Pseudomonas aeruginosa biofilm and planktonic bacteria display different virulence mechanisms when co-cultured with human A549 lung cells using the Calgary Biofilm Device co-culture system

By Laura Bowler

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements of the degree of

Doctor of Philosophy

The Department of Medical Microbiology and Infectious Diseases

University of Manitoba

Winnipeg

Copyright © 2015 by Laura Bowler

Abstract

Cystic Fibrosis (CF) is the most common hereditary genetic disorder among Caucasians. In CF patients over 85% of mortality will result from a progression of their lung disease. *Pseudomonas aeruginosa* is a major cause of morbidity in cystic fibrosis patients. Chronic infection with *P. aeruginosa* eventually occurs and is associated with a switch to biofilm formation of the bacteria. The symptoms and pathology of acute and chronic *P. aeruginosa* infections differ greatly. Chronic infections cause less cell damage and are characterized by substantial inflammation of the lungs. Chronic inflammation ultimately leads to lung fibrosis and a dramatic decrease in lung function. The first line of defense within the lung is the physical barrier of the lung epithelia. The lung epithelia provide a structural barrier, secrete cytokines for immune activations, and secrete defensive enzymes and antimicrobial peptides, including LL-37, that help protect against bacterial infection. The examination of established biofilm interactions with lung epithelia is difficult. Here, I use the Calgary Biofilm Device co-culture system to conduct the concurrent analysis of established biofilms and planktonic bacteria with A549 lung cells. I hypothesize that the pathogenicity of planktonic bacteria will involve a more direct interaction with the lung cells, stimulating more cell damage and death to the A549 cells, typical of an acute infection; while the pathogenicity of the biofilm will be indirect, stimulating less cell death and a stronger inflammatory response, characteristic of a chronic lung infection.

Comparison of *P. aeruginosa* biofilm and planktonic bacteria's effects on A549 lung cells showed that planktonic bacteria caused more A549 cell rounding and death, while biofilm stimulated more IL-8 release by epithelial cells. Biofilm was shown to secrete significantly more Pseudomonal Elastase than planktonic, causing A549 morphological changes and loss of tight junctions. This protein is associated with significant disease pathology but its regulation in biofilms is not fully understood. The function of the majority of the proteins secreted by planktonic bacteria was for metabolism and growth, while biofilm secreted more proteins involved in virulence and protection. The antimicrobial peptide LL-37 was shown to differentially affect biofilm and planktonic bacteria. LL-37 caused a decrease in twitching of planktonic bacteria and exposure to LL-37 for 48 hours resulted in a decrease in elastase secretion likely due to down-regulated type 2 secretion. When established biofilms were compared with newly adherent biofilms, young biofilms were shown to have characteristics similar to both planktonic bacteria and mature biofilms. From this data we can follow the pattern of bacterial virulence and pathogenesis as *P. aeruginosa* transitions from the planktonic mode of growth to forming a young biofilm and the eventual mature biofilm that is associated with chronic infection.

In conclusion, this study provides the foundation for a co-culture system that can be used to study the host-pathogen interactions of mammalian epithelia with established *P. aeruginosa* biofilms. This data provides new insights into the virulence mechanisms of *P. aeruginosa* and the interaction with host epithelial cells. By uncovering the virulence of biofilms we can begin to target chronic biofilm infections with new therapeutic development. The future adaptations of this model will better represent the *in vivo* characteristics of chronic lung infection to delineate ongoing virulence mechanisms of the bacteria causing host cell stimulation and damage.

Acknowledgements and Dedication

There are many people who have helped me along the way. I would like to thank my supervisors Laura Saward and Blake Ball, as well as my committee members George Zhanel, Neeloffer Mookherjee and Joanne Embree for providing guidance and ensuring that I stayed focused. This work would not have been possible without the financial contributions of NSERC, the University of Manitoba and Cangene/Emergent Biosolutions. I would like to thank Nyla Dil for her help with the cytokine bead assay and Peyman Ezzati for his help with the mass spec work and to Dr. De Kievit for her donation of the knockout strains. Antibiotic plates were graciously provided by Dr. George Zhanel's laboratory. I would also like to thank all of the people that I have shared lab space with throughout the years. I have gained friendships that will last a lifetime and together we were bonded forever through the loss of a dear friend. The scar of her passing will always remain and she still encourages me to struggle and overcome obstacles that seem insurmountable. Through my department I have made friends who were sharing in the same long graduate studies journey. No one can understand what we are going through. With this shared bond we have a friendship closer than any other.

When I look at how my life has changed during this long, winding, unending, continuous journey, it is quite remarkable. I started off as a young and naïve student and have grown into a mature and grounded scientist (albeit a touch pessimistic). I have gotten married, started a family and become a responsible home-owner, all while having the constant looming presence and stress of my thesis to keep me company. My family has been a pillar of support throughout this entire process. My husband has been so supportive even during particularly stressful periods where I may have become quite unreasonable. I'm glad that you were with me through thick and thin. To my daughter Anna. You are the light of my life and you have been a breath of fresh air when I could have suffocated under the weight of writing my thesis. Your smile melts my heart and all I want is to make you happy. I am so lucky that I can watch you grow and learn and play. Although I wish you would nap more. I love you more than words can say and I'm doing all of this for you and your new baby brother Jack, who waited until after my defense to make his arrival. I would like to thank my mom who drove in every Tuesday so that I could work on my thesis. Your constant support has strengthened me and helped me to surge forward in my studies.

I would like to thank Chris's family, who welcomed me into their lives and offered support without ever questioning my life decisions.

Through this entire process the only regret that I have is that I didn't finish sooner. My dad has been my biggest supporter and it breaks my heart that I couldn't finish in time for him to see. So, while this thesis is dedicated to everyone in my life, it is especially dedicated to my dad. My biggest fan.

Table of Contents

Abstract i

Acknowledgements and Dedication ii

Table of Contents iv

List of Figures xi

List of Tables xiv

1.0 Introduction 1

1.1 Cystic fibrosis *1*

1.1.1 Pseudomonas aeruginosa and Cystic fibrosis *2*

1.2 P. aeruginosa biofilms versus planktonic bacteria *5*

1.2.1 Two-component pathway *8*

1.2.2 Quorum-sensing *10*

1.2.3 P. aeruginosa biofilm regulation *12*

1.2.4 Host-pathogen interaction *13*

1.2.4.1 Lung epithelia *13*

1.2.4.2 P. aeruginosa infection of lung epithelia *15*

1.2.4.3 P. aeruginosa secreted factors *16*

1.2.4.4 The role of the antimicrobial peptide LL-37 in lung epithelial host defense *17*

1.2.5 Antibiotic resistance *20*

1.2.5.1 Antibiotic use in CF *21*

1.3 Biofilm/mammalian cell co-culture methods *23*

1.3.1 Advancements in cell culture for infection studies 23
1.3.1.2 Monolayers 24
1.3.1.3 Air-liquid interface 25
1.3.1.4 3-Dimesional cell culture 26
1.3.2 Advancements in biofilm culture for infection studies 27
1.3.2.5 Static model 28
1.3.2.6 Flow cell model 29
1.3.2.7 Co-culture of established biofilm with mammalian cells 30
1.4 Calgary Biofilm Device for biofilm co-culture 32
1.5 Rationale 34
1.6 Objectives and Hypothesis 35
2.0 Materials and Methods 39
2.1 Bacterial strains and cell culture 39
2.2 Biofilm formation and planktonic growth 39
2.2.1 Preparation of bacteria for co-culture 39
2.2.1.8 Growth in the presence of LL-37 41
2.2.1.9 Transition from planktonic to biofilm 42
2.3 A549 co-culture 46
2.3.1 96-well co-culture 46
2.3.2 8-well co-culture 46
2.4 Preparation of conditioned media from biofilm and planktonic bacteria 47
2.5 Biofilm formation curve 47

2.6 Crystal violet assay ... 48

2.7 Immunofluorescence ... 48

2.7.1 Actin and P. aeruginosa staining following bacterial co-culture ... 49

2.7.2 Actin and Occludin staining following incubation with bacterial CM ... 50

2.8 Adherence/internalization of P. aeruginosa biofilm and planktonic bacteria to A549 cells ... 50

2.9 WST-1 assay for metabolic activity ... 51

2.10 Cell death and apoptosis of A549 cells after exposure to biofilm and planktonic bacteria ... 52

2.11 Cytokine and chemokine measurement ... 53

2.11.1 Milliplex human cytokine/chemokine panels ... 53

2.11.2 IL-8 ELISA ... 54

2.12 Western blot analysis ... 55

2.12.1 Occludin staining ... 56

2.12.2 E-Cadherin staining ... 56

2.13 Wound healing assay ... 56

2.14 Mass spectrometry ... 57

2.15 Lactone extraction from biofilm and planktonic CM ... 59

2.16 Elastase activity of biofilm and planktonic CM ... 60

2.17 Minimum inhibitory concentration/minimum bactericidal concentration ... 60

2.18 β-Lactamase activity ... 61

2.19 RNA extraction/real-time PCR *62*

2.19.1 RNA extraction *62*

2.19.2 cDNA synthesis and real-time PCR *63*

2.20 Twitching assay on biofilm and planktonic bacteria *66*

3.0 Results 67

3.1 Development of a novel in vitro co-culture system allows the concurrent analysis of established biofilm and planktonic bacteria with human lung epithelia *67*

3.1.1 Characterization of a 24-hour biofilm grown on the Calgary Biofilm Device *69*

3.2 Planktonic P. aeruginosa and biofilm display different virulence mechanisms compared to biofilm when co-cultured with human A549 epithelial cells *73*

3.2.1 Planktonic exposure causes epithelial cell rounding and cytoskeletal dysregulation in A549 cells but not with biofilm exposure *73*

3.2.2 Biofilm and planktonic bacteria adhere equally to A549 cells but planktonic bacteria undergo 15-fold more internalization by epithelia *77*

3.2.3 Planktonic exposure causes epithelial cell death, while biofilm increases epithelial cell metabolism *80*

3.2.4 Biofilm exposure stimulates an increase in IL-8 production by epithelial cells *85*

3.2.5 Summary *88*

3.3 Biofilm and planktonic bacteria secrete different virulence factors and biofilm secretes significantly more elastase than planktonic *89*

3.3.1 CM from biofilm culture stimulates morphological changes in epithelial cells and activity is controlled by quorum-sensing *89*

3.3.2 The factor released by biofilms is controlled by quorum-sensing but is not a quorum-sensing lactone. 94

3.3.3 CM from biofilm cause A549 epithelial cells to lose their tight junctions through the loss of Occludin.. 96

3.3.4 Biofilm secreted factors caused increased cell death but reduced apoptosis than planktonic secreted factors.. 100

3.3.5 Biofilm secreted factors suppress immune factors in A549 cells while up-regulating IL-8. 104

3.3.6 A549 cells are not undergoing epithelial-to-mesenchymal transition following exposure to biofilm conditioned media 109

3.3.7 Secretomes of biofilm and planktonic bacteria contain differences in virulence factors. 114

3.3.8 Specifically inhibiting elastase activity eliminates the effect of biofilm CM exposure to A549 cells.. 116

3.3.9 Summary 120

3.4 4-hour and 24-hour biofilms differ in their β-lactamase and PE secretion but interact similarly with A549 epithelial cells 121

3.4.1 24-hour biofilms are more resistant to β-lactams than 4-hour biofilms................. 121

3.4.1.1 24-hour biofilms release bacteria that are more resistant to gentamicin, ceftazidime and meropenem than 4-hour biofilm 121

3.4.1.2 24-hour biofilms produce more β-lactamase than 4-hour biofilms 125

3.4.2 4-hour biofilms do not stimulate a significant increase in metabolic activation of A549 epithelial cells... 128

3.4.3 4-hour biofilms stimulate similar IL-8 release from A549 cells as 24-hour biofilms 130

3.4.4 4-hour biofilms produce significantly less elastase as compared to 24-hour biofilms and are unable to affect A549 epithelial cells *133*

3.4.5 Summary *136*

3.5 Growth of biofilm and planktonic bacteria in the presence of LL-37 has time-dependent effects on downstream virulence *137*

3.5.1 Growth in the presence of LL-37 decreases biomass, but not growth of biofilm ... *137*

3.5.2 Effect of LL-37 on virulence gene transcription with biofilms differs from 24-hours to 48 hours *143*

3.5.3 LL-37 pre-treatment has a greater effect on biofilm twitching than on planktonic twitching *153*

3.5.4 Biofilm and planktonic bacteria grown in the presence of LL-37 have a greater survival when exposed to ciprofloxacin *157*

3.5.5 Planktonic bacteria grown in the presence of LL-37 no longer stimulate A549 cell rounding while biofilm grown in the presence of LL-37 are internalized by A549 cells *159*

3.5.6 Growth of planktonic bacteria in the presence of LL-37 decreases the ability to cause cell death of A549 cells. *166*

3.5.7 LL-37 pre-treatment of bacteria does not affect IL-8 secretion by A549 cells. *169*

3.5.8 LL-37 exposure for 48 hours decreased elastase production from biofilms. *173*

3.5.9 Summary *176*

5.0 Discussion **178**

4.1 Major findings of thesis *178*

4.2 The importance of studying established biofilm ... 179

4.3 The Calgary Biofilm Device co-culture model ... 180

4.4 Characterization of the biofilm grown on the Calgary Biofilm Device ... 182

4.5 Planktonic P. aeruginosa and biofilm display different virulence mechanisms when co-cultured with human A549 epithelial cells as well as a differential secretion of virulence factors ... 185

4.6 4-hour and 24-hour biofilms differ in their β-lactamase and PE secretion but interact similarly with A549 epithelial cells ... 190

4.7 Growth of biofilm and planktonic bacteria in the presence of LL-37 has time-dependent effects on downstream virulence ... 193

4.8 Biofilm secretes more PE than planktonic bacteria and this acts as an important virulence factor ... 206

4.8.1 PE as a potential biomarker of transition to a chronic infection ... 209

4.8.2 PE is an important virulence factor in vivo ... 210

4.9 Summary/Conclusions ... 212

4.9.1 Major findings ... 212

4.9.2 Future directions ... 213

4.9.3 Conclusion ... 215

5.0 Reference List ... 217

Appendix ... 242

List of Figures

Figure 1. Schematic diagram of the cell culture plate and Calgary Biofilm Device peg-lid set-up. 45

Figure 2. The Calgary Biofilm Device produces uniform biofilms across the entire peg lid....... 68

Figure 3. Planktonic and biofilms grown for 24-hours on the Calgary Biofilm Device have different gene expression profiles. 71

Figure 4. Gene expression profiles of biofilm and planktonic bacteria grown for 48-hours show less significant differences. 72

Figure 5. Planktonic *P. aeruginosa* causes A549 cells rounding while biofilm had little effect on cell morphology. 75

Figure 6. Planktonic *P. aeruginosa* causes significant cytoskeletal dysregulation of A549 cells while established biofilm had little effect on A549 actin structure. 76

Figure 7. Biofilm and planktonic *P. aeruginosa* adhere equally to A549 cells while planktonic undergo 15-fold more subsequent internalization. 79

Figure 8. Biofilm and planktonic *P. aeruginosa* stimulate A549 apoptosis and cell death with planktonic stimulating significantly more cell death. 81

Figure 9. Planktonic bacteria cause a decrease in A549 cell metabolism after 1 and 4 hours while biofilm stimulates an increase in metabolic activity after 1 hour but this decreased after 4 hours. 84

Figure 10. *P. aeruginosa* biofilm stimulates significantly more IL-8 release from A549 cells than planktonic bacteria likely due to increased cell death with planktonic exposure. 87

Figure 12. Biofilm CM stimulates the loss of Occludin from A549 cells. 97

Figure 14. Biofilm CM stimulates A549 cell death after 48 hours of exposure. Planktonic CM stimulated an increase in A549 cell metabolism. 103

Figure 16. Planktonic CM stimulates significantly more IL-8 secretion from A548 cells than biofilm CM. 108

Figure 18. Neither biofilm CM not planktonic CM stimulates wound healing in A549 cells.... 113

Figure 19. Biofilm CM contains significantly more PE than planktonic CM. 118

Figure 20. PE inhibition studies indicated that the morphological changes in A549 cells following exposure to biofilm CM is caused by PE. 119

Figure 21. 24-hour biofilms show a greater resistance to antibiotics than 4-hour biofilms. 124

Figure 22. 24-hour biofilms secrete more β-lactamase than 4-hour biofilms allowing the growth of planktonic bacteria. 127

Figure 23. 4-hour biofilms do not stimulate a significant increase in A549 metabolism compared to 24-hour biofilms. 129

Figure 24. 4-hour biofilms are able to stimulate a significant increase in IL-8 release as compared to planktonic bacteria. 132

Figure 25. CM from 4-hour biofilms do not stimulate the morphological changes in A549 characteristic of PE secretion. 134

Figure 26. 4-hour biofilms do not secrete significantly more PE when compared to the control. 135

Figure 27. *P. aeruginosa* biofilm biomass was decreased with increasing concentration of LL-37. 139

Figure 28. Biofilm formation curve showing a lag in formation in the presence of LL-37 with equivalent bacterial numbers after 24 hours. 142

Figure 29. Physiological concentrations of LL-37 stimulated a 10-fold increase in selected gene expression following 24 hours of exposure to biofilms grown on the Calgary Biofilm Device. 146

Figure 31. Normalizing the gene expression of biofilms grown in the presence of increasing concentrations of LL-37 for 48 hours to *16S rRNA* results in an increased number of down-regulated genes. 152

Figure 32. Growing planktonic bacteria in the presence of LL-37 does not alter its downstream twitching ability. 154

Figure 33. Peg inoculation of twitching media showed opposing effects of LL-37 exposure between biofilm and planktonic bacteria. 156

Figure 34. Growing planktonic bacteria in the presence of LL-37 decreases their ability to stimulate A549 cell rounding. 161

Figure 35. Growth of planktonic bacteria in the presence of LL-37 reduces subsequent A549 cytoskeletal dysregulation. 162

Figure 36. Planktonic bacteria grown in the presence of LL-37 undergo less internalization by A549 cells compared to the untreated control. 165

Figure 37. Growing planktonic bacteria in the presence of LL-37 decreases virulence by decreasing A549 cells death. 168

Figure 38. Growth of bacteria in the presence of LL-37 does not affect subsequent IL-8 secretion from A549 cells. 171

Figure 39. Growth of biofilm in the presence of LL-37 caused an increase stimulation of IL-8 release from A549 cells after 7 and 24 hours. 172

Figure 40. Biofilm grown in the presence of LL-37 for 48 hours had a decrease in PE secretion. 175

List of Tables

Table 1. List of bacterial strains used in this study along with the source reference.................... 39

Table 2. Summary of primers and conditions used for each gene studied for relative quantitation. 65

Table 4. A549 cells undergo morphological changes following exposure to biofilm CM and CM from concentrated planktonic culture.. 94

Table 5. List of cytokines analyzed with the Multiplex bead assay. 104

Table 6. Planktonic *P. aeruginosa* secretes a larger array of proteins compared to biofilm...... 116

Table 7. A larger proportion of proteins unique or up-regulated in biofilm CM were categorized as for virulence and protection as compared to planktonic CM.. 116

Table 8. Biofilms grown in the presence of LL-37 are more resistant to ciprofloxacin treatment. 158

Table 9. Growth of biofilm in the presence of LL-37 for 48 hours slows the morphological changes in A549 cells.. 173

1.0 Introduction

*1.1 **Cystic fibrosis***

Cystic Fibrosis (CF) is the most common autosomal recessive hereditary genetic disorder among Caucasians. In Canada, 1 in 25 people carry a defective gene that leads to CF and 1 in 3400 babies are born with the disease. The defective gene in CF encodes for the Cystic Fibrosis transmembrane conductance regulator (CFTR). The CFTR protein is involved in maintaining ion gradients across membranes, thereby controlling the movement of water across membranes. The CFTR plays a crucial role in controlling sweat, saliva, mucus, tears and digestive enzymes. The three major organ groups that are impacted by CF are the respiratory, gastrointestinal and integumentary systems. There are nearly 2000 different mutations within this gene that lead to varying severities of CF (90). These mutations are classified into 5 different classes based on the functionality of the CFTR. A class 1 mutation results in no functional CFTR produced and results in the most severe form of the disease; a class 2 mutation results in misprocessed/misfolded proteins; class 3 mutations produce proteins that are unable to be properly activated; class 4 mutations result in reduced passage through the channel pore; and class 5 mutations result in reduced amounts of CFTR produced in the cell (187). The most common mutation, accounting for 70% of the CF alleles, is the ΔF508, which is the deletion of a phenylalanine residue at position 508 of the protein. This mutation results in a class 2 protein. In CF patients over 85% of mortality will result from a progression of their lung disease (210).

Within the lung, goblet cells secrete mucus, which functions to capture pathogens and foreign particles that enter the lung. The coordinated beating of the epithelial cilia sweep the mucus out

of the lung, a process termed mucociliary clearance. In CF, a mutated CFTR causes dehydrated mucus and impaired airway clearance making these patients vulnerable to infections such as *Pseudomonas aeruginosa, Burkholderia cepacia,* and *Staphylococcus aureus* infections (256). *P. aeruginosa* is a major cause of morbidity in CF patients. The appearance of *P. aeruginosa* in the lungs is associated with decreased lung function and allows for the subsequent infection with other opportunistic pathogens. Moreover, the nature of CF severely impairs the innate and adaptive immune systems in the lung. CF patients often suffer from chronic inflammation within the lung, leading to tissue damage and lung fibrosis (22,264,317). The lung of CF patients contains significantly increased levels of IL-8, a marker of inflammation, due to altered CFTR function (212). The establishment of a persistent infection results in a cyclic immune response and leads to chronic inflammation and tissue damage (35). In the 1970s the median age at death for patients with CF was 8 years old, primarily due to airway infections; however in 2011, advancements in medical practices and antibiotic treatment allowed most patients to survive into their 40s (68). Improvements in medical practices include methods to improve airway clearance including high frequency chest compressions (10,286). This is accompanied by therapeutics such as bronchodilators, mucolytics and osmotic agents that serve to improve sputum clearance and air exchange (142). While these practices may help inhibit *P. aeruginosa* infection, there are still no interventions that are able to eliminate the chronic infection.

1.1.1 Pseudomonas aeruginosa and Cystic fibrosis

P. aeruginosa is a Gram negative opportunistic pathogen. In North America this bacteria causes around 180 000 infections per year (123). *P. aeruginosa* is one of the leading causes of nosocomial infections and a major cause of morbidity in cystic fibrosis patients with nearly half of all patients infected with either *P. aeruginosa* or *S. aureus* (67). This opportunistic pathogen is

also associated with catheter and urinary tract and burn wound infections and infections in immunocompromised patients (69,102,145,299). *P. aeruginosa* infections are notoriously difficult to eradicate due to their inherent broad-spectrum antibiotic resistance. Furthermore, *P. aeruginosa* contains a very large genome with 6.3 million base pairs, 9.1% of which encode known and putative regulatory proteins (281). In fact the genome of *P. aeruginosa* encodes for 127 proteins that are members of two-component regulatory pathways (257). Two-component regulatory pathways consist of a sensor protein, which responds to stimuli, and a regulator protein, which is activated by the sensor. *P. aeruginosa* regulates the expression of its wide array of virulence genes through the quorum sensing and two-component regulatory pathways (303).

The transmission of *P. aeruginosa* occurs between CF patients as well as via environmental sources. Patient-to-patient transmission can occur during hospital stays when CF patients are in close proximity to each other, or during routine visits to CF clinics (66); however the main source of *P. aeruginosa* is from the environment (274). This early infection with *P. aeruginosa* is often acute and treatment with aerosol antibiotics is often sufficient to eliminate the infection (108). This initial infection is followed by a varying intermittent period where the patient is transiently colonized by *P. aeruginosa,* where approximately 50% of their sputum samples are positive for bacterial growth (176). Longitudinal studies show that 70-80% of CF patients have been colonized with *P. aeruginosa* by the time they reach their teens (46). Initial acquisition of *P. aeruginosa* in the lungs of CF patients usually results in transitioning through this intermittent phase and ultimately becoming chronically infected. In fact, in adults the presence of *P. aeruginosa* is associated with worse clinical scores than infection with *S. aureus* (1). This colonization with *P. aeruginosa* is associated with poorer disease outcomes including decreased age of survival, poorer chest x-ray scores and decreased performance in lung function tests (83).

A transition to chronic infection with *P. aeruginosa* eventually occurs and is often accompanied by the detection of excess polysaccharides in the patient sputum that are associated with the switch to a mucoid strain of *P. aeruginosa*. The emergence of the mucoid *P. aeruginosa* parallels the formation of bacterial biofilms (see below). The appearance of mucoid *P. aeruginosa* is associated with an increase in humoral response (130,137), and a decrease in lung function and survival (116). The conversion to a mucoid phenotype can be associated with genetic changes in the *algD* or *mucA* genes (193); but it can also occur in the absence of any genetic evolution. In this case it is simply the conversion to the biofilm mode of growth that accounts for the increase in exopolysaccharides.

Biofilms are 3-dimensional biofilm communities of *P. aeruginosa* that have been shown to be present in the respiratory zone of chronically infected CF patients. The regions containing the biofilm show tissue damage and pronounced inflammation with infiltrating immune cells (32). These immune cells produce reactive oxygen species kill and eliminate pathogens (325). Moreover, the chemokine IFN-γ, which is released by epithelial cells upon infection, serves to activate infiltrating macrophages to promote the phagocytosis of infecting bacteria. *P. aeruginosa* biofilms are protected against the reactive oxygen species within these inflammatory zones by their secretion of alginate, which serves to scavenge and neutralize these free radicals (115). The production of alginate also protects the *P. aeruginosa* biofilms from IFN-γ-mediated killing by alveolar macrophages (178), as well as increasing resistance to antimicrobials by impeding penetration through the extracellular matrix (220).

The increased vulnerability of CF patients to *P. aeruginosa* infection goes beyond impaired mucociliary clearance. A functional CFTR protein has been shown to be a receptor for the internalization and elimination of *P. aeruginosa* by epithelial cells (244,245). Moreover, the

increased salt concentration in the airway surface fluid of CF epithelia has been shown to have decreased bactericidal activity (268). Animal models have revealed that a mutated CFTR leads to increased bacterial burden within the lungs (243,244). Therefore, the CFTR-mediated internalization of *P. aeruginosa* by epithelial cells appears to be an important function of the host immune response.

1.2 ***P. aeruginosa biofilms versus planktonic bacteria***

The abundance of regulatory proteins allows *P. aeruginosa* to adapt and thrive in diverse environments. A crucial aspect of *P. aeruginosa*'s virulence and diversity is its ability to form complex biofilms (136). While most studies of the virulence of *P. aeruginosa* have focused on the planktonic form, the biofilm form is now known to be prominent in the majority of infections (31,104). Unlike swimming planktonic bacteria, biofilms are structured communities of bacteria encased in an extracellular polysaccharide matrix.

A typical *in vitro P. aeruginosa* biofilm contains a mushroom-cap architecture when grown under flow conditions with glucose as a nutrient source (161). The growth and formation of this biofilm architecture has been shown to require bacterial pili and twitching motility (226). Twitching motility uses the bacterial pili to pull along solid surfaces. It has been shown that *P. aeruginosa* is capable of using twitching motility to climb up its own newly formed biofilm to form the mushroom-cap morphology. *P. aeruginosa* lacking the ability to twitch will form uniform, flat biofilms (55,63,161,161). While twitching motility is required for this biofilm architecture, overactive twitching motility is also detrimental to biofilm formation (160). Overactive twitching motility also leads to flat biofilms, similar to those created by mutants incapable of twitching (160,161). Twitching motility by *P. aeruginosa* requires controlled

expression of its pili proteins (200). The importance of the mushroom-cap architecture is not fully understood and many have noted that this architecture has not yet been witnessed in clinical samples (30).

A defining characteristic of *P. aeruginosa* biofilms is the presence of a complex extracellular matrix as well as the formation of a heterogeneous bacterial population. Many secreted products, such as bacterial DNA become incorporated into the extracellular matrix (181,213,314). The DNA serves to chelate cations and is involved in resistance to antibiotics such as polymyxin B, colistin, gentamycin and tobramycin (213). The three main components of the extracellular matrix – alginate and the polysaccharides pel and psl – have also been shown to contribute to the formation of the architecture of the biofilm (71,221,313). As discussed, alginate also likely plays a key role in immune evasion and antimicrobial resistance. *P. aeruginosa* biofilms form multi-layered structures containing gradients in nutrients and oxygen leading to differential gene expression throughout the biofilm (71,99), which allows for a multitude of survival pathways to be active at one time including the formation of persister bacteria in the inner layer of the biofilm.

The formation of a persister bacterial population lends to its resistance to antimicrobials. These bacteria can be considered metabolically quiescent and can serve to repopulate the environment following antibiotic or immune assault (73). The formation of a persister population is controlled by several pathways in *P. aeruginosa*. Notably persister formation has been shown to be promoted by pyocyanin and high concentrations of quorum-sensing molecules (205). Moreover, the secondary metabolite ppGpp has been shown to be involved in the formation of the persister population (80). The persister population is not equivalent to the stationary phase of growth kinetics where the maintenance protein RpoS regulates cell function (215). Persister cells show a

down-regulation of both *rpoS* and *rpoD* along with a general cessation of transcription. Moreover, the structural nature of the biofilm lends to its resistance to effector immune functions as well as to its resistance to many antibiotics (see Antibiotic resistance).

There are four general stages of biofilm formation in *P. aeruginosa*: initial attachment, coordinated growth, biofilm maturation, and dispersion (65,72,75,105,136,173,311). The initial adhesion of the bacteria requires the flagella (161,226). Once attached the bacteria are able to move around the substrate surface using their twitching motility powered by the pilus. The control of pilus function is mainly at the mRNA level, and expression is controlled based on the two functional parts of the pilin, the major and the minor subunits (26,204,313). Each subunit is controlled by a separate two-component regulatory system. The major subunit includes the *pilA* gene and the minor subunit includes the *fimU-pilVWX* genes. The expression of the minor subunit has been shown to be particularly important in biofilm development (26,204). Thus, the ability to decrease the expression or activity of the pilus should consequently down-regulate the formation of biofilm.

The subsequent growth of the *P. aeruginosa* biofilm can occur through two processes. The first process is through the binary fission and growth of the bacterial population (131). This is what is thought to occur where free-floating bacteria are removed from the environment. An example would be infections of endothelial cells in blood vessels where bacteria that do not adhere to the endothelial cells are eliminated from the infection site. In these particular environments the biofilm is formed by the propagation of the initially adhering population. The second process is through the recruitment of bacteria within the environment (289). Compared to the propagation model, biofilms formed through recruitment have a larger genetic diversity. This process is thought to dominate in static environments where unattached bacteria remain in close proximity

to the surface. This may occur in the CF lung where impaired mucociliary clearance allows the bacteria to grow in the lumen of the lung. Thus far, many studies have formed *P. aeruginosa* biofilms using a flow system that continually replenishes the media and the biofilm is subjected to a high flow rate. In this thesis the Calgary Biofilm Device was used to form biofilms in a static environment more representative of the propagation model. This will serve to provide a new perspective on the characteristics of the infecting biofilm and compliment the current flow system data.

The maturation of the biofilm includes the formation of water channels and completion of the extracellular matrix, which is undertaken through cell-to-cell interactions via two-component regulatory systems and quorum-sensing (see below). The extracellular matrix of *P. aeruginosa* consists of the polysaccharides Pel and Psl. Mucoid strains of *P. aeruginosa* are also able to produce a significant amount of the exopolysaccharide alginate. The activation of genes involved in alginate synthesis is initiated within 15 minutes of initial attachment (71). This results in a step-wise production of biofilm-specific proteins. As part of the maturation process there is coordinated bacterial autolysis that helps create water channels (311). The release of genomic bacterial DNA helps strengthen the extracellular matrix by cross-linking the exopolysaccharides (213). The polysaccharide Psl also plays an early role by maintaining cell-to-cell adhesion (144,186). A mature biofilm maintains a subset of bacteria that remain motile and are released upon favorable conditions, such as a compromised host immune system (289).

1.2.1 Two-component pathway

The ability to sense and adapt to environmental changes is essential for survival. Bacteria contain two-component regulatory systems to sense changes in their environment and regulate gene

expression accordingly (95,114,241). Two-component systems typically involve a sensor kinase and a response regulator that mediates the transcription of various genes. The sensor kinase often has two effector regions. The input domain senses changes in the environment by binding ligands. This stimulates its autophosphorylation causing conformational changes in the transmitter domain, revealing its active site. This active site binds and phosphorylates the response regulator (194). There are three classes of sensor kinases. The largest class is a transmembrane kinase that binds ligands within the periplasm between the outer and inner membrane. The second largest class involves sensor kinases that lack a transmembrane domain. These kinases are either membrane-anchored or soluble in the cytoplasm. The last class of sensor kinases has multiple transmembrane domains (from 2 to 20) but do not bind to external stimuli. These kinases typically respond to mechanical stress or changes in ion gradients (194).

The PprA-PprB is an example of a two component regulatory system with a cytosolic sensor kinase, PprA (309). This system was originally identified for its importance in controlling the membrane permeability of *P. aeruginosa* (309). The response regulator PprB controls the gene expression of several membrane proteins including MexA and OprM. Since its initial discovery the PprA-PprB system has been implicated in a more global control of virulence gene expression (87). Interestingly, this system was shown to modulate the expression of 175 proteins, including several components of other two-component systems through down-stream activation of the gene expression of the sensor kinases and response regulators (87). Out of the genes that are activated by PprB, 85.5% overlapped with quorum-sensing-activated genes, including the autoinducer biosynthase LasI. In fact PprB positively regulates the expression of *lasI* as well as *rhlI,* the biosynthase of a secondary quorum sensing molecule in *P. aeruginosa* (87). Interestingly, PprB is suppressed in biofilms (315). Since this two-component regulatory system is important to

many aspects of *P. aeruginosa* virulence its down-regulation in mature biofilms indicates that there are likely differential virulence patterns between the two growth forms.

The ability to use two-component systems to sense environmental changes is crucial for opportunistic pathogens, such as *P. aeruginosa*. It can sense small changes in the host's immune system, such as changes in salt concentration, and up-regulate genes that allow it to activate virulence mechanisms to establish an infection. Factors that are able to change the expression of two-component systems can have significant effects on downstream virulence and are of particular interest in the development of new therapeutics. Due to the lack of *in vitro* co-culture systems it is difficult to examine the downstream virulence of *P. aeruginosa* biofilms following their growth in the presence of therapeutics, such as the cationic host defense peptide LL-37, that are able to affect two-component systems.

1.2.2 Quorum-sensing

With such a large genome in *P. aeruginosa*, the timing of virulence gene activation is critical. Expression of virulence genes too early in infection allows the host immune system to respond to a small number of bacteria. Instead, *P. aeruginosa* only activates these genes if there are high bacterial numbers. This ability to determine its density is termed quorum sensing. Quorum-sensing differs from two-component systems because quorum-sensing is specific to bacterial numbers, whereas two-component systems respond to all external stimuli. Quorum-sensing has been shown to be play a role in influencing *P. aeruginosa* biofilm structure (199). *P. aeruginosa* synthesizes and releases heat stable lactones that serve as autoinducers. As bacterial numbers increase, the concentration of autoinducers also increases. The autoinducer then binds to quorum-sensing regulators and activates or represses a series of target genes (76). However the

activation of genes through the quorum-sensing pathway is dependent on factors beyond bacterial numbers. Studies have shown that environmental conditions help guide the pattern of genes that are activated or deactivated through quorum-sensing (34). For example, conditions such as iron availability are able to promote biofilm-specific gene regulation.

