, whose transcription is controlled by LasR [71]. PCN is made from chorismate, an intermediate in salicylic acid production. It is mediated by two *phzsABCDEFG* operons and by *phzH, phzM, and phzS* gene products, which modify precursors into pyocyanin (reviewed in [62]).

An *in vivo* model of acute pulmonary infection caused by *P. aeruginosa* revealed that pyocyanin and other phenazine tox metabolites impede neutrophils through distinct mechanisms, most notably the reduction of chemokines and cytokines. The reduced number of neutrophils and acceleration of neutrophil apoptosis are linked to impaired bacterial clearance [2]. Mowat, et al. demonstrated that there is a direct link between pyocyanin overproduction and pulmonary exacerbation, suggesting that pyocyanin is a major contributor to symptoms associated with pulmonary exacerbations [72]. Furthermore, changes in the CF lung during exacerbation periods may favor the selection of pyocyanin overproducing isolates [72].

II. g. Proteases

P. aeruginosa produces a number of proteases capable of destroying host tissues. The most widely studied are elastase A, elastase B, alkaline protease, and protease IV, however, other exoproteases are in the process of being characterized [56]. Skim milk plates are often used to identify protease production, as many proteases secreted by *P. aeruginosa* are capable of degrading the milk protein, casein [98]. As classic virulence factors, the identification and study of proteases is important to understand *P. aeruginosa* infection in depth.

II. g. i. Elastase

Elastase is an enzyme capable of breaking down elastin, a fibrous glycoprotein found in connective tissue. *P. aeruginosa* secretes two kinds of elastase. The first, elastase A, is a metalloproteinase 27 kDa in size and also known as LasA or staphylolysin [56]. Elastase A is shown to increase the range of substrates degraded by elastase B and destroys host defense proteins [15]. The second is elastase B, a metalloproteinase 33 kDa in size and also called LasB or pseudolysin [56]. Encoded by the *lasB* gene, elastase B is thought to damage host tissue by hydrolyzing components of the extracellular matrix. Other studies suggest elastase breaches the endothelial and epithelial barriers by attacking tight junctions. A study by Kung et al. in 2011 showed that *P. aeruginosa* elastase degrades surfactant protein A (SP-A) in mouse lungs, a component of the innate immune system that contributes to pulmonary surfactant and reduces phagocytosis in the lungs [59]. SP-A also opsonizes microbes and permeabilizes membranes [59]. Further experiments by Kuang et al. showed that a Δ*lasB* mutant is attenuated in SP-A$^{+/+}$ mice lungs and virulent in SP-A$^{-/-}$ mice lungs [59]. Their work also showed that the inability to secrete elastase reduces *P. aeruginosa*'s ability to degrade SP-A and successfully colonize the lungs [59].

II. h. Hydrogen cyanide

The production of hydrogen cyanide (HCN) has long been identified as a major extracellular virulence factor contributing to the high mortality of burn wound patients [8]. It is a toxin against many eukaryotes, demonstrated in a *Caenorhabditis elegans* model [80]. Previous research has shown that mucoid *P. aeruginosa* actively transcribes the *hcnA* gene that encodes HCN synthase, the enzyme responsible for the conversion of glycine to hydrogen cyanide [8]. Cyanide (CN^-) is present in the sputum of CF and non-CF patients and subsequent breath analysis has also detected HCN gas present [8]. CN^- production occurs through the oxidative decarboxylation of glycine via HCN synthase. Immediately after synthesis, HCN gas diffuses and acts a direct respiratory inhibitor. The gas binds to cytochrome c oxidase and other metalloenzymes, resulting in the lethal inhibition of election transfer chains [4, 8, 80]. Production is controlled by a variety of factors, including oxygen availability, metabolic regulator compounds, transcriptional regulators including AHL quorum sensing regulators, and the presence of ANR (anaerobic regulator of arginine deaminase and nitrate reductase) [4, 8, 80]. Because it is such a potent toxin, the detection and quantification of CN^- levels in the lung are under consideration as a potential, non-invasive marker for *P. aeruginsoa* infection and the invention of rapid techniques to detect HCN production levels have been largely successful [4, 8].

II. i. Exotoxin A

One of the primary toxins of *P. aeruginosa* is exotoxin A, a single-chain polypeptide protein synthesis inhibitor with a molecular weight of approximately 66 kDa (reviewed in [81]). Exotoxin A shares biochemical properties with both diphtheria and cholera toxins and its mode of action is molecularly defined. Exotoxin A is an ADP ribosyl transferase dependent on nicotinamide adenine dinucleotide (NAD) to be enzymatically active and transfer an ADP-ribosyl

moiety from NAD to elongation factor 2 [76]. In mice, a 2.5 μg/kg dose of purified toxin is necessary for lethality [16, 17] and at sublethal doses, exotoxin A causes epithelial cell necrosis in rabbits [53]. Most clinical isolates of *P. aeruginosa* produce exotoxin A and the human immune system is capable of producing antibodies in response to toxin exposure [23, 83]. The presence of antibodies within the serum is usually indicative of bacteremia survival and their absence is correlated with poor survival rates. Unlike the similar toxin-mediated diseases diphtheria and cholera, whose etiological agents cause disease by releasing toxins that bind to host cells, *P. aeruginosa* is capable of invading host cells and the release of extracellular products like exotoxin A facilitate this invasion [81]. It is suggested that exotoxin A also acts as an immunotoxin due to its toxic effect on immune cells such as macrophages [82]. In a mouse study, infection of unimmunized mice with exotoxin A-producing strains of *P. aeruginosa* exhibited more bacteremia and tissue invasion than those who were immunized with exotoxin A-specific antibodies, suggesting that exotoxin A may play a role in interfering with bacterial clearance mechanisms [77].

III. Summary

Cystic fibrosis is a genetic disorder caused by mutations in the CFTR gene affecting 30, 000 individuals in the United States and treatment of secondary infections and symptoms is of high concern. CF individuals have highly viscous mucus secretions that result in poor pathogen clearance. The formation of a diverse polymicrobial community in the lower airways leads to chronic inflammation and tissue destruction.

P. aeruginosa is the dominant pathogen in CF adults and its ability to hypermutate and readily adapt to its environment makes it resistant to treatment even with antimicrobial agents. It produces a wide variety of virulence factors necessary for invasion and host cell destruction.

Virulence factors investigated in this study are siderophores, proteases, proteins for flagellar movement and adhesin, and toxins. An intricate hierarchy of quorum sensing systems mediates the regulation of most of these virulence factors and environmental conditions such as oxygen and nitrate levels also affect protein activity.

The relationship between virulence factor production in *P. aeruginosa* and the advancement of cystic fibrosis has been subject to much scrutiny in adults. This study aims to fill gaps in CF research regarding the variances in virulence factor expression in children and adolescent patients. Furthermore, this lays the foundation for further studies in the interactions of *P. aeruginosa* virulence factors and other members of the polymicrobial community present in the lungs.

CHAPTER III

METHODOLOGY

I. Repository

I.a. Collection

Sputum samples were collected from patients by the Oklahoma Cystic Fibrosis staff in 50 mL sterile conicals. The clinic staff provided information on the patient's age, sex, and medical condition upon admittance to the clinic, and a patient identification tag assigned to the sample. Approximately 150 µL of sputum was aliquotted into 150 µL of 50% glycerol and frozen on dry ice for transport back to Stillwater. Another 150 µL of sputum was saved into a cryovial and also frozen on dry ice for transport. If additional sputum remained after streaking on selective media and stabilization of two 150 µL aliquots of sputum in DNA/RNA Shield, then the remaining sputum was also added to the 50% glycerol and whole sputum stocks before freezing on dry ice for transport. Both sputum samples were stored long term at -80°C.

I.b. gDNA and RNA Extraction

Approximately two 150 µL aliquots of sputum were stabilized in DNA/RNA Stabilizer (Zymo) and frozen on dry ice for transport. gDNA was extracted using Quick-gDNA Extraction Kit (Zymo) and stored at 4°C. RNA was extracted using Quick-RNA Extraction Kit (Zymo) and stored at -80° C. In both kits, sterile water was used as the elution buffer instead of the provided solution to prevent downstream enzymatic processes.

I. c. Growth on Nutrient and Selective Media

Using a cotton swab, sputum was struck onto eight different nutrient and selective media: Brain Heart Infusion (BHI) agar (Criterion), Miller Luria-Bertani agar (LB) (Fisher), Nutrient agar (NA) (Difco), Skim milk protease agar (adapted from Sokol [98]), Mannitol Salts agar (MSA) (BBL), *Burkholderia cepacia* Selection agar (BCSA) (8.0 g peptones, 1.0 g $NH_4 SO_4$, 5.0 g $C_3H_3NaO_3$, 0.2 g Mg_2SO_4, 0.01 g $(NH_4)_2Fe(SO_4)_2$, 4.25 g KH_2PO_4, 1.42 g Na_2HPO_4, 0.5 g bile salts, 15.0 g agar in 1.0 L H_2O adjusted to pH 6.4. After autoclaving, solutions were allowed to cool, followed by the addition of 1 mL 0.05 g/50 mL 1000X crystal violet, 1 mL 1 g/50 mL1000X phenol red, 1 mL 2 g/20 mL 1000X ticarcillin and 1 mL 0.75 g/20 mL 1000X polymixin B was added to agar solution before pouring plates), MacConkey's agar (MAC) (Difco), and *Pseudomonas* Isolation agar (PIA) (Criterion). Plates were incubated at 37°C for 72-96 hours.

I. d. Colony Morphology

Colony morphology was recorded on all plates with recoverable growth. Other biochemical characteristics, such as protease production and mannitol fermentation, were also

noted at this time. All plates were photographed using a Canon EOS Rebel SL1 digital single lens reflex camera body and a Canon EF-S 18-55mm f/3.5-5.6 IS STM standard zoom lens.

I. e. Freezer Stocks

Recoverable growth from BHI, LB, NA, Skim Milk, MSA, BCSA, MAC, and PIA was harvested from plates and frozen at -80°C in 2 mL of 10% skim milk in a cryovial. Each vial is labeled with the patient identification tag, the media growth was collected from, and the date the freezer stock was made.

II. Freezer Block

II. a. Isolation

Patients were selected on the basis of age and growth on *Pseudomonas* Isolation agar. A child (under 13), two adolescent (13-18), and two adult (over 18) patients with growth on PIA were selected to make freezer blocks in order to test the variance in virulence factor expression. Using a wooden inoculating stick, the PIA freezer stock was serially struck on PIA to collect individual colonies. Plates were incubated at 37°C for 24-48 hours.

II. b. Block

A 96-well block was filled with 1 mL of BHI broth. 96 colony-forming units were picked using a wooden toothpick and inoculated the block. The block was sealed with aluminum sealing foil and incubated shaking overnight at 220 RPM and 37°C. To collect the cell pellet, the block

was centrifuged for 5 minutes at 4200 RPM at 20°C. The supernatant was discarded and the cell pellet resuspended in 900 μL of 10% skim milk. Patient blocks were stored long-term at -80°C.

II. c. Stamp Plate

Using a 48-pin replicator, half of the freezer block was stamped onto LB agar to create a working stock for phenotypic assays. Plates were incubated at 37°C for 48 hours. Thirty colonies were randomly selected from the stamp plate and consistently used throughout all phenotypic assays.

III. Controls

In all assays, *Pseudomonas aeruginosa* PAO1 was used as a control. For pigment production assays, *Pseudomonas fluorescens* ATCC 13525 was additionally used as a control. For motility assays, *Pseudomonas aeruginosa* pA1092-245 (Δ*filC*) [60] and pA4525-33348 (Δ*pilA*) [99] were also used. All strains were struck from freezer stocks using a wooden inoculating stick and incubated for 24-48 hours at 37°C, with the exception of *P. flurorescens* ATCC 13525 which was incubated at 30°C. PAO1 and ATCC 13525 were grown on LB agar. pA1092-0245 and pA4525-33348 were grown on LB agar containing 60 mg/mL tetracycline.

IV. Virulence Factors

IV. a. Hydrogen Cyanide

Hydrogen cyanide production was assessed according to Cody et al. [21]. A detection solution was made consisting of 40 mg 4,4'-methylenebis, 40 mg Cu(II)ethyl acetoacetate, and 20

mL chloroform. A 6mm filter disk was soaked in the detection solution and left to air dry on the plate lid. A lawn of each sample was inoculated on a small petri plate of LB agar using a cotton swab. Plates were incubated inverted at 37°C for 24 hours. The filter disk was checked for a blue color change.

IV. b. Pigment Production

IV. b. i. *Pseudomonas* Medium B for Pyoverdine Production

Pseudomonas Medium B (PMB) was made according to Atlas [6]: 20.0 g peptone, 15.0 g agar, 10.0 g glycerol, 1.5 g $MgSO_4$ 7 H_2O, and 900 mL of distilled water. One hundred mL of a K_2HPO_4 solution (1.5 g K_2HPO_4 dissolved in 100 mL distilled water) was also autoclaved with the media and added after sterilization. Samples were spot struck three per plate and incubated at 37°C for 24 hours. Color production and fluorescence under UV light was recorded.

IV. b. ii. Pigment Production during Denitrification

Denitrification media was made according to Atlas [5]: 10.0 g glycerol, 10.0 g KNO3, 3.0 g yeast extract, 1.5 g $(NH_4)_2SO_4$, 1.0 g agar, 0.8 g K_2HPO_4 3 H_2O, 0.5 g $MgSO_4$ 7 H_2O, 0.2 g KH_2PO_4, 0.1 g $CaCl_2$, 1 L distilled water. Samples were stab-inoculated and incubated 37°C. Growth was checked at 24 and 48 hours. Color production, fluorescence, and media liquification was recorded.

Media liquification as a positive indicator for denitrification may be explained by the stability of agar, which weakens at low pH values. In traditional soil pH experiments, the presence and level of nitrogen will increase the acidity of the soil [104]. For the *Pseudomonas* denitrification medium, the potassium nitrate served as a nitrate source and released nitrogen by

the denitrification process will lower the pH of the media. This in turn weakens the agar's ability to remain solid and liquifies the media.

IV. c. Siderophore Production

Chrome Azurol S media was prepared according to Schwyn and Neilands [93]. All glassware and utensils were first acid washed. 10 mLs of a Fe(III) solution (27.03 mg $FeCl_3$ $6H_2O$, 1 mL 1M HCl, and 99 mL of nanopure H_2O) was mixed with a CAS solution (60.5 mg of Chrome Azurol S dissolved in 50 mL of nanopure water). The CAS solution was added to a CTAB solution (72.9 mg CTAB/HDTMA dissolved in 40 mL nanopure H_2O) in a 250 mL bottle. In a separate 1L bottle, an agar solution (1 g NH_4Cl, 3.218 g Na_2HPO_4 $7H_2O$, 1.089 g KH_2PO_4, 5.0 g NaCl, and 30.24 g PIPES dissolved in 800 mL nanopure H_2O) was prepared and the pH adjusted to 6.8. The volume was adjusted to 890 mL with nanopure H_2O and both solutions were autoclaved for 20 minutes. The solutions were allowed to cool and the CAS solution was mixed into the agar solution. One mL of filter sterilized 1M $MgSO_4$ and 10 mL of filter sterilized 50% glucose was added to the media before aliquotting 15mL of media per plate. Plates were stored in foil at 4°C. Samples were spot struck eight per plate and incubated for 48 hours at 37°C. A positive recording for siderophore production was considered to be the agar color change from blue-green to orange. Growth and medium color change were recorded.

IV. d. Motility

IV. d. i. Swimming Motility

Swimming media was prepared according to Murray and Kazmierczak [73]. 0.3% (w/v) LB agar was made and left to air dry. Samples were spot inoculated with a toothpick and

incubated for 72 hours at room temperature with additional humidity. Growth diameter was measured in centimeters.

IV. d. ii. Swarming Motility

Swarming media was prepared according to Murray and Kazmierczak [73]. Two g glucose, 5 g agar, 0.5 g glutamate, 0.24 g $MgSO_4$, 1 L distilled water. After autoclaving, 100 mL of 10x M8 salts (12.8 g Na_2HPO_4 $7H_2O$, 7.2 g KH_2PO_4, 2.5 g NaCl, 500 mL H_2O) was added. Samples were spot inoculated with a toothpick and incubated for 48 hours at 37°C. Growth diameter was measured in centimeters.

IV. d. iii. Twitching Motility

Twitch media was prepared according to Darzins [26]. One % (w/v) LB agar was made and left to air dry. Samples were stab inoculated with a toothpick and incubated for 48 hours in 37°C. The agar was discarded and the plate bottom was left to air dry for two minutes. The twitch pattern was visualized with a Coommassie blue stain made of 0.3g Coomassie Brilliant Blue dissolved in 45 mL of methanol mixed with 10 mL of Glacial Acetic Acid mixed with 45 mL of H_2O for a minute and rinsed three times with distilled water. Pattern diameter was measured in centimeters.

IV. e. Biosurfactant Production

Biosurfactant production was assessed using the drop-collapsing test from Lindum et al. [64]. An overnight culture was grown in 5 mL of BHI broth and mixed. One mL was aliquotted

into a sterile tube and spun down 4200 RPM for 5 minutes. Ten μL of the supernatant was spotted in triplicate on a petri dish lid and allowed to incubate at room temperature for 10 minutes. The spreading diameter was measured in millimeters.

IV. f. Proteases

IV. f. i. Caesin Protease Production

A quantitative skim milk protease media was prepared according to Sokol et al. [98]. Using a dialysis tube, 9.25 g BHI was dialyzed in 25 mL of H_2O overnight at 4°C. The dialyzed BHI solution was mixed with 15.0 g agar, and 450 mL H_2O in a 1 L bottle and autoclaved for 30 minutes with a stir bar. In a separate bottle, 15.0 g skim milk mixed with 500 mL H_2O was autoclaved for 20 minutes and mixed with the BHI solution. Fifteen mL of media was poured per plate. Overnight cultures of each tested isolate were grown in 5 mL of BHI broth. A 1:10 dilution was made in BHI broth and the OD_{600} measured. The sample concentration was determined and normalized to an OD_{600} reading of 0.3. Three μL was spotted in triplicate onto a skim milk plate Spots air-dried and the plates were incubated for 24 hours at 37°C. The radius was measured from the center of the colony to the edge of the clearing in millimeters.

IV. f. ii. Elastase Production

The production of elastase was detected using media described by Rust et al. [88]. Reverse elastin media was prepared as a 5 mL overlay (0.4 g Nutrient Broth, 1.0 g agar, 0.25 g elastin, 50 mL H_2O) on nutrient agar (15.0 mL Nutrient Agar/plate). The overlay solution was autoclaved for 20 minutes and poured into individual petri plates using a serological pipette.

Samples were spot struck eight per plate and incubated for 48 hours at 37°C. Growth and clearing of the media was recorded.

IV.g. Cluster Analysis

Clustering of the results was done in Gene Cluster 3.0 program for Macintosh and viewed in Java Tree View. The data included the percent of positive results or average diameter measured. Data adjustments were made with the following parameters: mean center genes, and mean center arrays. In the hierarchical settings, the genes and arrays were clustered in hierarchy using average linkage clustering method.

CHAPTER IV

FINDINGS

I. Introduction to Repository Collection

One of the main objectives of the Lutter lab was to build and maintain a repository of CF sputa and isolates that can be accessed not only by the Department of Microbiology and Molecular Genetics, but also by other departments within and outside Oklahoma State University. The Cystic Fibrosis Foundation does have its own library of isolated strains but they impose stringent regulations regarding the use of these strains in research. Therefore, it became a high priority for this project to build an accessible research repository for future use in our laboratory and Department.