The two most prominent quorum-sensing systems in *P. aeruginosa* are the Las and Rhl systems (239). The Las system consists of a transcriptional regulator LasR, which binds the homoserine lactone 3O-C12-HSL; this lactone is synthesized by the LasI protein. Davies *et al* (72) showed that the Las system was critical for biofilm architecture and differentiation. The synthesis of LasI is controlled by the LasR regulator. This system is mirrored by the Rhl system containing the transcriptional regulator RhlR, which binds the homoserine lactone C4-HSL produced by RhlI synthase that is regulated by RhlR (239). Of the two, the Las system is at the top of the quorum-sensing hierarchy since Rhl is under its transcriptional control (239). However, downstream of the *lasR* gene and under the transcriptional control of LasI is the *rsaL* gene, whose protein functions as a negative repressor of LasI-activated genes (74). In fact, the transcription of *lasI* in biofilms has been shown to decrease over time, while the expression of *rhlI* has been shown to remain steady (75). Over 616 genes have been shown to be under quorum-sensing control in *P. aeruginosa* including the up-regulation of virulence genes and genes known to be involved in biofilm formation (239,315). The importance of quorum-sensing in biofilm formation has been shown *in vitro* as well as in mice models, where interference with quorum-sensing through azithromycin treatment was able to prevent the formation of biofilms (133).

1.2.3 P. aeruginosa biofilm regulation

P. aeruginosa contains two primary transcriptional housekeeping proteins, also known as σ-factors in *Escherichia coli*, RpoD and RpoS. RpoD functions as a general housekeeping factor and is expressed during logarithmic growth (101). As the bacteria enter stationary phase the expression of *rpoD* decreases and *rpoS* increases. RpoS regulates the expression of genes involved in nutrient acquisition, high oxidative stress and several virulence genes (283). Interestingly, the gene expression of *rpoS* as measured by qRT-PCR of 3-day old continuously-fed biofilms was 3 times higher than stationary phase planktonic bacteria; however the level of RpoS protein did not differ between the two forms (323). Moreover, it was shown that expression of *rpoS* within *P. aeruginosa* biofilms was higher within the actively metabolizing bacteria in the cap region of the biofilm (238). The expression of *rpoS* is controlled primarily through the global response regulator PsrA (163,164). Mutations in either *rpoS* or *psrA* results in biofilms that are thicker, but less differentiated (132). Therapeutics that are able to alter the expression of *rpoS* and *psrA* may cause dysregulation of the biofilm maturation, thereby decreasing the virulence of the biofilm.

Differences in biofilm and planktonic bacteria can be seen at the mRNA level (304,315), protein expression level (75,273), and the protein phosphorylation level (240,241). Gene expression changes are mediated through a complex network of two-component response pathways, quorum-sensing systems and other transcriptional regulatory proteins.

A microarray analysis of mature biofilms showed that 73 genes had differential gene expression compared to planktonic cells (306,315). Several studies have shown that the protein expression pattern of biofilms goes through a maturation process during biofilm development (259,273). However, a separate study also looked at gene and protein expression of biofilms in a different

system and found a completely different protein expression pattern (304). This paper went on to describe how every aspect of culture (strain, culture conditions and technologies) will yield different results because they will each stimulate a separate regulatory pathway leading to biofilm formation. This theory of no "universal biofilm" transcription profile has been echoed by other groups, highlighting the importance of growth conditions on biofilm phenotype (27,174,203,301). However, the development of biofilms always follows a step-wise process with young biofilms displaying differing protein profiles from mature biofilms. In fact, after the initial attachment of *P. aeruginosa* to the surface there is a production of 11 proteins at the onset of biofilm formation (42). Petrova and Sauer (240,241) have shown that 7 proteins are phosphorylated in *P. aeruginosa* biofilms and not in planktonic bacteria. Moreover they found a stepwise phosphorylation process of 7 proteins leading to the maturation of biofilms, along with 26 proteins constitutively phosphorylated in biofilms and planktonic bacteria. It is clear that *P. aeruginosa* biofilms possess intricate gene and protein regulation in the formation of biofilms. There are few studies examining the downstream effects of growing biofilms in the presence of factors that can perturb the biofilm gene expression. This opens the door for new therapeutics that may display a lower selective pressure for resistance.

1.2.4 Host-pathogen interaction

1.2.4.1 Lung epithelia

The first line of defense within the lung is the physical barrier of the lung epithelia (265). The epithelial cells form tight junctions and subjacent adherens that prevent the passage of extracellular pathogens (189). The lung epithelia within the conducting zone contains globular cells that secrete mucus which can bind *P. aeruginosa* and sweeps the pathogen out of the lungs through the beating of the epithelial cilia (252). Besides its role as a physical barrier, the lung

epithelia also plays a key role in the recruitment and stimulation of effector cells of the innate and adaptive immune systems (85). Lung epithelia are able to recognize foreign pathogens through specific pattern-recognition molecules. These proteins are typically transmembrane receptors, but are also located within the cell. An example of these pattern-recognition molecules are the family of Toll-like receptors (TLRs). Several bacterial factors interact with the epithelial cells, including lipopolysaccharide (LPS) and flagellin, through the cells' TLRs (121,166). Stimulation through the TLRs causes a cascade of phosphorylation events leading to the secretion of cytokines, chemokines and antimicrobial factors (156,242,249). This initiates two pathways of pathogen removal: direct killing of the pathogen through antimicrobial secretion, although rare; and secretion of chemokines that recruit effector cells resulting in pathogen removal. Moreover, the epithelia secrete defensive enzymes and peptides including defensins and LL-37 (53,56,233).

There is a marked difference in the characteristics of acute and chronic lung infections and the resultant host response. Acute infections are typrically characterized by significant cell damage, potential dissemination into the blood stream and pneumonia-like symptoms with significant morbidity (104). Chronic infections can last decades and are differentiated by a continual inflammatory state and subsequent decrease in lung function. The acute-infection is caused by bacteria that have several mechanisms to evade the host immune system; however they tend to be more susceptible to antibiotic treatment. This differs from the biofilm bacteria of chronic infections, which are not able to be eradicated by most antibiotic therapies (93). Moreover, the heterogeneity of the biofilm also mediates its resistance to the effector functions of resident and infiltrating immune cells especially phagocytosis by effector immune cells such as alveolar macrophages (48,167,201). Also, some have suggested that the alginate in the extracellular

polysaccharide matrix of the biofilm can reduce the activation of complement, as well as promote neutrophil oxidative burst, leading to epithelial cell damage (237). This contributes to chronic inflammation and subsequent lung damage. There is frequently an exacerbation of chronic infections which is possibly mediated by the release of planktonic bacteria from the biofilm that may take advantage of the damaged epithelial barrier. Understanding the difference between the interactions of biofilm and planktonic bacteria with the host immune system is critical. The virulence and growth kinetics are vastly different between biofilms and planktonic bacteria and therefore require different treatment strategies. The differences between the virulence of biofilms and planktonic *P. aeruginosa* with lung epithelia are not well characterized.

1.2.4.2 P. aeruginosa infection of lung epithelia

P. aeruginosa induces an immune response upon binding to lung epithelial cells (255). *P. aeruginosa* may also evade the immune system by binding to lung epithelia (23,47,49,326) and in rare cases can stimulate their own internalization (94,246). Bacteria typically bind to the epithelia through their pili and flagella and may enter the cells through receptor-mediated endocytosis (302). The purpose of entering the epithelia is two-fold: to survive within the lung epithelia and avoid the killing mechanisms of the effector cells, as well as to kill the lung epithelia and compromise the epithelial barrier. While in many cases internalization of *P. aeruginosa* results in death of the bacteria, a small subset of bacteria survive within the epithelial cells. *P. aeruginosa* has been shown to survive within host cells for as long as 7 days *in vitro* (246). The eventual release of bacteria potentially serves to establish an acute or chronic infection.

P. aeruginosa employs a type III secretion system (TTSS) that allows the injection of virulence factors directly into host cells (61,297). Among the injected toxins are the exoenzymes S, T, U

and Y. These enzymes cause cytoskeletal rearrangement, decreased cell adhesion and often result in cell lyses. The TTSS machinery and toxins are expressed in biofilms; however their role in pathogenesis is unknown since the virulence mechanisms are more characteristic of acute infections (202,292). *P. aeruginosa* is also able to permeate the barrier of the epithelial lining through the stimulation of apoptosis of the epithelial cells (120,253). Moreover, *P. aeruginosa* secretes proteases that are able to degrade the tight junction proteins and allow bacterial invasion (11,13,14). Differences in *P. aeruginosa* pathogenesis may be due to the different interactions between biofilms and planktonic bacteria with the lung epithelia.

1.2.4.3 P. aeruginosa secreted factors

There are many factors secreted by *P. aeruginosa* that interact with lung epithelia including quorum-sensing molecules (269,270), phenazines (84,152,172), proteases (12,179,190,234), and exotoxins (171,247,253). Comparing the secretomes of *P. aeruginosa* isolates from early and late infections of CF patients revealed significant differences (312). The proteins from cellular fractions showed over 80% similarity while the extracellular fractions contained more significant differences. This observation has been mirrored in other studies (225). This phenomenon has also been shown in other pathogens like *Bacillus cereus* where the secretomes of the same strain change significantly when grown in different environmental conditions, while the expression of cytosolic proteins were less perturbed (60,110). Thus, it makes sense that changes in virulence would be reflected in the extracellular proteins that interact with their environment. While the secretomes of different strains of *P. aeruginosa* grown under similar conditions have been reported, there have been few studies examining the differences in proteins secreted by biofilm and planktonic bacteria of the same strain. For example, we know that the LPS secreted by biofilms is structurally and functionally different than planktonic bacteria (59). Biofilms release

LPS with altered lipid A and polysaccharide moieties that induce more inflammation (122). The unique proteins in the secretome of biofilms could serve as biomarkers for biofilm infection, as well as providing targets for therapeutics for chronic infections.

1.2.4.4 The role of the antimicrobial peptide LL-37 in lung epithelial host defense

The human host defense peptide LL-37 is a cathelicidin that is a key component of the innate immune system in the lung (20). The peptide is the only known cathelicidin produced by humans and is first synthesized as the pro-peptide hCAP18. Upon secretion it is cleaved to the highly conserved functional 37 amino acid LL-37. LL-37 is an α-helical peptide with hydrophobic and cationic moieties, allowing for its interaction with many targets, including cell membranes, genetic material and various proteins. LL-37 is a polyfunctional peptide with both immunomodulatory and antimicrobial properties. The expression of LL-37 differs among cell types. Epithelia constitutively express LL-37 with capacity for up-regulation upon stimulation of cells by inflammatory cytokines. LL-37 is released by other immune cells including neutrophils and macrophages.

The importance of LL-37 in host immunity has been well described (25,222,251,308,319). Subjects that are unable to produce LL-37 are significantly more prone to infections, particularly bacterial infections (251). Beaumont *et al.* (25) found that mice which were deficient in cathelicidin had impaired neutrophil response and were unable to clear infections with *P. aeruginosa*. They also showed the important role that LL-37 plays in modulating the immune response, since they were unable to find any direct bactericidal activity of the peptide. Wnorowska *et al.* (319) found similar results with a highly pathogenic strain of *P. aeruginosa*. This strain contains a bacteriophage that is able to inhibit the bactericidal activity of LL-37; however patients containing high levels of LL-37 in their lungs were still able to clear infection.

The kinetics of LL-37 release during *P. aeruginosa* infection is complex. Initial exposure to bacteria stimulates an LL-37 "burst" from infiltrating neutrophils. However, the airway surface fluid of CF patients contains high levels of extracellular DNA and actin that likely sequesters the released LL-37 and may prevent its interaction with the bacteria (45). Interestingly, the current use of DNase treatment in CF patients may liberate this trapped LL-37 in a therapeutic-like manner (45).

LL-37 helps initiate an inflammatory response by binding to the Fprl2 receptor and acting as a chemoattractant for blood neutrophils, monocytes and T cells (81). Inactivation of the TLR4 signaling pathway by LL-37 also contributes to the modulation of the inflammatory response (206). Along with modulating the inflammatory response, LL-37 also plays a role in directing the effector functions of the infiltrating cells. In particular, LL-37 has been shown to promote the phagocytosis of bacteria by macrophages through TLR4 signaling and the FPR2/ALX receptor (308). This was true for IgG opsonized and non-opsonized bacteria. LL-37 has also been shown to be involved in epithelial wound healing, and it has been proposed that decreased secretion of LL-37 in chronic lung infection may lead to a decrease in wound repair and formation of lung fibrosis (129). While the role of LL-37 in immune modulation is clearly important, LL-37 has also been shown to directly interact with bacteria. The role of LL-37 as an anti-microbial peptide has been extensively studied; however even low concentrations of LL-37 have been shown to affect the virulence of *P. aeruginosa* (82,232,282).

LL-37 interacts with the outer membrane of Gram negative bacteria initially through interactions with negatively charged bacterial LPS (143,222). Once the concentration of LL-37 on the membrane reaches a critical point the peptide is able to form pores increasing membrane permeability. However the direct killing of bacteria by LL-37 *in vivo* has been questioned since

the pore-forming ability is abolished at physiological salt concentrations (78,123,232). Instead it seems likely that the anti-infective role of LL-37 lies in its immunomodulatory capability as well as through other non-killing interactions with bacteria. Constitutive exposure to LL-37 has been shown to down-regulate the quorum sensing *lasI* and *rhlI* systems in *P. aeruginosa* biofilms (82,232). These quorum sensing systems are crucial for bacterial virulence and pathogenesis. Many bacteria have been shown to secrete proteases that are able to degrade LL-37 (262). Whether the constant exposure to intact LL-37 is required for the down-regulation of virulence genes is unknown. The effects of continuous low concentrations of LL-37 were examined on biofilms using a flow-cell method, where media is continuously replaced (232). Interestingly architectural changes to established *P. aeruginosa* biofilms following exposure to LL-37 were noted. It was also found that LL-37 promotes twitching of planktonic *P. aeruginosa*; however the mechanism is still less understood. Exposure to LL-37 and other antimicrobial peptides has been shown to induce activity of the global response regulator PsrA, resulting in the stimulation of swarming and biofilm formation (113). Several studies have shown that LL-37 impairs the adherence of *P. aeruginosa* to abiotic surfaces, thereby decreasing subsequent biofilm formation (82,232). The flow-cell methods previously used to study biofilms have several draw-backs when studying LL-37 interactions. Importantly, the large amount of media required to pump through the system leads to only low concentrations (such as 4 µg/mL) of LL-37 for biofilm exposure to be studied. The local physiological concentration of LL-37 in the lungs of adults and neonates is estimated to be 2 µg/mL and 5 µg/mL respectively (21,261). This increases to approximately 6 µg/mL and 15 µg/mL during an infection (20,53,261). However other infection sites have reported local concentrations as high as 1500 µg/mL (230). Using a static system, such as the Calgary Biofilm Device, where the media is not replaced would allow for higher

concentrations of LL-37 to be studied; however the static system may not necessarily reflect the *in vivo* environment.

1.2.5 Antibiotic resistance

Antibiotic resistance is widespread in *P. aeruginosa* and is of particular importance in CF lung infections due to the repeated use of antibiotics as a common treatment regimen (57,112). This provides selective pressure ideal for promoting the development of resistant strains.
P. aeruginosa employs several strategies to combat antibiotic pressure. One strategy is the secretion of β-lactamases that degrade β-lactam antibiotics (112,155,223). In fact, high levels of β-lactamases can be recovered from the sputum of CF patients (112). An interesting characteristic of secreted resistance factors is that they benefit the entire bacterial community. The presence of one β-lactamase-producing strain allows the growth of other strains through the degradation of antibiotics. While it has been shown that β-lactamase production can be induced in biofilms, it is currently unknown whether the source of β-lactamases *in vivo* is from biofilm or planktonic bacteria.

Another strategy that is employed by *P. aeruginosa* biofilms is the separation into two different metabolic states. The layered community architecture of the biofilm results in two distinct bacterial populations. The external bacteria are actively growing cells that are likely susceptible to antibiotic treatment; however the internal cells are quiescent and thereby resistant to many antibiotics that interfere with metabolic pathways (41). Following the completion of the antibiotic course, these persister cells serve to re-seed the population. Therapeutics used in CF may have the unintended effect of skewing the bacterial distribution towards more quiescent bacteria. This would hamper the ability of antibiotics to eradicate the bacteria.

A resistance strategy employed by *P. aeruginosa* is the exclusion of antibiotic. This was long thought to be attributed to the reduced penetration of antibiotics through the extracellular matrix; however it is now known to be largely due to efflux pumps that actively pump the antibiotics out of the cell. Four efflux pumps – MexXY, MexCD-OprJ, MexEF-OprN and MexAB-OprM – have been shown to be important in *P. aeruginosa* resistance to several different classes of antibiotics (77,196). Of the four pumps the expression of *mexAB* is the only one that is constitutive. The expression of *mexX* is inducible by the presence of antibiotic. Interestingly, the expression of *mexX* was down-regulated in biofilms compared to planktonic bacteria and did not appear to be critical for biofilm resistance since biofilms lacking *mexX* were not more sensitive to any antibiotics (77). A component of the resistance of *P. aeruginosa* biofilms to antibiotics is still thought to lie in the ability of the polysaccharide matrix to impede the diffusion of the antibiotic (278). However even antibiotics that are able to penetrate the matrix are still unable to eradicate the infection. Thus further studies are still required to evaluate antibiotic resistance and the expression of efflux pumps within biofilm and planktonic *P. aeruginosa* and how this may be affected by LL-37.

1.2.5.1 Antibiotic use in CF

The lung environment includes two distinct zones: the conductive zone, including the trachea and bronchioles; and the respiratory zone, which includes the alveoli and is the location of air exchange. It has been shown that non-mucoid strains of *P. aeruginosa* are limited to the conductive zone, while mucoid biofilm-forming strains colonize both the conductive and respiratory zones (32,135). Each zone requires separate antibiotic delivery strategies with nebulized and inhaled antibiotics reaching high concentrations in the conductive zone and low penetration into the respiratory zone; and intravenous and oral antibiotics obtaining high levels in

the respiratory zone and low levels in the conductive zone. Therefore combination therapy is often used to treat infection. There have been attempts to use prophylactic antibiotic use to prevent the initial colonization of *P. aeruginosa* with no success (291). Once there is an initial acquisition of *P. aeruginosa* an aggressive antibiotic treatment occurs to attempt to eradicate the bacteria (19,108,296). When deciding between different antibiotic treatment strategies there are several factors that need to be taken into consideration, including: pharmacology and pharmacodynamics, toxicity, cost, lung function, symptoms and others (258).

An aminoglycoside often used in CF patients is aerosol nebulised tobramycin. The use of tobramycin inhalation solution has been associated with significantly reduced mortality among CF patients (260). Inhaled tobramycin is often alternated with fluoroquinolone treatment, including ciprofloxacin, on a monthly basis to combat bacterial resistance (91). Beta-lactams are often used to treat non-pseudomonal infections in CF patients; however this class of antibiotic is rarely used to treat *P. aeruginosa* infections due to widespread antibiotic resistance. Several of these include anti-pseudomonal carbapenems, such as meropenem, and cephalosporins, such as ceftazidime. These antibiotics are rarely used during acute exacerbations and are administered intravenously (330). Unfortunately there is still no antibiotic that is capable of eradicating a *P. aeruginosa* infection once it has become chronic. Many of these treatments are used to try to prevent chronic infection, or, once chronic infection is established, then to prevent exacerbations (98,108,111). Since *P. aeruginosa* biofilms have such an inherent resistance to antibiotics potentially some other mitigation strategy is necessary. Better understanding the interactions of the biofilm with the host immune system with antibiotic use will help guide the development of new therapeutic modalities.

Several clinical studies have identified antibiotic treatment strategies that improve patient lung function and decrease *P. aeruginosa* bioburden within the lung while also decreasing hospitalization due to acute exacerbations (228). The AIR-CF3 study was a phase III examination of inhaled aztreonam (228). Several years after the completion of this study the efficacy of inhaled aztreonam in comparison to and in synergy with inhaled tobramycin on *P. aeruginosa* biofilms was re-examined *in vitro* (327). It was found that tobramycin was more capable of preventing biofilm formation as well as eliminating established biofilms as compared to aztreonam; however tolerance to aztreonam was limited to the non-mucoid strains of *P. aeruginosa* with mucoid strains more susceptible to aztreonam than tobramycin (327). The tolerance of the non-mucoid strains appears to be linked to the exopolysaccharide Psl. Moreover, synergy between tobramycin and aztreonam was found for certain strains of *P. aeruginosa*, indicating that this treatment combination may be beneficial to some CF patients.

1.3 ***Biofilm/mammalian cell co-culture methods***

1.3.1 Advancements in cell culture for infection studies

In vitro culture methods are important for dissecting the pathogenicity of *P. aeruginosa* and its interaction with the host. The importance of these models have been acknowledged by others (30). The value of *in vitro* systems lies in their ability to create a simple environment to study individual aspects of the host-pathogen interaction. Once important aspects are identified it is important to develop animal models to confirm the results. While often considered over simplistic, *in vitro* co-culture methods have been crucial in highlighting key aspects of the host-pathogen relationship. In fact, several studies have gone on to confirm *in vivo* results that were initially identified using *in vitro* systems (44,64,192,244,245). Pier *et al* (243) used *in vitro* co-

culture assays to discover that CFTR is involved in the binding and internalization of *P. aeruginosa*. Their lab then went on to confirm these results *in vivo* using a murine model. The importance of *P. aeruginosa* in stimulating vascular endotheliall growth factor was also found using an *in vitro* co-culture assay (192). The long-term effects of azithromycin use on the formation of lung fibrosis in *P. aeruginosa* infections was initially found *in vitro* and confirmed *in vivo* (64). Each of these *in vitro* studies discovered key aspects of bacterial virulence that were reflective of the effects seen *in vivo*.

Efforts are constantly being made to advance co-culture systems to mimic the *in vivo* infection environment in order to better understand the host-pathogen interaction in a physiologically relevant environment. A co-culture system must also take into account the specific biological conditions at each infection site; one co-culture method may not reflect all infection environments. Further, having multiple co-culture systems to study host-pathogen interactions allows versatility in confirming findings. There have been major advances in the development of the mammalian epithelial side of this co-culture system that will be discussed below.

1.3.1.2 Monolayers

The simplest form of epithelial cell culture is to grow an immortalized cell line in monolayers. Examples of lung epithelial cell lines used include A549 pneumocyte cells and 16HBE cells. Due to its simplicity these immortalized cell lines are often used for the initial study of bacterial-epithelial interactions. A549 cells are a human lung carcinoma cell line derived from type II alveolar pneumocytes (109). Interestingly, recent characterization of this cell line has questioned the suitability of this cell line for representing type II alveolar epithelia; rather showing that this cell line is more characteristic of type I alveolar epithelia (284). Type I alveolar epithelia

constitute approximately 95% of the alveolar surface area while representing only approximately 40% of the alveolar epithelial cells (50).

Once the model system is established and unique characteristics of the host-pathogen interaction are identified then the findings can then be tested in primary cell lines. Primary cell lines are composed of a heterogeneous cell population of varying cell types and growth states that can give different responses and are more difficult to maintain than immortalized cell lines. Because of this it is significantly more cost effective to perform the initial infection studies on immortalized cell lines. Moreover, the heterogeneity of the primary cell growth can complicate the results and often requires a larger sample size to account for the variability. One of the major limitations of using cell monolayers, both immortalized cell lines and primary cell lines, is the lack of mammalian cell differentiation. This includes the lack of cell polarity and architecture. While primary cell lines include a heterogeneous cell population, those cells still lack the polarity and architecture of intact epithelia. Further, immortalized cell lines can also exhibit different characteristics than primary cell lines. When modeling the lung, this is evident by the lack of cilia, as well as by the lack of a mucus layer. Moreover, since the cells are not polarized they often do not display key markers that may play an important role in the host-pathogen interaction. As mentioned previously, cell monolayers also easily detach from the surface shortly after infections and long-term studies are limited (208).

1.3.1.3 Air-liquid interface

To better reflect the *in vivo* cell characteristics of the interaction of bacteria with lung epithelia an air-liquid interface system has been used (61,96,246,292). In this system, epithelial cells are grown to confluency on a semi-permeable surface. Once confluency is reached the media is removed from the apical side of the cells and nutrients are only available on the basal side

through the semi-permeable surface. The apical side of the cells is exposed to the air. Cells grown at an air-liquid interface become polarized and display strengthened tight junctions. Since the disruption of tight junctions is crucial to *P. aeruginosa* planktonic virulence this system has been beneficial for thoroughly characterizing the interaction of *P. aeruginosa* with lung epithelia. Further, the differentiation of the cells allows for a higher tolerance to bacterial exposure, allowing for longer incubation times and the formation of early biofilms (320). Moreover, the polarized cells also develop cilia and certain cell types, including Calu-3 cells, will secrete mucus (96). As with the monolayers, using primary cell lines at an air-liquid interface can also better reflect *in vivo* characteristics. These include a heterogeneous cell population, cilia and mucus production as well as the display of important cell markers. A major draw-back to this system is that it is expensive and time-consuming. Following the establishment of the air-liquid interface, the cells are grown for over 3 weeks to obtain proper differentiation. This method is not conducive to high-throughput infection studies and obtaining the proper controls is difficult. Moreover, time-course studies are difficult since a high-throughput system is ideal for these experiments.

1.3.1.4 3-Dimesional cell culture

Infection studies have also been performed using 3D cell culture to create organoid aggregates of cells that associate together. There are two primary methods that have been employed: spheroids and agitation. In the spheroid model, cells are grown on beads coated with extracellular matrix components such as collagen. The cells are grown under the gentle agitation of a rotating vessel wall. The spheroids display polarized cell characteristics including increased epithelial cell markers and mucus production (49). The spheroids are viable for up to 6 weeks using lung epithelial cells isolated from one patient. This allows for multiple experiments to be performed

using one patient sample. Unfortunately this method is not amenable to high-throughput studies since it is so labor intensive and is quite expensive.

The second method of 3D cell culture is to grow the cells under gentle agitation. The cells naturally aggregate and grow as 3D cell clumps (294,295). When the model is adapted to include primary cell lines then a heterogeneous cell population is obtained with functioning cilia (295). The aggregate model is more conducive to high-throughput studies; however there is less control over aggregate size and therefore produces relatively high variability. Both 3D models have been used to study various host-pathogen interactions using planktonic bacteria, including bacterial adherence and invasion (49). These studies have thus far been primarily limited to the planktonic bacteria and short incubation times.

1.3.2 Advancements in biofilm culture for infection studies

These advancements in cell culture methods are better reflecting the characteristics of lungs *in vivo*; however thus far co-culture has been largely limited to planktonic bacteria and therefore generally only reflects early infection interactions. There has been recent interest in altering the pathogen arm of this interaction to include biofilms. As described it is clear that pathogenesis and host-pathogen interactions differ between biofilm and planktonic bacteria. The pathology and pathogenesis of chronic infections differ greatly from acute infections and co-culture studies with planktonic *P. aeruginosa* are unable to replicate the characteristics seen *in vivo.* The interaction of biofilm with lung epithelia has been described previously using various models, which will be further summarized in the following sections (136,208,292). Each of these studies showed significant differences in the interactions of biofilm versus planktonic bacteria with lung epithelia. A greater understanding of the role of the innate immune system in regulating biofilm

development is also needed; however few models are available for studying the interaction between the innate immune responses elicited by host epithelial cells and the biofilm. Several studies have examined the interaction of A549 lung cells with isolated, secreted components of biofilm infections such as quorum-sensing molecules (56), and biofilm LPS (59). These studies use either *in vitro* analysis using cell lines such as A549 lung cells, or using *in vivo* methods by injecting biofilm components into animals. While these studies are interesting and can identify key virulence factors, they give little insight into the overall interaction of the biofilm with the host epithelium.

1.3.2.5 Static model

The direct interaction of *P. aeruginosa* biofilms with mammalian cells was described by Moreau-Marquis *et al* (208) using both static and flow cell culture. In the static model mammalian cells are grown as a monolayer to confluency and then seeded with bacteria. The bacteria are allowed to adhere for an hour and then the media is replaced to allow the bacteria to grow and form biofilms. This method is relatively simple and can be used to study the establishment of biofilm infection. This method is ideal for studying early infections and has been used to discover key components involved in the initial establishment of infection such as the flagella and pili (47,128). It has also been used to determine the efficacy of antibiotics on early biofilms adhered to mammalian cells, as well as in adherence studies of various mutants (5,7,292). While this method is simple, it has several drawbacks. The first issue is that only early biofilms can be studied with this method since death of the mammalian cells limits the length of co-culture time. Thus, these newly forming biofilms may not be the best representation of a chronic infection. It has been shown that the virulence mechanisms of these newly forming biofilms are more similar to that of planktonic bacteria, while established biofilms differ greatly

in their virulence mechanisms (37,226,259). Secondly, even though the media contains components that promote biofilm formation (such as arginine), planktonic bacteria will be released from the biofilm and will rapidly overgrow and cause cell death that may confound results; therefore only short incubation times may be used. Even when this model was adapted using mammalian cells grown at an air-liquid interface as described above, the biofilms were very young and only short incubation times could be used. However this adapted model was interesting since the mammalian cells were more phenotypically characteristic of *in vivo* lung cells (320). The Calgary Biofilm Device represents a static method of biofilm growth; however this system allows for the ability to control the growth and maturity of the biofilm, which can then be easily co-cultured with mammalian cells.

1.3.2.6 Flow cell model

The flow cell method is similar to the static method except that following initial bacterial incubation the supernatant is continually replaced (208). In this system mammalian cells are grown on glass coverslips. Once they reach confluency the coverslip is placed in a flow-cell chamber and inoculated with bacteria. The bacteria are allowed to adhere for a short period of time. Following a short incubation the media flow is initiated by a pump that moves the media at a fixed rate. Since the media is continually replenished there is more control over the environment. In the static model the media is the same throughout the incubation, leading to the depletion of nutrients and build-up of secreted products. In the flow model the nutrients are constantly replenished and the secreted products are removed. This method is far superior to the static method for the study of longer time-points; however the biofilm still does not usually have enough time to mature prior to the death of the mammalian cells. In flow models, biofilms take much longer to reach maturity since the biofilm growth is dependent on the bacterial doubling

time, unlike the static model where bacteria in the environment come together to form the biofilm. Moreover, since the media is constantly replaced the flow method is not ideal for studying secreted factors by the biofilm or mammalian cells. A second drawback is that it is labor intensive and experimental replicates are not easy to obtain. Also, time-course studies are difficult to perform with this procedure. It is also important to consider the *in vivo* environment when choosing a model system. The flow model is not necessarily reflective of the lung environment in chronic infections, particularly in CF, where the mucus is not replaced, there is very minimal shear force and it is characterized as a more nutrient-limiting environment (168,263). The flow cell model is potentially more representative of early infections of endothelial cells in blood vessels or epithelial cells in the intestine (117,154). A system that allows for the study of a chronic infection would involve the pre-maturation of the biofilm prior to exposure to the mammalian cells would be ideal. This system would be able to define differences between planktonic, young and established biofilms.

1.3.2.7 Co-culture of established biofilm with mammalian cells

There are currently few *in vitro* culture systems that allow the co-culture of established biofilms with human cells. One co-culture system involves the formation of biofilm on disks in a flow cell chamber (118). Once the biofilms have matured the disk is transferred to a 6-well cell culture plate containing mammalian cells. The disks are placed on a ring support that maintains a separation between the biofilm and the cell monolayer. While this system can be used to study the interaction of mature biofilm with mammalian cells, it is not currently amenable for assays in a 96-well format. Moreover, the growth of biofilm in a flow cell system is quite labor intensive and does not allow for many replicates to be performed.

A second co-culture system that has been used is the *ex vivo* infection of tissue samples (9). In this model, an excised biopsy tissue sample is maintained in a 6-well plate using an air-liquid interface. The tissue is infected with a small inoculum of bacteria and allowed to grow for up to 72 hours. In order to have such a long incubation period the tissue samples should contain a fully stratified epithelium, including the underlying connective tissue. Because such a long time-point can be used, the biofilm has an opportunity to mature. However, during the maturation of the biofilm the planktonic bacteria are able to cause significant tissue damage, which cannot be repaired. Therefore this method does not necessarily reflect the *in vivo* characteristics of chronic lung infection, where tissue repair can occur following the acute infection. The use of a tissue sample as opposed to cell culture allows for the study of differentiated host cells. However the control over the biofilm growth is extremely limited. Moreover this method is even more labor intensive and expensive and does not allow for subsequent assays that are best performed in 96-well plates.

Along with direct co-culture models, transwell systems have been used to examine the interactions of secreted factors between biofilms and airway epithelial cells (170). This model involves the growth of biofilm in alginate beads and then applying the biofilm beads to the upper chamber of a transwell system with the airway cells in the lower chambers. This system is ideal for studying secreted factors because the epithelia are not harmed by direct bacterial contact. Moreover, the secreted factors of the epithelia can impact the biofilm to better represent the *in vivo* infection conditions.

1.4 Calgary Biofilm Device for biofilm co-culture

The Calgary Biofilm Device is a method for studying the co-culture of mammalian cells with planktonic *P. aeruginosa* as well as an established biofilm (52). The Calgary Biofilm Device is a 96-peg lid that fits into a 96-well plate. Each peg is used to cultivate a biofilm. The peg-lid methods, including the Calgary Biofilm Device, allow for 96 uniform biofilms to be formed on pegs (3). The peg-lid can then be removed, washed and exposed to many different experimental conditions. Individual pegs can be removed at any point throughout the procedure to act as controls or for time-course studies (52). Using the Calgary Biofilm Device for co-culture with mammalian cells functions as a modified version of the static model since there is no media flow. This system allows *P. aeruginosa* biofilms to completely grow into matrix-encased communities which can then be used to inoculate human lung epithelia. Once the method has been fully developed and characterized, this system can be adapted using more advanced mammalian culture methods, including polarized cell monolayers. This is a new tool to study and compare the interactions of biofilm and planktonic bacteria with human lung epithelia. The nature of the system allows for appropriate replicates to be used and can also study many different physiological conditions in one experiment. The concurrent analysis of biofilm and planktonic bacteria highlights differences in the virulence of these two growth forms, which helps provide a better understanding in the host-pathogen interactions of chronic and acute lung infections. Comparison of biofilms grown on the Calgary Biofilm Device for 22 hours and biofilms grown on a flow cell device for 5 days in rich media resulted in biofilms with a flat architecture (125,285). Moreover, when these two biofilms were exposed to heavy metal cations they both showed killing of exterior bacteria and the presence of persister internal bacteria

(125,285). The presence of persister cells in biofilms grown on the Calgary Biofilm Device was confirmed by Spoering and Lewis (275).

The Calgary Biofilm Device co-culture method certainly has several limitations that must be taken into consideration when interpreting the results. The growth of biofilm on the abiotic surface of the pegs may not result in equivalent biofilms as those grown on biotic surfaces (8,207). Moreover, as in all static systems there is a depletion of nutrients and accumulation of waste products in the media that cannot be controlled for as in the flow cell model. Since the biofilms are formed on pegs it is questioned whether or not the biofilm would contact and directly interact with the mammalian cells seeded at the bottom of the wells. Importantly, it is unlikely that the biofilms formed on the Calgary Biofilm Device obtain the mushroom-cap architecture that many flow cell biofilms develop (126), even though this architecture has been seen with other static systems (39). In fact, Müsken *et al.* (216) showed that after 24 hours biofilms formed in a static model produced a flat lawn of biofilm which continued to develop over time. The final maturation of these static biofilms did occur at a shorter time-frame than with flow cell biofilms. The importance of this architecture in the interaction with host epithelial cells is not thoroughly understood and some have speculated whether or not this architecture occurs *in vivo* (30,158). Kirketerp-Møller *et al*. (158) attempted to replicate the mushroom-cap architecture with an *in vivo* model and were unable to view this structure in an animal model. Nonetheless, the difference in architecture between the biofilms grown with the Calgary Biofilm Device and flow cell systems should be noted.