Collaboration with Dr. James Royall, C.R. Anthony Professor and Chief of Pediatric Pulmonology, at the Oklahoma Cystic Fibrosis Center in Oklahoma City was established for the purpose of building such a repository. The sputum was collected by the nursing staff and brought to a separate room where two aliquots were taken and frozen on dry ice for preservation during

transport back to Stillwater. The sputum was then struck on eight different types of nutritional and selective media in order to collect as much diverse microbial populations as possible. Provided there was enough sputum left after plating, two aliquots were stabilized in Zymo DNA/RNA Shield and gDNA and RNA were extracted upon return to the laboratory.

This specific protocol was established to meet the demands of different research aims. Researchers in need of whole sputum to study its composition and effects on microbial populations have access to whole sputum, both as is and in 50% glycerol, in this repository. Those who study polymicrobial communities and need gDNA or RNA for sequencing have a diverse group of patients in all age categories to select from. These represent a few examples for which the creation and catalogue of a vast repository of isolates is beneficial to further CF research.

II. Results

It is unsurprising that most collected samples come from adult patients as seen in Table 1. There is a lack of children and adolescents whose parents or legal guardians consent to study participation. As such, the overrepresentation of adult samples in this study is unavoidable. Additionally, as the predominant pathogens in children are *S. aureus* and *H. influenzae*, the number of children infected with *P. aeruginosa* is few. Children also do not produce much sputum and throat swab cultures are easier to obtain from younger patients. This, however, prevents an accurate representation of the lower airways' polymicrobial community from being depicted. In an attempt to correlate plate growth with clinical status, there was no evidence that clinical status affects the bacterial population present in the lungs, as demonstrated by inconsistent recovery of microorganisms on selective media.

Table 1: Patient list

Patient ID Tag	Age	Condition	Sex
21314a	12	Exacerbation	Male
21314b	10	No exacerbation	Female
21314c	11	No exacerbation	Male
22014a	16	No exacerbation	-
22014b	39	No exacerbation	Male
22014c	23	Exacerbation	-
22014d	29	Exacerbation	-
22714a	55	No exacerbation	Male
22714b	27	Exacerbation	Female
22714c	12	No exacerbation	Male
22714d	31	No exacerbation	Male
22714e	26	Exacerbation	-
3614a	28	No exacerbation	Female
3614b	29	No exacerbation	Male
3614c	38	No exacerbation	Male
31314a	28	No exacerbation	Male
32014a	16	No exacerbation	Male
32014b	41	No exacerbation	Male
5814a*	6	No exacerbation	Female
5814b*	14	No exacerbation	Male
5814c*	5	No exacerbation	Male
5814d*	8	No exacerbation	Female
5814e	11	No exacerbation	Male
5814f*	32	Exacerbation	Male
5814g	20	No exacerbation	Female
5814h	32	Exacerbation	Female
51514a	28	No exacerbation	Male
51514b*	7	No Exacerbation	Female
51514c	16	Exacerbation	Female
51514d	20	Exacerbation	Female
6514a	19	Exacerbation	Female
6514b	28	No exacerbation	Female
6514c	55	Exacerbation	Male
6514d	20	No exacerbation	Male
6514e*	18	No exacerbation	Female
61214a	24	Exacerbation	Female
61214b	28	No exacerbation	Male
61214c	22	Exacerbation	Male
61214d	28	No exacerbation	Male
73114a	25	No exacerbation	Male
8714a	13	Exacerbation	Male
8714b*	10	No exacerbation	Female
8714c	32	No exacerbation	Female
8714d*	29	No exacerbation	Male
8714e	26	No exacerbation	Female
81414a*	10	No exacerbation	Female
81414b*	25	No exacerbation	Female
81414c	39	No exacerbation	Male

81414d	32	-	Female
9414a*	20	No exacerbation	Male
9414b	28	No exacerbation	Female
9414c	33	Exacerbation	Female
9414d	25	Exacerbation	Male
102314a*	7	No exacerbation	Female
102314b*	8	No exacerbation	Female
102314c*	14	No exacerbation	Male
102314d*	8	No exacerbation	Male
102314e*	16	No exacerbation	Male
102314f*	17	No exacerbation	Female
102314g	14	No exacerbation	Male
102314h*	28	No exacerbation	Female
102314i*	25	No exacerbation	Female
102314j*	12	No exacerbation	Male
102314k*	13	No exacerbation	Male
102314l*	18	No exacerbation	Male
102314m*	28	No exacerbation	Female
102314n*	36	No exacerbation	Female
102314o	~30/36	No exacerbation	Male
11614a*	7	No exacerbation	Female
11614b*	12	No exacerbation	Male
11614c*	20	Exacerbation	Female
11614d	10	No exacerbation	Female
11614e	16	Exacerbation	Male
112014a	13	No exacerbation	Male
112014b	24	No exacerbation	Male
112014c	32	Exacerbation	Male
112014d	26	Exacerbation	Male
112014e	10	No exacerbation	Female
2515a	39	Exacerbation	Male
2515b	30	Exacerbation	Male
2515c	30	Exacerbation	Male
2515d	17	No exacerbation	Male
21215a	13	Exacerbation	Male
21915a	33	Exacerbation	Male
21915b	40	No exacerbation	Male

* denotes a throat swab was collected instead of sputum
- indicates information was not provided

A total of 85 samples were collected from the Oklahoma Cystic Fibrosis Clinic at the University of Oklahoma Children's Hospital from February 2014 to February 2015 and are listed in Table 1. Of these, 28 were throat swabs and the remaining 57 are sputum samples. From the sputum samples, 7 were collected from children, 9 from adolescents, and 41 from adults. Among those samples, 1 child, 4 adolescents, and 19 adults were experiencing pulmonary exacerbations or receiving treatment for respiratory distress at the time of sampling.

As stated previously, one of the purposes in building a repository that includes sputum, gDNA, and RNA is to foster collaborations within and outside Oklahoma State University. Table 2 is a comprehensive list of those patients who have available sputum, in both 50% glycerol and without any additives, gDNA, or RNA for studies. Of the 85 collected samples, 52 patients have associated glycerol sputum stocks and whole sputum frozen at -80 °C. 43 patients have gDNA and RNA extracted from the sputum.

Each patient had sputum struck onto three nutrient media (BHI, LB, NA) and four types of selective media (MSA, BCSA, MAC, PIA). After incubation of all nutritional and selective media plates at 37°C, the plates demonstrating microbial growth were noted for colony morphology and photographed for record-keeping purposes. A plus mark in Table 2 indicates that the sputum cultured microorganisms on that particular media type. This growth was termed as "recoverable growth" and will be referred to as such throughout the course of this text.

Table 2: Patients with Sputum, gDNA, and RNA

Patient ID Tag	Sputum in 50% Glycerol	Sputum	gDNA	RNA
21314a	+	+	+	+
21314b	+	+		
21314c	+	+		
22014a	+	+	+	+
22014b	+	+	+	+
22014c	+	+	+	+
22014d	+	+	+	+
22714a				
22714b	+	+		
22714c			+	+
22714d	+	+	+	+
22714e	+	+		
3614a	+	+	+	+
3614b	+	+	+	+
3614c	+	+		
31314a	+	+	+	+
32014a				
32014b	+	+	+	+
5814a*				
5814b*				
5814c*				
5814d*				
5814e	+	+		
5814f*				
5814g	+	+		
5814h				
51514a	+	+	+	+
51514b*				
51514c	+	+	+	+
51514d	+	+	+	+
6514a	+	+	+	+
6514b	+	+	+	+
6514c	+	+	+	+
6514d	+	+	+	+
6514e*				
61214a	+	+	+	+
61214b	+	+	+	+
61214c	+	+	+	+
61214d	+	+	+	+
73114a	+	+	+	+
8714a	+	+	+	+
8714b*				
8714c	+	+	+	+
8714d*	+	+		
8714e	+	+	+	+
81414a*				
81414b*				

81414c	+	+		
81414d	+	+	+	+
9414a*				
9414b	+	+	+	+
9414c	+	+		
9414d	+	+		
102314a*				
102314b*				
102314c*				
102314d*				
102314e*				
102314f*				
102314g	+	+	+	+
102314h*				
102314i*				
102314j*				
102314k*				
102314l*				
102314m*				
102314n*				
102314o	+	+	+	+
11614a*				
11614b*				
11614c*				
11614d			+	+
11614e	+	+	+	+
112014a	+	+	+	+
112014b	+	+	+	+
112014c	+	+	+	+
112014d	+	+	+	+
112014e	+	+	+	+
2515a	+	+	+	+
2515b	+	+	+	+
2515c	+	+	+	+
2515d	+	+	+	+
21215a	+	+	+	+
21915a				
21915b	+	+	+	+

+ indicates the patient has sputum, gDNA, or RNA available for research

Table 3: Growth on Selective Media

Patient	BHI	LB	NA	S. Milk	MSA	BCSA	MAC	PIA
Under 13								
21314a	+	+		+			+	
21314b	+	+		+			+	
21314c	+							
22714c	+	+	+	+	+	+	+	+
5814e	+	+	+	+	+			
11614d	+	+	+	+	+			
112014e	+	+						
13-18								
22014a	+	+	+	+	+		+	+
32014a	+	+	+	+	+	+	+	+
51514c	+	+	+	+	+	+		
8714a	+	+	+	+	+		+	+
102314g	+	+	+	+				
11614e	+	+	+	+	+		+	+
112014a	+	+	+	+	+	+	+	
2515d	+	+	+	+	+	+	+	+
21215a	+	+	+	+	+	+	+	+
Over 18								
22014b	+	+	+	+	+		+	+
22014c	+	+	+	+	+	+	+	+
22014d	+	+	+	+	+		+	+
22714a	+	+	+	+			+	+
22714b	+	+	+	+	+	+	+	+
22714d	+	+	+	+	+		+	+
22714e	+	+	+	+	+		+	+
3614a	+	+	+	+	+	+	+	+
3614b	+	+	+	+	+	+	+	+
3614c	+	+	+	+	+		+	
31314a	+	+	+	+	+		+	+
32014b	+	+	+	+	+	+	+	+
5814g	+	+	+	+	+	+	+	+
5814h	+	+	+	+	+			
51514a	+	+	+	+	+			
51514d	+	+	+	+	+	+	+	+
6514a	+	+	+	+	+			
6514b	+	+	+	+		+	+	+
6514c	+	+	+	+	+	+	+	+

6514d	+	+	+	+	+	+	+	+
61214a	+	+	+	+	+		+	+
61214b	+	+	+	+	+			
61214c	+	+	+	+	+	+	+	+
61214d	+	+	+	+	+	+	+	+
73114a	+	+	+	+	+		+	+
8714c	+	+	+	+	+	+	+	
8714e	+	+	+	+	+		+	
81414c	+	+	+	+	+		+	+
81414d	+	+	+	+	+	+	+	+
9414b	+	+	+	+	+	+	+	+
9414c	+	+	+	+	+		+	+
9414d	+	+	+	+			+	+
102314o	+	+	+	+	+		+	+
112014b	+	+	+	+	+	+	+	+
112014c	+	+	+	+	+	+	+	+
112014d	+	+	+	+	+		+	+
2515a	+	+	+	+			+	+
2515b	+	+	+	+	+		+	+
2515c	+	+	+	+	+		+	+
21915a	+	+	+	+	+	+	+	+
21915b	+	+	+	+	+			

+ denotes the sputum cultured microorganisms on media

As seen in Table 3, patients are colonized with a diverse population of microorganisms. In addition to *Pseudomonas,* the salt-tolerant organisms that have also been isolated are most likely to belong to the *Staphylococcus* genus. Fungi have also grown on both nutrient and selective media in a select number of patients. Individuals isolates observed on BCSA have had their 16S gene sequenced by an undergraduate student and matched to *Achromobacter* spp., *Acidovorax* spp., and *Stentrophomonas maltophilia* sequences.

II. a. Clinical Status of CF patients

After observing recovered growth, we asked the question if growth recovered from selective media correlates with the clinical status of the patient. Table 4 is the same as Table 3 with an added field for clinical status. An "e" denotes that the patient was experiencing an exacerbation at the time of sputum collection.

The relationship between clinical status and recoverable growth on media does not appear to have any correlation with each other. There are inconsistencies in recoverable growth between patients experiencing exacerbations. As an example, Patient 61214c has recoverable growth on all nutrient and selective media but Patient 61214b does not have recoverable growth on BCSA, MAC, and PIA, yet both patients were experiencing exacerbations. This indicates that pulmonary exacerbation may not be dependent on the composition of the polymicrobial community, a finding that agrees with current literature [37].

Table 4: Clinical Status and Growth on Selective Media

Patient	Exacerbation	BHI	LB	NA	S. Milk	MSA	BCSA	MAC	PIA
Under 13									
21314b		+	+		+			+	
21314c		+							
22714c		+	+	+	+	+	+	+	+
5814e		+	+	+	+	+			
11614d		+	+	+	+	+			
112014e		+	+						
21314a	e	+	+		+			+	
13-18									
22014a		+	+	+	+	+		+	+
32014a		+	+	+	+	+	+	+	+
102314g		+	+	+	+				
112014a		+	+	+	+	+	+	+	
2515d		+	+	+	+	+	+	+	+
51514c	e	+	+	+	+	+	+		
8714a	e	+	+	+	+	+		+	+
11614e	e	+	+	+	+	+		+	+
21215a	e	+	+	+	+	+	+	+	+
Over 18									
22014b		+	+	+	+	+		+	+
22714a		+	+	+	+			+	+
22714d		+	+	+	+	+		+	+
3614a		+	+	+	+	+	+	+	+
3614b		+	+	+	+	+	+	+	+
3614c		+	+	+	+	+		+	
31314a		+	+	+	+	+		+	+
32014b		+	+	+	+	+	+	+	+
5814g		+	+	+	+	+	+	+	+
51514a		+	+	+	+	+			
6514b		+	+	+	+		+	+	+
6514d		+	+	+	+	+	+	+	+
61214d		+	+	+	+	+	+	+	+
73114a		+	+	+	+	+		+	+
8714c		+	+	+	+	+	+	+	
8714e		+	+	+	+	+		+	
81414c		+	+	+	+	+		+	+
81414d		+	+	+	+	+	+	+	+
9414b		+	+	+	+	+	+	+	+

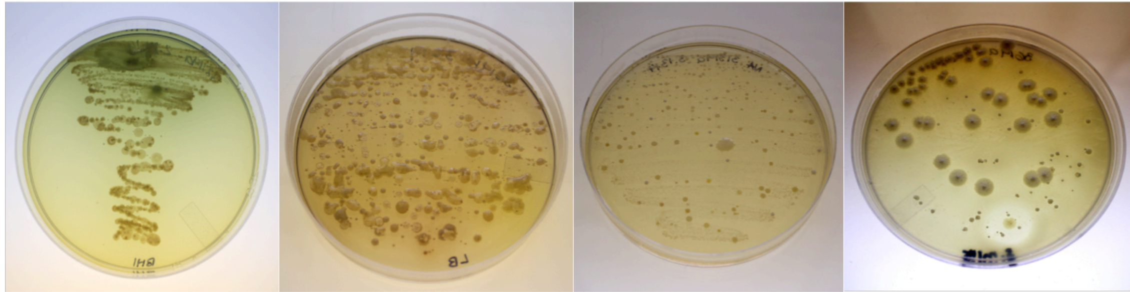
9414c		+	+	+	+	+		+	+
102314o		+	+	+	+	+		+	+
21915b		+	+	+	+	+			
22014c	e	+	+	+	+	+	+	+	+
22014d	e	+	+	+	+	+		+	+
22714b	e	+	+	+	+	+	+	+	+
22714e	e	+	+	+	+	+		+	+
5814h	e	+	+	+	+	+			
51514d	e	+	+	+	+	+	+	+	+
6514a	e	+	+	+	+	+			
6514c	e	+	+	+	+	+	+	+	+
61214a	e	+	+	+	+	+		+	+
61214b	e	+	+	+	+	+			
61214c	e	+	+	+	+	+	+	+	+
9414d	e	+	+	+	+			+	+
112014c	e	+	+	+	+	+	+	+	+
112014d	e	+	+	+	+	+		+	+
2515a	e	+	+	+	+			+	+
2515b	e	+	+	+	+	+		+	+
2515c	e	+	+	+	+	+		+	+
21915a	e	+	+	+	+	+	+	+	+

e indicates the patient experienced pulmonary exacerbation at time of sample collection
+ denotes the sputum cultured microorganisms on the media

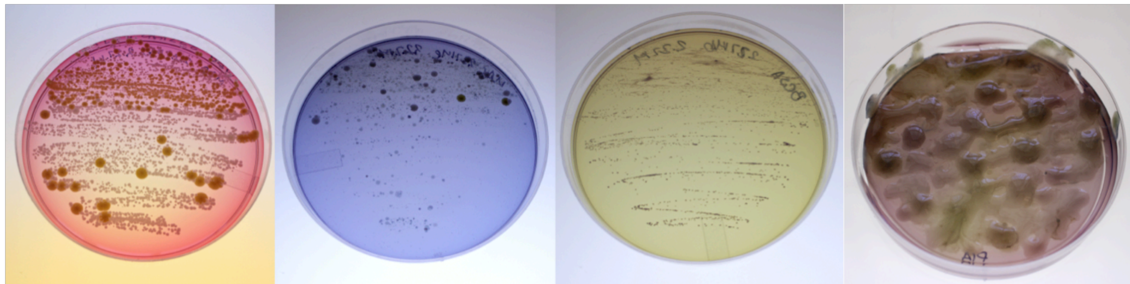
II. b. Inter-patient Colony Morphology Variability

A wide diversity of microorganisms has been isolated from sputa. The recoverable isolates show a wide range of colony morphologies, such as smooth and rough phenotypes, and biochemical characteristics that are either representative of different species or phenotypes of the same species. Most plates grow heavily mixed cultures after incubation and Figure 1 represents the vast diversity of the CF lung. Even in nutrient media like BHI and LB, the high degree of observed diversity between patients is remarkable because no two patients will cultivate growth on nutrient media with identical colony morphologies and the same amount of growth of each observed morphology. Figure 1 represents a sampling of the colony morphologies of the recovered microorganisms.

With special attention to the growth on selective media, a wide array of colony morphologies and biochemical characteristics have been observed. Both protease producers and non-protease producers have grown on skim milk agar and fungi typically grow the best on this media. Mannitol Salts agar (MSA), which is traditionally used for the isolation of Gram-positive, salt-tolerant organisms and the identification of *Staphylococcus aureus* by mannitol fermentation, shows both white and yellow colonies in large and pinpoint sizes in Fig, 1E. Colonies on *Burkholderia cepacia* Selection agar (BCSA) tend to be very small and purple in color, as seen in Fig. 1F, with occasional pink discoloration of the agar demonstrated in Fig. 2F. Colony growth observed on MacConkey's agar represents a potential niche for further study. These MacConkey's isolates are usually smaller in size and purple in color.



A. 22714a on BHI | B. 3614b on LB | C. 31314a on NA | D. 3614a on S. Milk



E. 22714b on MSA | F. 22714e on MAC | G. 22714b on BCSA | H. 3614b on PIA

Figure 1: A representation of the diversity of microbial growth seen on nutrient and selective media. A was growth on BHI that shows a mixed culture of smooth and rough colony types with some green pigment production seeping into the agar. B showed a mixture of flat and rough colonies with no agar discoloration. C was growth on NA showing translucent and light brown-pigmented colonies. D demonstrated growth of a variety of microorganisms and the production of casein proteases by a single colony. E was growth on MSA with small, white round colonies co-cultured with yellow, round colonies that ferment mannitol. F was growth on BCSA with pinpoint, purple colonies. G was mixed growth on MAC with pinpoint and large round colonies. H was heavily mucoid growth on PIA with a mixture of green and purple agar discoloration and pigment production.

II.c. Intra-patient Colony Morphology Variability

It is of interest to note that comparison of growth on all plate media from a single patient exhibits slight differences in recovered growth and morphology. As an example, Patient 32014b was chosen because all eight nutrient and selective media yielded recoverable growth. Focusing on the growth seen in Fig. 2A, Fig. 2B, and Fig. 2C, they appear to exhibit similar growth of a small, cream-colored, smooth colony, but how well they grow and the intensity of pigmentation varies from BHI, LB, to NA. This may be explained by the composition of the media themselves, as BHI is richer in nutrients than LB, and LB is richer than NA. Additionally, Fig. 2A and 2B appear to be single colony cultures but this is contradicted by the mixed culture observed on NA (Fig. 2C) and skim milk (Fig. 2D) plates. This apparent contradiction can also be explained by differences in nutrient richness. The available nutrients in BHI and LB that are absent in NA may encourage the growth of one microorganism over the other. This cream-colored smooth colony observed in all three plates may also be growing on top of the flat, translucent colonies seen in NA, therefore leading to the observation of a single colony type on BHI and LB.

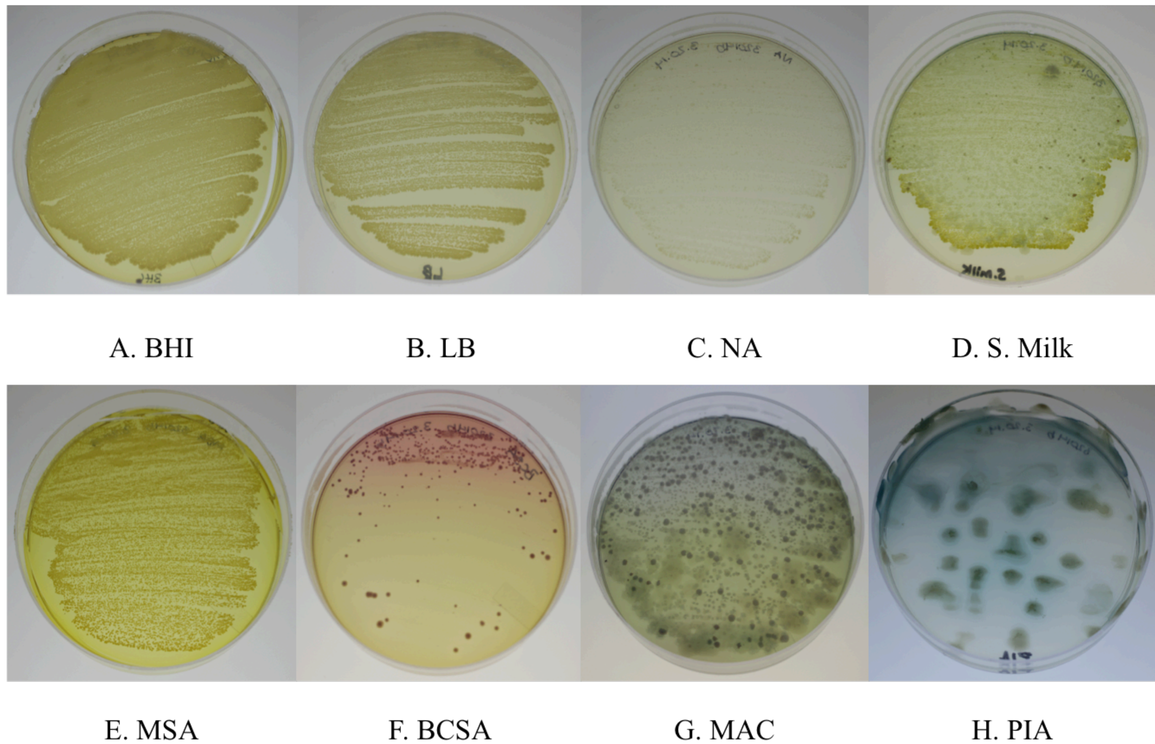


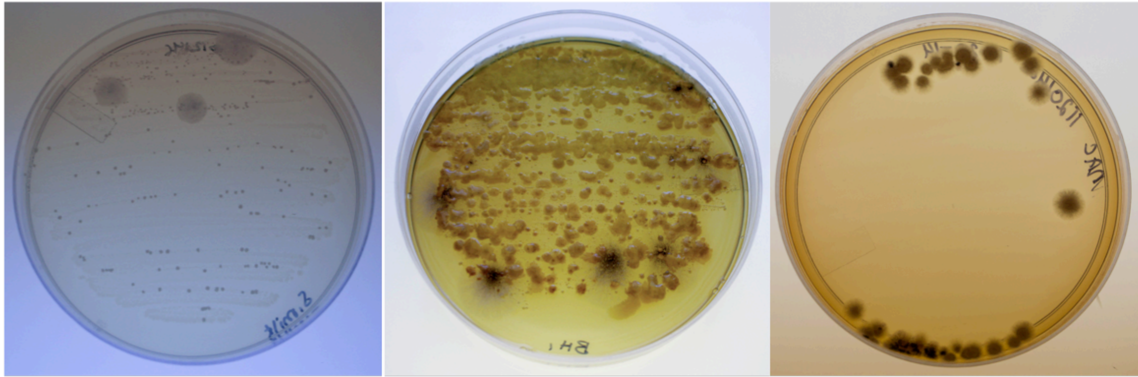
Figure 2: Growth on Selective Media from Patient 32014b. A appeared to be a single culture on BHI with smooth, dark cream colonies. B also appeared to be a single culture on LB with smooth, light cream-pigmented colonies. C was a mixed culture with smooth, cream-pigmented colonies and flat, translucent colonies. D was also mixed culture that cleared the entire plate and had green discoloration of the agar, containing flat, translucent colonies, brown, small growth, moist, green colonies, and shiny, yellow colonies. E contained light yellow, pinpoint colonies that turned the MSA plate yellow. F had pink colonies in large and small sizes with slight pinking of the agar in the initial streak. G showed mixed growth of large, yellow green, moist colonies, white, round shiny colonies, and translucent, round colonies that also tinted the agar green. Finally, H demonstrated growth of indistinguishable mucoid colonies that are blue, green, and cream-colored and heavy alginate producers.

II. d. Fungal Diversity

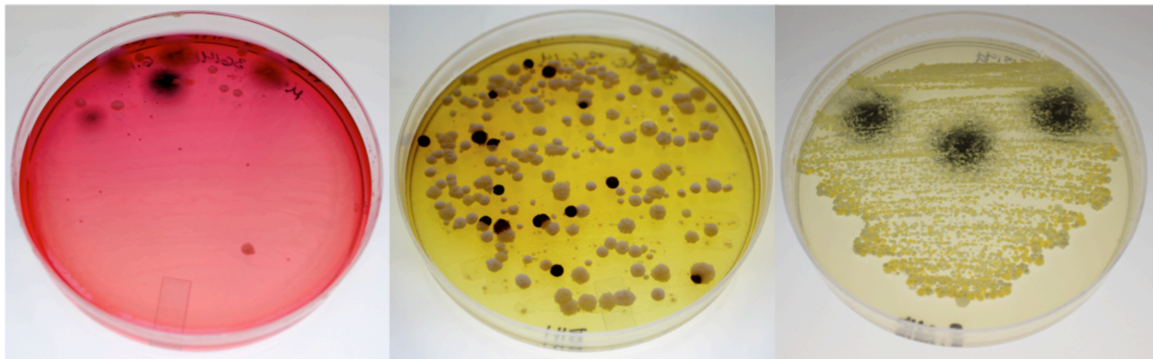
Filamentous fungi such as *Aspergillus fumigatus* and *Aspergillus flavus* have been previously identified in sputum cultures by pyrosequencing [28] and traditional plate culturing but their role in respiratory infections is unknown and controversial [47, 52]. Figure 3 demonstrates the diversity of recovered fungi on various plate media. Most fungal growth is either black or white in color and grows into the agar. Fungi isolates grow in filamentous patterns, suggesting the fungi cultured on plate media may be *A. fumigatus* or *A. flavus*.

II. e. *Pseudomonas* diversity

Finally, the growth on *Pseudomonas* Isolation agar (PIA) may be the most interesting of the recoverable diversity. As seen in Figure 4, some adult patients have distinct, cream-colored colonies with varying pigment production. Other adult patients produce such mucoid colonies that the alginate spills onto the plate lid during incubation. There is usually a bright array of colors from blue-green to purple observed.

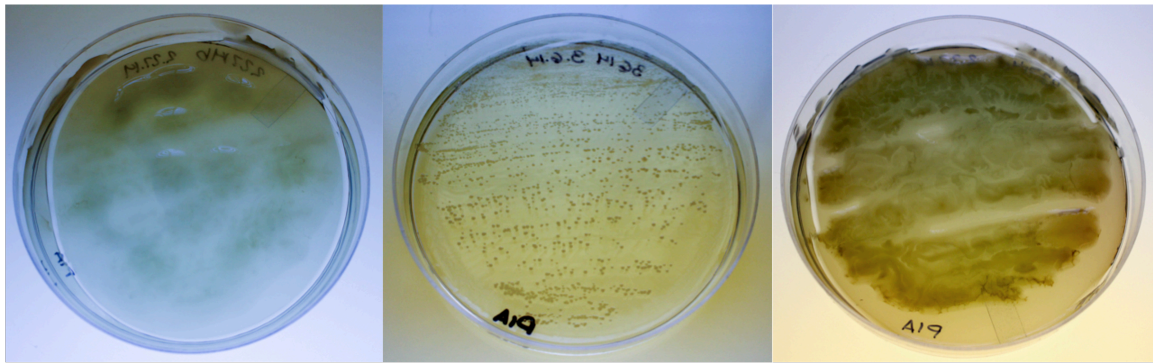


A. 61214c on S. Milk B. 3614b on BHI C. 112014a on MAC

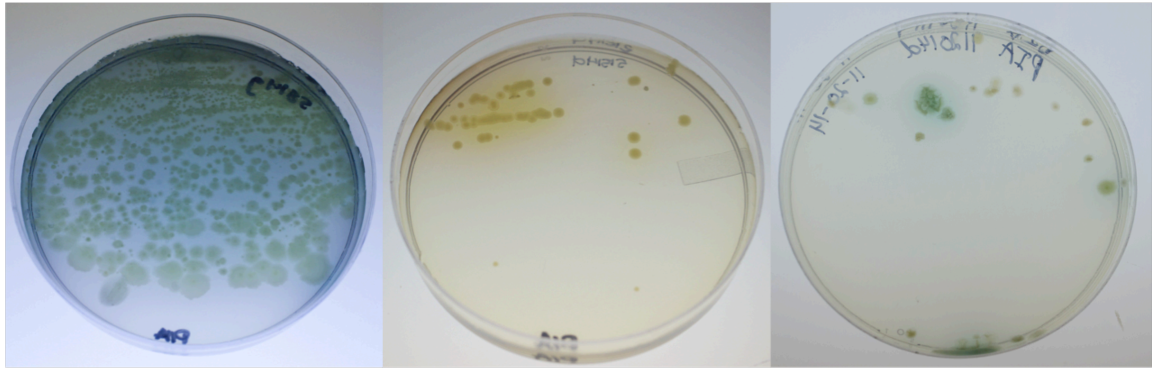


D. 3614b on MSA E. 3614c on BHI F. 51514d on S. Milk

Figure 3: Representation of Fungal Growth Seen on Selective Media. 3A demonstrates a white, filamentous growth. 3B shows black, filamentous growth. 3C is an example of a black, slightly filamentous fungus. 3D shows two types of fungi on MSA, a light gray filamentous growth and a black, filamentous fungus. 3E shows black, non-filamentous fungal growth. 3F demonstrates black, highly filamentous fungus growth in co-culture with *Pseudomonas*.



A. 22714b B. 3614a C. 22714d



D. 5814g E. 51514d F. 112014b

Figure 4: A representation of the diversity of *Pseudomonas* growth on PIA. A showed heavy mucoidy with green and yellow pigment production. B showed individual colonies with yellow pigmentation. C was also mucoid growth with green and purple pigment production. D demonstrated individual smooth colonies with blue-green pigmentation. E also demonstrated individual colonies with yellow pigmentation. F has a mixture of mucoid and smooth colony phenotypes with blue-green or yellow pigment production respectively.

III. Summary of Repository Collection

The collected repository to date represents a collection of sputum, polymicrobial communities, and strains from patients of all ages. This library was built to meet the needs of various research groups. Whole sputum, recoverable growth, and isolation of gDNA and RNA were catalogued within this large repository.

With special attention given to the recovered growth seen on selective media, there exists a wide diversity of recovered microorganisms on the plates. Although the precise make up of these recovered isolates are in need of sequencing to confirm their identities, their roles in advancing pulmonary decline and interspecies interactions are niches for potential studies.

IV. Introduction to the Virulence Factors of *P. aeruginosa*

The virulence factors of *P. aeruginosa* are well-studied and characterized for their role in pathogenesis. In CF, the expression of these virulence factors is instrumental for chronic infection and advancement in host pulmonary decline. In addition to possessing a dangerous set of virulence factors contributing to decreased lung function, the intrinsic antimicrobial resistance mechanisms of *P. aeruginosa* allow the bacterium to avoid clearance by antimicrobial means. The inability of the mucocilliary escalator to clear pathogens entrapped by mucosal secretions and the formation of polymicrobial biofilms allow for continued persistence of *P. aeruginosa* in the lungs.

In this study, ten virulence factors were assessed for differences in expression in relation to patient age. These factors were chosen on the basis of their contribution to pathogen colonization and host tissue destruction. Swim, swarm, and twitch motilities were assessed for their role in colonization of the lung. Similarly, the production of biosurfactants to lower surface tension allows for *P. aeruginosa* to establish and maintain proper biofilm architectures. After

colonizing the lung, secreted factors that assist in cell-to-cell communication and facilitate microbial growth are necessary to remain present in an otherwise hostile environment. These secreted factors include the production of quorum sensing signals and siderophores for iron uptake, however, this study assessed the production of siderophores only. Similarly, as the amount of oxygen present in the lung is highly variable, the use of nitrate or nitrite as an alternate electron acceptor is necessary for growth and advantageous for microorganisms residing deep within a biofilm that are more limited to oxygen. Finally, the secretion of exoproteins causing host damage is key in pathogenesis. The production of proteases, elastases, and the redox inhibitor hydrogen cyanide gas were also studied for differences in their expression in varying patient age groups. As such, this survey of a selection of virulence factors known to contribute to lung colonization and decline of pulmonary function provides the foundation for further studies in *P. aeruginosa* phenotypic expression in different age categories in the context of mixed infections and polymicrobial community interactions.

V. Results

V. a. Hydrogen Cyanide

The production of HCN is linked to lethal inhibition of cellular respiration as HCN gas binds to cytochrome c oxidase and other metalloenzymes to prevent electron transfer from occurring [4, 8, 80]. This leads to tissue death and results in pulmonary decline from loss of tissue. This study demonstrates that HCN is detected in all patients; however, the amount varies from patient to patient. Figure 5 suggests that HCN production peaks in the early stages of colonization as indicated by the increase in detected gas from child isolates compared to adolescent isolates. In particular, 32014a shows the highest percentage of gas production with all isolates testing positive (n=30). Additionally, the results suggest that after establishment in the lung, HCN production wanes, indicated by the decrease in the number of isolates testing positive

for gas production from adolescent isolates to adult isolates. That is not to say gas production ceases entirely—the amount of weak HCN gas-producing isolates, as indicated by partial blue coloration of the filter disk soaked in detection solution, increases from adolescent to adult isolates. It is of interest to note that 61214c exhibits the highest amount of non-HCN gas producing isolates (20%) and weak gas-producing isolates (63%) since the patient was experiencing pulmonary exacerbation at the time of sputum collection.

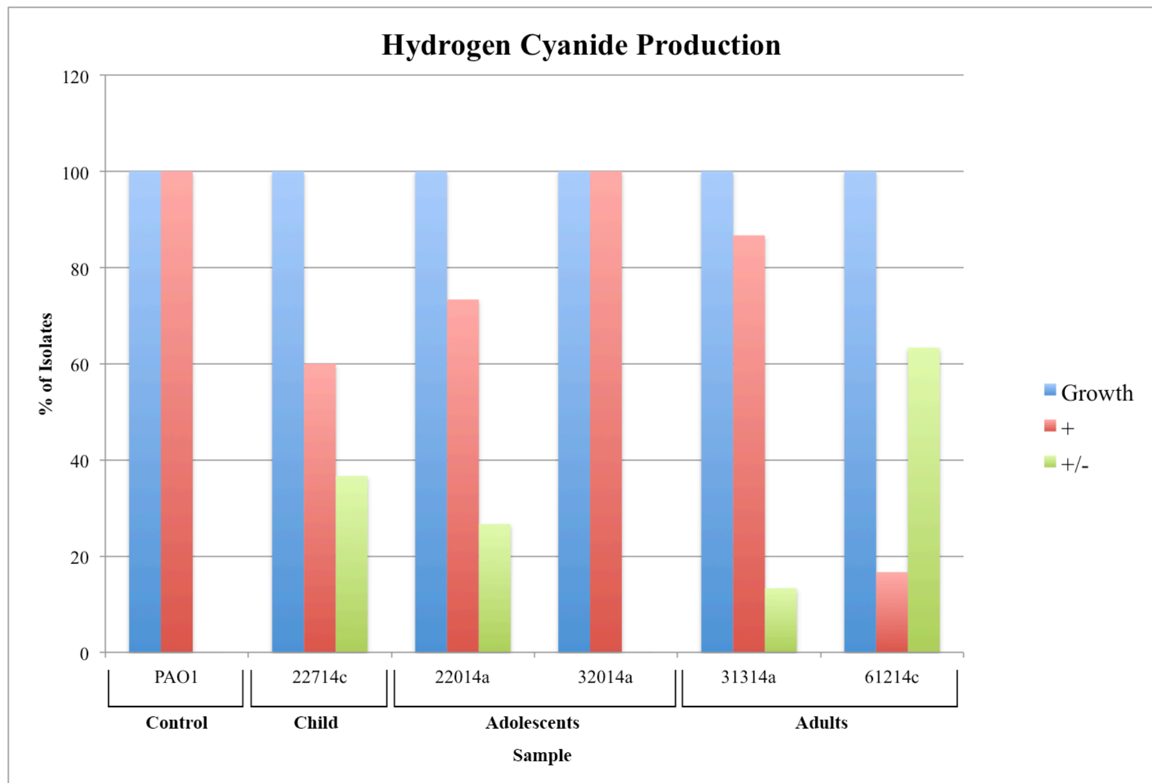


Figure 5: Hydrogen Cyanide Production: All isolates grew on LB plates. PAO1 was positive for HCN production. Sample size for patients was $n = 30$. 22714c was 60% positive, 37% weakly positive and 3% negative. 22014a was 73% positive and 27% weakly positive. 32014a was 100% positive. 31314a was 87% positive and 13% weakly positive. 61214c was 17% positive, 63% weakly positive and 20% negative.

V. b. Pigment Production

While the brightly colored pigments are characteristic of *P. aeruginosa*, the colored molecules also act as important virulence factors. Pyocyanin and pyoverdine are widely studied for their role as a secondary redox metabolite and a major siderophore respectively. In this study, pyoverdine production is assessed using *Pseudomonas* Medium B as described by Atlas. Most patient isolates were able to grow on PMB as indicated by the percentage of growth in Figure 6. A positive result was indicated by the presence of a green-yellow pigment, which is characteristic of pyoverdine. Other possible pigment colors that may be observed are blue-green for pyocyanin, red for pyorubin, and red-brown for pyomelanin [109]. Because pyoverdine is fluorescent, plates were also observed under UV light to confirm the production of a blue-green fluorescent pigment.