1.5 ***Rationale***

Advances in medical practices in CF have increased the life expectancy of patients. While these practices have helped delay the onset of *P. aeruginosa* infection, there are still no interventions that are able to readily eliminate the chronic infection. One hindrance in development of treatment for chronic *P. aeruginosa* infection stems from an incomplete knowledge of the interactions between biofilms with the lung epithelia.

Thus far there have been few co-culture systems that allow for the exposure of human lung epithelia to established biofilms. There are several characteristics that differ between planktonic bacteria and biofilms that have yet to be studied in co-culture with human lung epithelia; including the direct bacterial interactions with the epithelia as well as the secretion of proteins that affect the host cells. Determining the differences in how biofilms and planktonic bacteria interact with lung epithelia through adherence and subsequent internalization will help uncover explanations for how these two growth forms respond to antibacterial treatments. Moreover, an *in vitro* system that allows the characterization of the lung epithelia after contact with established biofilms and planktonic bacteria, as well as their secreted factors, can help guide therapeutic responses to chronic infections. While the secretomes of different strains of *P. aeruginosa* have been compared, there have been few studies examining the differences in biofilm and planktonic bacteria. The unique proteins in the secretome of biofilms could serve as biomarkers for biofilm infection, as well as providing targets for therapeutics of chronic infections.

It is clear that *P. aeruginosa* biofilms use intricate gene and protein regulation in the formation of biofilms. There are few studies examining the downstream effects on host-biofilm interactions of growing biofilms in the presence of factors that can perturb the biofilm gene expression. The human cationic peptide LL-37 has been shown to be expressed in high amounts upon infection

with *P. aeruginosa*. Moreover, significant levels of this immunomodulatory peptide have been shown to be sequestered by extracellular DNA in the lungs of CF patients, subsequently liberated with exogenous DNase treatment. The comparison of LL-37 treatment on virulence gene expression between planktonic bacteria and biofilm in a static system has also yet to be determined. This opens the door for new therapeutics that may display a lower selective pressure for resistance. Using a static system where the media is not replaced would allow for higher concentrations of LL-37 to be studied. However a major drawback to using a static system is the early degradation of the peptide by bacterial proteases.

The goal of this research was to study the interactions of established *P. aeruginosa* biofilms with human lung epithelia to help model chronic infections seen *in vivo.* The Calgary Biofilm Device was used to develop a host-pathogen co-culture method for the concurrent study of *P. aeruginosa* PAO1 biofilm and planktonic bacteria with human A549 lung cells. This method allows for the easy comparison of direct bacterial contact with the lung cells, as well as the comparison of the secreted bacterial factors. The ability to obtain multiple replicates as well as internal controls provides a unique opportunity to directly compare the virulence between established biofilm and planktonic bacteria.

1.6 ***Objectives and Hypothesis***

Hypothesis:

I hypothesize that *P. aeruginosa* biofilm will differ from planktonic bacteria in its direct and indirect interactions with A549 lung epithelial cells and further it will differ in its immune-regulatory properties, including release of different virulence factors than planktonic, and that

these responses will be differently modulated in the two bacterial forms by treatment with the antimicrobial peptide LL-37.

This hypothesis was determined in the following Specific Aims:

Aim 1: Compare the interactions of P. aeruginosa biofilms and planktonic bacteria with human A549 lung cells

To characterize the difference between the host-pathogen interactions of planktonic *P. aeruginosa* and biofilms grown on the Calgary Biofilm Device, an *in vitro* model system was used to compare the virulence of established biofilms and planktonic bacteria with A549 lung cells. The adherence of planktonic bacteria and biofilms to A549 lung cells and subsequent internalization of bacteria was compared. The response of A549 lung cells to biofilm and planktonic bacteria exposure was also determined through microscopy, metabolic and apoptosis assays, and induction of inflammatory cytokines (IL-8).

Aim 2: Compare the secreted factors from biofilm and planktonic P. aeruginosa and their effects on A549 lung cells

To investigate the indirect interactions with lung epithelia the Calgary Biofilm Device was used to compare the secreted factors produced by equivalent numbers of biofilm and planktonic bacteria, and determine their effects on A549 lung cells through microscopy, metabolic and apoptosis assays, and induction of inflammatory cytokines. Moreover, the proteins within the secretomes of biofilm and planktonic bacteria will be compared through shotgun proteomics.

Aim 3: Evaluate the change in host-pathogen interactions as bacteria transition from the planktonic growth form to established biofilms

The *P. aeruginosa*/A549 co-culture system was used to compare the virulence of bacteria grown on the Calgary Biofilm Device for 4-hours and 24-hours with A549 lung cells. The antibiotic resistance phenotype of 4-hour and 24-hour biofilms was also compared. This characterization allowed for the delineation of pathogenic interactions as *P. aeruginosa* transitions from planktonic to early biofilm formation.

Aim 4: Determine how LL-37 affects the downstream virulence of P. aeruginosa biofilm and planktonic bacteria when exposed to A549 lung cells

To evaluate the role of LL-37 in biofilm development and the regulation of downstream virulence pathways in planktonic and biofilms grown on the Calgary Biofilm Device, the expression of virulence genes by biofilms and planktonic bacteria grown in the presence of LL-37 for 24 and 48 hours was determined. The downstream effects of growth in the presence of LL-37 was determined through motility assays, resistance studies and co-culture studies.

The ultimate objective of this project was to study the difference in host-pathogen interactions between established *P. aeruginosa* biofilm and planktonic bacteria with human lung epithelia. There are currently few model systems that adequately allow the co-culture of established biofilms and planktonic bacteria with human lung epithelia in an *in vitro* system. The goal of this thesis was to characterize both the bacterial and the host cellular responses in various end-point assays that can provide better information to strengthen and support our understanding of the virulence of *P. aeruginosa* biofilms and extrapolate these findings to chronic *P. aeruginosa* lung infections.

2.0 Materials and Methods

2.1 Bacterial strains and cell culture

Pseudomonas aeruginosa strains are listed in Table 1. Each strain was cultured from a frozen stock aerobically on Tryptic Soy Agar (Sigma-Aldrich Canada Co., Oakville, ON, Canada) at 37° C for 24 hours prior to biofilm formation. A549 cells were obtained from American Type Culture Collection (ATCC CCL-185, Manassas, VA, USA) and were grown at 37°C with 5% CO_2 in high glucose Dulbecco's Modified Eagle Medium (DMEM) containing phenol red (Invitrogen, Burlington, ON) supplemented with 10% fetal bovine serum (FBS) (Sigma) and 1X Pen-Strep (Invitrogen).

Table 1. List of bacterial strains used in this study along with the source reference.

Strain	Description	Reference
PAO1 (ATCC 15692)	Wild-type	(138)
JP1	lasI mutant	(236)
PDO100	rhlI mutant	(40)
JP2	lasI/rhlI mutant	(236)
JP3	lasR/rhlR mutant	(236)

2.2 Biofilm formation and planktonic growth

2.2.1 Preparation of bacteria for co-culture

Bacterial biofilms were formed using the P&G Calgary Biofilm Device as previously described (52) with slight modifications. The Calgary Biofilm Device is a 96-peg-lid that is used to grow identical biofilms in a standard 96-well microplate (3). The pegs can be easily removed at any step to serve as controls or to fit the lid to different microplate shapes. The inoculum was prepared as described using a 1 in 30 dilution of a McFarland Standard Number 1. For assays to

be performed in 96-well plates all pegs were left on the lid (Figure 1A) and the plate was divided into three sections: biofilm, planktonic and control. The wells to be used for biofilm growth had 150 µL of inoculum added, the remaining wells were left empty with the exception of two which were inoculated and served as a biofilm control (Figure 1B). For assays to be performed in 8-well plates the biofilm lid had pegs removed to fit the plate. Pegs from columns 3, 4, 9, and 10; as well as from rows D and E were all removed (except for D12 and E12, which act as biofilm controls) (Figure 1C). There is a tiny peg in the corner of the plate for orienting that was also removed. This has divided the plate into 8 groups of 6 pegs. Three of the eight groups were selected for biofilm growth (as well as two pegs for control) and 150 µL of inoculum was added to each of the 20 wells (6 pegs/group plus two control pegs) (Figure 1D). The biofilms were grown for 24 hours at 37°C and 150 rpm as previously described (52). Following the incubation the biofilms were washed with PBS by dipping the peg-lid in three separate 96-well plates containing 200 µL of PBS in each well. The control pegs were removed and quantified according to Ceri *et al.* (52). Briefly, the removed pegs were placed into 200 µL of PBS-T and sonicated (Fischer Scientific M Series) for 5 minutes. Following sonication the pegs were removed and biofilm bacteria were vortexted and subjected to sequential 10-fold dilutions, which were spot plated and counted. While aggregates of biofilm bacteria may still be present, the sonication and vortexing of the sample does a sufficient job of distributing the aggregates. The biofilm peg lid was ready for co-culture.

Planktonic bacteria were grown from the same inoculum as the biofilm. Starstedt tubes were inoculated with 1 mL of the 1 in 30 inoculum and incubated horizontally at 37°C and 150 rpm for 24 hours. The bacteria were then washed thoroughly with PBS and re-suspended in 1 mL DMEM and further diluted 1 in 5 in DMEM. This was serially diluted and CFUs were

determined using spot plates. It was determined that 100 µL of this dilution gave similar CFUs as 1 biofilm peg. The planktonic bacteria were ready for co-culture.

2.2.1.8 Growth in the presence of LL-37

Bacterial biofilms were formed using the P&G Calgary Biofilm Device as described above with slight modifications. The inoculum was prepared as described using a 1 in 30 dilution of a McFarland Standard Number 1. Inoculums were prepared in TSB with or without the peptide LL-37 (amidated acetate salt, American Peptide Company, Sunnyville, CA). LL-37 was maintained as 2 mg/mL frozen stocks (in water). Thawed peptides were sonicated for 5 minutes and then thoroughly vortexed. Inoculating media was prepared by diluting the peptide in TSB to either 4 µg/mL or 16 µg/mL. For assays to be performed in 96-well plates all pegs were left on the lid (Figure 1A) and the plate was divided into three sections: biofilm, planktonic and control. The biofilm and planktonic sections were then further divided into two sections (A-D, E-H) with the top half of the plate containing control bacteria and the bottom half containing the LL-37-exposed bacteria. The wells to be used for biofilm growth had 150 µL of inoculum added with the TSB inoculums in rows A-D and LL-37-containing TSB in rows E-H, the remaining wells were left empty with the exception of four which were inoculated and served as a growth control for biofilm control (wells 11H and 12H) and LL-37-exposed biofilm (wells 11G and 12G) (Figure 1B).

For assays to be performed in 8-well plates the biofilm lid had pegs removed to fit the plate. Pegs from columns 3, 4, 9, and 10; as well as from rows D and E were all removed (except for G11, G12, H11 and H12, which act as biofilm growth controls) (Figure 1C). There is a tiny peg in the corner of the plate for orienting that was also removed. This had divided the plate into 8 groups of 6 pegs. Three of the eight groups were selected for biofilm growth (as well as four pegs for

control) and two 8-well plates were used. For each biofilm plate wells A1-C2 contain untreated biofilm, wells A5-C8 contain LL-37-treated biofilm. For growth controls, wells 11H and 12H had untreated biofilm and 11G and 12G had LL-37 exposed biofilm. Using two plates results in 2 biofilm control samples, 2 planktonic control samples, 4 LL-37-treated biofilm samples, 4 LL-37-treated planktonic samples, and 4 cell control samples. The 96-well plate had 150 μL of control or LL-37-containing inoculum added to each of the corresponding wells (6 pegs/group) (Figure 1D). The biofilms were grown for 24 hours at 37°C and 150 rpm as previously described (52). Control pegs were broken off following the 24 hour incubation and bacteria were quantified as described above.

Planktonic bacteria were grown from the same control and LL-37-containing inoculums as the biofilm. Starstedt tubes were inoculated with 1 mL of the 1 in 30 inoculum and incubated horizontally at 37°C and 150 rpm for 24 hours. The bacteria were then washed thoroughly with PBS and re-suspended in 1 mL DMEM and further diluted 1 in 5 in DMEM. This was serially diluted and CFUs were determined using spot plates. It was determined that 100 μL of this dilution gave similar CFUs as 1 biofilm peg. The planktonic bacteria were ready for co-culture.

2.2.1.9 Transition from planktonic to biofilm

The transition from planktonic bacteria to biofilms was examined by inoculating *P. aeruginosa* onto the P&G Calgary Biofilm Device as described above for 4 hours and 24 hours. The inoculum was prepared as described using a 1 in 30 dilution of a McFarland Standard Number 1. Inoculums were prepared in TSB. For assays to be performed in 96-well plates all pegs were left on the lid (Figure 1A) and the plate was divided into three sections: biofilm, planktonic and control. The biofilm and planktonic sections were then further divided into two sections (A-D, E-H) with the top half of the plate containing established biofilm and the bottom half containing the

newly adherent biofilm. The wells used for biofilm growth had 150 µL of inoculum added in rows A-D, the remaining wells were left empty with the exception of two which were inoculated and will serve as a growth control for mature biofilm (wells 11H and 12H). The biofilms were grown for 20 hours at 37°C and 150 rpm. Following 20 hours a new bacterial inoculum was prepared as previously described. This was used to inoculate wells E1-H4, as well as the growth control for newly adherent biofilm (wells 11G and 12G) (Figure 1B). This was placed in the incubator at 37°C and 150 rpm for a further 4 hours.

For assays to be performed in 8-well plates the biofilm lid had pegs removed to fit the plate. Pegs from columns 3, 4, 9, and 10; as well as from rows D and E were all removed (except for G11, G12, H11 and H12, which act as biofilm growth controls) (Figure 1C). There is a tiny peg in the corner of the plate for orienting that was also removed. This had divided the plate into 8 groups of 6 pegs. Three of the eight groups were selected for biofilm growth (as well as four pegs for control) and two 8-well plates were used. For each biofilm plate wells A1-C2 contain established biofilm, wells A5-C8 contain newly adherent biofilm. For growth controls, wells 11H and 12H had established biofilm and 11G and 12G had newly adherent biofilm. Using two plates results in 2 established biofilm samples, 2 24-hour planktonic samples, 4 newly adherent biofilm samples, 4 4-hour planktonic samples, and 4 cell control samples. The 96-well plate had 150 µL of inoculum added to each of the wells corresponding to established biofilms (6 pegs/group) (Figure 1D). The biofilms were grown for 20 hours at 37°C and 150 rpm (52). Following 20 hours a new bacterial inoculum was prepared as previously described. This was used to inoculate wells corresponding to newly adherent biofilm, as well as the growth control for newly adherent biofilm (wells 11G and 12G). This was placed in the incubator at 37°C and 150 rpm for a further

4 hours. Control pegs were broken off following the 24 hour incubation and bacteria were quantified as described above.

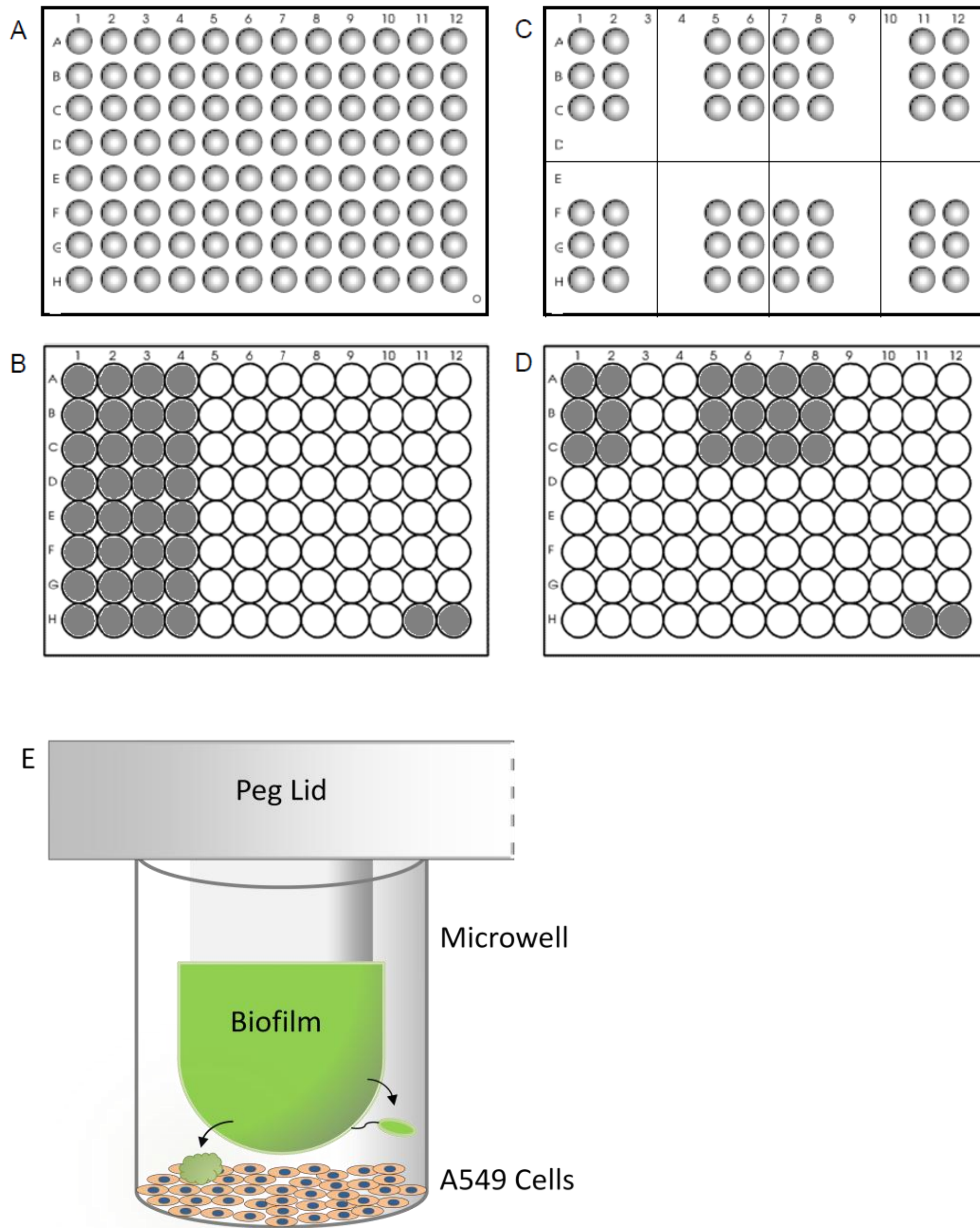


Figure 1. Schematic diagram of the cell culture plate and Calgary Biofilm Device peg-lid set-up. Diagram shows the peg lay-out (A, C), and biofilm inoculums lay-out (B, D) for the 96-well format (A, B) and 8-well format (C, D). Shaded areas represent wells that were inoculated for biofilm formation. Unshaded wells remained empty during biofilm formation. E) A schematic of one of the pegs in a well of a 96-well plate.

2.3 A549 co-culture

A549 human pneumocytes were seeded into either 96-well plates (Fisher Scientific, Toronto, ON) at 1 x 10^4 cells per well or 8-well plates (Nunc, Ottawa, ON) at 5 x 10^5 cells per well, unless otherwise indicated. The cells were allowed to adhere overnight. To ensure cells were in stationary phase, cells were rinsed with PBS and cultured in serum-free DMEM for 18 hours as previously described (47,54,127).

2.3.1 96-well co-culture

The media was removed from the plate and A549 cells were washed with PBS. Following Figure 1B the biofilm wells (A1-H4) and control wells (A9-H12) had 200 µL DMEM added while the planktonic wells (A5-H8) had 100 µL of DMEM and 100 µL of the planktonic culture added. The washed biofilm lid was placed on top and the plate was placed in an incubator at 37 °C with 5% CO_2 for the co-culture time period specified in each of the assays. This represents an approximate multiplicity of infection of 100 which was confirmed through spot plat analysis of both the biofilm and planktonic bacteria.

2.3.2 8-well co-culture

The media was removed from the plate and A549 cells were washed with PBS. Following Figure 1D the biofilm wells (A1-A3) and control wells (A4 and B4) had 5 mL of DMEM added while the planktonic wells (B1-B3) had 4.4 mL of DMEM added. These same wells then had 600 µL of the planktonic culture added. The biofilm peg lid was carefully placed onto the 8-well plate and the plate was placed in an incubator at 37 C with 5% CO_2 for the co-culture time period specified in each of the assays. This represents an approximate multiplicity of infection of 100 which was confirmed through spot plate analysis of both biofilm and planktonic bacteria.

2.4 Preparation of conditioned media from biofilm and planktonic bacteria

Biofilm and planktonic bacteria were prepared using the described Calgary Biofilm Device co-culture method according to the 8-well format. Following the initial growth of the biofilm and planktonic cells for 24 hours (4 hours for young biofilm preparation) the biofilm and planktonic bacteria were inoculated into an 8-well plate in serum-free DMEM, leaving 2 wells uninoculated for control. Following a 4-hour incubation the supernatant was removed and each well was sterile filtered through a fresh 0.2 µm syringe filter (Nalgene, Rochester, NY). The conditioned media (CM) was now ready for exposure to A549 cell culture.

2.5 Biofilm formation curve

To perform a biofilm formation curve the 96-well plate of a Calgary Biofilm Device was split into three columns. Columns 1-4 were inoculated with *P. aeruginosa* in TSB as previously described. Columns 5-8 were inoculated with *P. aeruginosa* in TSB containing 1 µg/mL LL-37 (prepared as previously described). Columns 9-12 were inoculated with *P. aeruginosa* in TSB containing the sub-MIC 16 µg/mL LL-37. The peg-lid was placed onto the plate and it was incubated at 37°C and 150 rpm. After each time-point pegs across the top-most row (4/condition) were removed, sonicated, diluted and spot plated as previously described. Pegs were removed after the following time-points: 0.5 hour, 1 hour, 1.5 hours, 2 hours, 3 hours, 4 hours, 6 hours, and 24 hours. The CFU were log transformed and graphed according incubation time. Differences in biofilm formation between the untreated and LL-37 treated bacteria were determined using an unpaired two-tailed Student's T-test.

2.6 *Crystal violet assay*

To determine biofilm biomass the crystal violet assay was performed. The 96-well plate of a Calgary Biofilm Device was split into 4 columns. Columns 1-3 were inoculated with *P. aeruginosa* in TSB as previously described. Columns 4-6 were inoculated with *P. aeruginosa* in TSB containing 2 µg/mL LL-37 (prepared as previously described). Columns 7-9 were inoculated with *P. aeruginosa* in TSB containing 4 µg/mL LL-37. Columns 10-12 were inoculated with *P. aeruginosa* in TSB containing 16 µg/mL LL-37. The peg-lid was placed on the plate and incubated at 37°C and 150 rpm for 24 hours. Following the incubation the lid was washed 3 times in PBS as previously described. The peg lid was then placed in a 96-well plate containing 4% crystal violet in water and incubated for 15 minutes. The peg lid was then rinsed twice in PBS and allowed to completely dry. The crystal violet was eluted from the biofilm by placing the peg lid in a 96-well plate containing 95% ethanol. The absorbance of the eluted crystal violet was determined at 600 nm. Differences in crystal violet staining between untreated and LL-37 treated samples were determined using an unpaired, two-tailed Student's T-test.

2.7 *Immunofluorescence*

For immunofluorescence studies sterile glass coverslips were placed in the 8-well plates prior to cell seeding. Immunofluorescence was performed on A549 lung epithelia following exposure to biofilm and planktonic bacteria (Actin and *P. aeruginosa* staining), as well as exposure to CM from biofilm and planktonic bacteria (Actin and Occludin staining), as well as controls for each.

Bacterial co-culture was prepared as described except A549 cells were seeded at 1×10^5 cells/well. Following a 1 hour incubation the cells were washed three times with PBS and fixed with 3% paraformaldehyde (Sigma) for 15 minutes, washed and then permeabilized with 0.1%

Triton-X-100 for 5 minutes. The coverslips were then washed 5 times and blocked with 1% BSA (Sigma) in PBS for 30 minutes, and used for staining.

For incubation with bacterial CM the A549 cells were seeded on coverslips at 1 x 10^5 cells/well. Following a 24 hour incubation with CM from biofilm and control media the cells were washed three times with PBS and fixed with 3% paraformaldehyde (Sigma) for 15 minutes, washed and then permeabilized with 0.1% Triton-X-100 for 5 minutes. The coverslips were then washed 5 times and blocked with 1% BSA (Sigma) in PBS for 30 minutes, and used for staining.

2.7.1 Actin and P. aeruginosa staining following bacterial co-culture

After bacterial exposure each slide was then stained for Actin using 5 µL Alexa-Fluor®-Phalloidin-488 (Invitrogen) in 45 µL blocking solution (1% BSA [Sigma-Aldrich Canada Co.] in PBS) for 20 minutes. After washing the slides were incubated in a 1 in 500 dilution of biotinylated-anti-*Pseudomonas* antibody (Applied Biological Mat. Inc., Richmond, BC) in blocking solution for 1 hour. After washing the slides were counter-stained with a 1 in 1400 dilution of streptavidin-Texas Red in blocking solution for 20 minutes. Each experiment contained control slides that were not stained to ensure no background fluorescence was taking place. Following a thorough washing of the coverslips they were mounted onto slides using Slowfade®-Gold with DAPI (Invitrogen). One drop of mounting solution was placed on the slide and a coverslip was carefully lowered, cell side down, onto the drop of mounting solution. The slides were imaged using an Olympus BX41 upright microscope and Retiga 2000R camera (QImaging, Surrey, BC).

2.7.2 Actin and Occludin staining following incubation with bacterial CM

Following exposure to bacterial CM each slide was then stained for Actin using 5 µL Alexa-Fluor®-Phalloidin-488 (Invitrogen) and 5 µL α-Occludin Alexa-Fluor®-594 (Invitrogen) in 40 µL blocking solution for 20 minutes. Following a thorough washing of the coverslips they were mounted onto slides using Slowfade®-Gold with DAPI (Invitrogen). One drop of mounting solution was placed on the slide and a coverslip was carefully lowered, cell side down, onto the drop of mounting solution. The slides were imaged using an Olympus BX41 upright microscope and Retiga 2000R camera (QImaging, Surrey, BC).

2.8 ***Adherence/internalization of P. aeruginosa biofilm and planktonic bacteria to A549 cells***

The adherence and internalization of *P. aeruginosa* biofilm and planktonic bacteria to A549 cells were compared using previously described methods in 96-well plates (49,97). For adherence studies, co-culture was carried out as previously described using the Calgary Biofilm Device for 1 hour. The control pegs were used to determine the inoculum concentrations of the biofilm and planktonic bacteria. Following the co-culture, the plate containing the A549 cells was rinsed three times with PBS and then overlaid with 200 µL of PBS-T and incubated for 30 minutes. Following the incubation the cells were thoroughly triturated with a P200 pipette to ensure complete dispersion of the bacteria making up the biofilm. The media was then removed, diluted and spot plated as previously described. The percent adherence was determined by dividing the CFUs remaining on A549 cells by the CFUs determined of each inoculum and multiplying by 100. Statistical differences between the adherence of biofilm and planktonic bacteria were determined using a two-tailed unpaired Student’s T-test.

The internalization of bacteria was determined using a gentamicin exclusion assay. The co-culture was performed similarly to the adherence assay. Following the hour incubation the external bacteria were killed using a concentration of 200 µg/mL of gentamicin for 1 hour, a high enough concentration to kill bacteria within the biofilm. A549 cell-free wells were used as controls to ensure complete killing of all external bacteria. The gentamicin was washed away using 3 rinses of PBS. The A549 cells were then lysed by adding 1% Triton X 100 (Sigma-Aldrich Canada Co.) in PBS and incubating for 1 hour. Following the incubation the media was then diluted and spot plated as previously described. The percent internalization was calculated by dividing the CFUs recovered from each well by the CFUs determined of each inoculum and multiplying by 100. Statistical differences between the internalization of biofilm and planktonic bacteria were determined using a two-tailed unpaired Student's T-test.

2.9 ***WST-1 assay for metabolic activity***

To determine if *P. aeruginosa* biofilm or planktonic bacteria stimulate A549 lung epithelial metabolism, the metabolic activity of the A549 cells was measured using the WST-1 reagent (Roche, Laval, QC) according to manufacturer's protocol with minor deviations. WST-1 is a tetrazolium salt that is enzymatically cleaved to formazen by the metabolic enzyme mitochondrial dehydrogenase. The product formazen absorbs light at 450 nm, and therefore mitochondrial dehydrogenase activity can be measured by examining an increase in absorbance at this wavelength. This assay was performed for both co-culture of A549 cells with bacteria, as well as for exposure of A549 cells to CM isolated from biofilm and planktonic bacteria. For the co-culture, the Calgary Biofilm Device protocol was used as previously described using the 96-well format. The A549 cells were exposed to bacteria for 1 hour and 4 hours. Following the

bacterial exposure the supernatant was removed, the cells were washed with PBS and the media was replaced with warm phenol-free DMEM. The plate was then ready for the WST-1 assay.

For exposure to CM, the A549 cells were grown in the 96-well format as previously described. The media was replaced and the cells were washed with PBS. Each well was then filled with 200 µL of either biofilm or planktonic CM. This was allowed to incubate for 24 hours. The plate was then ready for the WST-1 assay.

To perform the WST-1 assay, each well had 10 µL of WST-1 reagent added and the plates were incubated for 30 minutes prior to measuring at 450 nm. Every experiment included blank wells as well as A549-free biofilm and planktonic bacterial (or CM) controls to measure the background metabolic activity contributed by the bacteria. Statistical differences between biofilm, planktonic and control samples were determined using an unpaired, two-tailed Student's T-test.

2.10 ***Cell death and apoptosis of A549 cells after exposure to biofilm and planktonic bacteria***

To determine if *P. aeruginosa* biofilm and planktonic bacteria stimulate similar A549 cell death and apoptosis the Agilent Bioanalyzer (Mississauga, ON) cell pressure cartridge was used to measure cell death and apoptosis using flow cytometry. A549 cell death and apoptosis was determined following the co-culture with *P. aeruginosa* biofilm and planktonic bacteria, as well as following incubation with CM isolated from biofilm and planktonic bacteria. The A549 cells were grown in the 8-well format and exposed to bacteria using the Calgary Biofilm Device for 4 hours as previously described. For CM exposure, the A549 cells were grown in the 8-well format. Following the rinsing of cells in PBS the media was removed and replaced with 2 mL of

CM from either biofilm, planktonic bacteria, or control. Following exposure the supernatant was removed and placed into 15 mL tubes (to retain detached cells). The remaining cells were trypsinized for 5 minutes and then the typsin activity was neutralized by adding the DMEM with serum. The wells were gently scraped and the media was replaced into the corresponding 15 mL tube. The cells were then washed with PBS and re-suspended in 500 μL Binding Solution (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$, pH 7.4) containing 2.5 μL Calcein AM (Invitrogen) and 25 μL Annexin V-Alexa Fluor®-647 (Invitrogen) and incubated at room temperature for 15 minutes. The cells were washed three times with PBS and loaded onto the Agilent Cell Chip according to manufacturer's instructions. The data was analyzed using the Apoptosis Fast Protocol instructions. The cells were grouped based on their fluorescence gating. Cells that only showed Calcein AM staining were considered viable and non-apoptotic. Cells that did not show any Calcein AM staining were considered dead and non-apoptotic. Cells that showed Calcein AM staining as well as Annexin V staining were considered apoptotic. Statistical differences between biofilm, planktonic and control samples were determined using an unpaired, two-tailed Student's T-test.

*2.11 **Cytokine and chemokine measurement***

2.11.1 Milliplex human cytokine/chemokine panels

To determine if CM from *P. aeruginosa* biofilm and planktonic bacteria stimulated similar immune responses in A549 lung epithelia, chemokine levels were determined using the Milliplex MAP multiplex kit (Human Cytokine/Chemokine I, III from Millipore, Billerica, MA) and analyzed on the BioPlex-200 (Bio-Rad, Mississauga, ON, Canada). A549 cells were prepared as previously described using the 96-well format. CM was isolated from biofilm and planktonic

bacteria as previously described. The A549 cells were rinsed with PBS and then exposed to CM from biofilm, planktonic bacteria and control for 24 hours. The media was then transferred to a new 96-well plate and flash frozen with a dry ice/methanol bath. The supernatants were then thawed and used in the multiplex assay in collaboration with Dr. Nyla Dil. Cell culture supernatants were analyzed according to manufacturer's protocol. Lower detection limit was 10.6 pg ml^{-1} for Fractalkine, 40.6 pg ml^{-1} for IFN-α2, 0.3 pg ml^{-1} for IFN-γ, IL-8, and IL-17, 6.4 pg ml^{-1} for IL-1α and MIP-1α, 0.7 pg ml^{-1} for IL-1β, IL-6, and IL-15, 5.5 pg ml^{-1} for IL-1rα, 0.6 pg ml^{-1} for IL-2, 0.5 pg ml^{-1} for IL-10, 2.2 pg ml^{-1} for IP-10, 1.6 pg ml^{-1} for MCP-1, 3.7 pg ml^{-1} for MCP-3, 6.9 for macrophage-derived chemokine, 8.9 pg ml^{-1} for MIP-1β, 7.7 pg ml^{-1} for sIL-2Rα, 9.0 pg ml^{-1} for sCD40L, 19.4 pg ml^{-1} for MIG, 2.9 for MIP-3α, 0.8 for I-TAC, and 0.1 pg ml^{-1} for tumor necrosis factor-α. Samples below the lower detection limit were assigned a value of half of the lower detection limit in pg ml^{-1}. Statistical differences between biofilm, planktonic and control samples were determined using an unpaired, two-tailed Student's T-test.

2.11.2 IL-8 ELISA

IL-8 secretion by A549 cells was determined following exposure to biofilm and planktonic bacteria, as well as following exposure to CM from biofilm and planktonic bacteria. A549 cells were grown in the 96-well format and exposed to either bacteria or CM as describe above. At each time-point the first column of supernatant for each condition (biofilm, planktonic, control) was removed and placed at -80°C. The following time-points were used: 4 hours, 7 hours and 24 hours. A sandwich ELISA was performed with antibodies and standard obtained from Biolegend (San Diego, CA) with a biotinylated secondary antibody. A 96-well plate was coated with 1 μg/mL α-IL-8 antibody (Biolegend) diluted in carbonate buffer (Fisher Scientific) overnight at

4°C. The plate was then washed three times with PBS-T and blocked with blocking buffer (10% BSA in PBS) for 1 hour at room temperature. After 3 washes the samples and standards were then added. The samples were 2-fold diluted with 1% BSA blocking buffer. The standards were two-fold serially diluted from 2000 pg/mL to 33.5 pg/mL. The samples and standards were incubated for 2 hours at room temperature. Following three washes the detector α-IL-8-biotin antibody (Biolegend) was added (0.5 μg/mL) and incubated for 1 hour at room temperature. The antibodies were detected with 1/2000 dilution of streptavidin-POD (Roche) in blocking buffer for 1 hour at room temperature. Following the incubation the plate was washed twice and the peroxidase substrate (R&D Systems, Minneapolis, MN) was added and incubated for 30 minutes in the dark. The peroxidase stop solution (R&D Systems) was added and the absorbance was determined at 450 nm with a VersaMax platereader (Molecular Devices, Sunnyvale, CA). Statistical differences between biofilm, planktonic and control samples were determined using an unpaired, two-tailed Student's T-test.