In general, isolates from all patient ages produce pigments but the amount of pigment appears to decline with age, as seen in Figure 6. Comparison between adult isolates from 31314a and 61214c shows a drastic difference in pigment production. This raises the question of whether the host's clinical status affects the phenotypic expression of the colonized *P. aeruginosa,* as 31314a was clinically stable and 61214c was experiencing pulmonary exacerbation.

The final pigmentation assay addressed the question of pigment production during denitrification. In conditions of low oxygen or oxygen depravation, *P. aeruginosa* is able to substitute nitrate or nitrite as an electron acceptor [19, 102]. In the CF lung where higher amounts of nitrate or nitrite are present and the oxygen demand of the many microorganisms colonizing the lung reduces the amount of oxygen available, this ability is extremely important for successful chronic infection. Additionally, bacterium located deep within a mucus plug or biofilm matrix and not near the surface of a biofilm receives lower amounts of oxygen than those at the surface of the biofilm, necessitating the need for an alternate electron acceptor. In an environment where

the levels of oxygen, nitrate, and nitrite constantly fluctuate, denitrification plays an important role in pathogen survival.

Most patient isolates grow in the denitrification media and are capable of producing green-yellow pigments. Denitrification was assessed by checking the semi-solid media for liquification. Preliminary testing showed that PAO1, which is capable of performing denitrification, will completely liquify the media after incubation. In contrast, *P. fluorescens* ATCC 13525, which cannot perform denitrification, was able to grow in culture but unable to liquify the media.

It is of interest to note in Figure 7 that the ability to perform denitrification does not appear to correlate with pigment production, as many isolates that liquified the media did not produce pigments. Adolescent isolates are capable of performing denitrification but between 22014a and 32014a, the percentages of those producing pigments are different. As previously observed in Figure 6, pigment production appears to decline with age.

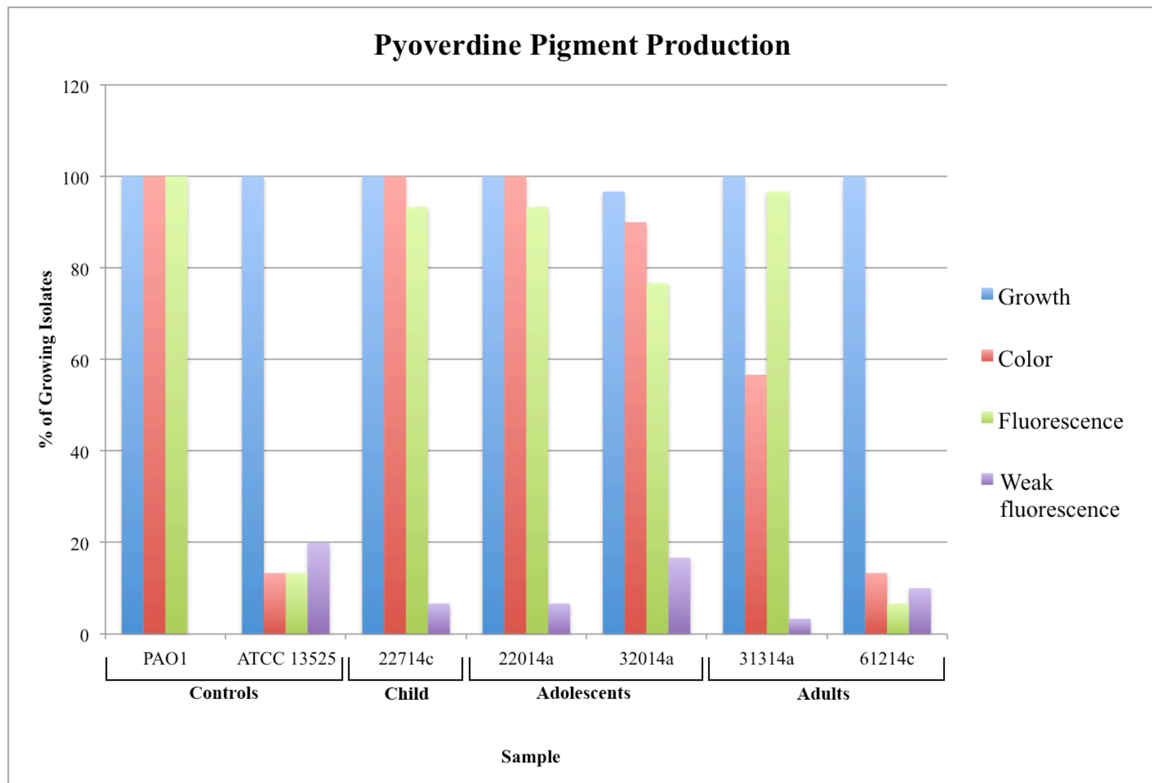


Figure 6: *Pseudomonas* Medium B for pyoverdine production: PAO1 grew on PMB and was 100% positive for color production and fluorescence. *P. fluorescences* ATCC 13525 also grew on PMB and demonstrated color production 13% of the time with 13% of isolates demonstrating fluorescence and 20% of isolates demonstrating weak fluorescence. For patients, a sample size of n = 30 was used. 100% of 22714c isolates grew on PMB with 100% exhibiting color production and 93% of isolates fluoresced while 7% of isolates weakly fluoresced under UV light. 100% of 22014a isolates grew with 100% of isolates producing color and 93% of isolates fluoresced while 7% of isolates demonstrated weak fluorescence. 97% of 32014a isolates grew on PMB with 90% of growing isolates produced color and 77% of growing isolates demonstrated fluorescence under UV light while 17% of growing isolates weakly fluoresced. 100% of 31314a isolates grew with 57% producing color, and 97% fluoresced under UV light and 3% weakly fluoresced. 100% of 61214c isolates grew with 13% producing color, and 7% fluoresced while 10% weakly fluoresced under UV light.

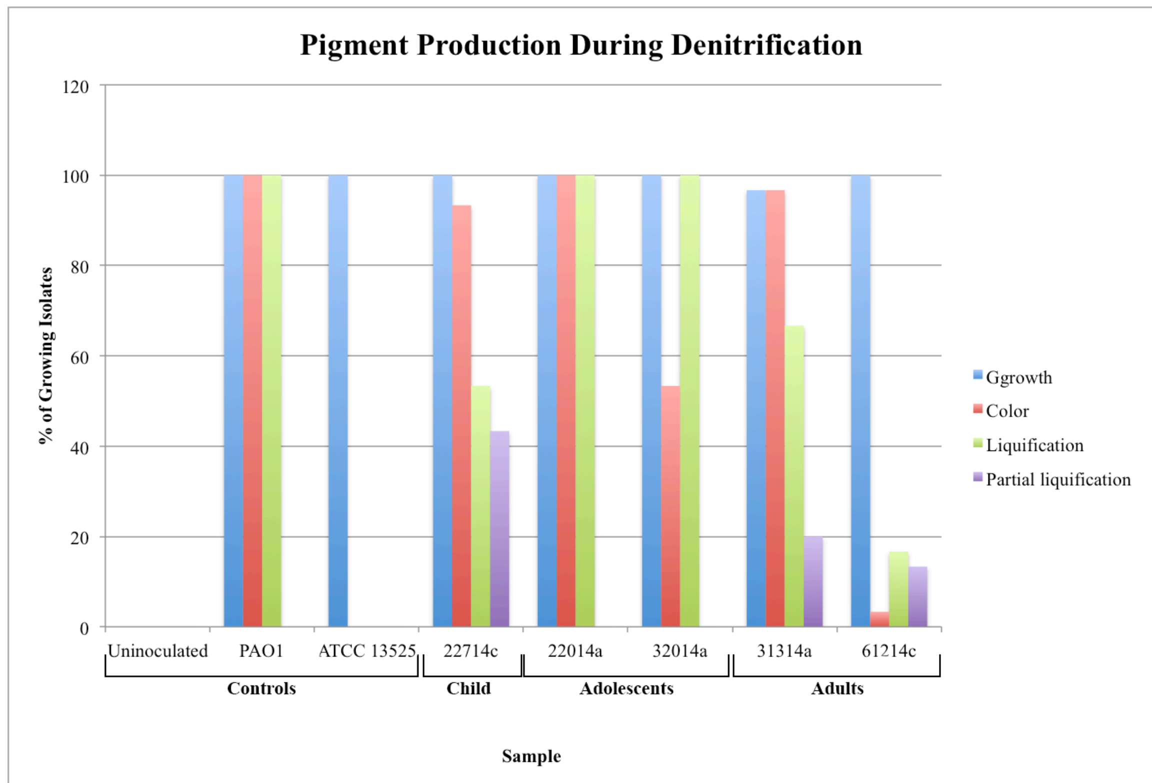


Figure: 7: Pigment production during denitrification: A sample size of n = 30 was used for patients. The uninoculated control demonstrated no growth, pigment production, or liquification. PAO1 grew in the media with 100% color production and liquification. *P. fluorescens* ATCC 13525 also grew 100% of the time but demonstrated no color production and liquification. All 22714c isolates grew with 93% producing color, and 53% of isolates completely liquified the media, 43% partially liquified the media, and 3% did not liquify the media. All 22014a isolates grew, with 100% of isolates producing color and liquifying the media. All 32014a isolates grew with 57% producing color and 100% of isolates liquified the media. 97% of 31314a isolates grew with 97% of growing isolates producing color, and 67% completely liquified the media, 20% partially liquified the media, and 13% did not liquify the media. All 61214c isolates grew with 3% producing color, and 17% completely liquified the media, 13% partially liquified the media, and 70% of isolates did not liquify the media.

V. c. Siderophore Production

The acquisition of iron is necessary for the survival of any bacterium. Unlike eukaryotes that are capable of ingesting iron from their food or recycling iron from cellular components to use in cellular processes, bacteria must obtain iron through other uptake methods. One iron uptake system is the secretion of iron-sequestering molecules called siderophores to scavenge free iron from the environment [13]. This is also important in pathogenic infections of host tissues where the production of such molecules contributes to successful invasion and colonization of host tissues [79]. The two major siderophores of *P. aeruginosa*, pyoverdine and pyochelin, are readily identified using Chrome Azurol S assay [93]. Although the plate does not identify which of the siderophores are being secreted, a presumptive identification can be made when the results are analyzed alongside PMB growth, which identifies fluorescent pigments of which pyoverdine is one.

As seen in Figure 8, PAO1 readily produces siderophores and is capable of growing on an iron-rich media. Most tested isolates also tested positive for siderophore production. 22014a, while all 30 tested isolates were capable of growing on the plate, had 3.33% test negative for siderophore production. When taken together with the results from Figure 6, the negative isolates of 22014a do not appear to produce siderophores at all. Additionally, of the 30 tested isolates of 61214c, only 83.33% were able to grow on media. From that, 76% of growing isolates tested positive for siderophore production and 24% tested negative.

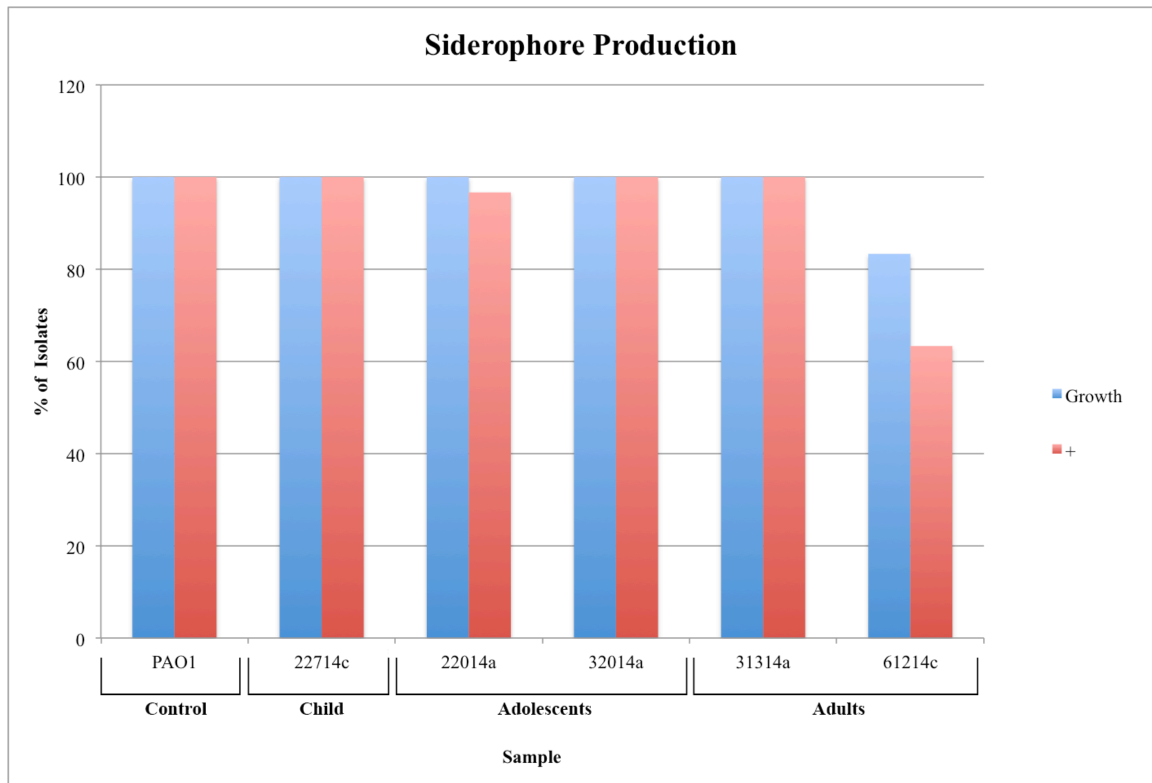


Figure 8: Siderophore production: A sample size of n = 30 was used for all patients. PAO1 grew 100% of the time and was 100% positive for siderophore production. All 22714c isolates grew and were all positive for siderophore production. 100% of 22014a isolates grew with 97% positive for siderophores and 3% negative for siderophore production. 32014a isolates grew 100% on media and were 100% positive for siderophore production. 31314a isolates grew 100% on media and were 100% positive for siderophore production. 83% of 61214c isolates grew with 76% of growing isolates positive for siderophore production and 24% of growing isolates were negative.

V. d. Motility

Prior studies have emphasized the importance of motility in the colonization of host tissues. Patients near the end of their lives show down-regulation of genes associated with flagellum synthesis, indicating a lack of need for them in late chronic infection [49]. pA1092-0245 is a Δ*fliC* mutant and pA4525-33348 is a Δ*pilA* mutant. It is expected that pA1092-0245 will exhibit decreased swim motility and pA4525-33348 will show decreased twitch motility.

The average colony diameter was measured in swim and swarm motility assays, and the average twitch pattern diameter was measured in the twitch motility assay. In comparison to PAO1, 32014a isolates exhibit greater motility for twitch (Fig. 11), are comparable for swarm (Fig. 10), and lesser motility for swim (Fig. 9). Isolates from the other adolescent patient, 22014a, also showed greater twitch motility (Fig. 11) in comparison to other patients, but showed lesser swim (Fig. 9) and swarm (Fig. 10) spreading diameters in comparison to 22714c. Isolates from child patient 22714c exhibited greater swarm (Fig. 10) and swim (Fig. 9) spreading diameters but not twitch (Fig. 11). Isolates from the adult patients, 31314a and 61214c, were hardly motile. Taken as a whole, CF isolates exhibit smaller or equal to PAO1 wild-type motility diameters. The lone exception is 32014a isolates, which on average have a greater twitch diameter than PAO1. This supports the overarching observation that adolescent isolates of *P. aeruginosa* may be actively invading host cells and establishing chronic infections in the epithelial cell layer. This supports previous work demonstrating active *P. aeruginosa* expression of virulence factors during the early years of colonization and the increase in the number of loss-of-function mutants frequently found in adult patients [49, 65, 72, 107], as demonstrated by phenotype assays [72, 107], proteome and transcriptome analysis [49], and animal infection models [65]. Student's t-tests were performed on data from isolates taken from children and adolescents, adolescents and adults, and adults and children, and all had a p-value < 0.05.

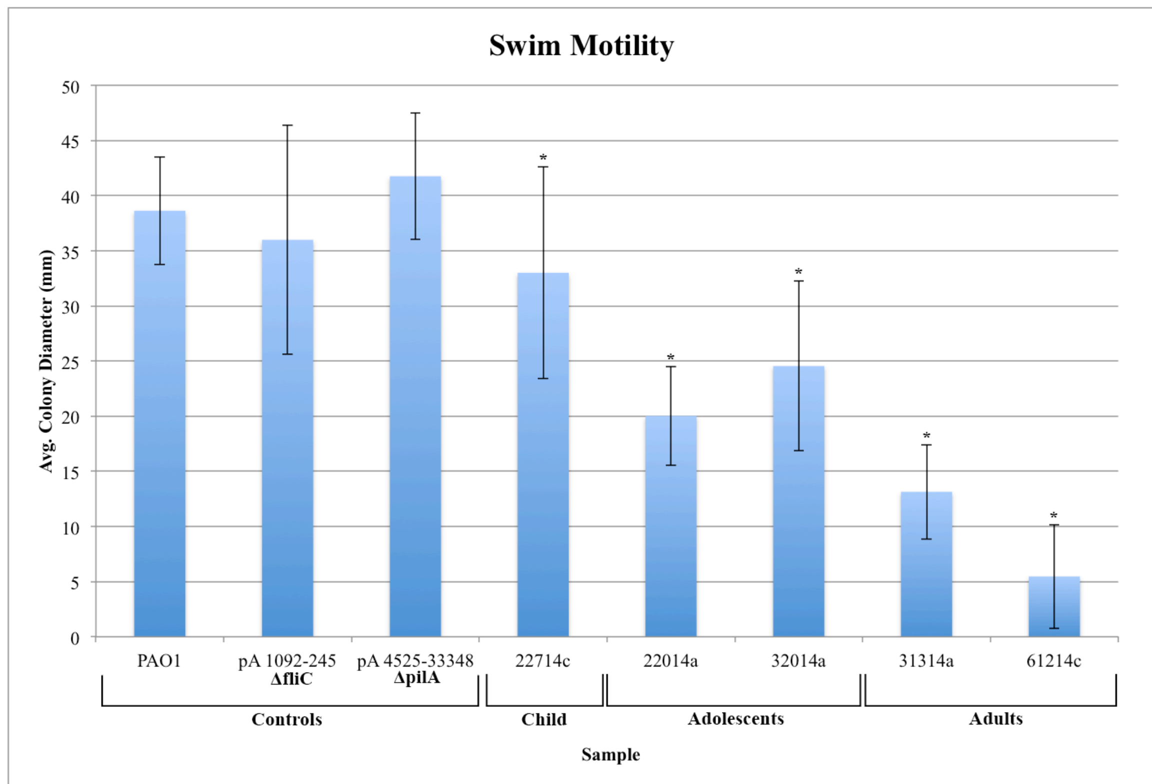


Figure 9: Swim Motility: A sample size of n = 30 was used for all patients. PAO1 had an average diameter of 38.98 mm (s = 4.87). pA1092-0245 had an average diameter of 35.98 mm (s = 5.72). pA 4525-33348 had an average swim diameter of 41.75 mm (s = 5.72). 22714c had an average diameter of 33.00 mm (s = 9.58). 22014a had an average diameter of 20.03 mm (s = 4.50). 32014a had an average diameter of 24.54 mm (s = 7.68). 31314a had an average diameter of 13.14 mm (s = 4.29). 61214c had an average diameter of 5.47 mm (s = 4.70). Error bars represent standard deviation. A single asterisk represents a p-value < 0.05 in comparison to controls. Double asterisks represent a p-value > 0.05 in comparison to controls.

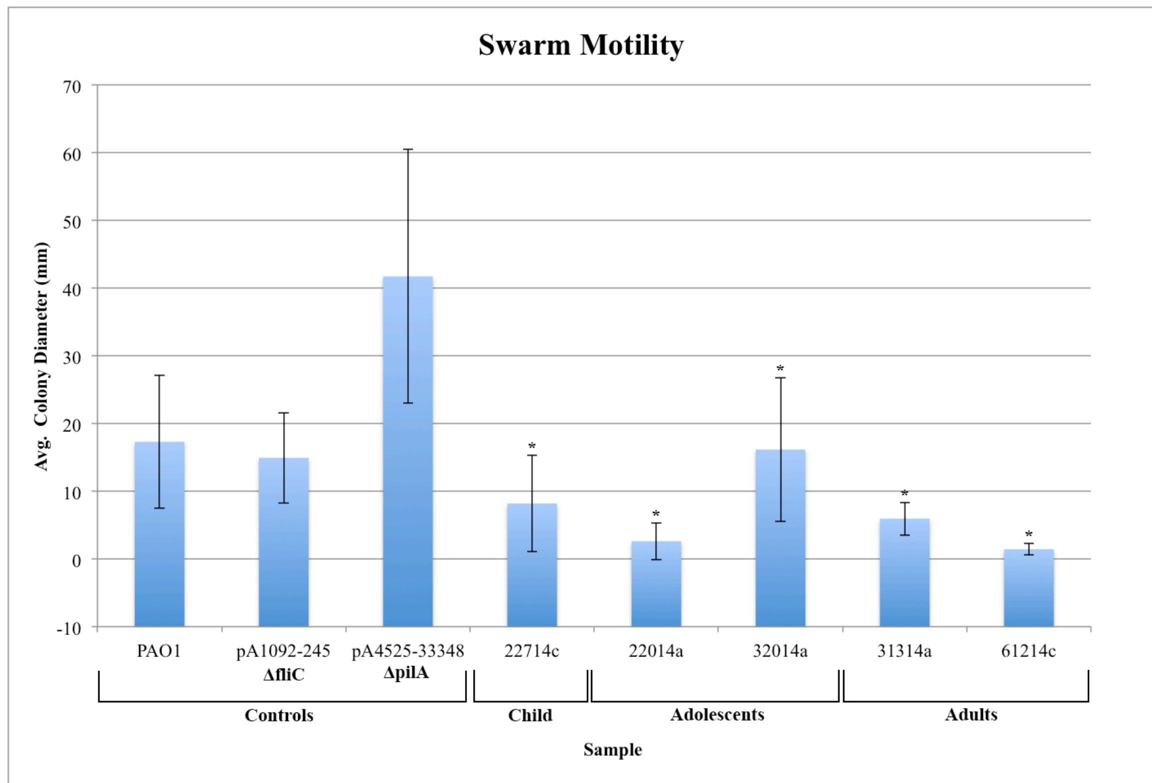


Figure 10: Swarm Motility: A sample size of n = 30 was used for all patients. PAO1 had an average diameter of 17.30 mm (s = 9.80). pA1092-0245 had an average diameter of 14.93 mm (s = 6.68). pA 4525-33348 had an average swim diameter of 41.72 mm (s = 18.74). 22714c had an average diameter of 8.18 mm (s = 7.11). 22014a had an average diameter of 2.62 mm (s = 2.72). 32014a had an average diameter of 16.15 mm (s = 10.60). 31314a had an average diameter of 5.95 mm (s = 2.43). 61214c had an average diameter of 1.42 mm (s = 0.86). Error bars represent standard deviation. A single asterisk represents a p-value < 0.05 in comparison to controls. Double asterisks represent a p-value > 0.05 in comparison to controls.

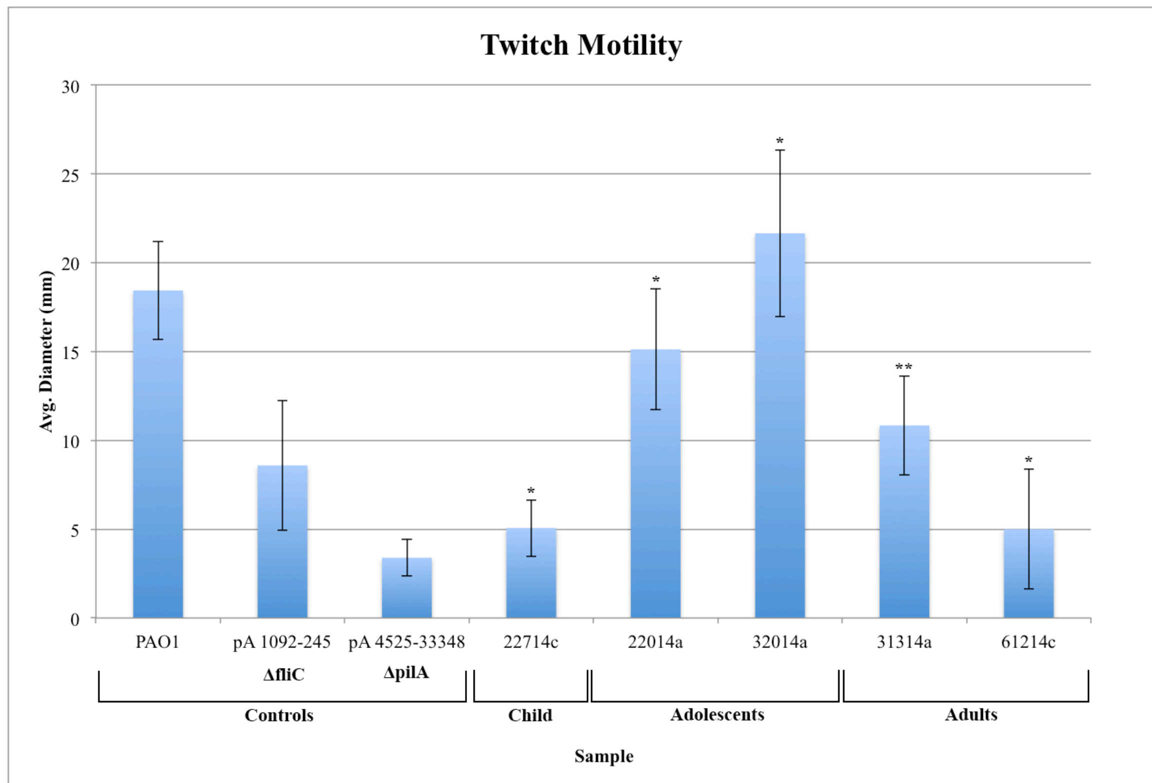


Figure 11: Twitch Motility: A sample size of *n* = 30 was used for all patients. PAO1 had an average diameter of 18.43 mm (*s* = 2.75). pA1092-0245 had an average diameter of 8.58 mm (*s* = 3.64). pA 4525-33348 had an average swim diameter of 3.83 mm (*s* = 1.01). 22714c had an average diameter of 5.06 mm (*s* = 1.59). 22014a had an average diameter of 15.11 mm (*s* = 3.40). 32014a had an average diameter of 21.64 mm (*s* = 4.70). 31314a had an average diameter of 10.83 mm (*s* = 2.79). 61214c had an average diameter of 5.00 mm (*s* = 3.36). Error bars represent standard deviation. A single asterisk represents a p-value < 0.05 in comparison to controls. Double asterisks represent a p-value > 0.05 in comparison to controls.

However, the negative motility controls, pA1092-0245 (Δ*fliC*) and pA4525-33348 (Δ*pilA*), did not behave as expected for the swim and swarm motility assays. Subsequent control experiments varying in the age of the control strains and in the freshness of the motility media consistently produced similar colony diameters for both motility assays. On the other hand, the negative controls behaved as expected in the twitch motility assay, with pA4525-33348 (Δ*pilA*), producing the smallest twitch diameter of the three controls. Taken together, this suggests that the age of the control cultures and the freshness of the prepared motility media are not contributing factors to the explanation of why the negative motility controls were not behaving as expected. Further tests are needed to confirm the insertion of the mutation in both control strains prior to performing further motility experiments.

V. e. Biosurfactant Production

The biosurfactant of *P. aeruginosa* is a class of molecules known as rhamnolipids [27, 44, 97]. They are essential for proper biofilm formation and play a role in maintaining open channels and architecture [27]. The drop-collapsing test qualitatively assesses the production of biosurfactant in the supernatant of a liquid culture [64, 111]. The larger the spreading diameter, the more biosurfactant is produced.

In Figure 12, PAO1 had an average spreading diameter of 4.96 mm (s =0.77). Using this as a threshold, isolates from children and adolescents produced on average higher amounts of biosurfactant, indicated by the larger average spreading diameters. In contrast, adult isolates produced lesser amounts of biosurfactant than PAO1, indicated by the smaller average spreading diameter. Student's t-tests were performed on data from isolates taken from children and adolescents, adolescents and adults, and adults and children, and all had a p-value < 0.05. This

observation lends support to the idea that biofilm establishment early in infection is necessary for *P. aeruginosa* persistence and the development of a chronic infection.

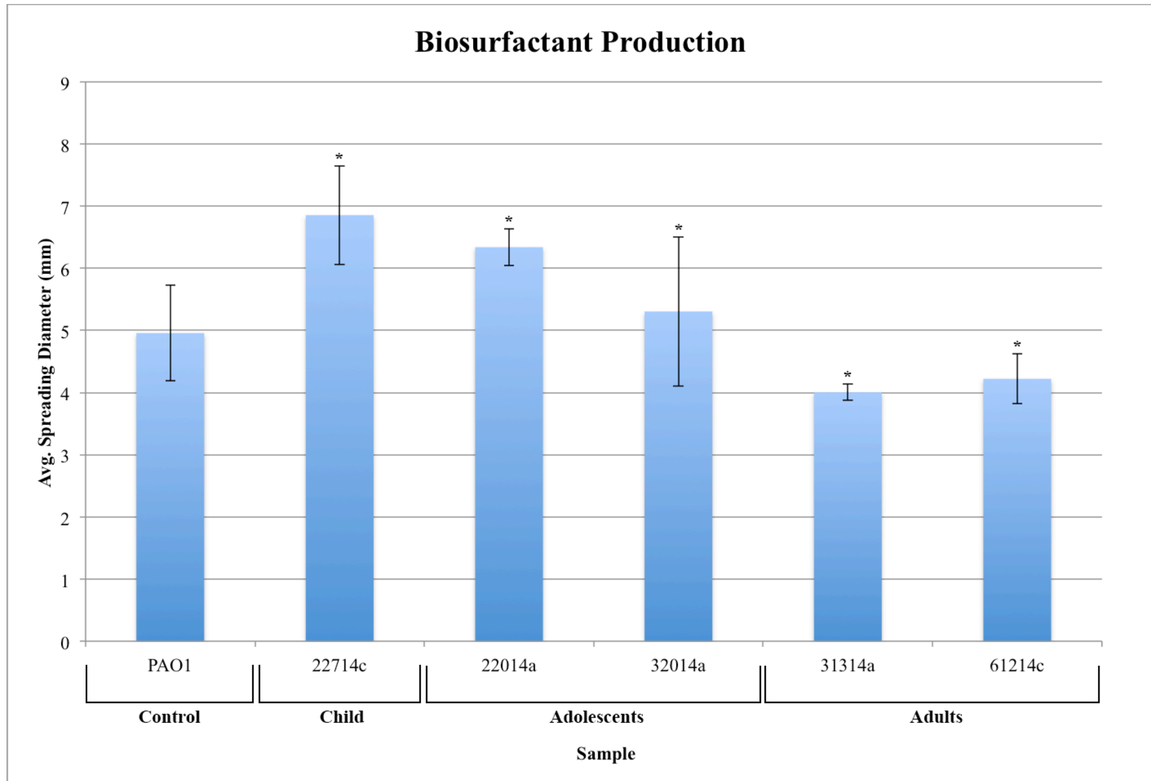


Figure 12: Biosurfactant Production: A sample size of $n = 30$ was used for all patients. PAO1 had an average spreading diameter of 4.96 mm ($s = 0.77$). 22714c had an average spreading diameter of 6.86 mm ($s = 0.79$). 22014a had an average spreading diameter of 6.34 mm ($s = 0.29$). 32014a had an average spreading diameter of 5.31 mm ($s = 1.20$). 31314a had an average spreading diameter of 4. ($s = 0.13$). 61214c had an average spreading diameter of 4. ($s = 0.40$). Error bars represent standard deviation. A single asterisk represents a p-value < 0.05 in comparison to control. Double asterisks represent a p-value > 0.05 in comparison to control.

V. f. Proteases

The proteases of *P. aeruginosa* are classic virulence factors important in pathogenesis for host tissue degradation. Several proteases have been thoroughly studied, notably elastase A, elastase B, protease IV, and alkaline protease, and their role in infection is well-understood. Proteolytic activity is often assessed using casein degradation [86] and this study uses a modification of dialyzed BHI with added 10% skim milk [98] to determine protease activity. The clearing radius was measured in millimeters from the center of inoculation and the average of all isolates for a patient was calculated. Student's t-tests were performed on data from isolates taken from children and adolescents, adolescents and adults, and adults and children, and all had a p-value < 0.05.

22714c, the child patient, had an average clearing radius comparable to wild-type PAO1. Adolescent isolates also had a comparable clearing radius to PAO1. However, adult isolates had noticeable decrease in protease production, with several isolates from 61214c failing to clear the agar entirely. Figure 13 suggests that protease production decreases as a chronic infection is established. Furthermore, during periods of exacerbation, protease production appears to halt completely.

A similar trend was observed for elastase production. While the reverse elastin media does not specifically detect LasA or LasB, it will detect both [88]. Elastase was produced by all growing isolates with no correlation to patient age (Fig. 14). However, the amount of positive elastase producers drops during exacerbation. This is of interest since elastases are known to destroy host proteins [15].

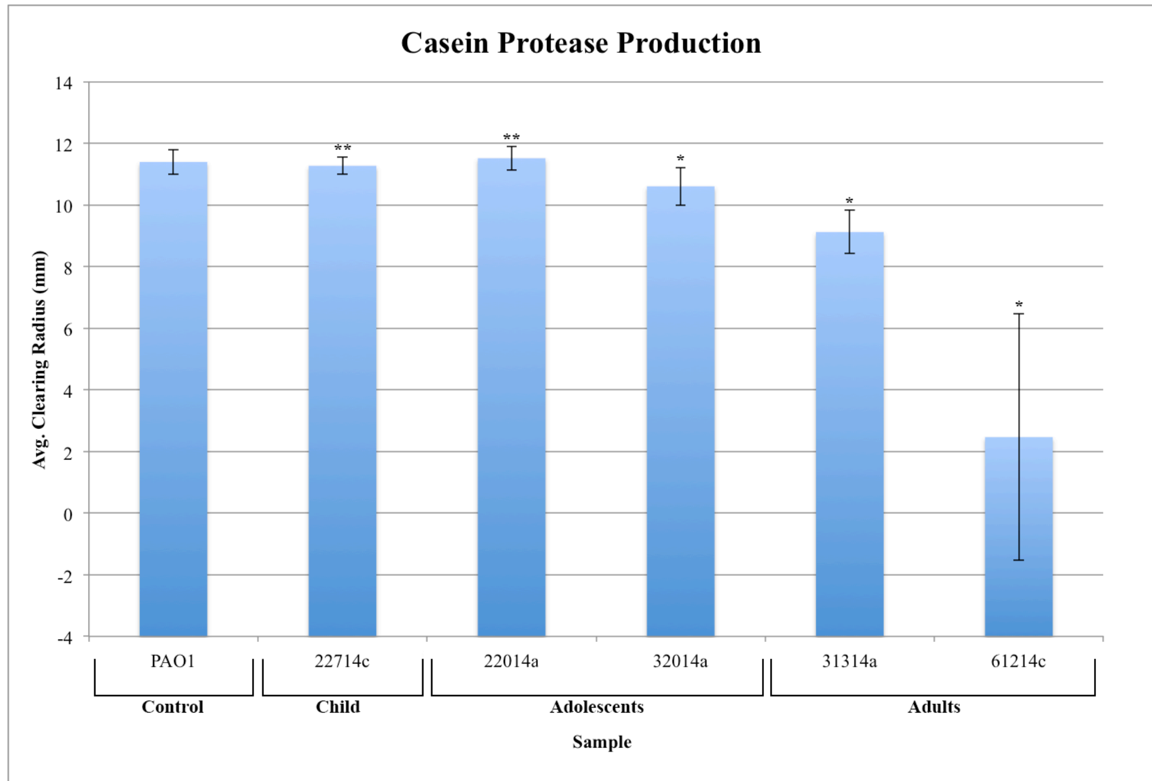


Figure 13: Casein Protease Production: A sample size of n = 30 was used for all patients. PAO1 had an average clearing radius of 11.40 mm (s = 0.41). 22714c had an average clearing radius of 11.27 (s = 0.27). 22014a had an average clearing radius of 11.52 mm (s = 0.38). 32014a had an average clearing radius of 10.61 mm (s = 0.62). 31314a had an average clearing radius of 9.12 (s = 0.71). 61214c had an average clearing radius of 2.47 mm (s = 4.00). Error bars represent standard deviation. A single asterisk represents a p-value < 0.05 in comparison to control. Double asterisks represent a p-value > 0.05 in comparison to control.

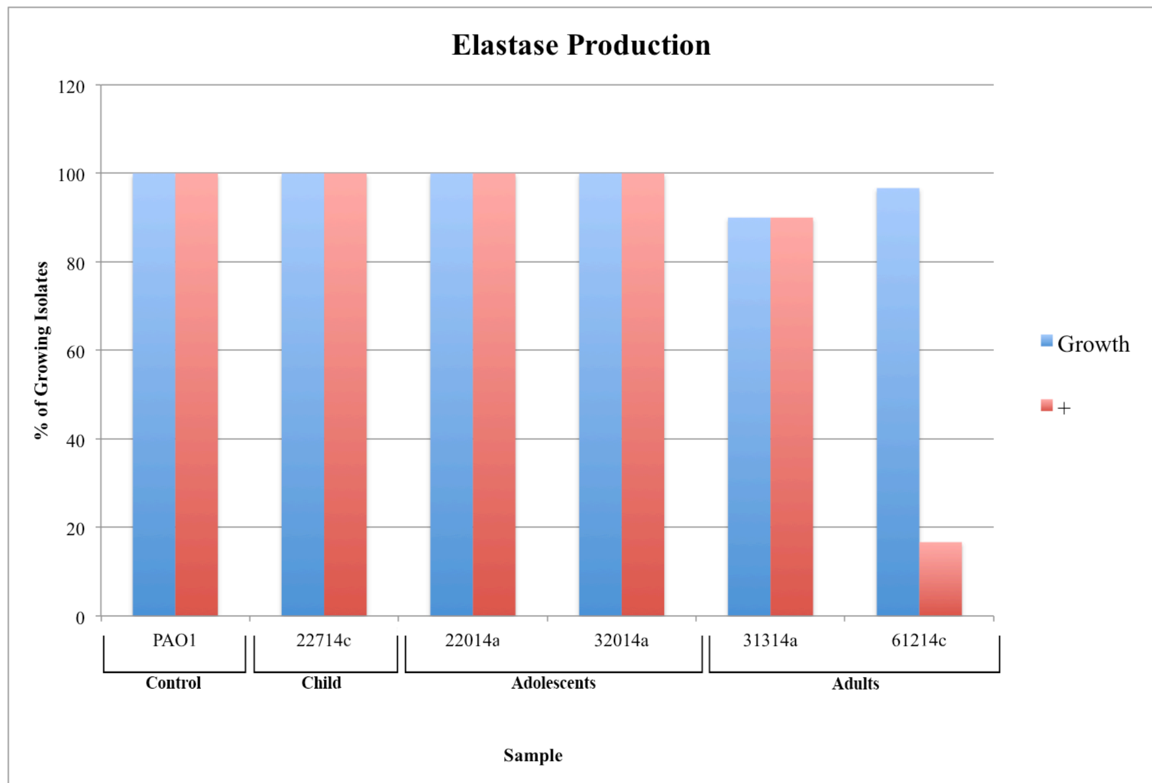


Figure 14: Elastase Production: A sample size of n = 30 was used for all patients. PAO1 grew 100% of the time on elastase media and was 100% positive for elastase production. 100% of 22714c isolates grew with 100% of isolates positive for elastase production. 100% of isolates from 22014a grew on media with 100% positive for elastase production. 100% of 32014a isolates grew with 100% positive for elastase production. 90% of isolates from 31314a grew on media and 100% of growing isolates produced elastase. 97% of 61214c isolates grew with 17% of growing isolates producing elastase and 83% of growing isolates negative for elastase production.