2.12 ***Western blot analysis***

Western blots were performed on protein samples isolated from A549 cells exposed to CM from *P. aeruginosa* biofilm and planktonic bacteria. Two Western blots were performed, one detecting Occludin and one detecting E-Cadherin. Both blots were initially prepared the same and only differed once primary antibody was added. The protein samples from A549 cells that were exposed to CM from biofilm and planktonic bacteria were heated in SDS sample buffer (Bio-Rad, Mississauga, ON) with 2.5% 2-mercaptoethanol at 100°C for 5 minutes. Following centrifugation, 3 μg of protein (as determined through BCA [Thermo-Fisher, Burlington, ON] following manufacturer's instructions) and Kaleidoscope ladder (Bio-Rad) was separated by

SDS 4 – 20% PAGE (Bio-Rad) and transferred to nitrocellulose membranes in 25 mM Tris (Sigma), 192 mM glycine (Sigma), 20% methanol (Thermo-Fisher), 0.2% SDS at 100 V, at 4°C for 1 hour. The membranes were blocked with 5% skim milk (in TBS-T) for 1 hour at room temperature. The membranes were now ready for primary antibody treatment.

2.12.1 Occludin staining

The membrane was incubated in a 1/5000 dilution of mouse-α-Occludin (Invitrogen) in blocking buffer overnight at 4°C. The membrane was rinsed 3 times in TBS-T then incubated in a 1/10000 dilution of secondary antibody (donkey α-mouse-alkaline phosphatase [Cedarlane, Burlington, ON]) at room temperature for 1 hour. Protein bands were then detected by incubation in SIGMA*FAST*™ BCIP®/NBT (Sigma).

2.12.2 E-Cadherin staining

The membrane was incubated in a 1/1000 dilution of goat-α-E-Cadherin (Human EMT, 3-Color Immunohistochemistry Kit, Cedarlane) in blocking buffer overnight at 4°C. The membrane was rinsed 3 times in TBS-T then incubated in a 1/10000 dilution of secondary antibody (rabbit-α-goat-alkaline phosphatase [Cedarlane, Burlington, ON]) at room temperature for 1 hour. Protein bands were then detected by incubation in SIGMA*FAST*™ BCIP®/NBT (Sigma).

2.13 ***Wound healing assay***

The wound healing assay was performed as previously described (70) with A549 cells in 8-well plates to determine if the A549 cells become motile following exposure to CM from *P. aeruginosa* biofilm or planktonic bacteria. The cells were prepared as described in section 2.1. After 24 hours the media was removed and replaced with serum-free DMEM and incubated for

another 24 hours. The cells were then scratched with a sterile 200 μl pipette tip in 3 separate places in each well and medium containing 2 mL of control, biofilm or planktonic CM in duplicate was added. After 24 hr, the wounds were observed to see if the A549 cells were closing the gap of the wound and images were taken in 20× magnification. Results are from three independent experiments.

2.14 ***Mass spectrometry***

Mass spectrometry was performed on CM from biofilm, planktonic bacteria and control media with two replicates per treatment. The CM was obtained as previously described using the same concentration of biofilm and planktonic bacteria. Each sample had a total of 30 mL and each treatment was run in duplicate. The CM was concentrated using Amicon Ultra-15 10k Centrifugal filter units (Millipore, Etobicoke, ON). Each wash and concentration step included centrifugation at 4,000 × g for 20 minutes (Beckman Coulter, Allegra®). The samples were then brought up to 15 mL with 8M urea (Sigma-Adlrich) in 100mM Tris, pH 8. This was centrifuged and samples were treated with 100mM DTT (Life Technologies) in 8M urea for 1 hour. Following concentration an equal volume of 500mM iodoacetimide (in 8M urea) was added and left for 30 minutes in the dark. Two volumes of 100mM DTT were then added and the volume was brought up to 1 mL with 1M urea in 100mM Tris. Then 1 μg of Sequencing Grade Modified Trypsin (Promega, Madison, WI) was added to each Amicon tube and digested the samples over night at 37°C. On the following day, peptides were eluted from the Amicon filters by centrifugation at 4000 x g by using 100mM ammonium bicarbonate and dried using a speed vac. Samples were desalted using C18 ZipTip® Pipette Tips (Millipore). Peptide concentrations were

measured using a NanoDrop spectrophotometer (Fisher Scientific Inc) for subsequent mass spectrometry analysis.

In collaboration with the Manitoba Centre for Proteomics and Systems Biology, the desalted samples were analyzed by nano-RPLC-MS/MS using an A splitless Ultra 2D Plus (Eksigent, Dublin, CA) system that is coupled to a high speed Triple TOF™ 5600 mass spectrometer (AB SCIEX, Concord, Canada) as described previously (33,318). Each sample had approximately 3 µg of peptides injected via a PepMap100 trap column (0.3 × 5 mm, 5 µm, 100 Å [Dionex Sunnyvale, CA]), and a 100 µm × 150 mm analytical column packed with 5 µm Luna C18(2) was used prior to MS/MS analysis. Eluents A (water) and B (99% acetonitrile) both contained 0.1% formic acid as an ion-pairing modifier. The peptides were analyzed with a 180 minute gradient. Eluent B had a gradient from 0%-35% over 165 minutes, 35%-85% in 1 minute and was kept at 85% for 5 minutes at a flow rate of 500 nL/min. Parameter settings for the TripleTOF 5600 mass spectrometer were: ionspray voltage floating (ISVF) 3000 V, curtain gas (CUR) 25, interface heater temperature (IHT) 150, ion source gas 1 (GS1) 25, declustering potential (DP) 80 V. All data was acquired using information-dependent acquisition (IDA) mode with Analyst TF 1.5 software (AB SCIEX, USA). For IDA parameters, 0.25 s MS survey scans in the mass range of 400–1250 were followed by 20 MS/MS scans of 100 ms in the mass range of 100–1600 (total cycle time: 2.3 s). Switching criteria were set for ions greater than mass to charge ratio (m/z) 400 and smaller than m/z 1250 with a charge state of 2–5 and an abundance threshold greater than 150 counts. Former target ions were excluded for 5 seconds. A sweeping collision energy setting of 37 ± 15 eV was applied to all precursor ions for collision-induced dissociation.

For each LC/MS/MS run, spectra files were generated using Analyst® TF 1.6.2 Software and converted into mascot generic file format (.mgf) using AB SCIEX MS Data converter (AB SCIEX). The following parameters were used: (i) enzyme, trypsin; (ii) one missed cleavage allowed; (iii) fixed modification, carbamidomethylation of cysteines (iv) variable modification, oxidation of methionine; (v) peptide tolerance, 20 ppm; and (vi) MS/MS tolerance, 20 ppm. LC/MS/MS data of were searched against the *P. aeruginosa* PAO1 proteome. Proteins were ranked based on log(e) values. For a protein to be considered present in a growth condition, it had to have acceptable log(e) values in both replicates. Log(e) is calculated within the software and represents the probability that a peptide is matched to a protein randomly. A log(e) score of -2 represents a probability of 1/100 of the protein being selected by random chance.

2.15 Lactone extraction from biofilm and planktonic CM

Lactones were extracted from the biofilm, planktonic and control CM samples. For each sample 10 mL of each treatment were placed in volumetric flasks. Each had 10 mL of acidified ethyl acetate added (70 uL glacial acetic acid in 70 mL ethyl acetate). The flasks were hand-shaken for 5 minutes and then poured into 100 mL Pyrex bottles and allowed to settle. Once fully settled the bottom aqueous layer was removed and placed back into the corresponding volumetric flask. This was repeated a second time and the two organic layers were combined and dried using anhydrous sodium sulfate. The solutions were decanted into Erlenmeyer flasks and allowed to dry under a stream of nitrogen. The dried material was re-suspended with 3 washes of ethyl acetate and placed in screw-cap tubes and allowed to dry. The lactones were re-suspended in 1 mL DMEM.

2.16 Elastase activity of biofilm and planktonic CM

Elastase activity in the CM from biofilm and planktonic *P. aeruginosa* was compared using the Elastin Congo Red (ECR) assay as previously described with slight modifications (236). ECR is a water-insoluble substrate for elastase. Once cleaved its product becomes soluble and can be detected at 485 nm. ECR was used at a concentration of 20 mg/mL and 75 µL was combined with 125 µL of CM in a round bottom plate. This was incubated overnight at 37°C at 700 rpm. Following centrifugation 100 µL of supernatant was transferred to a flat-bottomed plate and read at 485 nm with a VersaMax platereader (Molecular Devices). To ensure that the enzymatic activity was due to elastase, inhibition studies were performed similarly with the addition on 2 mM EDTA or the elastase-specific substrate 1 mM phosphoramidon. Statistical differences between biofilm, planktonic and control samples were determined using an unpaired, two-tailed Student's T-test.

2.17 Minimum inhibitory concentration/minimum bactericidal concentration

The Calgary Biofilm Device was used for concurrent MBEC (minimum biofilm eradication concentration) and MIC determination as has been described (4). The Calgary Biofilm Device was used to examine antibiotic susceptibilities of both newly adherent (4-hour) biofilms and established (24-hour) biofilms of *P. aeruginosa* PAO1; as well as for 24-hour biofilms grown in the presence (16µg/mL) or absence of LL-37. The biofilm was established as previously described for 4-hour and 24-hour biofilms, with rows A-D containing mature biofilm and rows E-H containing young biofilms. After the biofilm was established the pegs in column 12 were removed and the biofilm was quantified as previously described. The peg lid was exposed to increasing concentrations of gentamicin, ciprofloxacin, ceftazidime and meropenem (obtained as

analytical grade powders from Sigma-Aldrich Canada) in cation-adjusted Mueller-Hinton broth and the MIC and MBEC values were determined in quadruplicate as previously described (4). The inoculums of the 4-hour and 24-hour biofilms were standardized by using planktonic inoculums of identical concentrations to each stage and comparing the fold-increase in MIC values. The minimum bactericidal concentration (MBC) was determined by transferring 10 μL from each well of the challenge plate to a plate containing fresh media to facilitate the growth of all bacteria not eradicated by the antibiotic. The presence or absence of growth was determined visually by the presence of turbidity. Statistical differences between samples were determined using a two-tailed Mann-Whitney rank U-test using an α-value of 0.05.

2.18 ***β-Lactamase activity***

To determine if β-lactamase expression is similar for newly adherent and established biofilms, the activity of β-lactamase by 4-hour and 24-hour biofilms was measured using a nitrocefin colorimetric assay as previously described (6). Briefly, after 5 hours and 24 hours of antibiotic exposure biofilm pegs exposed to 1 μg/mL, 16 μg/mL, and 512 μg/mL of ceftazidime or meropenem were broken off and immersed in sodium phosphate buffer. The supernatant from the corresponding well was spun down to isolate the released planktonic bacteria. The planktonic bacteria and the biofilm were lysed by freeze-thaw and lysate was recovered after centrifugation. The nitrocefin assay to determine β-lactamase concentration was performed by mixing 10 pM nitrocefin in 50 mM sodium phosphate buffer (pH 7.0) and 20 μL of lysate or supernatant together to give a total volume of 100 μL. The plate was incubated at 37°C for 30 minutes and the absorbance was read at 482 nm (VersaMax platereader) determining the amount of β-lactamase present. The protein concentration of the biofilm and planktonic bacteria was

normalized using BCA (bicinchoninic acid) assay as previously described (321). Statistical differences between biofilm, planktonic and control samples were determined using an unpaired, two-tailed Student's T-test.

2.19 ***RNA extraction/real-time PCR***

2.19.1 RNA extraction

Biofilms and planktonic bacteria were grown as previously described in either the presence or absence of 4 µg/mL, 16 µg/mL and 64 µg/mL LL-37 for 24 and 48 hours. All three of these concentrations were sub-inhibitory in the growth conditions used. For each sample three biological repeats were performed. After the indicated incubation time the peg-lid was removed and quickly rinsed in PBS (200 µL/well) and placed into a microplate containing Bacterial RNA Protect Reagent (Qiagen, Toronto, Ontario) and sonicated for 15 minutes. Following sonication the peg-lid was discarded and 6 identically-treated-samples were combined into one tube. For planktonic samples the tubes were centrifuged at 13 200 rpm (Eppendorf Microcentrifuge, Model 5415D) for 5 minutes and the media was replaced with 1 mL Bacterial RNA Protect Reagent. The tubes were left to incubate for 5 minutes. Both the biofilm and planktonic tubes were centrifuged at 13 200 rpm for 10 minutes to pellet the bacteria and the supernatant was completely removed. RNA extraction was then performed with a Qiagen RNeasy kit (Qiagen, Toronto, Ontario) following protocol 1, which uses the enzymatic disruption of bacterial cell wall using lysozyme. RNA integrity and quantification was determined with an Agilent Bioanalyzer RNA chip (Agilent Technologies, Mississauga, Ontario). Only RNA samples with an RIN above 9.0, as determined by the Bioanalyzer, were used for cDNA synthesis.

2.19.2 cDNA synthesis and real-time PCR

cDNA was prepared with the Transcriptor First Strand cDNA kit (Roche, Laval, Quebec) following the manufacturer's instructions using the random hexamer primers and the optional denaturation step. The reaction was allowed to incubate for 30 minutes.

Primers for real-time PCR were designed in order for optimal quantitation with a LightCycler® 2.0 (Roche, Laval, Quebec). Primers were initially found using the open-source software Primer3. Primer pairs were analyzed for secondary structure using Beacon Designer™ and the associated amplicon was analyzed for secondary structure using Mobyle (331). The primers used for each gene are shown in Table 2.

Real-time PCR was performed using 20 µL capillaries and the Fast start DNA Master Plus SYBR Green I kit (Roche, Laval, Quebec). PCR master mixes were prepared according to the manufacturer's instructions. Each cDNA sample was diluted 1/100 and run in triplicate. Standards were prepared by combining cDNA from every sample and diluting 1/10, 1/100, and 1/1000 and a sample calibrator was included in each run. Real-time PCR incubation parameters were optimized and shown in Table 2. Every run ended with a melting curve to ensure proper binding and to rule out primer-dimer formation. Each run had random samples selected and ran on a DNA chip with the Agilent Bioanalyzer to confirm that only one band of DNA was amplified. The concentration of mRNA was determined by the software using the standard curve and calibrator sample. Each gene was normalized using *rpoD* and *16S rRNA*. Unfortunately it is difficult to identify a suitable housekeeping gene for the expression analysis in biofilms. Normalizing gene expression to the sigma factor *rpoD* is not ideal since it is differentially expressed between growth phases (101,232); however this gene has been used by others to compare gene expression changes between biofilms that are at a similar maturation phase

(43,328). Here gene expression is being compared between bacteria within the same growth phase as well as comparing biofilm and planktonic bacteria in stationary phase, which has been shown previously to have similarly expressed levels of *rpoD* (305). Using the calculated mRNA concentrations the fold changes of the LL-37 treated samples were compared to the control, which contained no LL-37 treatment. This was performed by dividing the concentration of mRNA for LL-37 treatment by the mRNA concentration of the untreated control.

Table 2. Summary of primers and conditions used for each gene studied for relative quantitation.

Function Class	Gene name	Protein/Function	Primers (Forward, Reverse)	Length	Annealing Temperature (°C)	Annealing Time (Seconds)	Extension Time (Seconds)
Regulatory Systems	*psrA*	Transcriptional regulator (involved in quorum sensing and motility)	GGCGGCGGTGAATTATCACT GCGACGATCCAGCTCCTTTT	109	55	2	5
	pprA	LytTR family two component transcriptional regulator	CCCTTCTTCACCACCAAGGC CAAGCGAACCACGAACAACG	135	55	3	6
	pprB	Two component response regulator	CTACCGTTGCGTAACCAGCA CGATTCGAAGAACCTGCCGT	166	55	2	7
	lasI	Quorum sensing regulator	TGGCCCCTACATGCTGAAGA AGCGTACAGTCGGAAAAGCC	141	55	5	6
Antibiotic Resistance	*mexA*	RND multidrug efflux membrane fusion protein	GTGCGTCCCCAGGTGAACGG TGCAGGTAGGCGGCATTGGC	233	59	3	10
	oprM	Major intrinsic multiple antibiotic resistance efflux outer membrane protein	CCATGAGCCGCCAACTGTC CCTGGAACGCCGTCTGGAT	180	56	3	8
	mexX	Multidrug efflux protein	CAAGGCGGAGACCCTTGGCG CACCTGGCTGCCGTGCATGA	103	60	3	5
	ampC	β-lactamase	GCCGTACAACCGGTGATGAA TCTTTCGAGGCCAGCCCATA	104	55	2	5
Motility	*fimU*	Type 4 fimbrial biogenesis protein FimU	GCGCCGCCGAGGAACTCAAT GCCGGAGGCCAGGACACCTA	137	60	2	6
	pilV	Type 4 fimbrial biogenesis protein PilV (involved in motility)	CGCCATGCAGGGCAAGACCA CGTCGTACAGCGCCTTCGGG	119	58	3	5
Housekeeping	*rpoD*		GGGCGAAGAAGGAAATGGTC CAGGTGGCGTAGGTGGAGAA	178	60	5	7
	16SRNA		TGGCTCAGATTGAACGCTGG GCCTTGGTAGGCCTTTACTCCT	253	60	5	11

2.20 Twitching assay on biofilm and planktonic bacteria

To compare the twitching abilities of biofilm and planktonic bacteria grown in the presence of LL-37, a twitching assay was performed. Twitching was also assessed by adding LL-37 to the twitching media. Agar plates were prepared for twitching experiments with 1% tryptic soy agar either in the presence or absence of 16 µg/mL LL-37. For plates containing LL-37, 20 mL of molten agar was poured into Petri dishes containing 16 µg/mL of the peptide. The plates were gently swirled and left to solidify for 24 hours. To compare the effects of pretreatment of planktonic bacteria with LL-37 versus having the peptide in the agar, the bacteria were grown in tubes as previously described and washed once with PBS. The bacteria were then brought to the density of McFarland Standard Number 1 and stab inoculated into the agar. To compare the twitching ability of growing biofilm and planktonic bacteria in the presence of LL-37 the Calgary Biofilm Device was used as previously described. A sterile peg from a biofilm peg lid was immersed in the planktonic suspension and then used to stab the twitching agar. Biofilms that were grown on the pegs were removed and rinsed once in PBS and then stabbed into the agar. It was determined that the inoculation concentration was the same for both biofilm and planktonic bacteria. Following 24 hours of growth at 37°C the plates were removed from the incubator. The agar was removed and the twitching zone was stained with 4% crystal violet for 5 minutes. The plates were rinsed with water and the twitching zones were measured.

3.0 Results

3.1 Development of a novel in vitro co-culture system allows the concurrent analysis of established biofilm and planktonic bacteria with human lung epithelia

There are currently few protocols that allow the study of human lung epithelia with established biofilms. This is crucial since mature biofilms are believed to be representative of the chronic infection state in cystic fibrosis patients (298). This study used a novel *in vitro* co-culture system that allows the interactions of established *P. aeruginosa* biofilm with human A549 lung epithelial cells to be examined. A549 cells were grown to confluency then serum-deprived for 18 hours as previously described to ensure the cells were in stationary phase (47,54,127).

P. aeruginosa PAO1 were cultured using the Calgary Biofilm Device for 24 hours at 37°C at 150 rpm. Initially procedures were optimized to produce uniform biofilm across the peg lid with replicable bacterial numbers. To test for uniformity biofilm was grown across the entire device and the amount of biofilm on each of the 96 pegs was quantified by calculating the CFUs within each biofilm. Figure 2 shows the log transformed colony forming units (CFU) that were obtained for each row and each column across the plate. Uniform growth of approximately 10^7 CFU was observed along each row and each column and when variability was examined the standard deviation between replicates was less than 1 log value and there was no significant difference in bacterial numbers between the rows or columns. This indicates that the biofilm growth is uniform across the entire peg lid of the Calgary Biofilm Device.

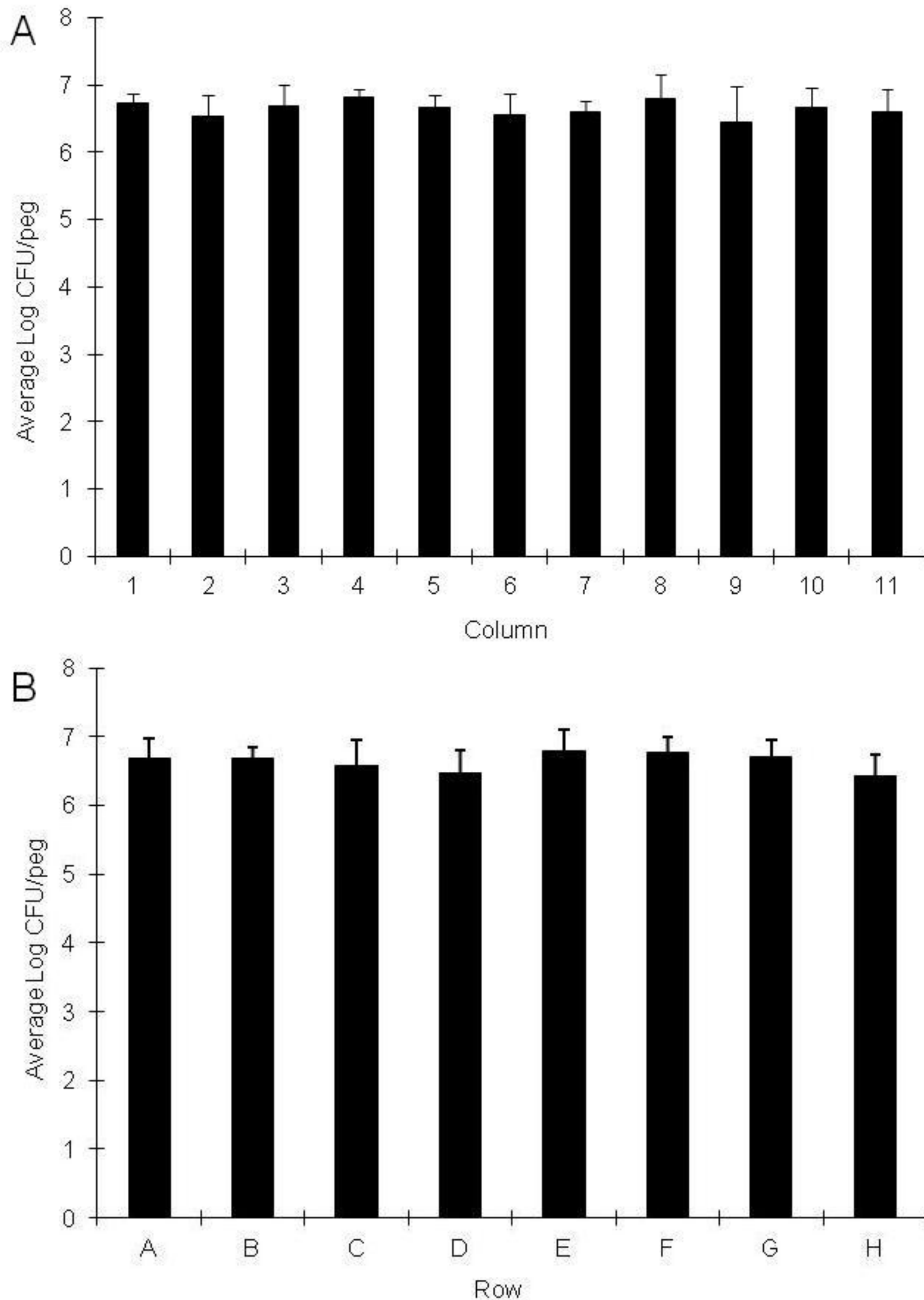


Figure 2. The Calgary Biofilm Device produces uniform biofilms across the entire peg lid. Biofilms were grown on the Calgary Biofilm Device for 24-hours at 37°C and 150 rpm. After the incubation the peg lid was placed in PBS-T and sonicated to remove the biofilm. Each well was serially diluted and spot plated to calculate the CFUs. Analysis of bacterial cell uniformity grown on pegs was determined by the average CFU/peg across each column and row. Results were obtained from three lids with each lid containing 8 pegs per column and 11 pegs per row. Error bars indicate standard deviation. Any differences between the rows or columns was calculated using a paired, two-tailed Student's T-test.

3.1.1 Characterization of a 24-hour biofilm grown on the Calgary Biofilm Device

The examination of gene expression of mature biofilms has been performed using flow cell and static biofilm growth methods. Each of these methods has shown a different temporal gene expression pattern, with static biofilms reaching maturity faster than flow cell biofilms. Gene expression patterns of selected genes were examined from biofilms grown on the Calgary Biofilm Device and planktonic bacteria to determine if there was a difference between the two populations. The genes were selected based on previous data and the importance in biofilm virulence (259,315) and not necessarily because they represent important markers of biofilm formation. Biofilm and planktonic bacteria were grown for 24 and 48 hours, after which the RNA was isolated and qRT-PCR was used to quantify the gene expression of *fimU, mexX, lasI* and *psrA* using the sigma-factor *rpoD* and *16S rRNA* as normalizers. The fold change in gene expression between biofilm and planktonic bacteria was determined and compared to previous data. After 24 hours expression of the motility gene *fimU* and quorum-sensing regulator *lasI* genes were not significantly different between planktonic and biofilm and the outer membrane protein *mexX* was significantly lower in the biofilm compared to the planktonic with only low concentrations detected in the biofilm (10.8-fold, p=0.008) (Figure 3). This was comparable to the results found for biofilms grown in a flow cell for 5 days representing mature biofilms (315). Moreover, architectural studies of 24-hour biofilms grown on the Calgary Biofilm Device indicate that this incubation period produces biofilms that are multilayered and contain cation gradients (126,181,235). However, in disagreement with my data, in that study by Whitley *et al.* (315) there was no difference in the expression of the global regulator *psrA* between biofilm and planktonic; while the biofilm grown on the Calgary Biofilm Device for 24 hours showed 15.1-fold less expression of *psrA* compared to the planktonic bacteria (p=0.013). When the incubation

time on the Calgary Biofilm Device was extended to 48 hours the gene expression profile of the biofilm changed (Figure 4). There was only one gene observed to have greater than 2-fold differential expression between the biofilm and planktonic bacteria. This was the *mexX* gene which showed more expression in the biofilm compared to the planktonic bacteria (26.7-fold, $p<0.001$). The data for 48-hour biofilms grown on the Calgary Biofilm Device supports the results of Sauer *et al.* (259) and are consistent with late, mature biofilms, but not at the dispersion phase where the bacteria re-enter the planktonic state to re-colonize. This data supports the designation of biofilms grown for 24-hours as fully established biofilms and biofilms grown for 48-hours are consistent with late/mature biofilms.

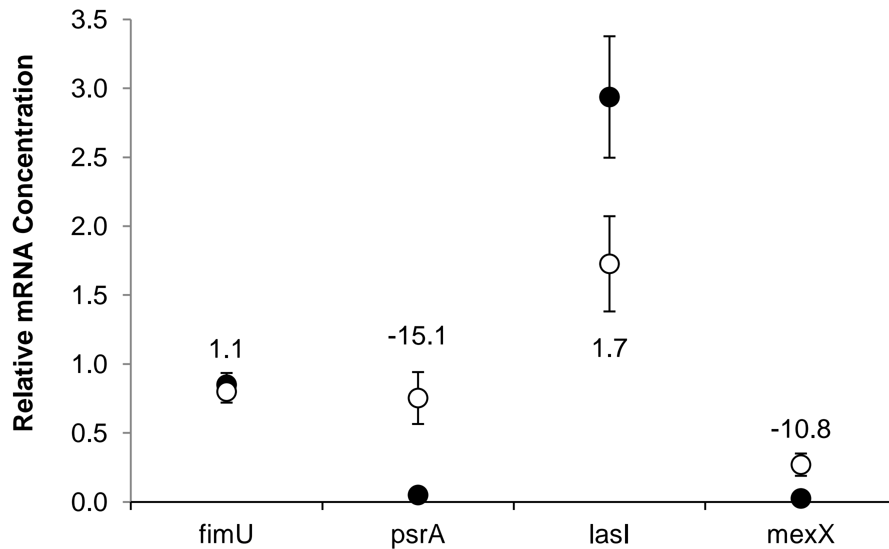


Figure 3. Planktonic and biofilms grown for 24-hours on the Calgary Biofilm Device have different gene expression profiles. Concentration of mRNA expressed by biofilm (closed circles) and planktonic bacteria (open circles) grown for 24 hours normalized using *rpoD*. Data represents the average of three replicates with standard error indicated. Data labels represent fold difference of the biofilm compared to the planktonic. The difference in the level of gene expression between biofilm and planktonic bacteria for *fimU* and *lasI* were not statistically significant ($p>0.05$), *psrA* and *mexX* were statistically significant ($p<0.05$).

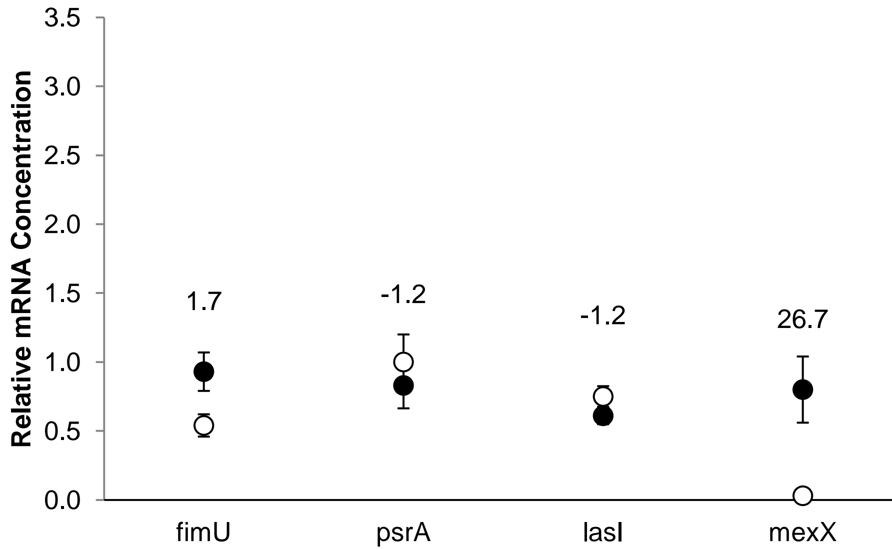


Figure 4. Gene expression profiles of biofilm and planktonic bacteria grown for 48-hours show less significant differences. Concentration of mRNA expressed by biofilm (closed circles) and planktonic bacteria (open circles) grown for 48 hours normalized using *rpoD*. Data represents the average of three replicates. Data labels represent fold difference of the biofilm compared to the planktonic. The difference in the level of gene expression between biofilm and planktonic bacteria for *fimU, psrA* and *lasI* were not statistically significant ($p>0.05$), but *mexX* was statistically significant ($p<0.05$).

3.2 ***Planktonic P. aeruginosa and biofilm display different virulence mechanisms compared to biofilm when co-cultured with human A549 epithelial cells***

There is currently minimal *in vitro* data comparing the virulence mechanisms of established *P. aeruginosa* biofilms and planktonic bacteria with human lung epithelia. Here, the Calgary Biofilm Device co-culture assay was used to concurrently examine the different interactions of 24-hour established biofilms and planktonic bacteria with A549 lung epithelia to better understand the potential mechanisms involved in chronic and acute lung infections.

3.2.1 Planktonic exposure causes epithelial cell rounding and cytoskeletal dysregulation in A549 cells but not with biofilm exposure

The ability of infecting bacteria to disrupt epithelial morphology and cell-to-cell interactions allows for the invasion of bacteria into the host. Acute infections with *P. aeruginosa* often involve lung tissue damage and a disruption of the physical barrier provided by the epithelia. To determine if biofilm and planktonic bacteria cause different epithelial morphological changes when co-cultured with A549 cells, the Calgary Biofilm Device was used to co-culture A549 cells with planktonic bacteria and established biofilm at an MOI of 100. Following a 1-hour co-culture incubation the A549 cells were examined for morphology. As expected, epithelial cells grown in the presence of planktonic bacteria were rounded and lost cell-to-cell interactions as seen through phase contrast microscopy (Figure 5B), which has been shown previously (49,54,61). A549 cells grown in the presence of biofilm did not have any marked cell rounding compared to the control (untreated) and the cell junctions appeared to remain intact (Figure 5C). This shows that planktonic bacteria are likely more capable of disrupting the A549 cytoskeletal structure.

To confirm that cytoskeletal dysregulation occurred following A549 cell exposure to planktonic bacteria an immunofluorescent analysis of the A549 cells was performed. A549 epithelial cells

were grown using the 8-well co-culture format with glass coverslips in each well. The A549 cells were exposed to established biofilm and planktonic bacteria at an MOI of 100 for 1 hour. The cells were then stained for Actin, *P. aeruginosa* and DNA. Staining for Actin confirmed that cytoskeletal dysregulation of epithelial cells occurred following exposure to planktonic bacteria (Figure 6). Planktonic exposure caused A549 cells to lose cell-to-cell contact and Actin filaments were disassembled. Planktonic bacteria can be seen localized on the A549 cells as well as within many of the rounded cells. The A549 epithelial cells exposed to biofilm did not show the same rounding as those exposed to the planktonic bacteria. Following exposure to biofilm, cells maintained some cell-to-cell contact and Actin filaments were still visible in most of the A549 cells. Staining for *P. aeruginosa* showed biofilm bacteria also coating the outer surface of the A549 cells. The different response by the A549 cells to bacterial exposure could be due to the different virulence mechanisms or possibly different secreted factor(s) of biofilm and planktonic bacteria. However, the differences could also be due to differential bacterial adherence, since much of the biofilm remains on the pegs during the co-culture.

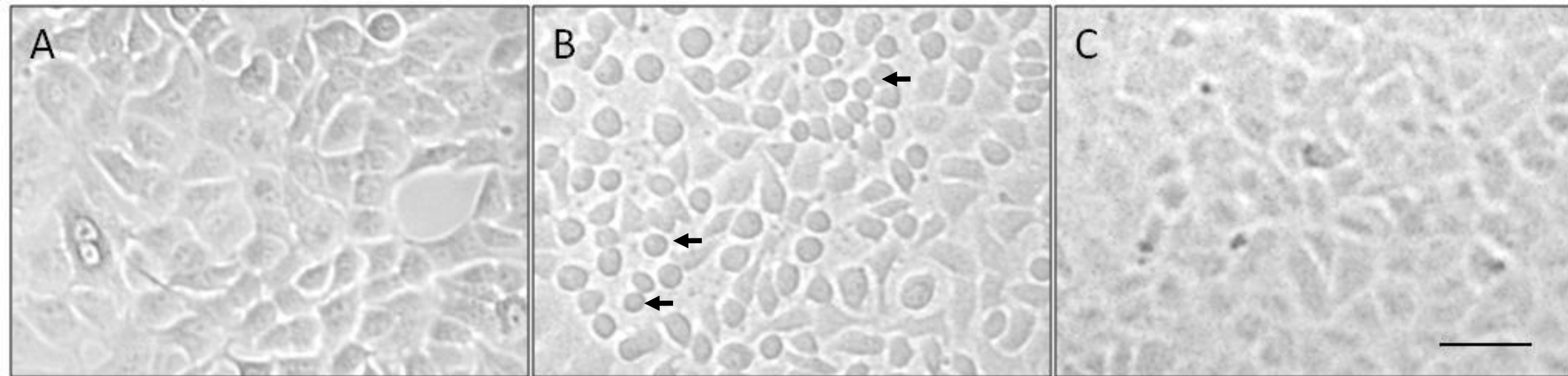


Figure 5. Planktonic *P. aeruginosa* causes A549 cells rounding while biofilm had little effect on cell morphology. A549 cells were exposed to cell media (A), planktonic bacteria (B), or established biofilm (C) at an MOI of 100 for 1 hour. The cells were then viewed with phase contrast microscopy. The A549 cells exposed to established biofilms showed very few changes in morphology, while the cells exposed to planktonic bacteria had marked rounding (arrows) and a loss of cell-to-cell contact. Selected images are representations of four replicates. Size bar represents 100 µm.

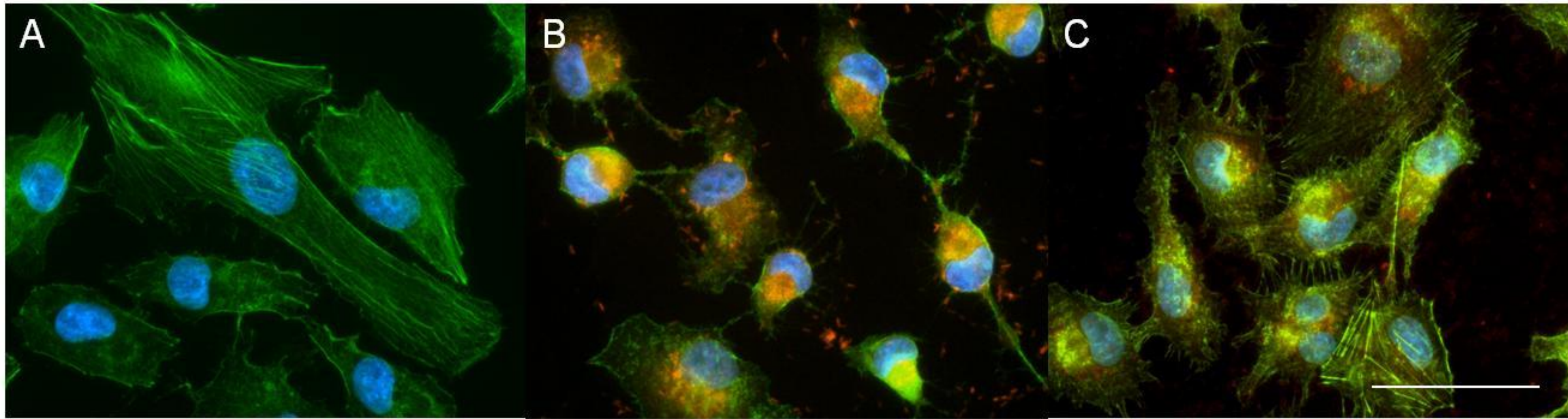


Figure 6. Planktonic *P. aeruginosa* causes significant cytoskeletal dysregulation of A549 cells while established biofilm had little effect on A549 actin structure. A549 cells were co-cultured with cell media alone (A), with planktonic *P. aeruginosa* bacteria (B), and with *P. aeruginosa* biofilm (C) at an MOI of 100 for 1 hour. Following the incubation the cells were washed and fixed with 3% PFA and permeabilized with 0.1% Triton-X-100. After blocking with 1% BSA the cells were stained for actin using phalloidin (green), anti-*Pseudomonas* (red) and DAPI (blue). Slides were imaged using an Olympus BX41 and Retiga 2000R camera. The A549 cells exposed to planktonic bacteria had significant cytoskeletal dysregulation as shown by the rounding and decrease in phalloidin staining of actin filaments (green). Bacteria (red) can be seen coating the A549 cells. Biofilm did not cause the same cytoskeletal dysregulation as shown by a strong staining of the actin filaments. The A549 cells were also coated with bacteria following exposure to established biofilms. Selected images are representations of four replicates. Size bar represents 100 μm.

3.2.2 Biofilm and planktonic bacteria adhere equally to A549 cells but planktonic bacteria undergo 15-fold more internalization by epithelia

In the Calgary Biofilm Device co-culture system the biofilm is inoculated on pegs and exposed to the epithelial cells. The difference in interactions could be due to differences in direct bacterial contact with the epithelial cells between the established biofilm and planktonic bacteria. It was initially assumed that the dynamic biofilm would be constantly releasing planktonic bacteria; moreover, microscopic analysis also showed bacterial aggregates of the biofilm sloughed off and contacting the A549 cells and A549 cells were coated with biofilm bacteria as shown with immunofluorescence staining (Figure 6). To determine if the differing effects on A549 morphology were caused by differential contact and adherence of the biofilm and planktonic bacteria to the cells, the Calgary Biofilm Device co-culture system was used to compare the adherence of the two bacterial growth forms. Biofilm was grown on the Calgary Biofilm Device and the quantity of biofilm per peg was calculated. An equivalent number of planktonic bacteria was then used to co-culture with A549 cells. A549 epithelial cells were exposed to biofilm and planktonic bacteria at an MOI of 100 for 1 hour and then the bacterial adherence was determined by rinsing the cells and quantifying the adhered bacteria through spot plate analysis. The percent adherence was calculated by determining the ratio of adhered bacteria to number of bacteria contained in the inoculum. Results show that both the biofilm and the planktonic bacteria have no significant difference in adherence to A549 cells (11.2% vs 11.0%, $p=0.943$) (Figure 7A). This indicates that the morphological effects on A549 cells by planktonic bacteria was not due to increased bacterial contact with the A549 cells and instead due to differences in another factor involved in bacterial virulence.

Since the adherence of the two forms was similar I also wanted to examine if biofilm and planktonic bacteria are also subsequently internalized by the epithelia to the same degree. Bacterial internalization by lung epithelia has been theorized to be beneficial to the host early in infection; however once an acute infection has been established it could be considered an important virulence factor for *P. aeruginosa.* A549 epithelial cells were exposed to biofilm and planktonic bacteria at an MOI of 100 for 1 hour and then external bacteria were eliminated by treating with 200 μg/mL gentamicin. The remaining bacteria, residing in the A549 cells were then quantified by lysing the A549 cells and performing a spot plate analysis. While the adherence of planktonic and biofilm showed no significant difference, following adherence it was shown that the planktonic bacteria were internalized by the epithelial cells significantly more than the biofilm bacteria (0.189% vs 0.004%, $p<0.001$) (Figure 7B). This indicates that this potential virulence mechanism is more prevalent in planktonic bacteria rather than biofilm.

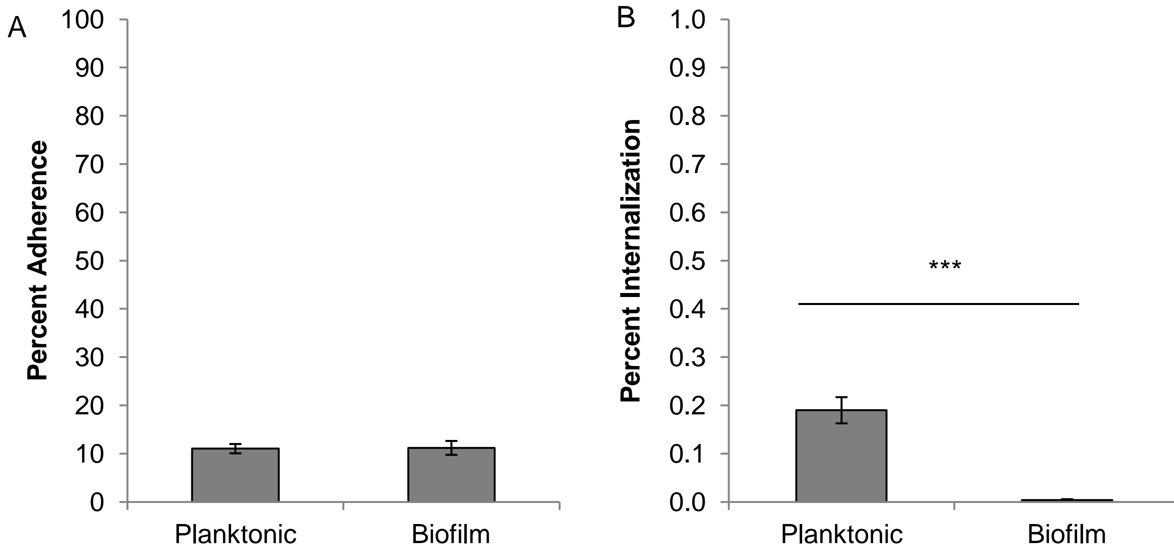


Figure 7. Biofilm and planktonic *P. aeruginosa* adhere equally to A549 cells while planktonic undergo 15-fold more subsequent internalization. *P. aeruginosa* biofilm was grown using the Calgary Biofilm Device for 24 hours and then rinsed three times. Biofilm and planktonic bacteria were exposed to A549 cells in DMEM at an MOI of 100 for 1 hour. Following the incubation the media was removed and the A549 cells were rinsed three times with PBS. A) Adherence of bacteria was determined by treating the A549 cells with PBS-T and resuspended with a pipette. The media was then diluted and spot plated. Percent adherence was calculated by dividing the number of recovered bacteria from the number of inoculating bacteria and multiplying by 100. B) Internalization of the bacteria was determined by treating the A549 cells with 200 µg/mL gentamicin for 1 hour. The A549 cells were rinsed three times with PBS then lysed with 1% Triton X 100 for 1 hour. The media was then diluted and spot plated to enumerate the recovered bacteria. Percent internalization was calculated by dividing the number of recovered bacteria by the number of inoculating bacteria and multiplying by 100. Results are from 20 replicates and error bars represent standard deviation. *** indicates a significant difference ($P<0.001$) between the two growth forms as determined with an unpaired, two-tailed Student's T-test.

3.2.3 Planktonic exposure causes epithelial cell death, while biofilm increases epithelial cell metabolism

The cell rounding and cytoskeletal dysregulation of A549 cells following exposure to planktonic bacteria indicates that the epithelial cells are likely in distress. The co-culture system was used to determine whether biofilm or planktonic *P. aeruginosa* were causing A549 cell death or apoptosis. Cell death and apoptosis was examined by flow cytometry using the 8-well co-culture format and exposure to biofilm and planktonic bacteria for 4 hours. Using an early indicator of apoptosis (phosphatidylserine translocation) and the cell viability marker Calcein AM, A549 cells exposed to bacteria were categorized as alive and non-apoptotic, alive and apoptotic or dead based on their staining. The percentage of cells undergoing apoptosis and the percentage of cells that were dead were then calculated (Figure 8). There was no significant difference in the number of apoptotic epithelial cells following biofilm or planktonic bacteria exposure for 4 hours (9.7% vs 10.25%, p=0.635) (Figure 8A); however both biofilm and planktonic bacteria caused a significant increase in apoptosis as compared to the control (biofilm: 9.7% vs 2.5%, p=0.043; planktonic: 10.25% vs 2.5%, p=0.006). Planktonic exposure caused significantly more A549 cell death than biofilm (40.8% vs 23.2%, p=0.007) as indicated by a decrease in Calcein AM staining (Figure 8B). Both biofilm and planktonic bacteria caused an increase in A549 cell death compared to the control (biofilm: 23.3% vs 5.1%, p=0.001; planktonic: 40.8% vs 5.1%, p=0.002). Planktonic bacteria may be employing virulence strategies including the type III secretion system that directly interact with the A549 cells.

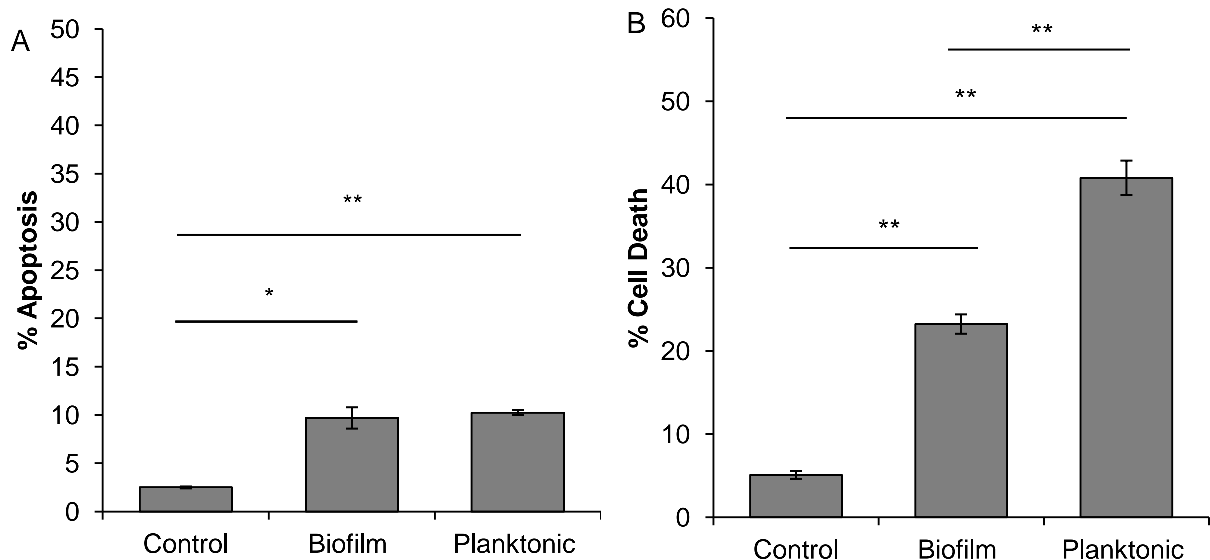


Figure 8. Biofilm and planktonic *P. aeruginosa* stimulate A549 apoptosis and cell death with planktonic stimulating significantly more cell death. A549 cells were co-cultured with biofilm and planktonic bacteria at an MOI of 100 for 4 hours. The supernatant was retained to include detached A549 cells and the adhered cells were treated with trypsin and added to the supernatant portion. The cells were washed and then treated with binding solution containing Calcein AM (live cell indicator) and Annexin V-Alexa Fluor®-647 (apoptosis indicator). The cells were analyzed using the Agilent Cell Chip using the Apoptosis Fast Protocol and were assessed for their a) apoptosis, and b) cell death. Error bars represent standard error. Significant differences compared to the control are indicated by *p<0.05, **p<0.01, ***p<0.001 as determined using an unpaired Student's T-test, n=3.

Planktonic bacteria caused more cell death in A549 cells than biofilm; however it is still unknown whether *P. aeruginosa* biofilms are able to stimulate an increase in A549 metabolism and activity. To examine the effects of biofilm and planktonic bacteria on epithelial metabolism, the Calgary Biofilm Device co-culture system was used for subsequent metabolic analysis. To test for metabolic activity the 96-well format of the assay was used and A549 cells were exposed to biofilm and planktonic bacteria at an MOI of 100 for 1 hour and 4 hours and then cell metabolism was determined using the WST-1 assay. These time-points were chosen based on the results of the previous experiments. After 1 hour the planktonic bacteria caused damage to the A549 cells. After 4 hours the A549 cells exposed to the biofilm were beginning to undergo cell rounding (data not shown). Moreover at 4 hours both biofilm and planktonic bacteria caused equivalent apoptosis of A549 cells with planktonic bacteria stimulating significantly more A549 cell death. WST-1 is a substrate for the metabolic enzyme mitochondrial dehydrogenase. An increase in A549 cell metabolism results in an increase in mitochondrial dehydrogenase activity and a subsequent increase in WST-1 hydrolysis, resulting in a larger accumulation of the chromatic product formazen. I found that planktonic bacteria caused a decrease in epithelial cell metabolism as measured by WST-1 after 1 hour (0.779 OD units vs 1.281 OD units, $p<0.001$) and 4 hours (0.008 OD units vs 1.231 OD units, $p<0.001$) of exposure, partially due to cell death (Figure 9). The low value following 4-hours of exposure to A549 cells could be attributed to cell loss from the three washing steps. After 1 hour the biofilm stimulated an increase in A549 cell metabolism compared to the uninfected control cells (1.910 OD units vs 1.281 OD units, $p<0.001$). This is potentially due to the presence of immune-activating factors in the biofilm. After 4 hours of biofilm exposure the metabolic activity of A549 cells was no longer significantly higher than the untreated A549 cells (1.034 OD units vs 1.231 OD units, $p=0.853$).

At this time point there is a large amount of released motile planktonic bacteria. This shows that under these conditions this co-culture cannot be used for incubation times longer than 4 hours for biofilm or planktonic bacteria. This data shows that planktonic bacteria cause an increase in A549 cell death and an associated decrease in A549 cell metabolism. Biofilm, on the other hand, does not cause the same high levels of A549 cell death after 1 hour of exposure while instead stimulating an increase in A549 cell metabolism.

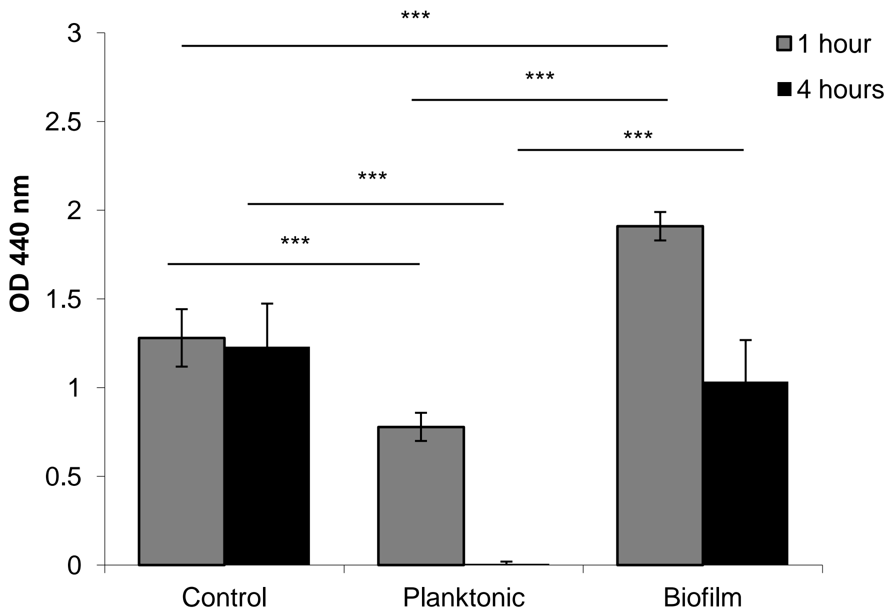


Figure 9. Planktonic bacteria cause a decrease in A549 cell metabolism after 1 and 4 hours while biofilm stimulates an increase in metabolic activity after 1 hour but this decreased after 4 hours. A549 cells were co-cultured with biofilm and planktonic bacteria at an MOI of 100 for 1 hour and 4hours. The cells were then washed with PBS and incubated with WST-1 for 30 minutes and then metabolism was detected at 450 nm. ***indicates a significant difference $p < 0.001$ using a two-tailed Student's T-test, n=12.

3.2.4 Biofilm exposure stimulates an increase in IL-8 production by epithelial cells

In chronic lung infections there is a constitutively high production of IL-8 (269). There is little data directly comparing IL-8 secretion stimulated by established biofilms and planktonic *P. aeruginosa*. Therefore, the Calgary Biofilm Device co-culture system was used to determine if the established biofilm stimulated more IL-8 release by A549 cells than planktonic bacteria. A549 cells were grown in a 96-well plate and exposed to biofilm and planktonic bacteria at an MOI of 100 for 24 hours with supernatant removed at 4 hours, 7 hours and 24 hours for IL-8 ELISA analysis. From the ELISA it was found that there was an increase in IL-8 production by A549 cells following exposure to biofilm and planktonic bacteria, compared to the control untreated cells (Figure 10). The planktonic bacteria caused a significant increase in IL-8 production; however biofilm stimulated higher levels of IL-8 release from A549 cells. At 4 hours, 7 hours and 24 hours there was significantly more IL-8 released by A549 cells following biofilm exposure compared to planktonic exposure (biofilm vs planktonic: 4 hours – 612.18 pg/mL vs 148.42 pg/mL, $p<0.001$; 7 hours – 1344.93 pg/mL vs 76.35 pg/mL, $p<0.001$; 24 hours – 463.26 pg/mL vs no detectable IL-8). After 24 hours of exposure to both the biofilm and the planktonic bacteria the A549 cells were completely lysed (as determined with phase-contrast microscopy, data not shown). The decrease in IL-8 at this time-point is due to A549 cell death. The fact that the IL-8 is still high in the biofilm sample at 24 hours indicates that either these A549 cells lysed much later than the A549 cells exposed to planktonic, or that the half-life of IL-8 is longer in the presence of biofilm as compared to planktonic bacteria.

These results indicate that both biofilm and planktonic bacteria are able to stimulate IL-8 release, which is a marker of an inflammatory response; however biofilm is able to stimulate a larger inflammatory response than the planktonic bacteria. This increase in IL-8 secretion by biofilms compared to planktonic bacteria could not be attributed solely to differences in A549 cell death, since at 4 hours the biofilm stimulated a 6-fold increase in IL-8 production, compared to only a 2-fold difference in A549 cell death.

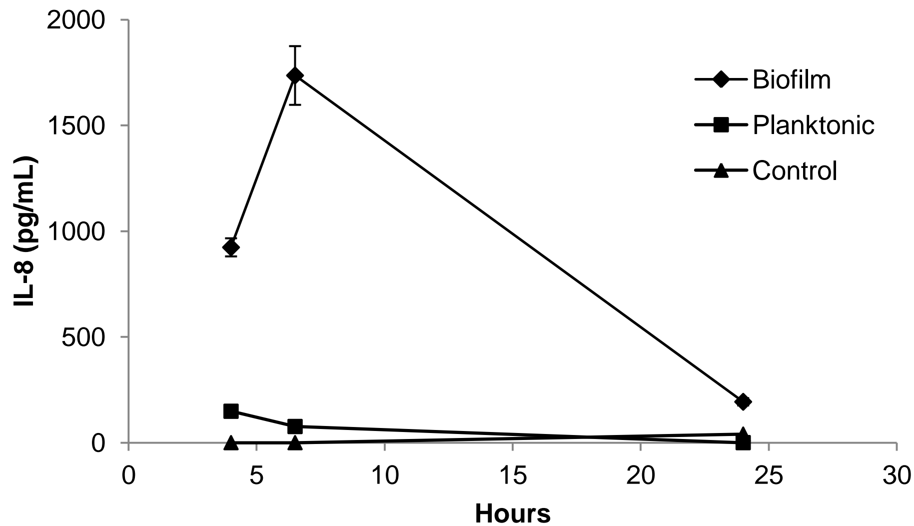


Figure 10. ***P. aeruginosa*** **biofilm stimulates significantly more IL-8 release from A549 cells than planktonic bacteria likely due to increased cell death with planktonic exposure.** A549 cells were exposed to biofilm and planktonic *P. aeruginosa* at an MOI of 100 for 4 hours, 7 hours and 24 hours and IL-8 secretion was measured by ELISA. Planktonic bacteria stimulated A549 cell rounding and death at all time-points. After 24 hours of co-culture all A549 cells were dead and lysed. Error bars indicate standard deviation of four biological repeats. Statistical differences were determined using an unpaired, two-tailed Student's T-test.

3.2.5 Summary

The Calgary Biofilm Device was used to successfully create multiple replicates of uniform biofilm across the entire peg lid. Characterization of the gene expression of 4 genes confirmed that growing the biofilm for 24 hours resulted in a biofilm with distinct gene expression compared to planktonic bacteria and extending the growth to 48 hours resulted in a biofilm that was consistent with the late phase of maturity but not yet entering the dispersion phase. Therefore, to study the interactions of a biofilm with A549 lung epithelia, the biofilm needed to be grown for 24 hours.

The optimized established biofilm grown on the Calgary Biofilm Device was used for the concurrent co-culture of biofilm and planktonic *P. aeruginosa* with A549 lung epithelia. From this it was determined that planktonic bacteria caused epithelial cell rounding and cytoskeletal dysregulation, while biofilm did not, supporting my original hypothesis. Biofilm and planktonic bacteria adhered to A549 lung epithelia equally. However, planktonic bacteria were internalized by A549 lung epithelia 15-times more than biofilm, indicating another difference in virulence between the two growth forms. While apoptosis of A549 cells following bacterial exposure did not differ between biofilm and planktonic *P. aeruginosa*, planktonic bacteria caused twice as much A549 cell death than biofilm. Following biofilm exposure the epithelial cells were stimulated resulting in the hypothesized increase in metabolism and release of IL-8, suggesting an increased ability to induce inflammation.

3.3 Biofilm and planktonic bacteria secrete different virulence factors and biofilm secretes significantly more elastase than planktonic

There is clearly a difference in the interaction of biofilm and planktonic bacteria with A549 cells when the bacteria are in contact with the A549 epithelial cell line. Planktonic bacteria stimulated morphological changes and cytoskeletal dysregulation in A549 cells. This was associated with increased A549 cell death following exposure to planktonic bacteria. Moreover, planktonic bacteria were internalized by A549 cells 15-fold more than biofilm, despite having equal adherence to the epithelial cells. The biofilm, on the other hand, stimulated a greater increase in IL-8 secretion by A549 cells. To determine the difference in the effects of secreted factors produced by biofilm and planktonic *P. aeruginosa*, A549 cells were exposed to conditioned media (CM) from either biofilm or planktonic bacteria. The Calgary Biofilm Device was used to culture biofilm and planktonic bacteria for subsequent collection of CM using controlled bacterial numbers.

3.3.1 CM from biofilm culture stimulates morphological changes in epithelial cells and activity is controlled by quorum-sensing

CM from biofilm and planktonic bacteria cultured for 4 hours were exposed to A549 epithelial cells for 24 hours to examine any effects on the cells. When cell morphology was examined using phase contrast microscopy periodically throughout the incubation it was found that the biofilm CM caused significant morphological changes in the A549 cells between 18 and 20 hours of exposure, while the planktonic CM stimulated no morphological changes in A549 cells (Figure 11). These changes appeared to be different than the cell rounding that was seen with planktonic bacterial exposure. The morphological changes caused by biofilm CM resulted in the A549 cells losing their cell-to-cell interactions and forming cellular extensions and spreading. As

the A549 cells spread it appeared as if neighboring cells were lifted off of the bottom of the plate.

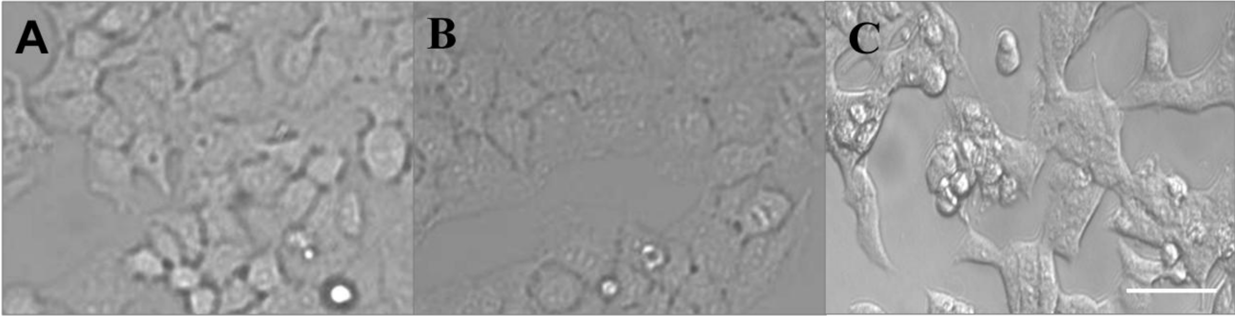


Figure 11. Biofilm CM stimulated A549 cell spreading and lifting while planktonic CM exposure resulted in no apparent morphological changes. A549 cells were exposed to CM from cell media (A), planktonic bacteria (B), or biofilm (C) for 24 hours and then viewed under phase contrast microscopy to detect any morphological changes. Biofilm caused A549 cells to lose cell-to-cell contact and to spread out and eventually lift off the plate. Selected images are representations of four replicates. Size bar represents 100 μm.

As described above, the morphological changes in A549 cells caused by biofilm CM took between 18 and 20 hours to occur. When the cells were examined at 18 hours no morphological changes were apparent. Even though no apparent changes had occurred earlier than 20 hours, it was possible that the biofilm CM was already actively affecting the A549 cells. To test this hypothesis A549 cells were exposed to biofilm CM for 4, 8, 11, 15, 18, and 20 hours. Following each incubation the CM was removed and the cells were rinsed twice with cell media. The A549 cells were then left to incubate in cell media for a total of 24 hours and examined for morphological changes. Interestingly, even when the biofilm CM was removed after 11 hours (but not before) the morphological changes still occurred when examined after 24 hours (Table 3). This indicates that the CM does not need to be present for the entire incubation to have already stimulated changes in the epithelia which lead to the changes in morphology.

Table 3. Biofilm CM required 11 hours of incubation with A549 cells to stimulate morphological changes at 24 hours. A549 cells exposed to biofilm CM for the indicated time period. The cells were subsequently washed and incubated in DMEM for a total of 24 hours.

Biofilm CM Incubation Time (Hours) Prior to Washing	Incubation Time (Hours) in Cell Media After Washing	Morphological Change in A549 Cells After 24 hours
4	20	No
8	16	No
11	13	Yes
15	9	Yes
18	6	Yes
20	4	Yes (Change had occurred before media change)

Many virulence factors are controlled by either the LasI or RhlI quorum-sensing systems. To determine whether the factor causing the morphological changes is controlled by either of these two systems studies using knockout strains were performed. *P. aeruginosa* knockout strains were grown as biofilms on the Calgary Biofilm Device as described. The strains that were examined

were the wild-type PAO1, a *lasI* mutant, *rhlI* mutant, *lasI* and *rhlI* double mutant, as well as a *lasR/rhlR* double mutant. LasI and RhlI are the enzymes that produce the corresponding quorum-sensing lactones, while LasR and RhlR are the transcriptional activators that respond to the increase in quorum-sensing lactones. The biofilm control pegs confirmed that all 4 mutant strains were able to form biofilm with no statistical differences in bacterial numbers. Following biofilm growth CM was isolated from each of the strains. The CM was then exposed to A549 lung epithelial cells for 20 hours to determine if each of the CMs were able to stimulate the morphological changes in the epithelial cells. After the incubation the morphological changes of the A549 cells only occurred following exposure to CM from the wild-type PAO1 (Table 4). CM from all 4 of the mutant isolates were unable to stimulate any epithelial morphological changes. This indicates that the active factor in the CM from *P. aeruginosa* biofilms is likely positively controlled by both the LasI and RhlI quorum-sensing systems.

Planktonic bacteria are able to activate their quorum-sensing systems in response to very large bacterial numbers. Since the active factor from biofilm CM is likely induced by quorum sensing, obtaining CM from planktonic bacteria grown at very high concentrations would likely contain the same active factor. To test this, an inoculum of planktonic bacteria was prepared that was 100 times more bacterial CFUs than previous studies. CM was isolated from this concentrated inoculum after 4 hours as previously described and A549 epithelial cells were exposed to this CM. As expected, the CM from a high concentration of planktonic bacteria was able to stimulate the morphological changes in A549 epithelial cells (Table 4). This further suggests the theory that the active factor is controlled by quorum-sensing.

Table 4. A549 cells undergo morphological changes following exposure to biofilm CM and CM from concentrated planktonic culture. A549 cells exposed to CM for 20 hours from wild type, quorum-sensing knockouts, concentrated planktonic *P. aeruginosa* PAO1 and heat-treated CM from wild type biofilm. Lactones were extracted from CM of biofilm, planktonic and control samples. The lactones were resuspended in DMEM to bring them to the original concentration.

Strain	**Description**	**Morphological Change in A549 Cells**
PAO1	Wild-type	Yes
JP1	lasI mutant	No
PD0100	rhlI mutant	No
JP2	lasI/rhlI mutant	No
JP3	lasR/rhlR mutant	No
Planktonic (x100)	Wild type at 100x concentration	Yes
Heat-treated biofilm CM	-	No
Heat-treated planktonic CM	-	No
Biofilm lactones	-	Cell necrosis
Planktonic lactones	-	Cell necrosis
Control lactones	-	No

3.3.2 The factor released by biofilms is controlled by quorum-sensing but is not a quorum-sensing lactone.

The results of the studies using knockout strains indicated that the active factor mediating the effects of biofilm CM exposure was likely controlled by quorum-sensing. The active factor could be a protein up-regulated by quorum-sensing, or it could be a quorum-sensing lactone. To determine if a quorum-sensing lactone was directly interacting with the A549 epithelial cells and causing the morphological changes, the lactones were extracted from both biofilm and planktonic CM through organic extraction. The extracted lactones were then exposed to A549 cells for 24 hours at a biological concentration based on the original CM volume. The A549 cells were examined microscopically for changes in cell morphology. There was a significant amount of cell death caused by both the biofilm and planktonic lactones, but not control, which was evident by cell necrosis (Table 4). This result was not seen when the A549 cells were incubated with whole CM. The highest amount of cell death occurred following exposure to the planktonic lactones. The cells did not show the expected morphological changes shown in Figure 11

following exposure to the biofilm lactones. This indicates that the active factor is not likely a lactone.

As lactones are heat stable and proteins are heat sensitive, to confirm whether the active factor was a protein the biofilm CM was boiled. The loss of activity following heat exposure would further confirm that the active factor was a protein. Biofilm and planktonic CM were prepared as previously described and then was boiled for 5 minutes. This boiled CM was exposed to A549 cells for 24 hours. The A549 cells were then examined for morphology. There was no morphological change following exposure to heat-treated CM from either biofilm or planktonic bacteria (Table 4). This further confirms that the active factor in the biofilm CM is a heat labile protein that is controlled by quorum-sensing and not a quorum-sensing lactone.

3.3.3 CM from biofilm cause A549 epithelial cells to lose their tight junctions through the loss of Occludin

The A549 cells exposed to biofilm CM for 20 hours appeared to lose their cell junctions and began to spread out and lift off of the plate. Since Occludin is an important protein in the tight junctions of epithelial cells I wanted to test whether the loss of tight junctions was linked to the loss of the expression of Occludin in the membranes of the A549 epithelial cells. A549 cells were exposed to CM isolated from biofilm for 24 hours and then subjected to immunofluorescent staining. The cells were stained for DNA, Actin and Occludin. Immunofluorescent staining for the tight junction protein Occludin showed a loss of this protein following biofilm CM exposure, but not control cell media (Figure 12). This confirmed that Occludin expression in the tight junctions of A549 cells is lost following exposure to CM from *P. aeruginosa* biofilm.