V. g. Clustering Analysis

The virulence factors of *Pseudomonas aeruginosa* play pivotal roles in CF infection and decline. A total of ten virulence factors were chosen for this survey and thirty isolates from each of five selected patients, one from children and two from adolescents and adults, were tested for variances in virulence factor expression. On a whole, the chosen set of virulence factors are expressed regardless of patient age, however, what does vary is the amount the particular phenotype is expressed.

Several trends can be seen in the data. Hydrogen cyanide production and swimming, swarming, and twitching motilities tend to peak at adolescence. Protease and siderophore production show a decrease in production as age increases. Pigment production is relatively constant with no correlation to patient age. However, a larger sample size taken from more patients in each age category will be necessary to determine if these observed trends are truly representative of *P. aeruginosa* host adaptation. In order to best summarize the results of this survey, a cluster diagram based on the percentage of isolates testing positive and the average diameter was generated (Fig. 15). Patients from the same age category did cluster together, which lends support to the idea that phenotypic expression may be related to patient age. It is interesting that some factors related to each other did not cluster together. The clustering of biosurfactant and protease production was expected since they are extracellular products, however, elastase, a type of protease, was not grouped with biosurfactant and proteases. HCN gas production and denitrification were grouped together, which was also expected since HCN synthase activity and the utilization of denitrification are dependent on oxygen availability. Lastly, pigment production did cluster together, as did swim, swarm, and twitch motility.

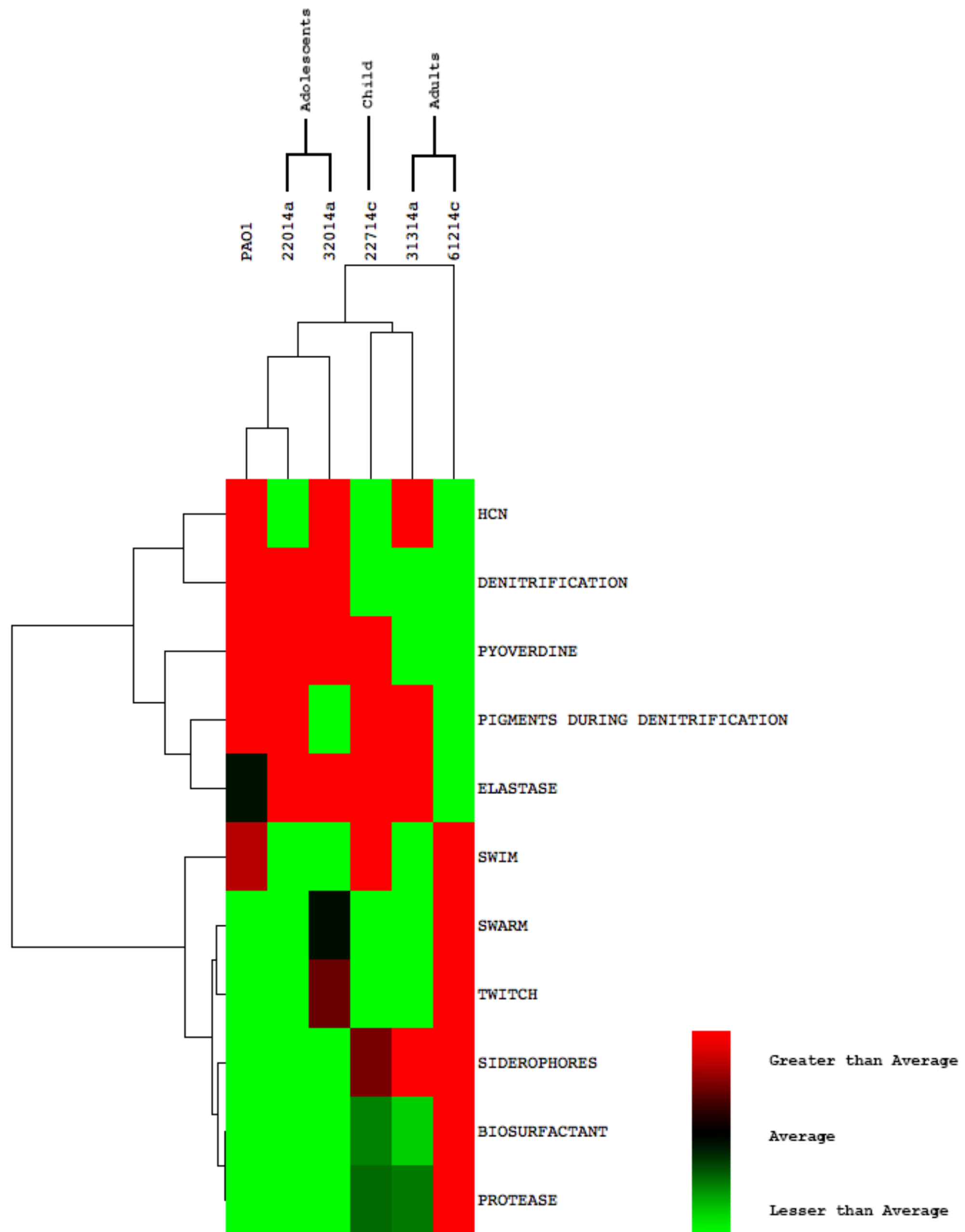


Figure 15: A Cluster Analysis of Results from Phenotypic Assays. Red squares indicate greater than average values, black squares represent average values, and green squares are lesser than average values.

VI. Summary

This survey on a selection of *P. aeruginosa* virulence factors related to pulmonary infection supports previous research that comments on the broad phenotypic diversity of *P. aeruginosa* CF isolates [72, 107]. Most isolates taken from CF patients in all age groups express the chosen virulence factors and their trends are summarized in Fig. 15.

There is one consistent anomaly in all data sets. Patient 61214c is an adult who experienced pulmonary exacerbation at the time of sputum collection. In contrast to the rest of the patients who are all clinically stable at the time of sampling, 61214c isolates show a surprising lack of virulence factor production in all tested areas. This leads to an interesting question: does the expression of particular virulence factors and phenotypes not only depend on the surrounding polymicrobial community and lung environments, but also on the clinical status of the host? Furthermore, what occurs during pulmonary exacerbation that consequently inhibits or down-regulates the expression of such virulence factors, and does this suppression ensure *P. aeruginosa* persistence?

Fodor, et. al suggested that exacerbations may represent the spread of infection in the lungs or be a consequence of changes in virulence factor expression by the resident polymicrobial community [37]. Since *P.* aeruginosa utilizes three different quorum-sensing systems to regulate the production of its many virulence factors, any mutations in the genes transcribing autoinducers, receptors, or other involved signaling molecules will result in the expression of different phenotypes. It has been established that *lasR* mutants are associated with growth advantages in times of oxidative stress and protection during antibiotic treatment [51]. It has also been suggested that *lasR* mutants may act as social “cheats” using extracellular products, such as siderophores, from those isolates in possession of functioning *lasR* protein [25]. Alternatively, the persistence of this particular genotype in the CF lung may be due to it apparent growth advantage

in an established infection but there is little evidence to support this hypothesis [46]. Further work to address these questions will need to include a larger number of patients in each age category. Additionally, the sample size should include both clinically stable and pulmonary distressed patients to determine if host clinical status truly does have an impact on virulence factor expression.

CHAPTER V

DISCUSSION AND FUTURE DIRECTIONS

Science has advanced our understanding of CF as a disease at the genetic level and from the perspective of its alimentary and respiratory manifestations. It is of great importance that further work is done to elucidate as many avenues of possible therapy in order to continue improving the livelihood of those afflicted with CF.

Antimicrobial treatments have proven to be effective in treating symptoms of pulmonary infection but often fail to clear the lungs of microorganisms entirely [37]. This is due to several adaptive strategies of the polymicrobial community. First, the transfer of plasmids between organisms to confer antimicrobial resistance is not uncommon. Several bacteria, including *P. aeruginosa* and *B. cepacia* complex, possess beta lactamases and efflux pumps to prevent susceptibility to prescribed antibiotics. The high pressure of antibiotics used in the lung also selects for those isolates capable of surviving repeated treatment. The emergence of the small-colony variant phenotype, particularly in *S. aureus*, is another sub-population that evades

chemical therapy and clearance [110]. Third, the formation of polymicrobial biofilms, most likely initiated by *S. aureus* and further developed by *P. aeruginosa,* allows for many microbial populations to remain protected from clearance in the lung. These strategies and many other factors contribute to persistence and chronic infection.

The creation of a repository of CF isolates accessible to interested research groups opens avenues for further exploration into polymicrobial communities. As CF research shifts from focus on a single pathogen to a polymicrobial infection, access to clinical samples becomes necessary to further work in this field. The broad microbial diversity cultured from the sputum emphasizes the unique profiles of the polymicrobial community present within an individual. Therefore, the extraction of gDNA and RNA shortly following sputum collection is necessary for pyrosequencing and transcriptomic studies. Performing sequencing on gDNA allows us to glimpse what organisms, dead and alive, are found in the CF lung [28, 37]. At the same time, RNA sequencing gives us information on what organisms are metabolically active [87]. Similar studies have been performed by other research groups, but their research was limited to adult patients [28, 36, 87]. These two pieces of information together allows us to paint a comprehensive view on the composition of the polymicrobial community, including those pathogens that cannot be cultured by the methods outlined in Chapter 3 such as anaerobic microorganisms.

In creating a repository and collecting recoverable aerobic growth, we have cultured a large number of microorganisms on nutrient and selective media. No two sputum samples present identical colony morphologies on plate media and this suggests there is a broad diversity of microorganisms present within the CF patient community. To confirm this assumption, we will need to perform sequencing on sputum gDNA to identify the members of the microbial community. Additionally, gDNA and RNA have been extracted from a total of 43 samples and a preliminary pyrosequencing study has been completed.

Because *B. cepacia* complex infection risks the development of fatal cepacia syndrome, the unusual growth observed on BCSA plates was a cause for concern. Subsequent sequencing identified these colonies as members of the *Acidovorax, Achromobacter,* and *Stenotrophomonas* genus. Their growth on BCSA is unexpected, but not unusual, since prior studies demonstrate an intrinsic high resistance to multiple antibiotics. More recent microbiome studies have identified fungi belonging to the *Aspergillus* genus and our preliminary sputum cultures lends support to their findings. Future studies on fungal growth and infection in the CF lung are possible with the isolation of at least three different fungal morphologies from the sputum.

Identifying the members of this complex polymicrobial community is the first step in understanding what roles they may play in CF infections. Pathogens such as *Streptococcus milleri* group (SMG), *Stentrophomonas maltophilia, Achromobacter xylosoxidans, Aspergillus fumigatus,* and *Candida albicans* have been identified in the sputum yet their possible role in infection is subject to controversy [28, 85]. *Streptococcus* was identified as the second most common bacterial genus in adult CF patients in a recent study by Delhaes and was assumed to be species belonging to the SMG [28]. The importance of SMG in CF is its association with pulmonary exacerbation and its production of quorum-sensing signals [43]. It is known that *A. xylosoxidans* and *S. maltophilia* utilize multiple resistance mechanisms including synthesis of B-lactamases and efflux pumps, and enzyme modifications to avoid antimicrobial clearance [1].

The virulence factors of potential pathogens such as *Acidovorax* spp. must be identified in order to understand what roles they may play in pulmonary infection and overall decline of host health. From there, mixed infection studies will elaborate on how the potential pathogen interacts with different clones and other CF pathogens such as *S. aureus* and *P. aeruginosa*. Insect and animal models such as *Drosophila* [58, 96], murine [9], and porcine [46] models have been used in prior studies to understand *P. aeruginosa* infections and their effect on host health. These

same models can be used to understand community interactions and their overall contribution to pulmonary decline.

With the collection of whole sputum samples from a variety of patients in different age categories, studies can be performed on the composition of the sputum itself. For example, ion concentrations of biologically relevant molecules such as Ca^{2+} or Fe^{3+} can be measured by separating the pathogens from the extracellular fluid by centrifugation. The extracellular fluid contains all extracellular products, including secreted proteases and ions, and the composition of the extracellular fluid can be elucidated by mass spectrometry or a similar method. Similarly, the identification of what cytokines and chemokines are present in the sputum will aid in understanding the relationship between innate host response and infection.

P. aeruginosa is widely recognized as the predominant pathogen in the adult lung and interacts with other key species such as *S. aureus* and *B. cepacia* complex. The virulence factors of *P. aeruginosa* have been studied and characterized in depth. Current CF research focuses heavily on adult isolates, a serious knowledge gap exists for early infections in childhood and adolescence. Previous studies demonstrate the vast diversity of *P. aeruginosa* isolates even within a patient. Workentine, et al. noted that within multiple isolates stemming from the same patient, there is no consistent expression of a single phenotype across all studied isolates [107]. Mowat, et al. attributes this high phenotypic diversity between isolates to rapid turnover of haplotypes due to bacterial dynamics within the lung [72]. This necessitates the use of a large sample size to create a more accurate representation of *P. aeruginosa* behavior in the lung. Throughout this study, a sample size of $n = 30$ was used for each surveyed patient and five patients, one from children and two from adolescents and adults, were utilized. This study surveys the production of ten virulence factors important for pulmonary pathogenesis in three different age groups—children, adolescents, and adults—in order to better understand shifts in virulence factor production in relation to age. These virulence factors are swim, swarm, and twitch motilities, and

the production of biosurfactant, proteases, hydrogen cyanide, siderophores, and fluorescent pigments during growth and aerobic denitrification. Other research groups have addressed the variability of these virulence factors in adult isolates and their research supports our findings [10, 49, 65, 72, 107], namely that there is a broad phenotypic diversity among isolates from the same patient and patients with chronic infections show suppression of virulence factor expression.

In summary, the production of virulence factors associated with biofilm attachment and maintenance—swim, swarm, and twitch motilities and biosurfactant production—are highest in adolescents. This observation may correlate with the increase in the number of patients infected with *P. aeruginosa* in this age category. It reasons that isolates from adolescent patients, which may be primed for *P. aeruginosa* colonization, would have the highest amount of isolates expressing swim, swarm, and twitch phenotypes. Similarly, isolates from child patients that express motile phenotypes do not have as great of swimming and swarming spreading diameters as isolates from adolescents. This may be indicative of the early stages of infection and adolescents may be at the peak of *P. aeruginosa* colonization. It was also observed that isolates from adults consistently produce a lower number of isolates testing positive for virulence factor production. This agrees with transcriptomic studies that show down-regulation of virulence factors at the end stages of life [49]. The sole exception to this observation is the production of siderophores, which are necessary for continued survival within the lung.

The final relationship observed in this study is the link between expressed virulence factors and a patient's clinical status. Patient 61214c was experiencing pulmonary exacerbation at the time of sputum collection and tested isolates from this patient consistently demonstrate a low number of isolates expressing the tested phenotype, if not entirely absent. The suppression of virulence factors such as protease production during exacerbations may be a mechanism to ensure persistence in the lungs, however, the lower percentage of isolates testing positive for siderophore production raises a few questions. First, are the non-growers auxotrophic for a substrate present in

other media such as LB or PMB that is absent in CAS? What is that substrate and is it present in the CF lung? Also, do the siderophore-negative isolates from 61214c represent a previously unconsidered phenotype? What advantage does suppression of siderophore production and secretion confer on the bacterial fitness, especially during exacerbations? It is known that iron is abundant in the sputum as a result of chronic inflammation and that *P. aeruginosa* down-regulates siderophore production after the onset of pulmonary infection and the establishment of a stable biofilm [79], but determining if this relationship holds true during exacerbations will be necessary to validate the suppression of siderophore production in this circumstance.

Other factors will need to be considered to explain the differences in virulence factor production by isolates from 61214c. For example, does a patient's age affect the expression of *P. aeruginosa* virulence factors? Similarly, does a patient who has been recently colonized by *P. aeruginosa* show differences in phenotypic expression in comparison to a patient of the same age that has been colonized for a longer period of time? Moreover, do shifts in *P. aeruginosa* isolate populations affect clinical status? Fodor, et al. demonstrated that antibiotic treatment during exacerbations temporarily improved clinical status but did little in eradicating bacterial populations [37]. This leads us to question if the antibiotics used to treat pulmonary exacerbations causes shifts in *P. aeruginosa* virulence factor production.

The relationship between exacerbations and *P. aeruginosa* virulence factor production is not well understood. Two hypotheses exist to explain this relationship. The first hypothesis suggests that exacerbations, which include an increase in airway inflammation, causes *P. aeruginosa* to produce quorum sensing molecules to initiate the synthesis of virulence factors in order to adapt to its new environment. The changes in virulence factor production cause an inflammatory response from the destruction of host tissues or the recognition of PAMPs by airway epithelial cells. Since the synthesis of the majority of *P. aeruginosa* virulence factors is dependent on environmental signals, such as oxygen concentrations affecting HCN synthase

activity [4, 8, 80] and iron availability controlling the production of siderophores, there is strong support for this first hypothesis.

The second hypothesis suggests that virulence factor changes in isolates cause exacerbations [37]. This hypothesis postulates that changes in virulence factor expression would lead to changes in PAMPs and elicit pro-inflammatory cytokine production by epithelial cells. The production of cytokines then leads to increased inflammation and causes exacerbations. Prior research demonstrated that overproduction of alginate, a polysaccharide found in *P. aeruginosa* biofilms, initiates the conversion from the smooth to mucoid colony phenotype [42]. This mucoid phenotype is associated with a rapid decline in lung function and a hyperimmune response [63, 78].

While both hypotheses must be studied in depth, current research appears to favor the first hypothesis over the second. Figure 16 graphically represents the first hypothesis as a cycle between inflammation and virulence factor production. A previous study showed that *P. aeruginosa* gene products stimulate and increase IL-8 production by epithelial cells [32], which leads to an increase in activated NF-κB [31], thereby increasing inflammation in the lungs. Because quorum-sensing molecules are produced in response to environmental signals and the three quorum-sensing systems control the expression of over 300 genes, it is more likely that environmental changes are prompting changes in the phenotypic expression of *P. aeruginosa* isolates.