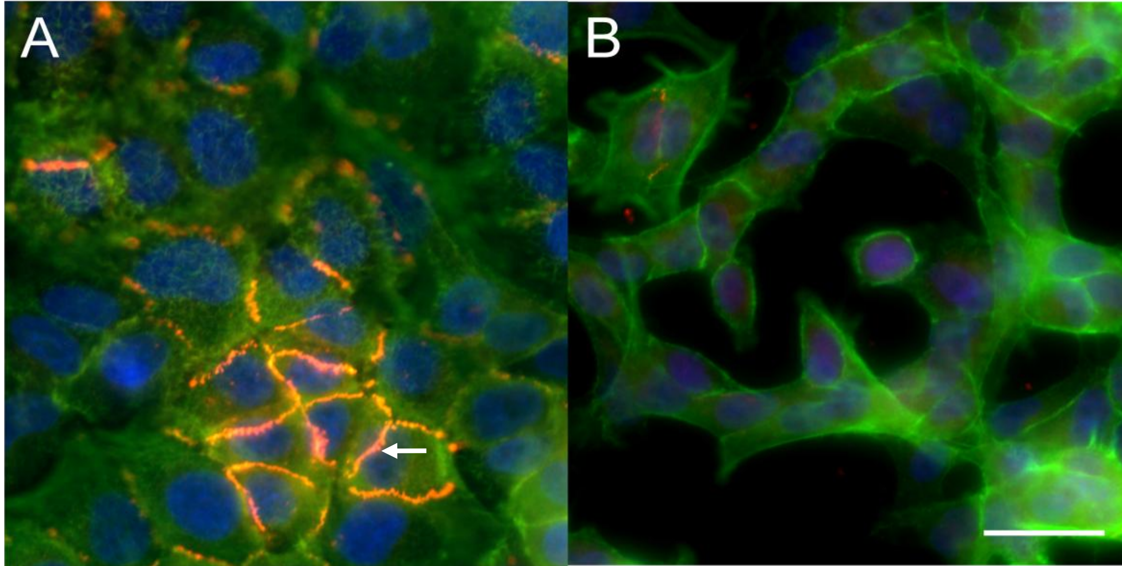


Figure 12. Biofilm CM stimulates the loss of Occludin from A549 cells. A549 cells were incubated with control media (A), and biofilm CM (B) for 24 hours. The cells were washed and fixed with 3% PFA and permeabilized with 0.1% Triton-X-100. The slides were blocked with 1% BSA and stained with phalloidin (green), anti-Occludin (red, arrow) and DAPI (blue). Slides were imaged using an Olympus BX41 microscope and a Retiga 2000R camera. The cells incubated with biofilm CM showed strong actin staining and a loss of Occludin. Selected images are representations of four replicates. Size bar represents 100 µm.

The loss of Occludin expression after 24 hours is clear; however it is not known if the loss of Occludin is the result of cell spreading, or rather part of the cause of the cell spreading. If Occludin expression is decreased prior to morphological changes then it is possible that the loss of Occludin is at least partly responsible for the A549 cell spreading. To test this A549 cells were exposed to biofilm and control CM for 4 hours, 18 hours and 24 hours. Following each incubation A549 cell proteins were extracted and resolved by Western blot analysis and probed for Occludin. Western blot analysis of the A549 cells exposed to biofilm CM showed that 4 hours of exposure did not affect the expression of Occludin; however, a loss of Occludin occurred at 18 hours, prior to the morphological changes in A549 cells (Figure 13). Moreover, in the CM-replacement study removing and replacing the biofilm CM with cell media after 11 hours still resulted in morphological changes with a further 13 hours of incubation. This suggests that changes in cellular proteins could be occurring as early as 11 hours. Since Occludin expression in the tight junctions of A549 epithelial cells was lost prior to cell spreading, it is possible that Occludin loss leads to the morphological changes.

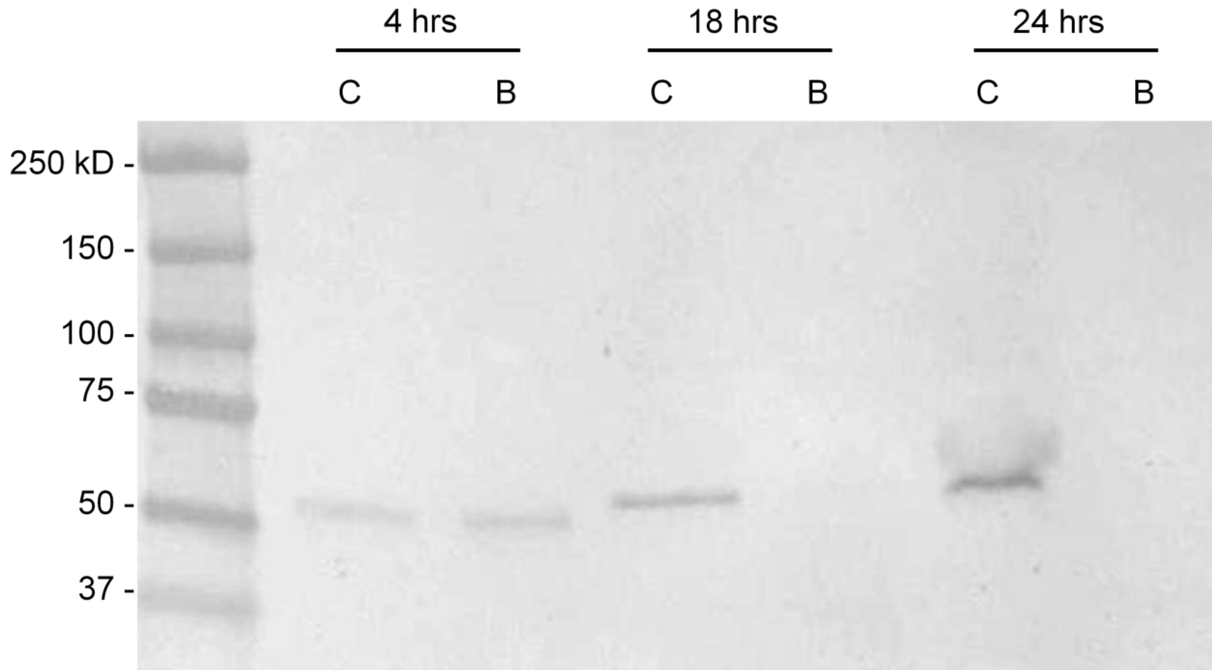


Figure 13. A549 cells exposed to biofilm CM had a loss of Occludin at 18 hours. Western blot analysis of crude plasma membrane extracts of A549 cells following exposure to media control (C) or biofilm CM (B). Cells were incubated with the CM for increasing time points, 4 hours, 18 hours and 24 hours prior to protein extraction. Protein extracts (3 μg) were separated with SDS-PAGE using a 4-20% gel. Bands were transferred to a nitrocellulose membrane at 100V. Membranes were blocked with 5% BSA and stained with mouse-α-Occludin overnight. After rinsing the bands were probed with donkey-α-mouse-alkaline-phosphatase and developed with SIGMA*FAST*™ BCIP®/NBT.

3.3.4 Biofilm secreted factors caused increased cell death but reduced apoptosis than planktonic secreted factors.

To determine whether the morphological changes induced by biofilm CM in the A549 cells were resulting in cell death or apoptosis, flow cytometry was performed using Annexin V binding to translocated phosphatidylserine as an apoptosis indicator. A549 cells were exposed to CM from biofilm and planktonic bacteria, as well as the positive apoptosis control camptothecin, for 24 and 48 hours. Following the exposure the cells were harvested and stained with Annexin V (binds to translocated phosphatidylserine) and Calcein AM (viability stain). The stained cells were then passed through a flow cytometer and gated based on their staining pattern. Cells that stained with only Calcein AM were gated as alive and non-apoptotic, cells stained with only Annexin V were considered dead and non-apoptotic, cells stained with both Calcein AM and Annexin V were considered alive and apoptotic. After 24 hours there was very little change in cell death of A549 cells stimulated by either biofilm or planktonic CM compared to the control (biofilm 2.3% p=0.179, planktonic 3.4% p=0.5) (Figure 14). Following 48 hours of incubation the biofilm CM caused significantly increased A549 cell death compared to the planktonic CM (biofilm 19.5% vs planktonic 3%, p=0.01); unlike the direct bacterial contact where planktonic caused a 2-fold increase in A549 cell death compared to biofilm exposure. This is not surprising since the cells were completely non-adherent at this time-point. It was thought that the increase in cell death in the cells exposed to biofilm CM could be due to anoikis, or apoptosis because of detachment (100). While changes in apoptosis were not significant at either time point (biofilm 24 hours: 2%, 48 hours: 2%; planktonic 24 hours: 6.6%, 48 hours: 2.6%; p=0.09, p=0.12), the cells exposed to biofilm CM had consistently less apoptosis than following exposure to the planktonic CM or control cell media (Figure 14A). This shows that after 24 hours of CM exposure, immediately following the morphological changes in A549 cells exposed to biofilm

CM, there is no significant increase in cell death or apoptosis. This data indicates that early exposure to biofilm CM does not stress A549 cells, immediately following their cell spreading. When the incubation time is extended to 48 hours the A549 cells are lifting off of the surface of the plate and there is an increase in A549 cell death. At this point it is unclear whether the A549 cell death is a result of lifting off the surface and losing its cell-to-cell interactions, since in this case cell death through apoptosis would be expected, or whether the biofilm CM is directly causing epithelial cell death.

The morphological changes in A549 cells following exposure to biofilm CM occurs after 20 hours of exposure. However the CM-replacement studies showed that removing the CM at 11 hours still resulted in the downstream morphological changes. Moreover, the tight junction protein Occludin was shown to be lost from A549 cells after 18 hours of biofilm CM exposure, prior to the morphological changes. Since the active factor in biofilm CM is interacting with the A549 cells prior to the morphological changes it was theorized that biofilm CM was stimulating A549 cell metabolism. Following direct biofilm contact with A549 cells for 1 hour there was an increase in epithelial metabolic activity as shown with the WST-1 assay. To determine if the same increase in metabolic activity occurred following biofilm CM exposure the WST-1 assay was used once again. A549 cells were exposed to biofilm and planktonic CM for 18 hours (prior to morphological changes) and then the reagent WST-1 was used as before to measure an increase in the metabolic enzyme mitochondrial dehydrogenase. Interestingly there was no change between the metabolism of A549 epithelial cells exposed to control media or biofilm CM (2.246 OD units vs 2.260 OD units, $p=0.916$) (Figure 14C). There was a significant increase in metabolism with exposure to planktonic CM compared to control (2.993 OD units vs 2.246 OD units, $p<0.05$). These results confirm the cell death data since there was no drop in A549 cell

metabolism following exposure to biofilm or planktonic CM. This also indicates that, while the active factor in biofilm CM was interacting with A549 cells after 18 hours of exposure, this did not result in an increase the metabolic activity.

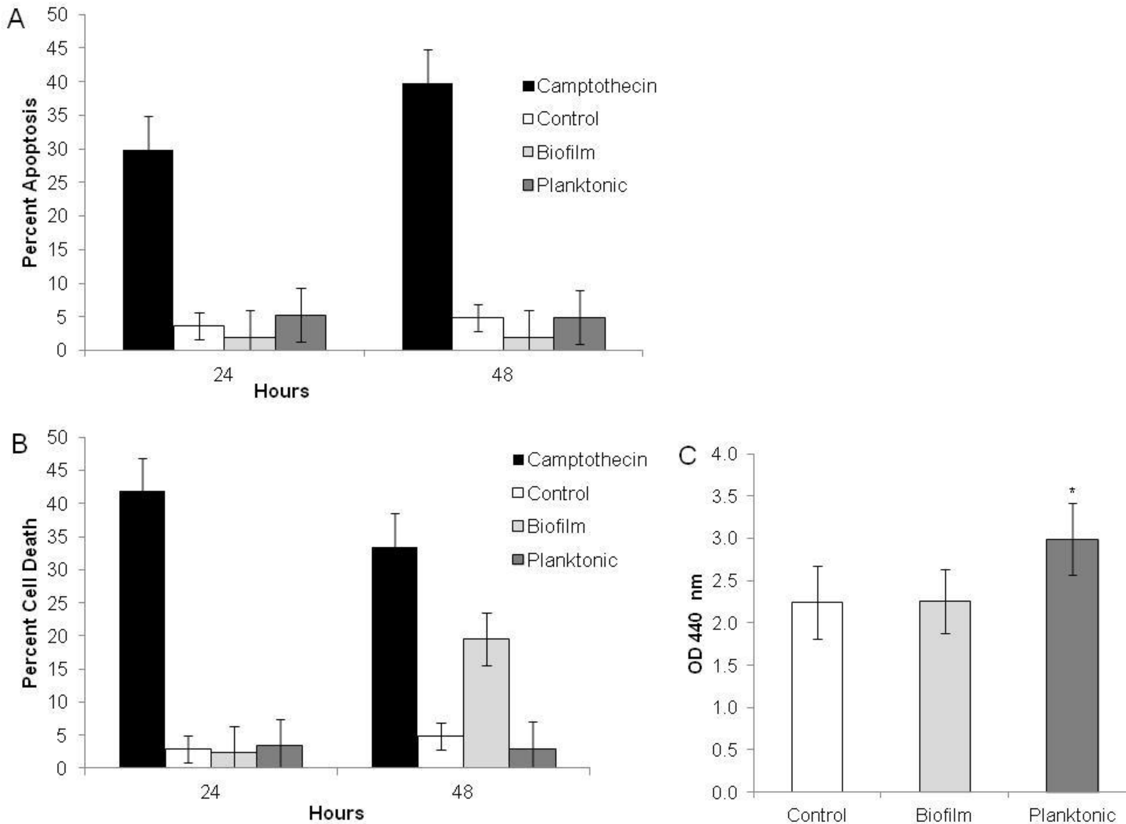


Figure 14. Biofilm CM stimulates A549 cell death after 48 hours of exposure. Planktonic CM stimulated an increase in A549 cell metabolism. A549 cells were incubated with CM from biofilm, planktonic bacteria and control for 24 and 48 hours. Following the incubation the supernatant was retained to include detached A549 cells and the adhered cells were treated with trypsin and added to the supernatant portion. The cells were washed and then treated with binding solution containing Calcein AM (live cell indicator) and Annexin V-Alexa Fluor®-647 (apoptosis indicator). The cells were analyzed using the Agilent Cell Chip using the Apoptosis Fast Protocol and were assessed for their a) apoptosis, and b) cell death. The positive control camptothecin was included since it stimulates apoptosis through phosphatidylserine translocation following 24 hours of exposure. c) Metabolic activity of A549 cells incubated with biofilm and planktonic CMfor 18 hours. The cells were then washed with PBS and incubated with WST-1 for 30 minutes and then metabolism was detected at 450 nm. Error bars represent standard error. *Significant change compared to the control are indicated by $p<0.05$ as determined using an unpaired Student's T-test, n=3.

3.3.5 Biofilm secreted factors suppress immune factors in A549 cells while up-regulating IL-8.

Chronic infections with biofilm are known to stimulate a robust IL-8 response from epithelia (36,65,86). To compare the immune activation in A549 cells by biofilm and planktonic CM, a Multiplex cytokine bead assay analysis system was used to measure cytokine responses in addition to IL-8. The Multiplex bead system combines principles from ELISA and flow cytometry assays. In one experimental well there are several beads each coated with a different cytokine antibody. The beads specific to each cytokine are differentiated based on a fluorophore. As in a typical sandwich-based ELISA, each bead binds to its specific cytokine. A cocktail of detector antibodies is then added to the well and bind to the captured cytokines. The detector antibodies are labeled with a unique fluorophore. The beads are then separated using flow cytometry. If a specific cytokine was present in the sample, then the corresponding bead will have its fluorophore detected, along with the fluorophore of the detector antibody. If the bead does not contain a detector antibody fluorophore then no cytokine was present in the sample. There were a total of 21 cytokines examined and listed in Table 5. The cytokines and chemokines were selected based on the availability in the kit panels. The panels were selected in order to examine as many epithelial secreted factors as possible. This work was done in collaboration with Dr. Nyla Dil of the University of Manitoba who used supernatants that I provided to conduct the Milliplex cytokine bead assays using equipment from Dr. Ball's laboratory at The Public Health Agency of Canada.

Table 5. List of cytokines analyzed with the Multiplex bead assay. Cytokines are listed in no specific order.

Cytokines Analyzed with the Multiplex Bead Assay						
GM-CSF	EGF	IFN-γ	IFNα2	RANTES	IL-1β	IL-2
IL-6	IL-7	IL-8	IL-10	IL-12p40	IL-12p70	IL-17
MCP1	IP-10	MIP1α	MP1β	MDC (CCL22)	MCP3	TNFα

A549 cells were exposed to CM from biofilm, planktonic bacteria as well as a media control for 24 hours. The supernatant was then collected and subjected to the Multiplex bead assay. The results of the assay showed that the planktonic CM stimulated an up-regulation of 9 immune markers of the 21 examined: GM-CSF (10.56 pg/mL, $p=0.003$) IL-6 (22.58 pg/mL, $p=0.004$), IL-8 (3465.81 pg/mL, $p=0.004$), IP-10 (71.87 pg/mL, $p<0.05$), MCP1 (5377.12 pg/mL, $p<0.05$), MDC (36.77 pg/mL, $p=0.001$), MIP1β (10.15 pg/mL, $p<0.05$), RANTES (951.39 pg/mL, $p<0.05$), and TNFα (6.59 pg/mL, $p=0.006$) (Figure 16). While not being significantly increased over the control, planktonic CM stimulated the release of significantly more IFNα2 than biofilm CM (10.46 pg/mL vs 2.19 pg/mL, $p=0.03$). Biofilm CM showed a marked decrease in stimulation compared with planktonic CM with only 4 of the 25 analytes being increased: GM-CSF (1.44 pg/mL, $p=0.017$), IL-8 (1148.48 pg/mL, $p=0.001$), MCP1 (3189.54 pg/mL, $p<0.05$) and RANTES (24.92 pg/mL, $p<0.05$). While biofilm CM stimulated the release of 4 cytokines, the levels secreted were less than those stimulated by planktonic CM. This shows that planktonic bacteria secrete factors that are more able to stimulate an immune response in A549 cells as compared to the factors secreted by biofilm.

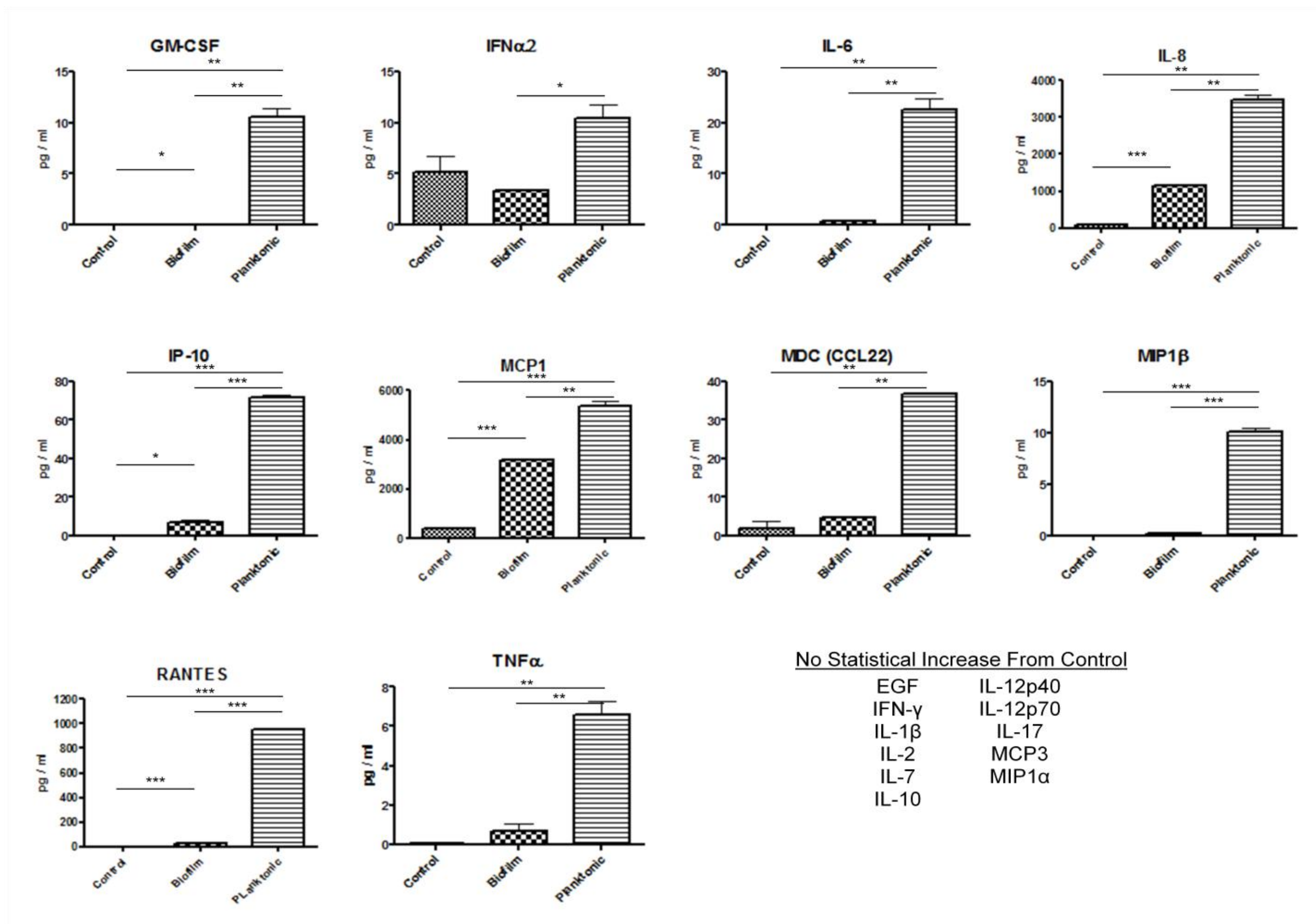


Figure 15. Planktonic CM stimulates A549 cells to release a larger array of cytokines and chemokines than biofilm CM. A549 cells were incubated with CM from biofilm, planktonic bacteria and control for 24 hours. Supernatant was removed and subjected to the Milliplex bead assay for the detection of cytokines and chemokines. Significant differences are indicated with a *$p<0.05$, **$p<0.01$, ***$p<0.001$ as determined with an unpaired, two-tailed Student's T-test. Error bars indicate standard error of three biological repeats.

The Multiplex assay showed IL-8 was released by A549 cells following exposure to both biofilm and planktonic CM with planktonic CM stimulating increased IL-8 compared to biofilm CM. IL-8 is often used as a biomarker for inflammation and as shown earlier, IL-8 release was also stimulated by direct biofilm contact; whereas direct contact with planktonic bacteria did not stimulate the same increase in IL-8 secretion. To determine if the levels of IL-8 release by A549 cells was similar between biofilms CM and direct biofilm contact, an ELISA was performed. A549 cells were exposed to CM from biofilms and planktonic bacteria for a total of 24 hours, with supernatant removed after 4 hours, 8 hours and 24 hours. IL-8 was then measured in the supernatant using a sandwich-based ELISA as previously described. The time-course study showed that the release of IL-8 by A549 cells was linear for stimulation with either planktonic CM (4 hours: 1072.46 pg/mL, 8 hours: 1913.95 pg/mL, 24 hours 4819.00 pg/mL; $p<0.001$ at all three time-points) and biofilm CM (4 hours: 98.12 pg/mL, $p=0.020$; 8 hours: 344.86 pg/mL, $p<0.001$; 24 hours: 1647.59 pg/mL, $p<0.001$) (Figure 16). At all three time-points the levels of IL-8 released were significantly higher following planktonic CM exposure versus biofilm CM ($p<0.001$). This differs from the results obtained using direct bacterial contact, where the levels of IL-8 released were higher with biofilm exposure. Here, with exposure to planktonic CM there is significantly less cell death of the A549 cells as compared to direct contact with planktonic *P. aeruginosa*. The levels of IL-8 released by A549 cells following biofilm CM exposure at 8 hours are comparable to those being released following direct exposure of biofilm to A549 cells; however there was no decrease at 24 hours as was seen with direct bacterial exposure (36). Instead the IL-8 level continued to increase, likely due to less A549 cell death. This indicates that the IL-8 response stimulated by biofilms as seen in chronic infection is likely mediated by secreted factors rather than direct bacterial contact.

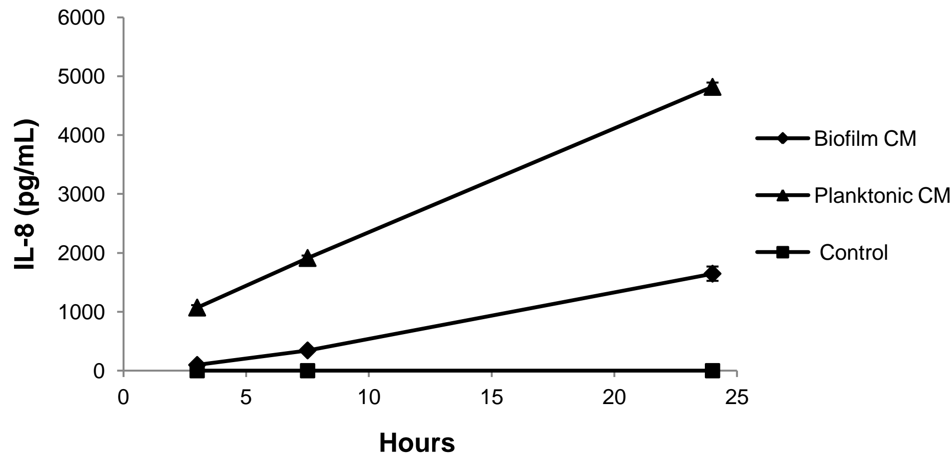


Figure 16. Planktonic CM stimulates significantly more IL-8 secretion from A548 cells than biofilm CM. A549 cells were incubated with CM from biofilm, planktonic bacteria and control for 24 hours with supernatant collected at 4 hours, 7 hours and 24 hours and IL-8 secretion was measured by ELISA. Error bars indicate standard deviation of four biological repeats. Statistical differences were determined using an unpaired, two-tailed Student's T-test.

3.3.6 A549 cells are not undergoing epithelial-to-mesenchymal transition following exposure to biofilm conditioned media

The morphological changes occurring in the A549 cells following exposure to biofilm CM could indicate epithelial-to-mesenchymal transition (EMT) (153). EMT occurs when the epithelial cells change cell type and become mesenchymal cells. These mesenchymal cells are unable to carry-out the same critical epithelial cell function within the lung. The transition to mesenchymal cells results in the permanent fibrosis of the lung and dramatic decrease in lung function. Along with morphological cell spreading changes associated with EMT, there are other characteristic changes of EMT including: cytoskeletal rearrangement, decrease in E-Cadherin, decrease in tight junction proteins such as Occludin, increase in Vimentin or Fibronectin, increase in signaling proteins, decrease in apoptosis, and an increase in wound healing (151). My previous data has shown that biofilm CM causes cytoskeletal rearrangement and loss of Occludin in epithelial cells, consistent with EMT; however, further evidence of EMT would be required to make this conclusion (Figure 12 and Figure 13).

To determine if EMT was an explanation of the observed changes following exposure to biofilm CM, E-Cadherin, Vimentin and the signaling marker Snail were analyzed. Cells undergoing EMT would have a loss of E-Cadherin and an increase in Snail and Vimentin expression. A549 cells were exposed to CM from biofilm and control media for 20 hours. When I examined E-Cadherin, Snail and Vimentin by immunofluorescence no increase in Snail and Vimentin in A549 cells exposed to biofilm CM was detected; however there may have been a slight decrease in E-Cadherin in the membrane of the cells (data not shown). To confirm that E-Cadherin was not being lost, a Western blot was performed. A549 cells were exposed to biofilm and control CM for 2 hours, 4 hours, and 18 hours. After each time point the A549 cell proteins were extracted, separated by Western blot and probed for E-Cadherin. Western blot analysis

confirmed that E-Cadherin was not lost in A549 cells incubated with biofilm CM, even after 18 hours (Figure 17); however a small amount of degraded protein was visible following 4 hours and 18 hours with biofilm CM (Figure 17). This confirms that the expected loss of E-Cadherin was not occurring, suggesting that EMT was not taking place.

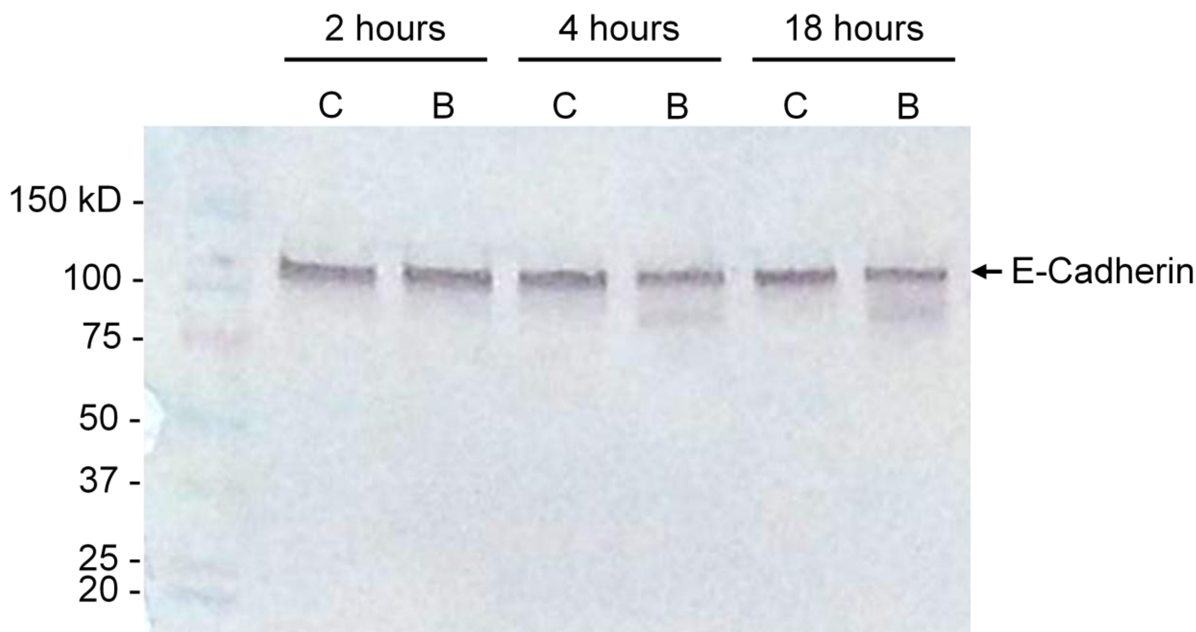


Figure 17. Biofilm CM does not cause a loss of E-Cadherin after 18 hours of incubation. A549 cells were exposed to CM from media control (C) or biofilm (B) for 2 hours, 4 hours, and 18 hours. After each time-point proteins were extracted and separated with SDS-PAGE using a 4-20% gel. Bands were transferred to a nitrocellulose membrane at 100V. Membranes were blocked with 5% BSA and stained with goat-α-E-Cadherin overnight. After rinsing the bands were probed with rabbit-α-goat-alkaline phosphatase and developed with SIGMA*FAST*™ BCIP®/NBT.

The analysis of E-Cadherin, Snail and Vimentin expression suggests that EMT is not taking place. However the morphological changes, cytoskeletal dysregulation and loss of Occludin are indicative of EMT but could also be attributed to other phenomena. A characteristic function of mesenchymal cells is the ability to migrate in response to wounds. To confirm that EMT was not taking place a wound healing assay was performed. For this assay, a scratch is placed in a monolayer of A549 cells while being exposed to CM. In a positive result the cells will become migratory and fill in the scratch, this would indicate that wound healing occurred and EMT could be taking place. A549 cells were grown to confluency in an 8-well plate and a scratch was created to disrupt the monolayer of cells. The wounded epithelial layer was then exposed to biofilm, planktonic and control CM for 18 hours and 40 hours. Following 18 hours of incubation with CM there was no wound healing observed from the control or planktonic CM (Figure 18). The biofilm CM caused the A549 cells to form some cellular extensions that could be considered motility from wound healing; however after 40 hours of incubation the epithelia had lifted off the plate and rounded indicating that no wound healing was actually taking place. This further confirms that EMT is not taking place following exposure to biofilm CM.

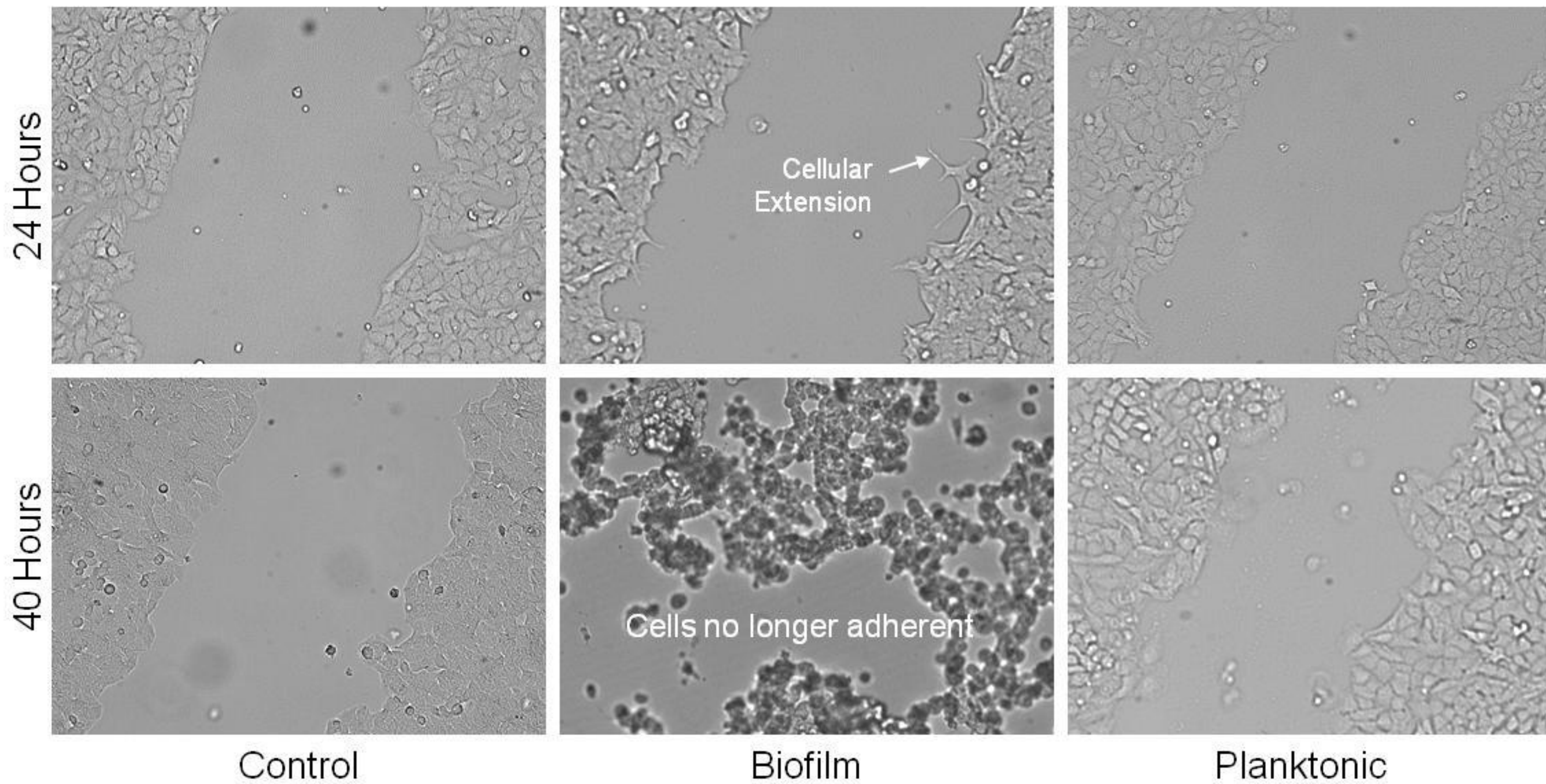


Figure 18. Neither biofilm CM not planktonic CM stimulates wound healing in A549 cells. Phase contrast images of A549 cells following the wound healing assay. Confluent A549 cell monolayers were scratched and then exposed to CM from cell media, planktonic bacteria, or biofilm. The scratches were observed following 24 hours and 40 hours of incubation to determine if the cells were migrating to close the wound. After 24 hours of exposure there was no cell migration of A549 cells following exposure to control or planktonic CM; A549 cells exposed to biofilm CM demonstrated some cellular extensions. After 40 hours of incubation there was still no wound healing with control and planktonic CM exposure and cells exposed to biofilm CM were no longer adherent. Selected images are representations of three replicates.

3.3.7 Secretomes of biofilm and planktonic bacteria contain differences in virulence factors.

Thus far it had been established that a quorum-sensing-controlled protein factor or factors in the biofilm CM was responsible for causing morphological changes in A549 cells . Therefore, comparisons of the secretomes of biofilm and planktonic bacteria may help identify the active protein. In collaboration with the Manitoba Centre for Proteomics and Systems Biology, biofilm and planktonic secretomes were examined using shotgun proteomics LC/MS/MS to identify proteins differentially abundant between the two growth forms. Proteins in each of the CM samples were digested with trypsin and separated with liquid chromatography. The peptides were directly injected into the mass spectrometer where they were subjected to tandem MS. The molecular weight of each peptide was compared to the Uniprot database containing proteomic data for *P. aeruginosa* PAO1. Datasets from each of the 2 replicates from biofilm CM, planktonic CM and control media (6 samples total) were combined. Following the elimination of duplicates and implementation of either a minimum criterion of two unique peptides or greater than 10% protein coverage (to ensure inclusion of small proteins); as well as a log(e) score below −2 a list of 237 proteins was identified. The results of the LC/MS/MS showed that planktonic bacteria secreted 219 proteins and biofilm secreted 190 proteins. There were no proteins identified in either of the control samples that met the minimum criteria. The secretomes were then analyzed and separated into five categories: common proteins (similar secretion in biofilm and planktonic) (Appendix 1), up-regulated in biofilm (present in both secretomes but significantly higher in biofilm) (Appendix 2), up-regulated in planktonic (Appendix 3), unique to biofilm (Appendix 4), and unique to planktonic (Appendix 5). For a protein to be considered present in either biofilm or planktonic CM it had to appear in both replicates of the sample. Also, for a protein to be considered unique to either biofilm or planktonic CM it could not be present in

either replicate of the opposite sample. Table 6 shows that the majority of proteins were similar between both growth phases (121 proteins out of 237 proteins). Planktonic bacteria secreted 44 unique proteins and had 44 proteins up-regulated in comparison to biofilm. Biofilms were found to secrete 15 unique proteins and had 10 proteins up-regulated compared to planktonic CM. The proteins were then categorized into four groups by the function indicated in the Uniprot protein database through manual curation: motility, metabolism and growth, virulence and protection, and other (Table 7). Of the proteins up-regulated and unique to biofilm, 12% were for motility (3 proteins), 36% were for metabolism and growth (9 proteins), 32% were for virulence and protection (8 proteins), and 20% were classified as other (5 proteins). In comparison planktonic bacteria secreted unique and up-regulated proteins where 2% were for motility (2 proteins), 57% for metabolism and growth (50 proteins), 14% virulence and protection (12 proteins), and 27% were other (24 proteins). The abundance of internal proteins identified indicates that bacterial lysis took place either during the incubation or more likely during the protein isolation process. It was expected that planktonic bacteria would express more proteins involved in metabolism and growth compared to a stable biofilm because a large portion of the biofilm is not actively metabolizing or growing (307). Both planktonic and biofilm bacteria had Flagellin B in their top 2 ranked proteins (based on estimated quantity using log(e)). This indicates that Flagellin B is produced in considerable quantity by both biofilm and planktonic bacteria. The top ranked protein in both replicates of biofilm secretome was Pseudomonal Elastase (PE) LasB. The two replicates of planktonic had this protein ranked 51 and 55 out of total protein produced. This protein is of interest because it is controlled by quorum sensing and is part of the LasI operon and known to be involved in epithelial cell destruction (29,107,236).

Table 6. Planktonic *P. aeruginosa* secretes a larger array of proteins compared to biofilm. Categorization of proteins identified in CM through LC/MS identification.

Description	Number of Proteins Identified
Unique to biofilm	15
Up-regulated in biofilm	10
Common to both biofilm and planktonic	121
Up-regulated in planktonic	44
Unique to planktonic	44

Table 7. A larger proportion of proteins unique or up-regulated in biofilm CM were categorized as for virulence and protection as compared to planktonic CM. Categorization of proteins unique or up-regulated in biofilm and planktonic CM based on function stated in the Uniprot and NCBI databases.

Function Category	Biofilm (%)	Planktonic (%)
Motility	3 (12%)	2 (2%)
Metabolism and Growth	9 (36%)	50 (57%)
Virulence and Protection	8 (32%)	12 (14%)
Other	5 (20%)	24 (27%)

3.3.8 Specifically inhibiting elastase activity eliminates the effect of biofilm CM exposure to A549 cells

Pseudomonas Elastase (PE) (also known as pseudolysin) is a metalloprotease that is translated as a large protein (proelastase) that undergoes autocleavage to the active form (29,197,198). To confirm that the presence of increased PE in the biofilm correlates with increased enzymatic activity, elastase activity was determined using an Elastin Congo Red assay. Elastin Congo Red is a substrate for all elastase enzymes. Once cleaved, the product can be measured with a spectrophotometer. CM was isolated from biofilm and planktonic bacteria and mixed with the substrate Elastin Congo Red. Elastase activity was significantly higher in the biofilm CM

compared to control (0.019 OD units vs 0.001 OD units, $p<0.001$), but not in the planktonic CM (0.003 OD units vs 0.001 OD units, $p=0.134$) (Figure 19).

Since Elastin Congo Red is not specific to PE, inhibition studies were performed to confirm that the enzymatic activity in the biofilm CM was due to PE. PE is a metalloprotease and is therefore inhibited by the addition of EDTA. Moreover, the PE inhibitor phosphoramidon was also used to confirm that the morphological changes were due to elastase. A549 cells that were exposed to biofilm CM treated with EDTA or phosphoramidon no longer underwent the expected morphological changes (Figure 20). Since the elastase inhibitor phosphoramidon and non-specific EDTA were both able to eliminate the activity, along with the prevalence of PE in the biofilm secretome and absence of other suspected enzymes, this data further supports that PE is the protein produced by biofilm bacteria that stimulates morphological changes in A549 cells.

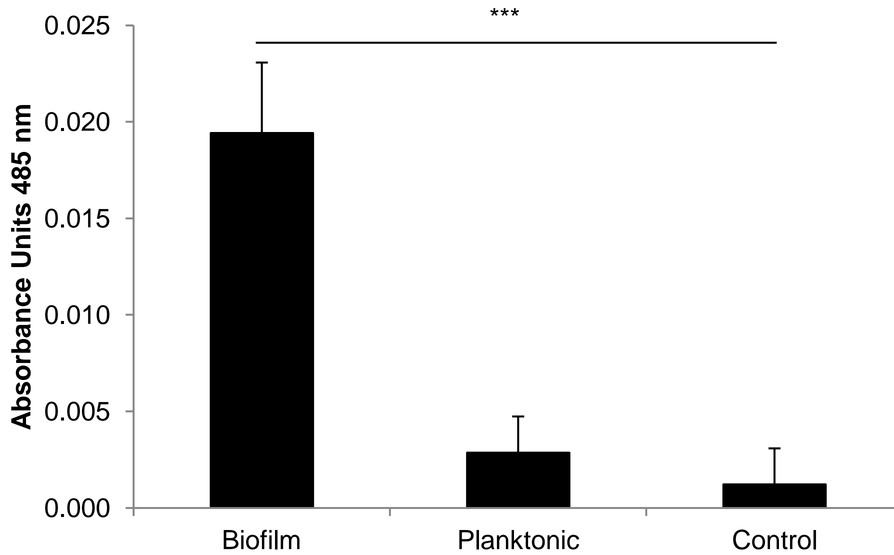
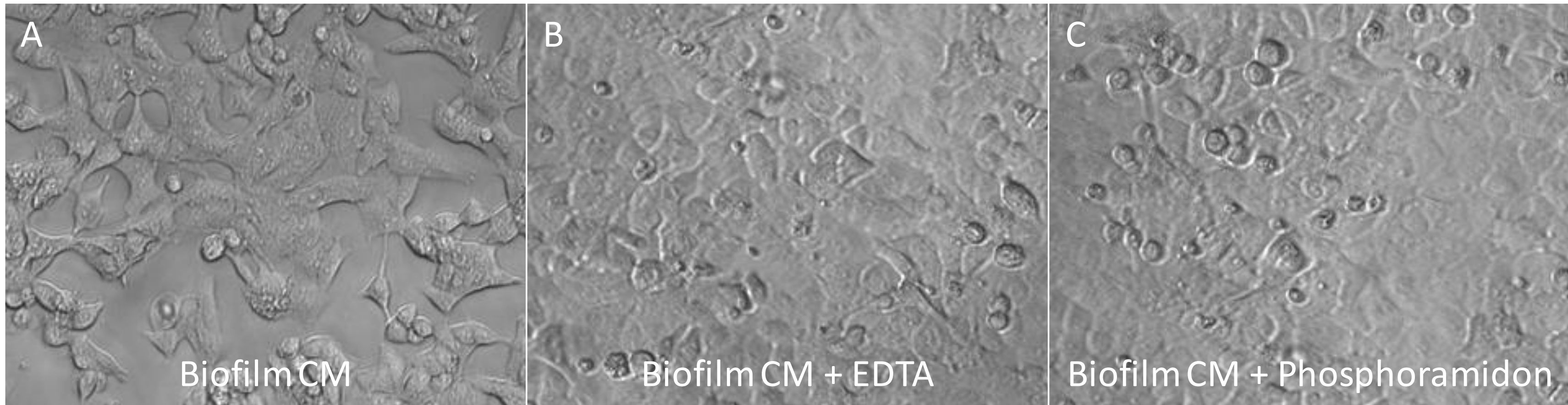


Figure 19. Biofilm CM contains significantly more PE than planktonic CM. Elastase activity was determined in the CM of biofilm and planktonic bacteria compared to control media as measured by the Elastin Congo Red assay. CM was incubated with Elastin Congo Red overnight at 37°C at 700 rpm. The plate was centrifuged and the supernatant was transferred to a microplate and absobance was detected at 485 nm. Each condition was tested in triplicate. *** Indicates a significant difference compared to the control as determined with an unpaired, two-tailed Student's T-test, $p<0.001$.



Inhibitor	Activity
None	Yes
EDTA	No
Phosphoramidon	No

Figure 20. PE inhibition studies indicated that the morphological changes in A549 cells following exposure to biofilm CM is caused by PE. Phase contrast microscopy showing the morphology of A549 cells following a 24 hour incubation with biofilm CM containing A) no inhibitor, B) non-specific inhibitor EDTA, and C) specific inhibitor phosphoramidon. The images are representations of 16 biological repeats. The ability of the CM to cause morphological changes in A549 cells is summarized in the table (bottom). A549 cells underwent the morphological change of cell spreading and loss of cell-to-cell contact following exposure to biofilm CM. Incubating the A549 cells with EDTA or phosphoramidon eliminated the ability of biofilm CM to cause the morphological changes.

3.3.9 Summary

In summary, I found that the CM from biofilms, but not planktonic bacteria, caused A549 epithelial cells to undergo morphological changes that were not linked to epithelial-to-mesenchymal transition, though it did cause a loss of the tight junction protein Occludin. Moreover, the CM from biofilm and planktonic bacteria caused an increase in the release of IL-8 from A549 epithelial cells, while the planktonic CM also stimulated the release of an array of cytokines and chemokines, such as GM-CSF, IL-6, IP-10, MCP1, MDC, MIP1β, RANTES, and TNFα. Along with IL-8 the biofilm CM stimulated the release of GM-CSF, MCP1 and RANTES. I had predicted that the secreted factors from biofilm would stimulate the most IL-8 release from A549 cells since biofilms are associated with inflammation; however it was the planktonic secreted factors that stimulated the most IL-8 release. As hypothesized, examination of the secretomes of biofilm and planktonic bacteria showed that planktonic bacteria released a larger number of proteins as compared to biofilm. The up-regulated proteins secreted from planktonic bacteria were categorized as being mostly for metabolism and growth, with a smaller proportion designated as virulence and protection as compared to proteins up-regulated in biofilm. One third of the secreted proteins unique or up-regulated in biofilms were classified as being for virulence and protection as compared to 12% of planktonic secreted proteins. One of the virulence factors up-regulated by biofilms was PE, which is part of the LasI quorum-sensing operon. Inhibition studies revealed that PE was likely responsible for the morphological changes of A549 cells and enzymatic studies confirmed that there was significant PE activity in biofilm CM and no significant PE activity in planktonic CM.

3.4 4-hour and 24-hour biofilms differ in their β-lactamase and PE secretion but interact similarly with A549 epithelial cells

Previous co-culture studies have examined the initiation of biofilm formation by planktonic bacteria (47,208,320). These co-culture studies have short incubation times and epithelial cell death often occurs before a biofilm is formed. Therefore there is little information directly comparing the changing virulence as a biofilm transitions from newly adherent planktonic bacteria to fully formed biofilms. Using the Calgary Biofilm Device for co-culture allows the concurrent comparison of newly adherent 4-hour and established 24-hour biofilms interactions with A549 lung epithelia.

3.4.1 24-hour biofilms are more resistant to β-lactams than 4-hour biofilms

Chronic lung infections with *P. aeruginosa* are known to be highly resistant to treatment with antibiotics. Previous studies with the Calgary Biofilm Device have examined the antibiotic sensitivity of 4-hour biofilms and have shown these biofilms to be resistant to many antibiotics (52). Since I have shown 24-hour biofilms are phenotypically distinct from planktonic bacteria, it was of interest to determine if the antibiotic resistance of these 24-hour biofilms is more pronounced than 4-hour biofilms.

3.4.1.1 24-hour biofilms release bacteria that are more resistant to gentamicin, ceftazidime and meropenem than 4-hour biofilm

The Calgary Biofilm Device was used to simultaneously determine the MIC, MBC (minimum bactericidal concentration) and MBEC (minimum biofilm eradication concentration) to different antibiotics that are used in the treatment of lung infections in CF patients. The MIC represents the minimum concentration of antibiotic required to prevent the growth of bacteria, while the MBC is the concentration required to kill the bacteria. The MBEC is the concentration of

antibiotic required to eliminate the biofilm. 4-hour and 24-hour biofilms were grown on the Calgary Biofilm Device and then exposed to increasing concentrations of gentamicin, ciprofloxacin, ceftazidime, and meropenem. The MIC is determined by visually examining the wells. The MBEC is determined by transferring the peg lid to a plate containing fresh media and incubating to stimulate growth, if no growth occurred then the corresponding concentration of antibiotic was sufficient to eradicate the biofilm. The MBC is determined similarly by transferring a small volume of media from the antibiotic plates to a plate containing fresh media. Once again, no growth indicates that the corresponding concentration of antibiotic was sufficient to kill the planktonic bacteria that were released from the biofilm.

The MIC and MBC results from 4-hour biofilms was similar to MICs determined by Ceri *et al.* (52) (gentamicin: MIC 4 μg/mL, MBC 28 μg/mL; ciprofloxacin: MIC 0.25 μg/mL, MBC 0.78 μg/mL; ceftazidime: MIC 2 μg/mL, MBC 60 μg/mL; meropenem: MIC 2 μg/mL, MBC 20 μg/mL) indicating that the methods used were consistent with other labs (Figure 21A). When the experiments were repeated using a 24-hour biofilm, I observed MIC and MBC results significantly greater than those for the 4-hour biofilm for all four antibiotics (gentamicin: MIC 8 μg/mL, $p<0.001$, MBC 87 μg/mL, $p=0.004$; ciprofloxacin: MIC 4 μg/mL, $p<0.001$, MBC 2 μg/mL, $p=0.025$; ceftazidime: MIC >2048 μg/mL, $p<0.001$, MBC >2048 μg/mL, $p<0.001$; meropenem: MIC 32 μg/mL, $p<0.001$, MBC 448 μg/mL, $p=0.007$) (Figure 21B). This indicates that the planktonic bacteria released from a 24-hour biofilm are more resistant as compared to planktonic bacteria released from a 4-hour biofilm.

The differences in antibiotic resistance of 4-hour and 24-hour biofilms were compared for the same four antibiotics. The MBEC was not significantly different for ciprofloxacin (4-hour 60 μg/mL, 24-hour 40 μg/mL, $p=0.101$).The MBECs were significantly greater for 24-hour

biofilms for gentamicin, ceftazidime and meropenem (gentamicin: 4-hour 128 μg/mL, 24-hour 384 μg/mL, $p<0.001$; ceftazidime: 4-hour 1024 μg/mL, 24-hour >2048 μg/mL, $p<0.001$; meropenem: 4-hour 288 μg/mL, 24-hour 896 μg/mL, $p=0.01$). Resistance to ceftazidime is usually mediated through the increased release of β-lactamases by *P. aeruginosa*. Meropenem is a carbapenem with increased resistance to β-lactamases (195). While being more resistant to β-lactamases, meropenem has also been shown to induce β-lactamases to a greater degree than ceftazidime (223). These results suggest that there may be differences in the expression of factors involved in β-lactam resistance between the 4-hour and 24-hour biofilms and that this may be a characteristic that should be examined *in vivo*.

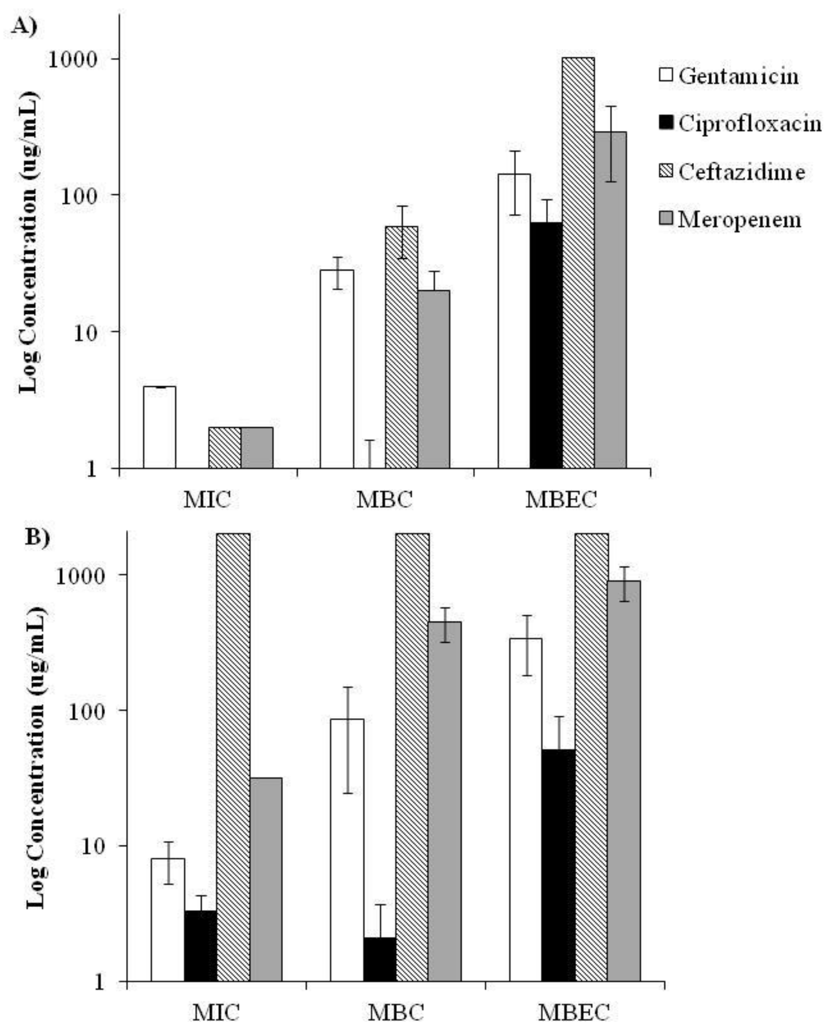


Figure 21. 24-hour biofilms show a greater resistance to antibiotics than 4-hour biofilms. Biofilms were formed on the Calgary Biofilm Device for 4-hours (a) and 24-hours (b) and then exposed to increasing concentrations of gentamicin, ciprofloxacin, ceftazidime and meropenem. MIC was determined as the lowest concentration of antibiotic without any visible bacterial growth. MBC was determined by transferring 10 µL of supernatant to a 96-well plate with fresh TSB and examining for growth. The MBEC was determined as the lowest concentration of antibiotic that eradicated biofilm after incubating the peg-lid overnight in a plate containing fresh TSB. The graph is showing the log transformed MBEC of the biofilm and the MIC and MBC values of the bacteria released from the biofilm following 24 hour exposure to antibiotics. Each condition was run in quadruplicate.

3.4.1.2 24-hour biofilms produce more β-lactamase than 4-hour biofilms

The lower phenotypic tolerance to meropenem by 24-hour biofilms further suggests that β-lactamases are crucial for tolerance to ceftazidime in *P. aeruginosa* biofilms. In the Calgary Biofilm Device there are two populations of bacteria in each well: the bacteria that make up the biofilm, as well as the planktonic bacteria that have been released from the biofilm. Therefore the growth of planktonic bacteria released by 24-hour biofilms could be the result of increased β-lactamase activity in either the bacteria making up the biofilm, or the planktonic bacteria released from the biofilm. To determine the quantity and source of β-lactamase in the experimental well, a nitrocefin assay was performed. 4-hour and 24-hour biofilms were grown as previously described and exposed to 1 μg/mL, 16 μg/mL, and 512 μg/mL of ceftazidime or meropenem for 5 hours and 24 hours. After each time-point the biofilm, planktonic bacteria as well as the supernatant were separated and assayed for β-lactamase activity using nitrocefin as a substrate. The activity of β-lactamase was normalized using total protein concentration in each sample. It was found, as previously reported (106), that meropenem exposure stimulated significantly more β-lactamase production than ceftazidime. After 5 hours of exposure to 512 μg/mL of either meropenem or ceftazidime, the 4-hour biofilms produced less β-lactamase than the 24-hour biofilms (meropenem – 5.1 units, 18.3 units, $p=0.03$; ceftazidime – 6.4 units, 12.5 units, $p=0.005$) (Figure 22A); however, after 24 hours of meropenem exposure, the 24-hour biofilm had also decreased its production of β-lactamase (6.4 units, $p=0.040$) (Figure 22B). There was decreased β-lactamase production by all planktonic bacteria exposed to ceftazidime or meropenem after 24 hours ($p<0.001$). β-lactamase production by released planktonic bacteria appears to drop significantly after 24 hours, however the amount of β-lactamase activity in each sample maintained high levels and only appears lower after protein normalization due to the high growth of bacteria. This indicates that the planktonic bacteria maintain a high level of β-

lactamase secretion after 24 hours, while the amount released per bacteria may decrease; or this may reflect an upper limit to the nitrocefin assay.

The antibiotic challenge assay revealed that planktonic bacteria released from 24-hour biofilms were able to survive in high concentrations of ceftazidime, while planktonic bacteria released from 4-hour biofilms were not. Using the nitrocefin assay it can be concluded that the survival of the planktonic bacteria is associated with the amount of β-lactamase released from the biofilm and not the planktonic bacteria themselves. The 24-hour biofilms produce significantly more β-lactamase than the 4-hour biofilm; which breaks down the ceftazidime in the environment and allows for the survival of the released planktonic bacteria. This result has significance for CF infections because it indicates that a chronic biofilm is able to break down β-lactam antibiotics and create an environment that facilitates acute exacerbations of *P. aeruginosa* and allows the survival of many different species of infecting bacteria.

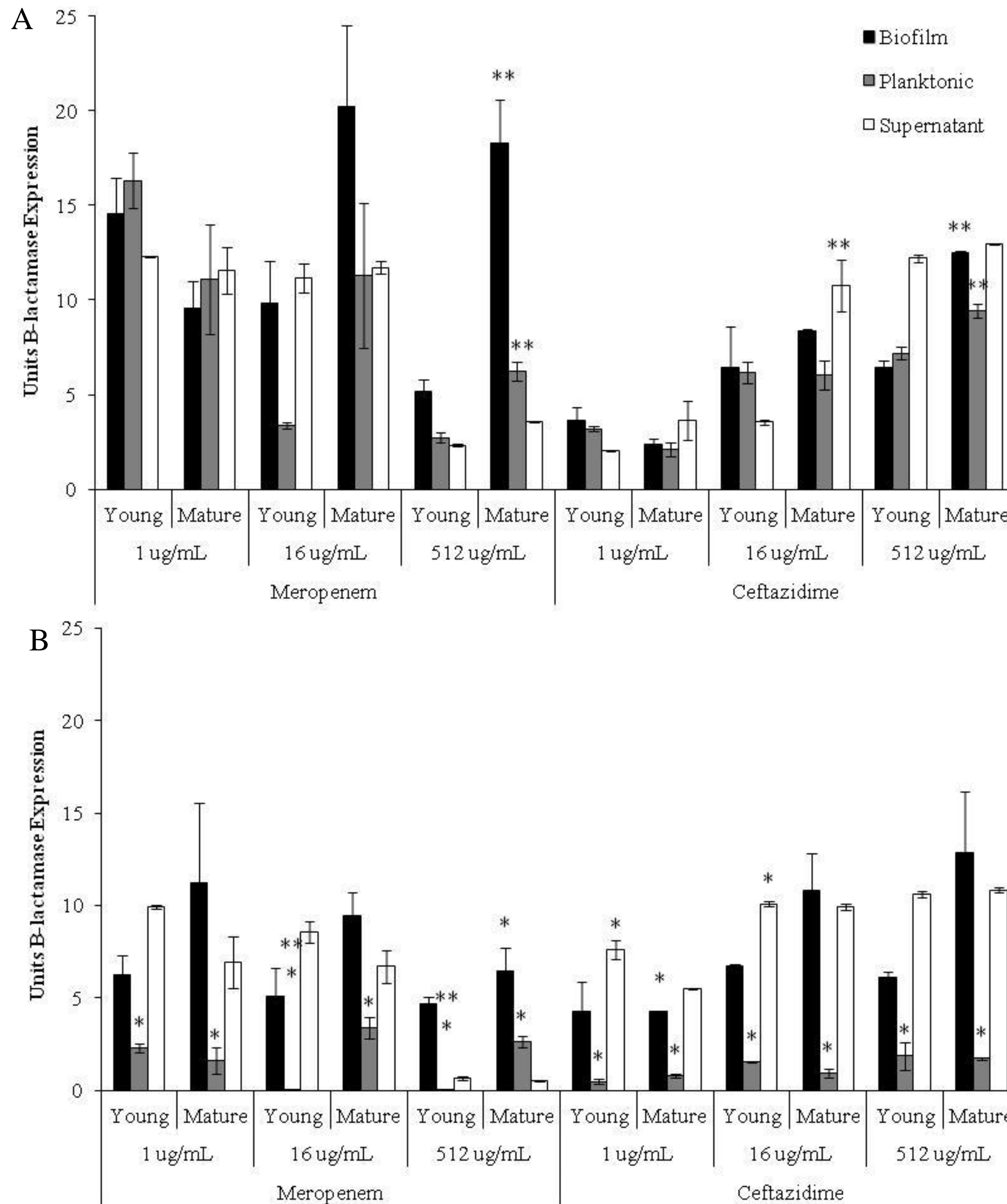


Figure 22. 24-hour biofilms secrete more β-lactamase than 4-hour biofilms allowing the growth of planktonic bacteria. 4-hour 24-hour biofilms were grown on the Calgary Biofilm Device and then exposed to ceftazidime or meropenem for 5 hours (a) or 24 hours (b). Following antibiotic exposure β-lactamase activity was detected in the biofilm, their released planktonic bacteria and the supernatant per μg/mL protein using the nitrocefin assay. All values are the averages of three replicates with error bars indicating standard error. * indicates a significant difference between 5-hour and 24-hour antibiotic exposure, ** indicates significant difference between 4-hour and 24-hour biofilms, as determined using an unpaired, two-tailed Student's T-test $p<0.05$.

3.4.2 4-hour biofilms do not stimulate a significant increase in metabolic activation of A549 epithelial cells

The antibiotic resistance studies show that newly forming 4-hour and established 24-hour biofilms have different susceptibility to antibiotic use. As acute infections shift into the chronic stage there is a transition from planktonic bacteria, to newly adherent forming biofilms, and finally to established biofilms. Using the Calgary Biofilm Device for co-culture allows the comparison of 4-hour and 24-hour biofilms' interactions with A549 lung epithelia. 4-hour biofilms exhibit a phenotype that is similar to both planktonic and 24-hour biofilms based on the results of the previous antibiotic study. To determine if established and newly forming biofilms of *P. aeruginosa* differ in their stimulation of A549 metabolism a WST-1 assay was performed. Since there is no way to infect A549 cells with an equivalent MOI of 4-hour and 24-hour biofilms the results were compared to equivalent MOI of planktonic bacteria. 4-hour and 24-hour biofilms were grown on the Calgary Biofilm Device. A549 cells were then infected with 24-hour biofilms and planktonic bacteria at an MOI of 100; and 4-hour biofilm and planktonic bacteria at an MOI of 10 for 1 hour along with a media control. Following the bacterial exposure the A549 cells were rinsed and incubated with the WST-1 reagent as previously described to measure the amount of the metabolic enzyme mitochondrial dehydrogenase. 24-hour biofilms stimulated the characteristic increase in metabolic activity in A549 cells compared to the control (1.921 OD units vs 1.281 OD units, $p<0.001$), while there was no significant increase when exposed to 4-hour biofilm compared to the control (1.651 OD units vs 1.281 OD units, $p=0.390$) (Figure 23). This was similar to the planktonic bacteria infected at the same MOI that showed no significant change with the control (1.454 OD units vs 1.281 OD units, $p=0.390$). This suggests that 4-hour biofilm may have characteristics in-between planktonic and 24-hour biofilms.

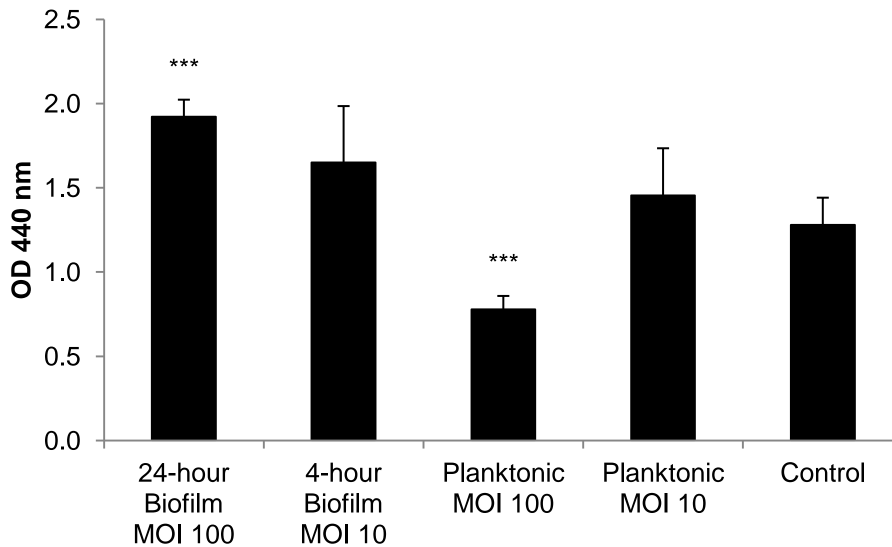


Figure 23. 4-hour biofilms do not stimulate a significant increase in A549 metabolism compared to 24-hour biofilms. 24-hour biofilms and planktonic bacteria were used to infect A549 cells at an MOI of 100; 4-hour biofilms and planktonic bacteria were infected at an MOI of 10. After a 1 hour co-culture metabolic activity of A549 cells was measured through conversion of WST-1 and absorbance was determined at an OD of 440 nm. Error bars represent standard deviation of 12 replicates. *** indicates a significant difference from the control as determined using an unpaired, two-tailed Student's T-test, $p<0.001$.

3.4.3 4-hour biofilms stimulate similar IL-8 release from A549 cells as 24-hour biofilms

Established biofilms grown on the Calgary Biofilm Device stimulate A549 cells to produce high levels of IL-8 (Section 3.2.3). Increased IL-8 release is characteristic of a chronic infection. The timing of IL-8 release and development of biofilm is not completely understood. I wanted to determine if IL-8 release by A549 cells occurs after exposure to a 4-hour biofilm, or if a 24-hour biofilm is required to stimulate the release of high levels of IL-8. The Calgary Biofilm Device co-culture assay was used to expose A549 cells to 4-hour (MOI 10) and 24-hour (MOI) biofilms and planktonic bacteria (MOI 10 and 100) for 4 hours, 7 hours, and 24 hours. The supernatant was removed and IL-8 was measured by ELISA as previously described. After 4 and 7 hours of incubation there was no difference between the IL-8 release stimulated by 4-hour and 24-hour biofilms (4 hours – 806.99 pg/mL vs 923.32 pg/mL, $p=0.094$; 7 hours – 2055.39 pg/mL vs 1735.78 pg/mL, $p=0.101$) (Figure 24); however both were significantly different than the control ($p<0.001$). The 4-hour biofilm stimulated significantly more IL-8 release from A549 cells as compared to planktonic bacteria infected at the same MOI (planktonic MOI 10: 4 hours – 13.72 pg/mL, $p<0.001$; 7 hours – 32.29 pg/mL, $p<0.001$; 24 hours – 9.86 pg/mL, $p<0.001$). This indicates that after adhering to the peg lid for 4-hours, *P. aeruginosa* has an altered ability to induce IL-8 compared to planktonic bacteria. After 24 hours of incubation with either biofilm or planktonic bacteria, the A549 cells were dead and lifted from the plate (data not shown), explaining the decrease in IL-8 compared to the 8 hour time-point. Even though IL-8 dropped after exposure to 24-hour and 4-hour biofilms after 24 hours, there was still significantly more IL-8 present after 4-hour biofilm exposure compared to 24-hour biofilm (463.26 pg/mL vs 193.10 pg/mL, $p=0.018$). Once again, the characteristics of 4-hour biofilm seem to lie on a

continuum between planktonic bacteria and established biofilms since 4-hour biofilms are able to stimulate A549 cells to release equivalent, if not slightly more, IL-8.

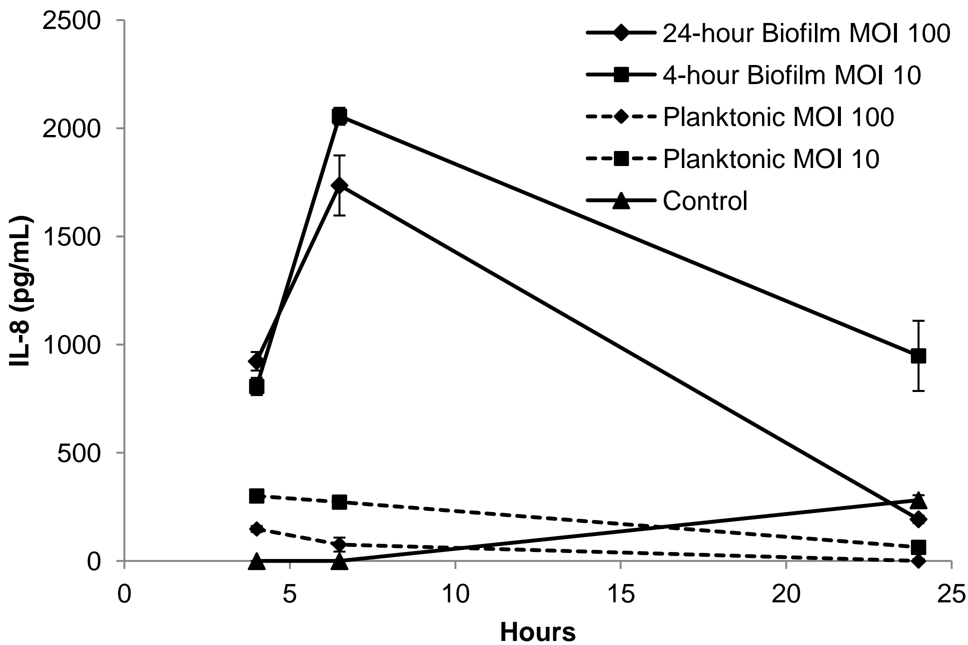


Figure 24. 4-hour biofilms are able to stimulate a significant increase in IL-8 release as compared to planktonic bacteria. A549 cells were infected with 24-hour biofilms and planktonic bacteria at an MOI 100; as well as 4-hour biofilm and planktonic bacteria at an MOI of 10 for 4 hours, 7 hours and 24 hours. After each time-point supernatant was isolated and IL-8 was detected through an ELISA. Error bars indicate standard deviation of four biological repeats and statistical differences were determined using an unpaired, two-tailed Student's T-test.