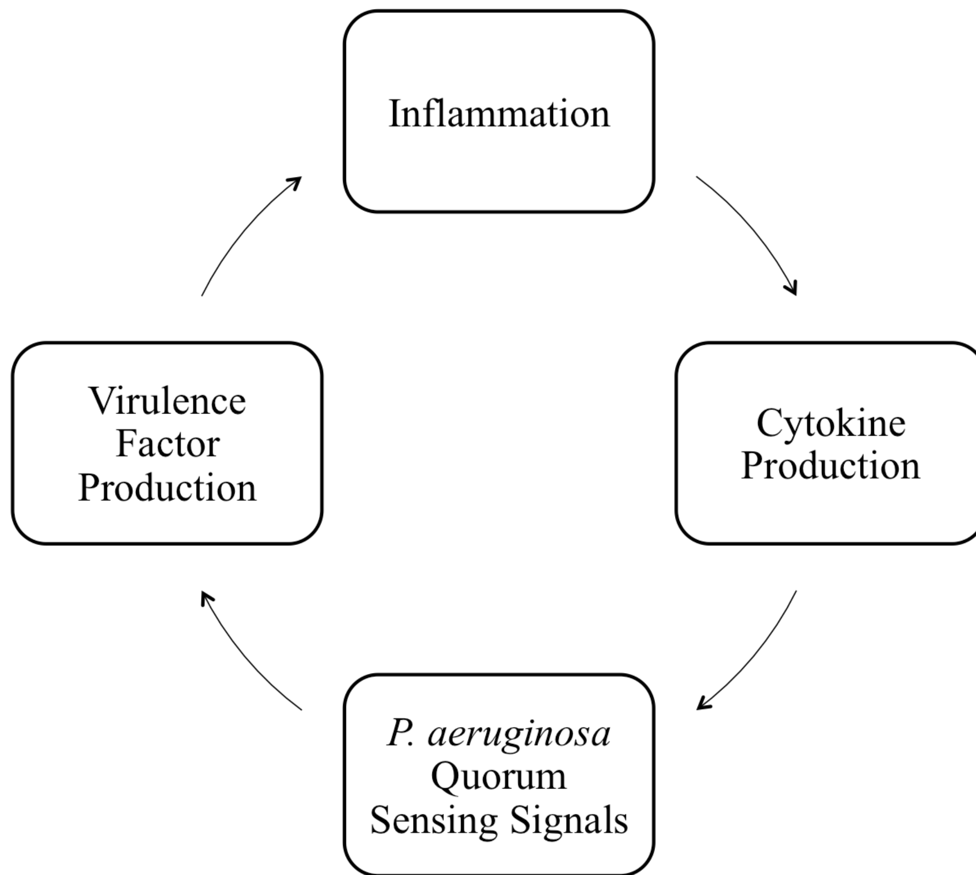


Figure 16: Cycle between Inflammation and Virulence Factor Production. Increased airway inflammation constitutes part of the clinical symptoms associated with pulmonary exacerbations. The increase in inflammation leads to an increase in cytokine production, which then initiates *P. aeruginosa* quorum sensing signal production. Quorum sensing controls the expression of several virulence factors and shifts in virulence factor production, such as an increase in pyocyanin, then causes tissue damage and leads to further airway inflammation.

Despite the importance of assessing variability in *P. aeruginosa* phenotypes in children, adolescents, and adults, CF is not caused by infection with a singular pathogen. The rapid identification of the polymicrobial community is essential for proper diagnosis and the prescription of effective treatment [28, 37, 85]. Because these polymicrobial infections are present in the CF lung as a biofilm, it is necessary to elucidate virulence factor heterogeneity between age groups in a situation that mimics *in vivo* conditions. The thick mucus in the lungs is sufficient for biofilm establishment and this environment can be emulated with artificial sputum media (ASM). However, this media has limitations for *in vivo* work because it is a homogenous liquid media that does not reproduce the compositional fluctuations of sputum faithfully [33]. Environmental signals like calcium (Ca^{2+}) have previously been shown to increase *P. aeruginosa* biofilm thickness through the increase in alginate production and is related to higher secretions of alkaline protease, elastase B, and PrpL proteases to the extracellular biofilm matrix [90]. Not only does the composition of the present polymicrobial community affect *P. aeruginosa* virulence, but also any external factors in the sputum play a role in virulence factor production especially within a biofilm.

Therefore, in order to determine if the relationships between patient age and the expression of the *P. aeruginosa* virulence factors surveyed in this study, it is necessary to assess phenotypic heterogeneity in mixed biofilms and in experimental conditions that mimic the CF lung environment. Additionally, a larger sample size for all age groups is necessary to determine if the observed trends are representative of the shifts in virulence factor expression and host adaptation strategies utilized by *P. aeruginosa*. Future studies will address these questions in depth.

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APPENDICES

Appendix I: Representation of Results from Phenotypic Assays

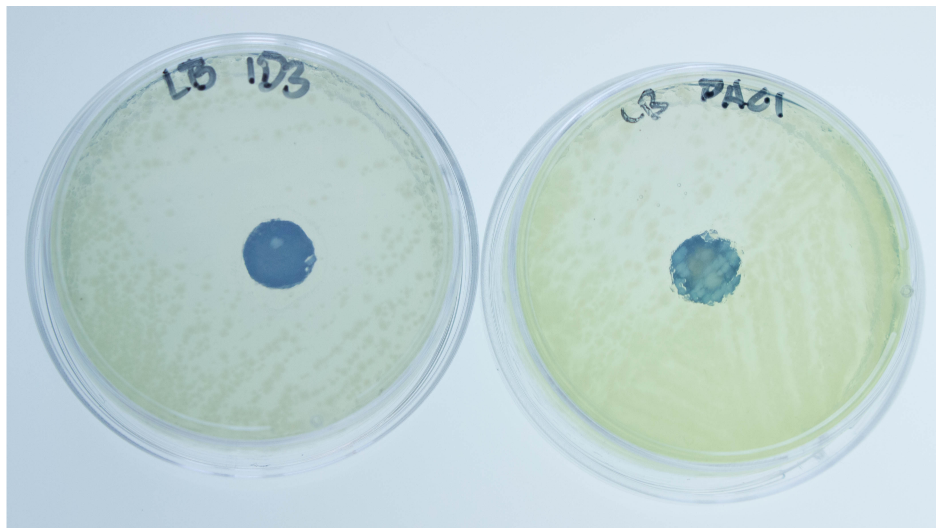


Figure 17: Representation of Hydrogen Cyanide Production Assay. After following inoculation and incubation procedures outlined in Chapter 3, a positive result is determined by the blue color change of a filter disk soaked in detection solution. The plate on the left is from an isolate used in this study and is positive for HCN gas production. The plate on the right is PAO1 and is positive for HCN gas production.

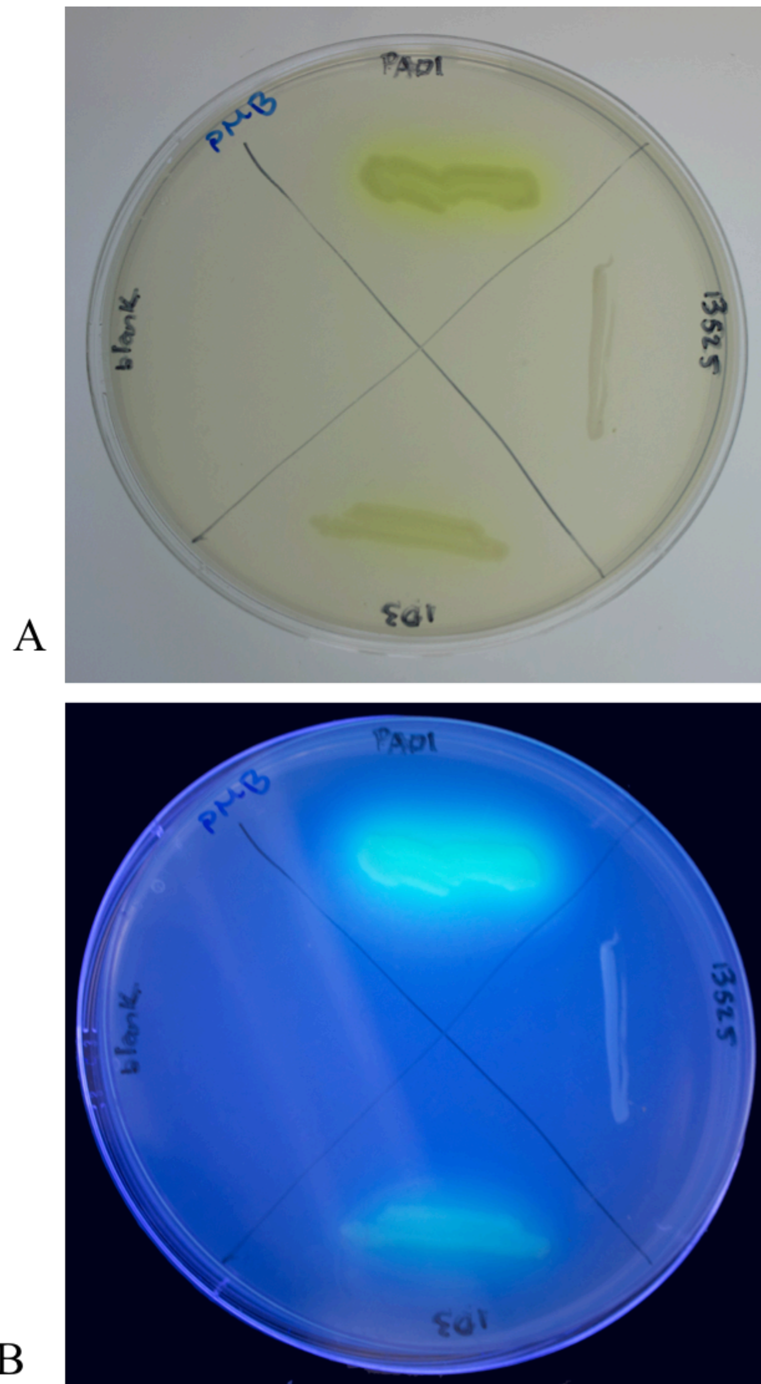


Figure 18: Representation of Pyoverdine Production by PMB. Shown is a single PMB plate inoculated with PAO1, *P. fluorescens* ATCC 13525, a clinical isolate, and an uninoculated control section. Both PAO1 and the clinical isolate produce a green-yellow pigment on PMB as seen A. PAO1 and the clinical isolate also fluoresce blue-green under UV light as seen in B. On the other hand, ATCC 13525 does not produce pigments (A) or fluoresce under UV light (B).

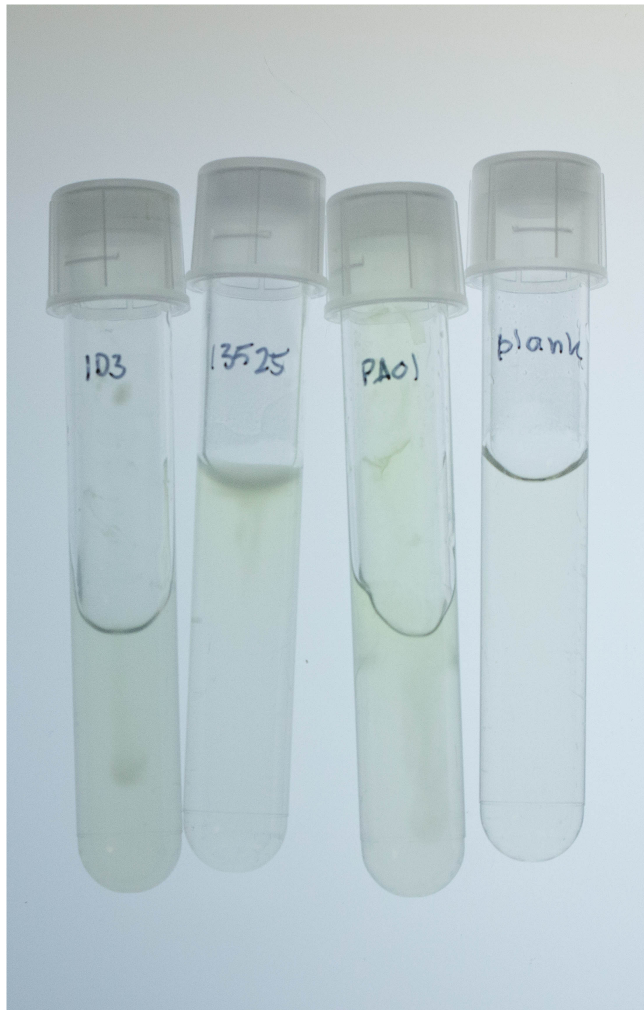


Figure 19: Representation of Pigment Production during Denitrification. A clinical isolate (1D3), *P. fluorescens* ATCC 13525, *P. aeruginosa* PAO1, and an uninoculated control (blank) have been inoculated following the methods outlined in Chapter 3. PAO1 and the clinical isolate both produce faint green-yellow pigments and completely liquify the media. ATCC 13525 grows well in the media with cream-colored grow but the medium remains solid. The uninoculated control shows no grow or pigment production and also remain solid.

Figure 20: Representation of the CAS Assay for Siderophore Production. PAO1, *P. fluorescens* ATCC 13525, a clinical isolate (1D3), and an uninoculated control (blank) were struck onto the medium. A positive result for siderophore production was assessed with the color change of the medium from green-blue to orange. All three inoculated samples turned the medium orange and are positive for siderophore production.

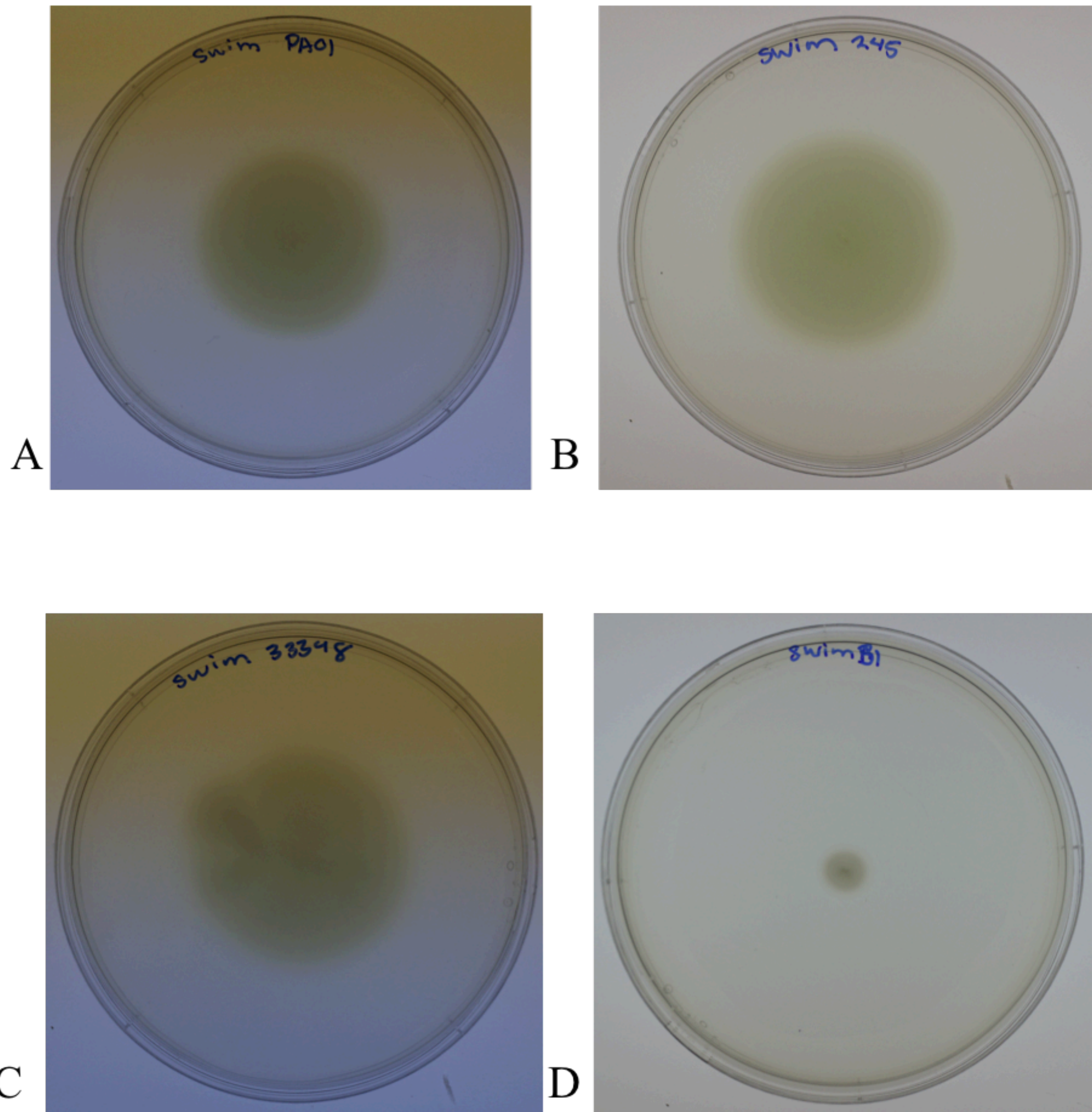


Figure 21: Representative plates demonstrating the Swim Motility Assay. A shows the swimming diameter of PAO1. B is an example of pA1092-0245 swimming diameter. C shows the swimming motility of pA4525-33348. Finally, D is an example of the swimming diameter of a clinical isolate. In this case, D demonstrates the small swimming diameter frequently observed in clinical isolates.

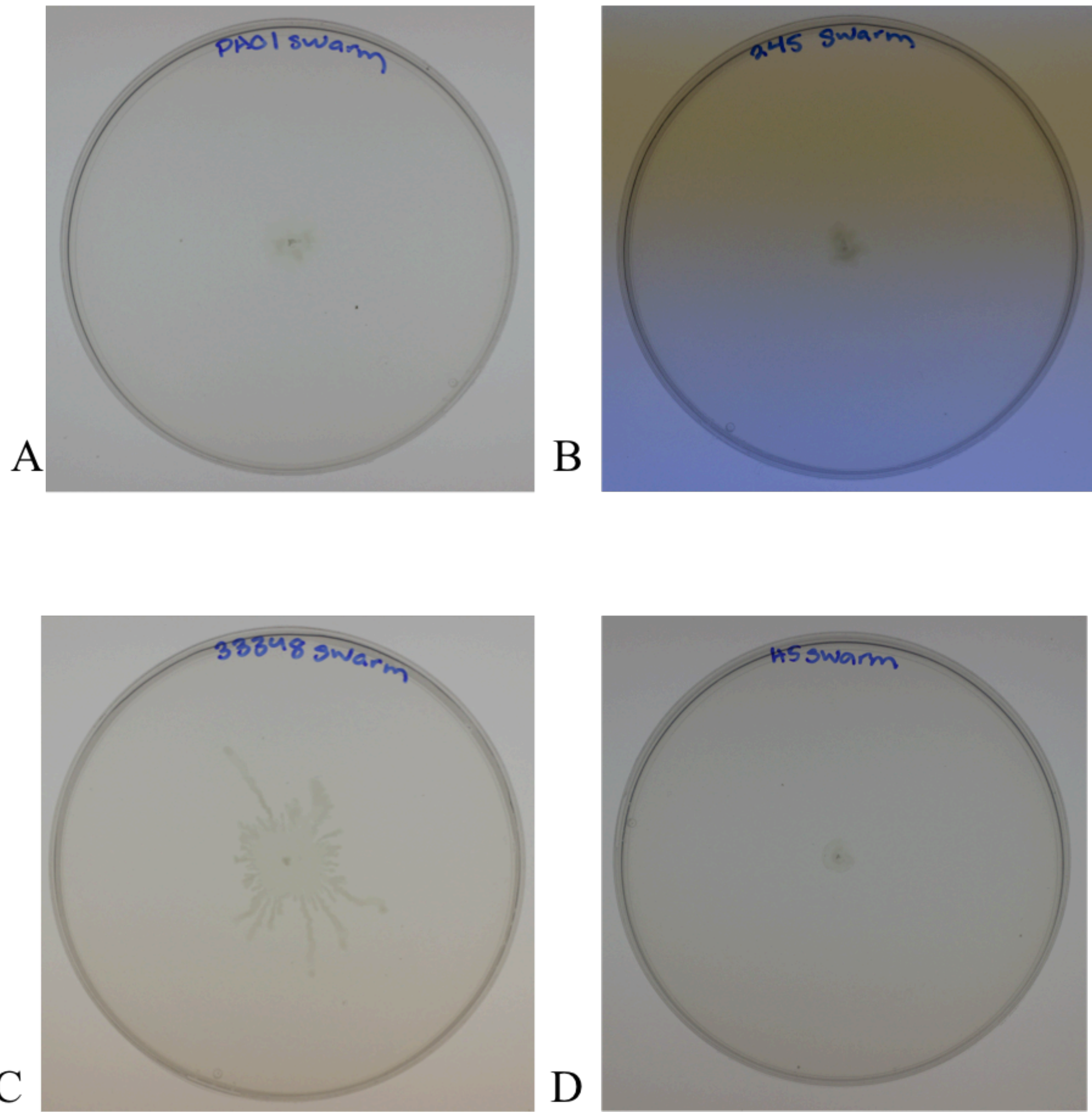


Figure 22: Representative plates demonstrating Swarming Motility. A demonstrates the swarming pattern of PAO1. B is a swarm motility plate inoculated with pA1092-0245, which shows little swarming activity. C shows the swarming motility and tendril pattern of pA4525-33348. Finally, D is an example of the swarming diameter of a clinical isolate that does not have a large swarming diameter.

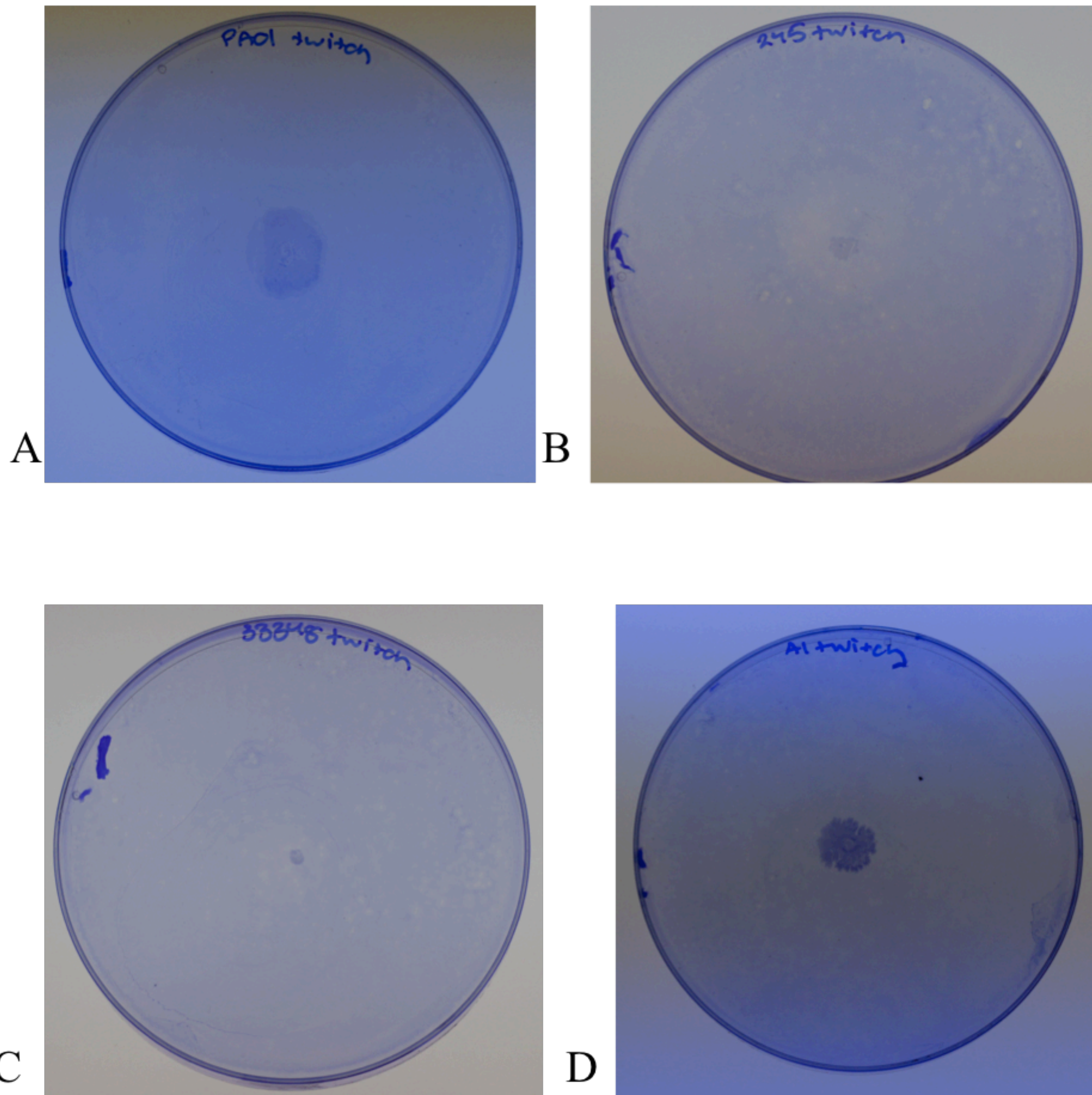


Figure 23: Representative plates demonstrating Twitch Motility. A shows the twitching diameter of PAO1 as stained by Coomassie Blue. B is an example of pA1092-0245, which does not show a large twitching diameter. C shows the twitching motility of pA4525-33348, and in this case, it does not utilize twitching motility either. Lastly, D shows a dark twitching pattern of a clinical isolate and is indicative of some utilization of twitch motility by that isolate.

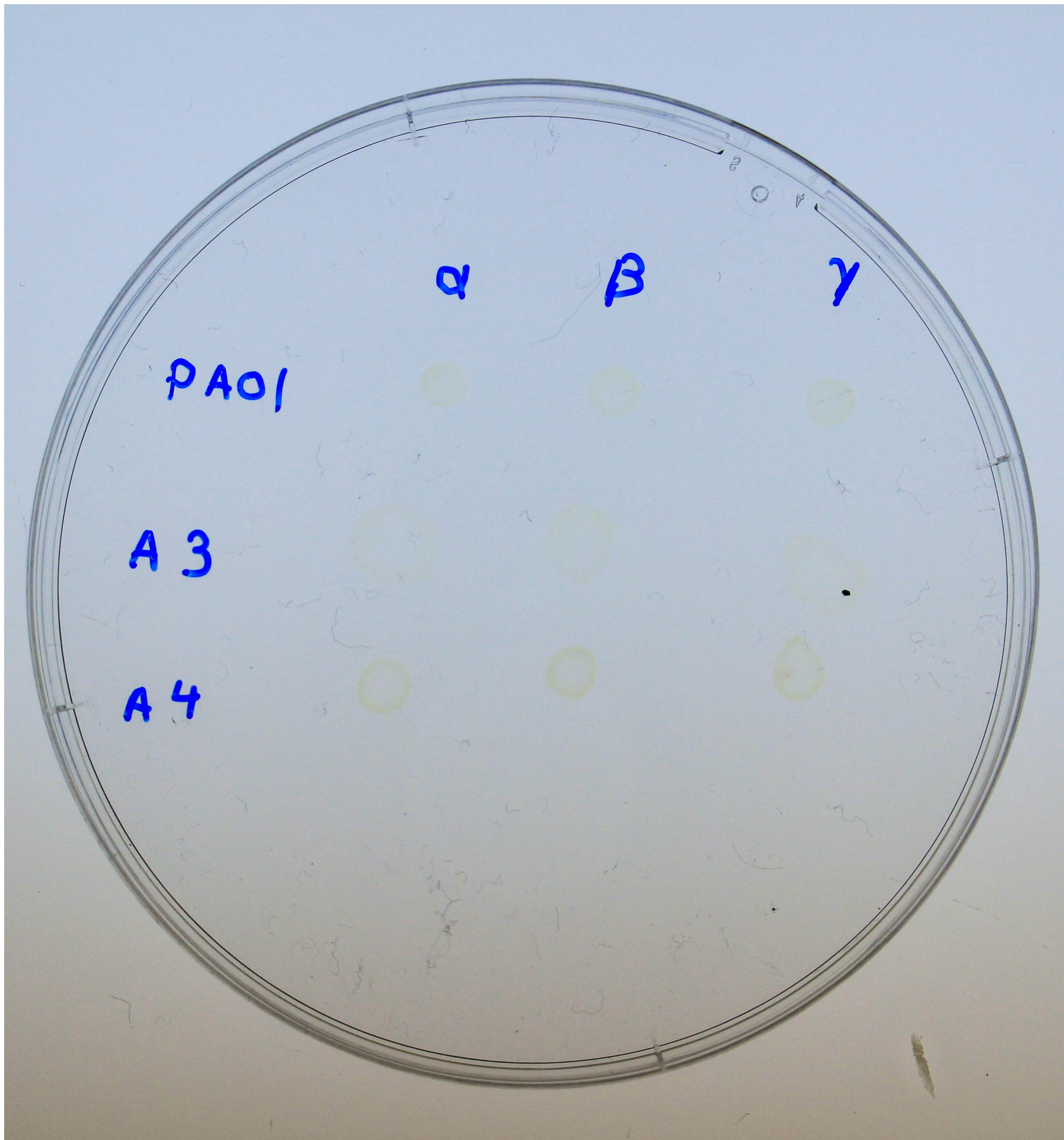
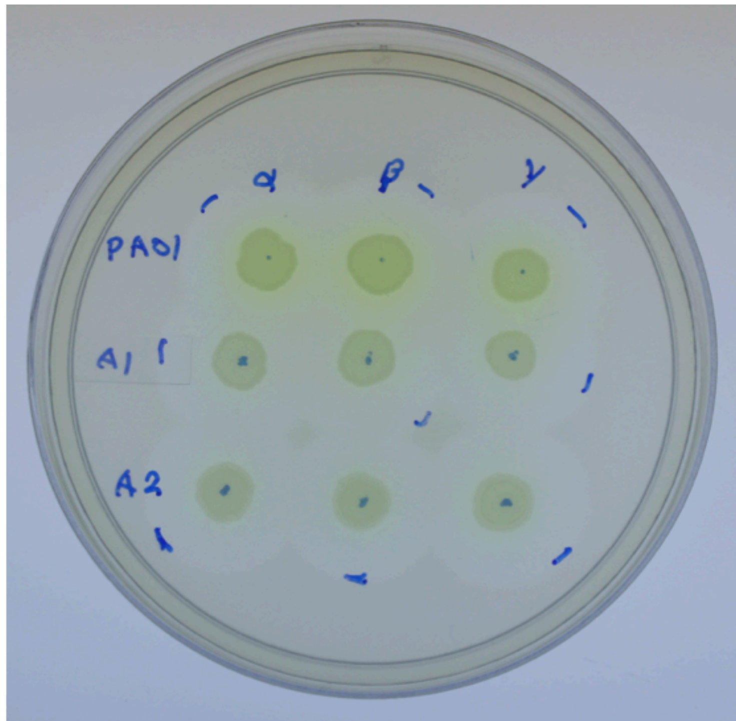
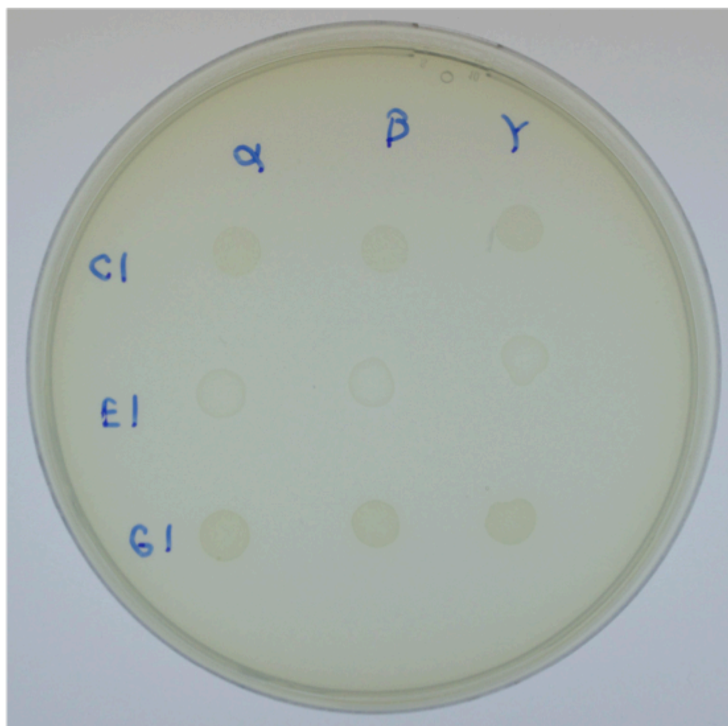


Figure 24: Representative plate demonstrating the Drop-Collapsing Test to Assess Biosurfactant Production. The spreading diameter of a drop of supernatant from PAO1 and two clinical isolates is measured after incubation at room temperature. In Fig. 23, the supernatant is light yellow in color on the petri plate lid.



A



B

Figure 25: A Representation of Casein Protease Production on D-BHI Skim Milk agar. A shows clearing of the agar by PAO1 and two clinical isolates. B also shows clearing of the agar by PAO1 but the inoculated clinical isolates on the plates fail to break down casein, indicating a lack of protease production.

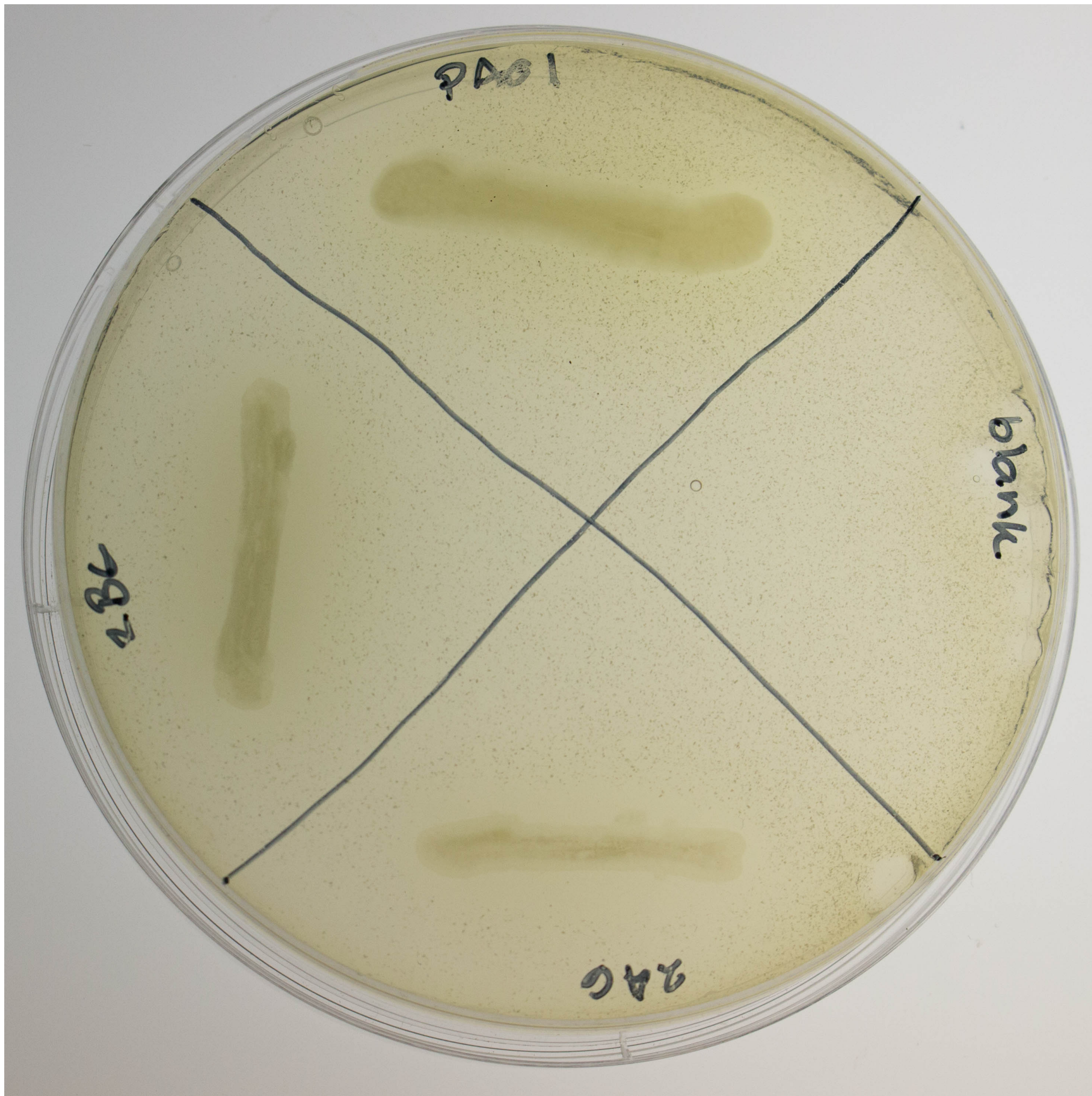


Figure 26: Representative plate demonstrating Reverse Elastin Media to Assess Elastase Production. An uninoculated control, PAO1, and two clinical isolates were inoculated on the plate and incubated for 48-72 hours. Clearing of the elastin in the media was indicative of elastase production.

Appendix II: Institutional Review Board Letter

Institutional Review Board for the Protection of Human Subjects

Human Research Determination

Date: February 18, 2014

To: Jodine S. Fox, BA, RN

Project Title: Pseudomonas aeruginosa microbiome and intra-species interactions

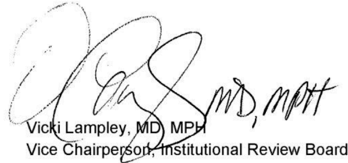
Reference Number: 580467

On behalf of the Institutional Review Board (IRB), I have reviewed the Determination of Human Research Worksheet for the above-referenced project. Based on the information provided, I have determined this ***does not meet*** the criteria for human subjects research.

You may begin your project without further review or approval from the IRB.

If you have questions about this notification or using iRIS, contact the HRPP office at (405) 271-2045 or irb@ouhsc.edu.

Sincerely,



Vicki Lampley, MD, MPH
Vice Chairperson, Institutional Review Board

1000 Stanton L Young Blvd, LIB 176, Okla City, OK 73117 (FWA 00007961)

VITA

Elizabeth Grace Ochavillo Pascual

Candidate for the Degree of

Master of Science

Thesis: A SURVEY ON THE PHENOTYPIC VARIABILITY OF *PSEUDOMONAS AERUGINOSA* IN CYSTIC FIBROSIS

Major Field: Microbiology and Molecular Genetics

Biographical:

Education:

Completed the requirements for the Master of Science in Microbiology and Molecular Genetics at Oklahoma State University, Stillwater, Oklahoma in May, 2015.

Completed the requirements for the Bachelor of Science in Biochemistry at Oklahoma State University, Stillwater, Oklahoma in 2013.

Experience:

Graduate Teaching Assistant:
Introduction to Microbiology Laboratory
Oklahoma State University, Stillwater, Oklahoma

Mentoring:
Rachel McAllister, Undergraduate Research Student
Project Title: Induction of Small-Colony Variant *Staphylococcus aureus*

Link Zheng, Undergraduate Research Student
Project Title: New Emerging Pathogens of CF Sputa

Professional Memberships:

American Society of Microbiology