3.4.4 4-hour biofilms produce significantly less elastase as compared to 24-hour biofilms and are unable to affect A549 epithelial cells

4-hour biofilms seem to interact similarly with A549 cells as 24-hour biofilms by stimulating an increase in IL-8 secretion; however they secrete less β-lactamases than 24-hour biofilms and are unable to stimulate an increase in A549 metabolism characteristic of the 24-hour biofilm (Section 3.4.1.2). Since the LasI operon is activated during the biofilm maturation stage, it was hypothesized that this quorum sensing system was not required for the initial biofilm formation. If this is true then it would be expected that 4-hour biofilms would secrete less PE and have less of an effect on A549 cell morphology as compared to 24-hour biofilms, since PE is controlled by the LasI quorum sensing system. To test this hypothesis, CM was isolated from 4-hour and 24-hour biofilms inoculated at the same CFU/mL to determine if newly adhering biofilms secrete less PE and therefore have a reduced effect on A549 cell morphology than established biofilms. A549 cells were exposed to CM from 4-hour and 24-hour biofilms and examined microscopically for the characteristic morphological changes of PE exposure. The CM from 4-hour biofilm did not cause morphological changes in A549 cells (Figure 25). This indicates that 4-hour biofilms likely do not produce sufficient PE to stimulate the morphological changes in epithelial cells.

Newly adhering biofilms appear to have less of an effect on A549 cells and may secrete less PE than established biofilms. To quantify PE activity CM was isolated from 4-hour and 24-hour biofilms and used in the Elastin Congo Red assay as previously described. The Elastin Congo Red assay revealed that the CM from 4-hour biofilms contained significantly less PE activity than 24-hour biofilms (0.013 OD units vs 0.0680 OD units, $p=0.008$).

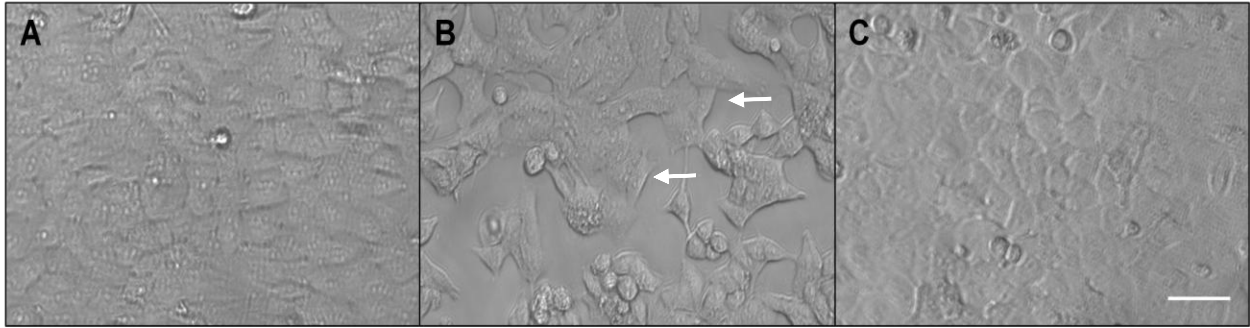


Figure 25. CM from 4-hour biofilms do not stimulate the morphological changes in A549 characteristic of PE secretion. A549 cells were exposed to CM from cell media (A), 24-hour biofilm (B), or 4-hour biofilm (C) for 24 hours and assessed for morphological changes using phase contrast microscopy. Arrows indicate A549 cells undergoing the morphological changes of losing cell-to-cell contact, spreading and eventually lifting off the plate. Experiment was performed in the 96-well format and selected images are representations of four replicates. Size bar represents 100 µm.

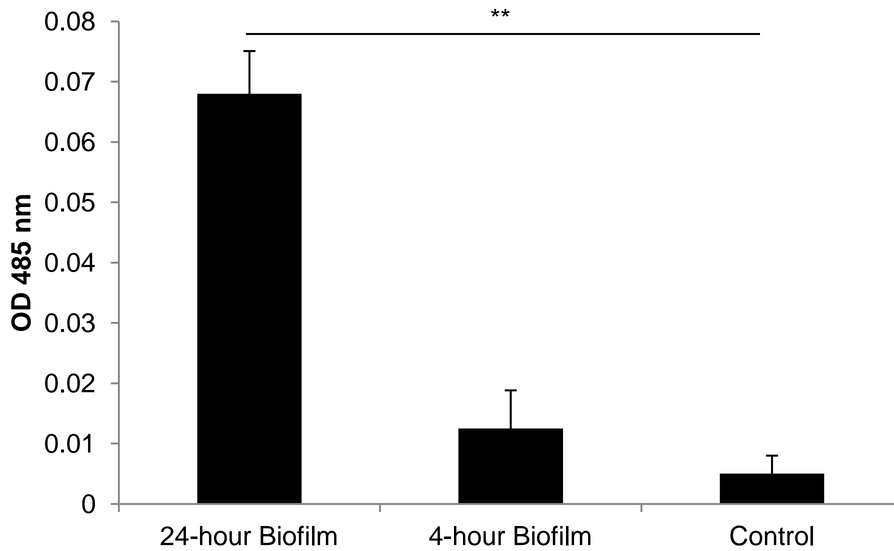


Figure 26. 4-hour biofilms do not secrete significantly more PE when compared to the control. CM was isolated from an equivalent CFU/mL of 24-hour and 4-hour biofilm and PE activity was quantified as measured by the Elastin Congo Red assay. Each condition was run in triplicate. ** Indicates a significant difference compared to the control using an unpaired, two-tailed Student's T-test, $p<0.01$.

3.4.5 Summary

Using the Calgary Biofilm Device co-culture system to compare newly forming and established biofilms revealed that 4-hour biofilms have characteristics similar to both planktonic bacteria and 24-hour biofilms. I had predicted that 4-hour biofilms would behave more similarly to 24-hour biofilms; however this was not the case when different aspects of virulence were assessed. 4-hour biofilms were found to secrete proteins similar to planktonic bacteria, with 24-hour biofilms secreting more β-lactamase and PE than 4-hour biofilms. However, 4-hour biofilms stimulated similar IL-8 release from A549 lung epithelia while not causing a significant increase in metabolic activity, as originally hypothesized.

3.5 Growth of biofilm and planktonic bacteria in the presence of LL-37 has time-dependent effects on downstream virulence

The cationic peptide LL-37 is important for epithelial host defense against infecting bacterial pathogens. This peptide and its derivatives have recently been examined as a potential therapeutic for the treatment of infections in CF (25,82,248). With models such as the flow cell method, the high volume of media required limits the analysis of external factors to very low concentrations and not the higher concentrations thought to be present in the local environment. The cationic peptide LL-37 has been studied for its effects on pre-grown biofilms, as well as on growing biofilms, but in very small concentrations (4 μg/mL) (82,149,232). Moreover, the downstream effect of bacterial LL-37 treatment on the host-biofilm interactions has not been thoroughly studied *in vitro*. Here, the Calgary Biofilm Device was used to study the effects of increasing LL-37 concentrations (up to 64 μg/mL) on growing biofilms at concentrations that are proposed to be present in the *in vivo* environment. The peptide is not replenished in the system to determine any downstream effects on bacterial virulence and represent a potential therapeutic scenario. It is important to note that the culture conditions used in the following experiments 64 μg/mL of LL-37 was below the MIC of planktonic bacteria (data not shown).

3.5.1 Growth in the presence of LL-37 decreases biomass, but not growth of biofilm

It has been previously shown using flow cell and static biofilm growth that small concentrations of LL-37 are able to decrease *P. aeruginosa* biofilm biomass. A vital component of biofilm biomass is the extracellular matrix that is important for the protection of the biofilm. Since the Calgary Biofilm Device is a different biofilm formation method than previously studied, I wanted to confirm that growth in the presence of LL-37 decreased biofilm biomass. The biofilms were grown on the Calgary Biofilm Device in the presence of 2 μg/mL, 4 μg/mL, and 16 μg/mL

of LL-37 for 24 hours and then a modified version of the crystal violet assay was used to assess biofilm biomass (3,232). Similar to previous studies (78,232) there was a decrease in biomass in the presence of increasing concentration of LL-37 treatment after the biofilm was allowed to grow for 24 hours, with a significant decrease in biomass after exposure to 4 μg/mL and 16 μg/mL (control – 1.140 OD units; 2 μg/mL – 0.692 OD units, p=0.082; 4 μg/mL – 0.545 OD units, p=0.038; 16 μg/mL – 0.522 OD units, p=0.035) (Figure 27). This indicates that LL-37 may decrease the biomass of *P. aeruginosa* biofilms which may be beneficial *in vivo* to help the host immune system combat the infection.

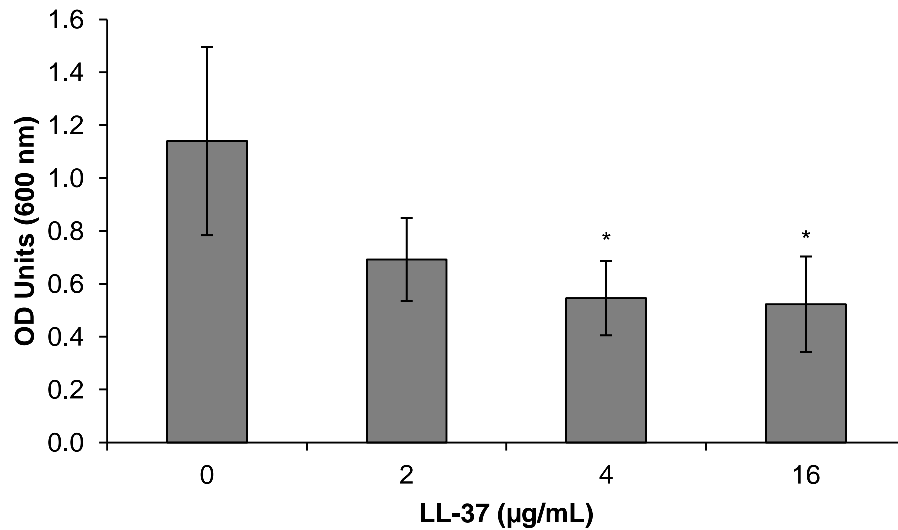


Figure 27. ***P. aeruginosa*** **biofilm biomass was decreased with increasing concentration of LL-37.** Absorbance values following the crystal violet assay on biofilms grown with the Calgary Biofilm Device in the presence of increasing concentrations of LL-37 for 24 hours. Each value is the average of 3 experimental repeats with 4 technical replicates in each experiment. Error bars indicate standard deviation. *Represents a condition statistically different ($P<0.05$) from the sample not containing peptide as determined with a two-tailed unpaired Student's T-test.

Biofilm biomass differs from biofilm bacterial numbers. The biomass of a biofilm includes the extensive extracellular matrix of the biofilm, which is independent of bacterial numbers. The crystal violet assay indicated that LL-37 treatment decreases biofilm biomass; however it isn't known whether the decrease in biomass is due to decreased bacterial numbers or a decrease in extracellular matrix. To quantify the bacterial numbers – or colony forming units (CFU) – within the biofilm a formation curve was obtained for biofilm forming in the presence and absence of LL-37. A formation curve is similar to a growth curve only it measures the rate that bacteria are assembled into a biofilm instead of the growth rate of the bacteria.

Biofilm was grown on the Calgary Biofilm Device in the presence of 1 μg/mL and 16 μg/mL LL-37 for 30 minutes, 1 hour, 1.5 hours, 2 hours, 3 hours, 4 hours, 6 hours, and 24 hours. After each time-point 4 pegs from each treatment were removed and the CFUs were quantified. The results of this experiment showed that the presence of LL-37 (16 μg/mL) seemed to delay the formation of biofilm after treatment causing significantly less biofilm formation for the first 1 hour compared to the control (0.5 hours – 4.60 logCFU/mL vs 5.48 logCFU/mL, $p=0.021$; 1 hour – 4.42 logCFU/mL vs 5.29 logCFU/mL, $p=0.028$) (Figure 28). Time-points after 1 hour showed that there was no statistical difference between the CFU/peg between the untreated biofilm compared to the LL-37-treated biofilm. After the full 24 hour incubation there was no statistical difference between the control and either 1 μg/mL or 16 μg/mL LL-37 exposure (8.28 logCFU/mL; 8.08 logCFU/mL, $p=0.416$; 8.08 logCFU/mL, $p=0.976$).

It is important to note that these are log transformed values. Using the original CFU/mL values results in a 1.6-fold decrease in bacterial numbers compared to the control. The crystal violet assay indicated that with growth in the presence of 16 μg/mL of LL-37 there was a 2.2-fold decrease in biofilm biomass. This confirms that the decrease in biomass with LL-37 treatment

indicated by the crystal violet assay was likely due to the decrease in extracellular matrix in addition to a decrease in bacterial numbers within the biofilm.

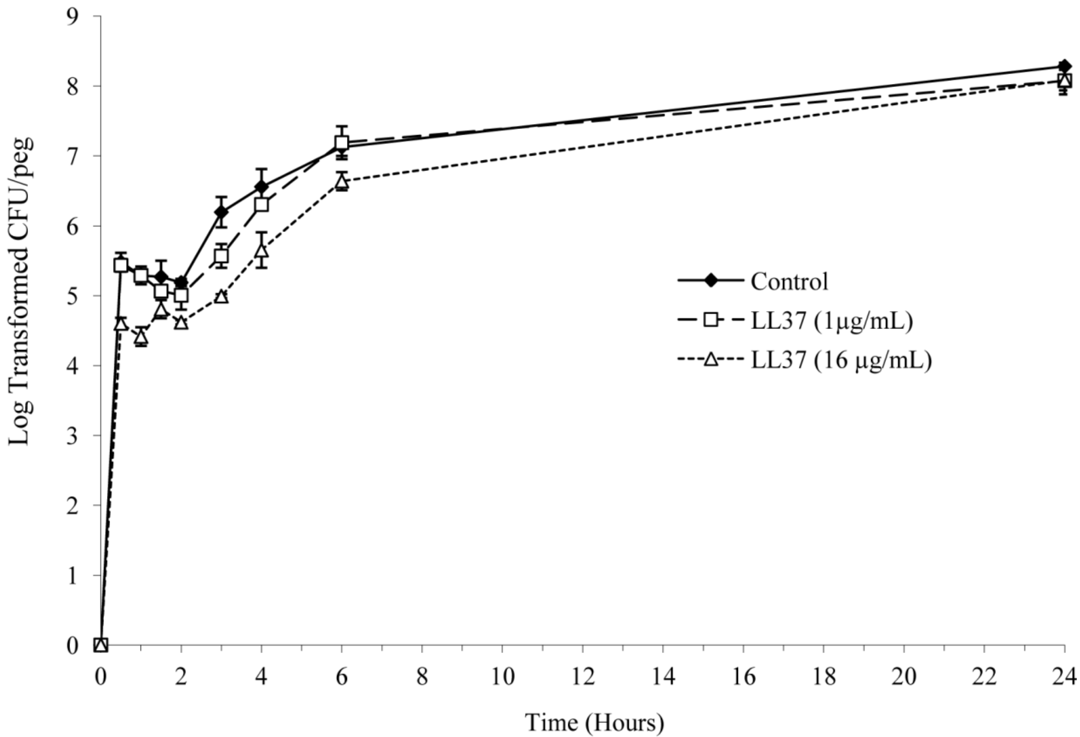


Figure 28. Biofilm formation curve showing a lag in formation in the presence of LL-37 with equivalent bacterial numbers after 24 hours. Log transformed CFU/peg measuring biofilm formation in the presence and absence of LL-37 using the Calgary Biofilm Device. Following each time-point three pegs were removed and the CFUs were enumerated by performing colony counts. Standard error is represented by the error bars. Each condition was run in triplicate. The first two time-points for exposure to 16 μg/ml of LL-37 shows biofilm CFU/peg that are statistically significant ($p<0.05$) from the control as determined by the Student's T-test.

3.5.2 Effect of LL-37 on virulence gene transcription with biofilms differs from 24-hours to 48 hours

There are dozens of genes that have been shown to be important for the formation of *P. aeruginosa* biofilm. These include the motility genes *fimU* and *pilV*, which are required for pilus function. The gene for the quorum sensing enzyme *lasI* has been shown to be required for biofilm maturation. There are also several genes whose importance in biofilm formation and virulence are unknown. These include the two-component regulatory genes *pprA* and *pprB*. The gene *pprB* has been shown to be down-regulated in biofilm, while the sensor gene *pprA* has not been shown to be decreased. This suggests that it may be important for biofilm virulence. The global gene regulator *psrA* was shown to be down-regulated in biofilm (Figure 3), which is interesting since this regulatory protein enhances the expression of *rpoS* as bacteria enter stationary phase (163). Dysregulation of the expression of this regulator could have implications on the differentiation of the biofilm. The expression of antibiotic resistance genes, such as *oprM, mexX, mexA,* and *ampC,* within *P. aeruginosa* biofilms is of interest because of increased drug resistance of *P. aeruginosa* biofilm. Therapeutics that can decrease the expression of these genes without directly killing the bacteria would be of interest to work synergistically with antibiotics to restore the sensitivity of the bacteria. The host defense peptide LL-37 was used to attempt to perturb the gene expression of *P. aeruginosa* biofilms. The downstream effects of these perturbations were then assessed through twitching assays, antibiotic sensitivity and co-culture studies.

Gene expression responses of *P. aeruginosa* biofilm and planktonic bacteria following exposure to 4 μg/mL, 16 μg/mL, and 64 μg/mL of LL-37 for 24 hours and 48 hours of growth was determined for the following genes: *lasI, pprA, pprB, fimU, pilV, oprM, mexX, mexA*, and *ampC*. In most LL-37 studies the biofilm was in constantly exposed to fresh peptide in a flow cell

system. Since the Calgary Biofilm Device is a static system it is expected that the effect of initial LL-37 exposure without replenishment would result in different gene expression changes. Biofilm and planktonic bacteria were grown in the presence of LL-37 for 24 hours and 48 hours. After each time-point the RNA was isolated from the bacteria and converted to cDNA. The expression of each gene was normalized to the gene *rpoD* and verified with *16S rRNA*. The fold change of the gene expression with each concentration of peptide was then compared to the gene expression of the control biofilm and planktonic bacteria grown in the absence of peptide. It has been shown previously that the expression of *rpoD* is regulated between the two growth forms. However, since the fold-change was calculated using similar growth phases (24-hour biofilm vs 24-hour biofilm) it was assumed that the expression of *rpoD* would be unchanged; however it is noted that Overhage *et al.* (232) found that *rpoD* was increased 1.8-fold with constant peptide exposure for 4 days. The dysregulation of *rpoD* in this system was determined by comparing the expression of *rpoD* to *16S rRNA* with increasing peptide exposure.

Following 24 hours of biofilm formation in the presence of 4 µg/mL, 16 µg/mL and 64 µg/mL of LL-37 there was a universal increase in expression of all the genes examined with exposure to 16 µg/mL (*lasI* – 8.07-fold, p=0.008; *pprA* – 9.43-fold, p=0.015; *psrA* – 8.80-fold, p=0.018; *fimU* – 10.24-fold, p=0.018, *pilV* – 10.89, p=0.019, *oprM* – 8.02-fold, p=0.006; *mexX* – 8.64-fold, p=0.016; *mexA* – 8.93-fold, p=0.019; *ampC* – 9.44-fold, p=0.007) and 64 µg/mL (*lasI* – 7.8-fold, p=0.049; *pprA* – 9.00-fold, p=0.054; *psrA* – 8.05-fold, p=0.049; *fimU* – 8.42-fold, p=0.051, *pilV* – 8.45, p=0.056, *oprM* – 8.07-fold, p=0.040; *mexX* – 10.37-fold, p=0.035; *mexA* – 6.37-fold, p=0.021; *ampC* – 8.62-fold, p=0.039) of LL-37, but not 4 µg/mL (Figure 29). Previous studies have reported significant gene expression changes with exposure to 4 µg/mL LL-37; however this was with continual replenishment (232). In a static system it is likely that this low

concentration of peptide is quickly degraded by the bacteria, resulting in minimal effects. The gene expression was highest with exposure to 16 μg/mL of peptide. There was no change in the gene expression of planktonic bacteria in the presence of any of the three peptide concentrations despite bacterial growth being unaffected (data not shown). The gene expression was normalized using *rpoD*. To confirm the universal expression of this gene its expression was compared to *16S rRNA*. The ratio of *rpoD/16S rRNA* did not change with increasing peptide exposure, indicating that *rpoD* was an appropriate normalizing gene.

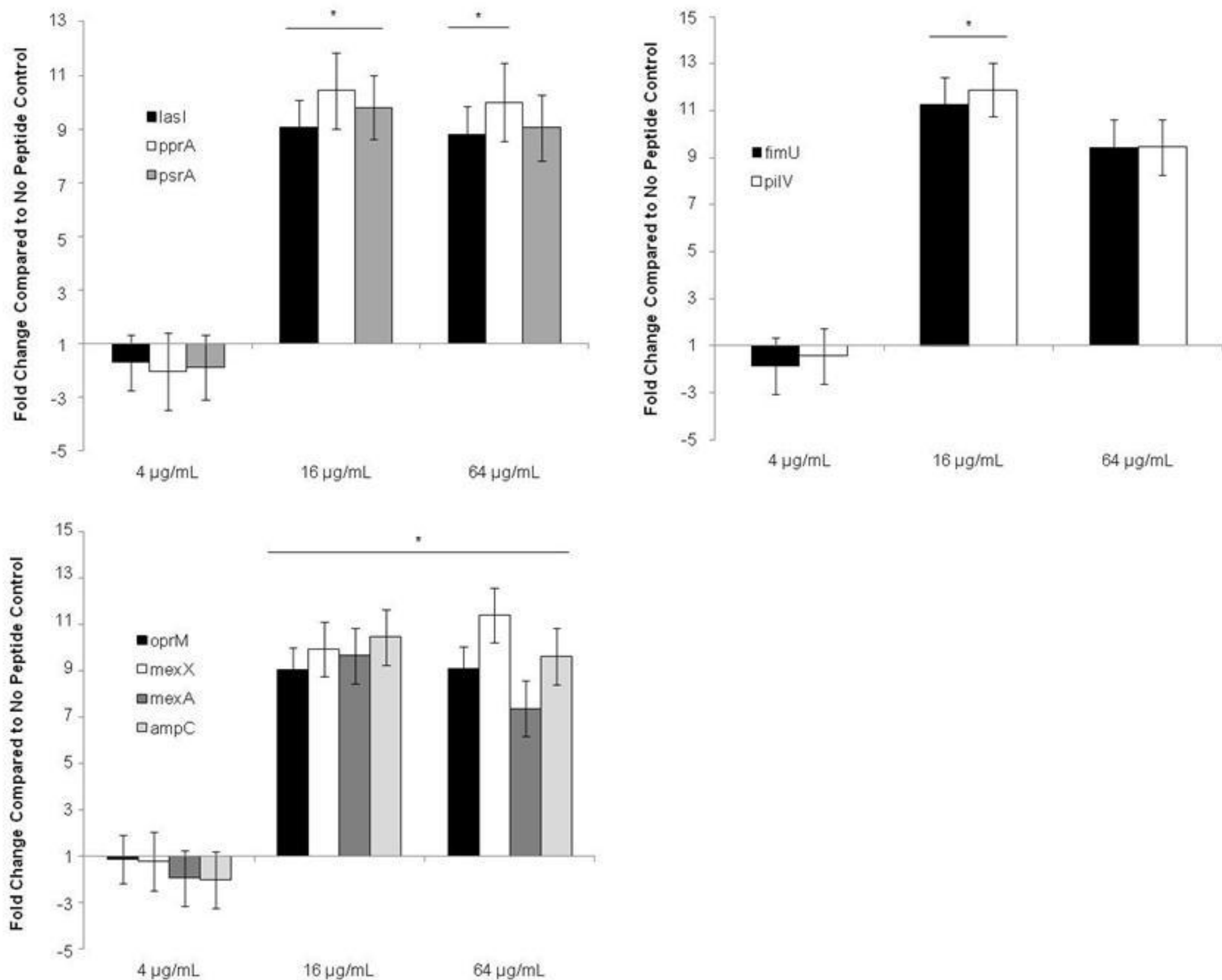


Figure 29. Physiological concentrations of LL-37 stimulated a 10-fold increase in selected gene expression following 24 hours of exposure to biofilms grown on the Calgary Biofilm Device. Gene expression from *P. aeruginosa* biofilm following 24 hours of growth in the presence of 4 µg/mL, 16 µg/mL and 64 µg/mL of LL-37. Data are presented as fold changes compared to a control without any peptide present. Genes are normalized using the *rpoD* gene. Error bars represent the standard error of 3 replicates. * Indicates a condition significantly different than the no peptide control as determined with an unpaired, two-tailed Student's T-test ($p<0.05$).

When the data was examined at 48 hours of exposure to the three concentrations of LL-37 there was differential gene expression in both the planktonic and biofilm bacteria (Figure 30). For planktonic bacteria there was an approximate 3-fold increase (p=0.031) in the expression of *lasI* with exposure to 4 µg/mL of LL-37, but at 16 µg/mL and 64 µg/mL of LL-37 the increase dropped to less than 1.5-fold and was not significant. In biofilms the fold change in *lasI* expression was negligible at all three peptide concentrations. The global regulatory gene *psrA* was decreased approximately 2-fold with 16 µg/mL (p=0.048) and 64 µg/mL (p=0.049) peptide exposure in planktonic bacteria; in biofilms the expression decreased to approximately 4-fold (p=0.024) and 9-fold (p=0.018) with these two peptide concentrations. In planktonic bacteria the change in *pprA* was negligible at all three peptide concentrations; while in biofilm its expression was decreased 9-fold at the two highest concentrations of peptide (p=0.018, p=0.016). The other half of this two-component regulatory system, *pprB* showed the opposite trend: the change in its expression was negligible in biofilm, while in planktonic being down-regulated 2.5-fold (p=0.035) with exposure to 64 µg/mL peptide exposure.

The two motility genes *fimU* and *pilV* showed a similar downward trend with increasing peptide concentration in both planktonic bacteria and biofilm. At the lowest peptide concentration there was a slight but insignificant increase in the expression of these two genes in planktonic bacteria; while there was little change in biofilm. The down-regulation of both of these genes at 16 µg/mL and 64 µg/mL was more pronounced in the biofilm, reaching 3-fold (p=0.028) and 9-fold (p=0.014) for *fimU* and 5-fold (p=0.038) and 7-fold (p=0.024) for *pilV*.

For the antibiotic resistance genes, the significant transport protein *oprM* was unchanged in biofilm and planktonic bacteria. Interestingly, the efflux protein *mexX* was up-regulated in planktonic bacteria with increasing peptide concentration (4 µg/mL – 2.3-fold, p=0.047;

16 μg/mL – 2.3-fold, p=0.043; 16 μg/mL – 4-fold, p=0.030); while being down-regulated with increasing concentration in biofilm (4 μg/mL – 3.05-fold, p=0.049; 16 μg/mL – 11-fold, p=0.006; 64 μg/mL – 21-fold, p<0.001). The efflux gene *mexA* (whose protein associates with OprM to form an efflux pump) was down-regulated in planktonic bacteria with increasing peptide concentration (16 μg/mL – 2.4-fold, p=0.044; 64 μg/mL – 3.7-fold, p=0.039); but it remained unchanged in biofilm. Lastly, the β-lactamase gene *ampC* was unchanged in planktonic bacteria, while being down-regulated approximately 6-fold in biofilm with exposure to 16 μg/mL (p=0.040) and 64 μg/mL (p=0.042) of LL-37.

This data shows that both biofilm and planktonic bacteria respond differently to LL-37 exposure. Moreover, the effects of the previously studied concentration of 4 μg/mL of LL-37 differ greatly from the effects of 16 μg/mL and 64 μg/mL.

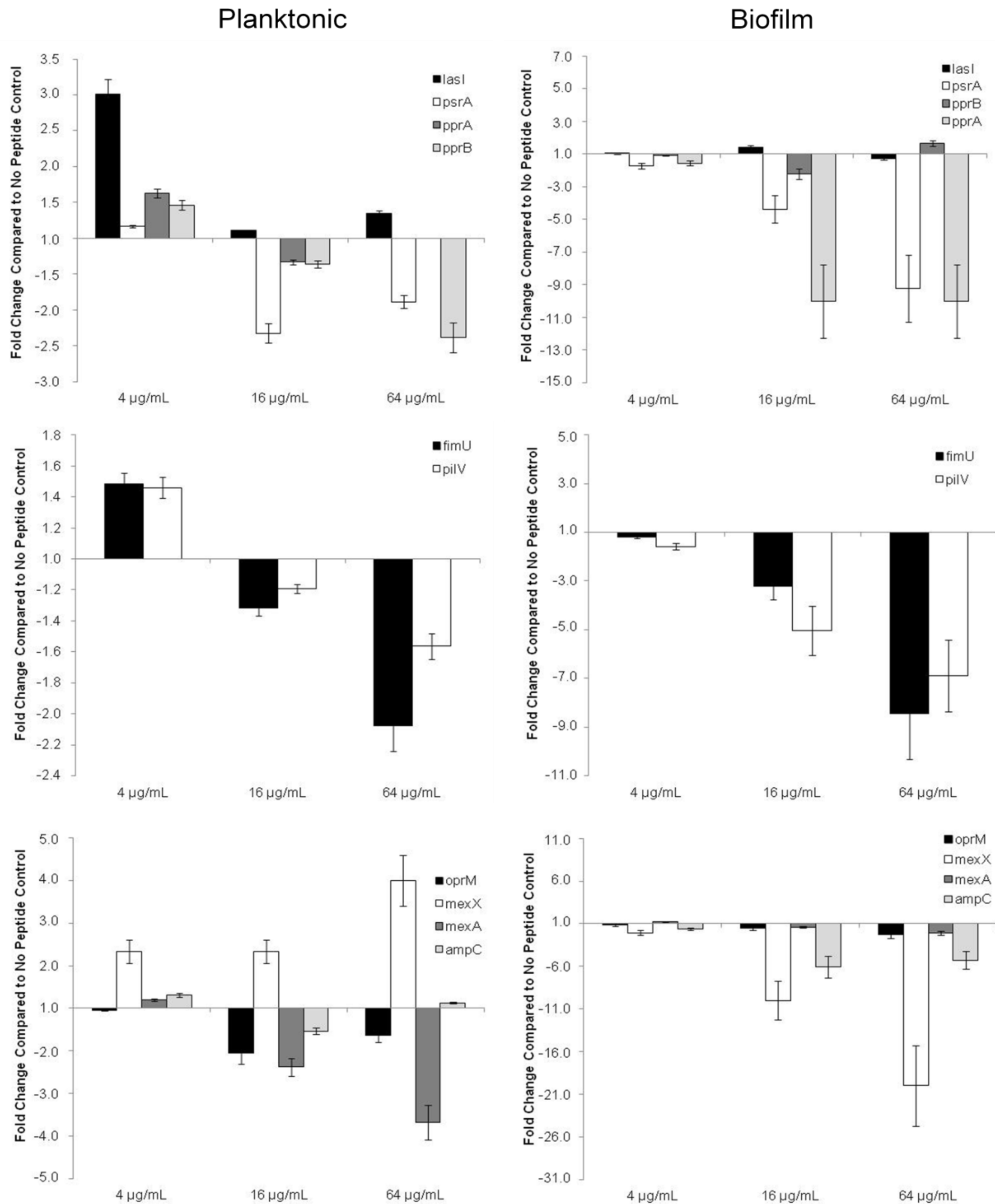


Figure 30. Exposure to physiological concentrations of LL-37 for 48-hours caused a down-regulation of selected gene expression in biofilms. Gene expression of regulatory (top), motility (middle) and antibiotic resistance (bottom) genes from *P. aeruginosa* planktonic (left) and biofilm (right) following 48 hours of growth in the presence of 4 µg/mL, 16 µg/mL and 64 µg/mL of LL-37. Data are presented as fold changes compared to the untreated control. Genes are normalized using the *rpoD* gene. Error bars represent the standard error of 3 replicates. Statistical significance was determined using an unpaired, two-tailed Student’s T-test.

The data described was obtained using the logarithmic growing phase housekeeping gene *rpoD*. After incubation for 48 hours the concentration of *rpoD* was compared to the concentration of *16S rRNA* with increasing peptide concentration. When the expression of *rpoD* was normalized to the concentration of *16S rRNA*, there was a down-regulation of *rpoD* in biofilms with increasing peptide concentration (Figure 31). However this was not the case with planktonic bacteria where the expression of *rpoD* remained constant (data not shown). The gene *rpoD* is expressed by bacteria that are actively metabolizing. Since the expression of *rpoD* decreases with increasing LL-37 concentration it is possible that LL-37 treatment promotes more bacteria within the biofilm to enter the persister phase. This would likely decrease the virulence of biofilms grown in the presence of LL-37 because there would be more bacteria that are dormant rather than expressing virulence genes; however antibiotics are also less effective on bacteria that are in persister phase. Because the expression of *rpoD* changed with peptide concentration, the gene concentrations and fold-changes for biofilm were also determined using *16S rRNA* (Figure 31). The results were an exaggerated down-regulation of gene expression with increasing peptide concentrations (16 µg/mL: *lasI* – 2.7-fold, p=0.045; *pprA* – 41-fold, p<0.001; *pprB* – 9.5-fold, p=0.013; *psrA* – 17.4-fold, p<0.001; *rpoD* – 4.3-fold, p=0.036; *fimU* – 13.7-fold, p=0.001; *pilV* – 21.5-fold, p<.001; *oprM* – 6.8-fold, p=0.035; *mexX* – 42.9-fold, p<0.001; *mexA* – 5.9-fold, p=0.029 *ampC* – 26-fold, p<0.001; 64 µg/mL: *lasI* – 27.3-fold, p<0.001; *pprA* – 400-fold, p<0.001; *pprB* – 10.5-fold, p=0.002; *psrA* – 295-fold, p<0.001; *rpoD* – 17.1-fold, p<0.001; *fimU* – 131-fold, p<0.001; *pilV* – 111-fold, p<.001; *oprM* – 37.9-fold, p<0.001; *mexX* – 342-fold, p<0.001; *mexA* – 37.6-fold, p<0.001; *ampC* – 84.5-fold, p<0.001). This shows that *16S rRNA* is not an ideal normalizing gene because of its abundance in the cell. The change in the *rpoD/16S*

rRNA ratio may indicate that more bacteria in the biofilms exposed to 16 and 64 μg/mL of LL-37 may be entering the persister population resulting in a cessation of gene expression.

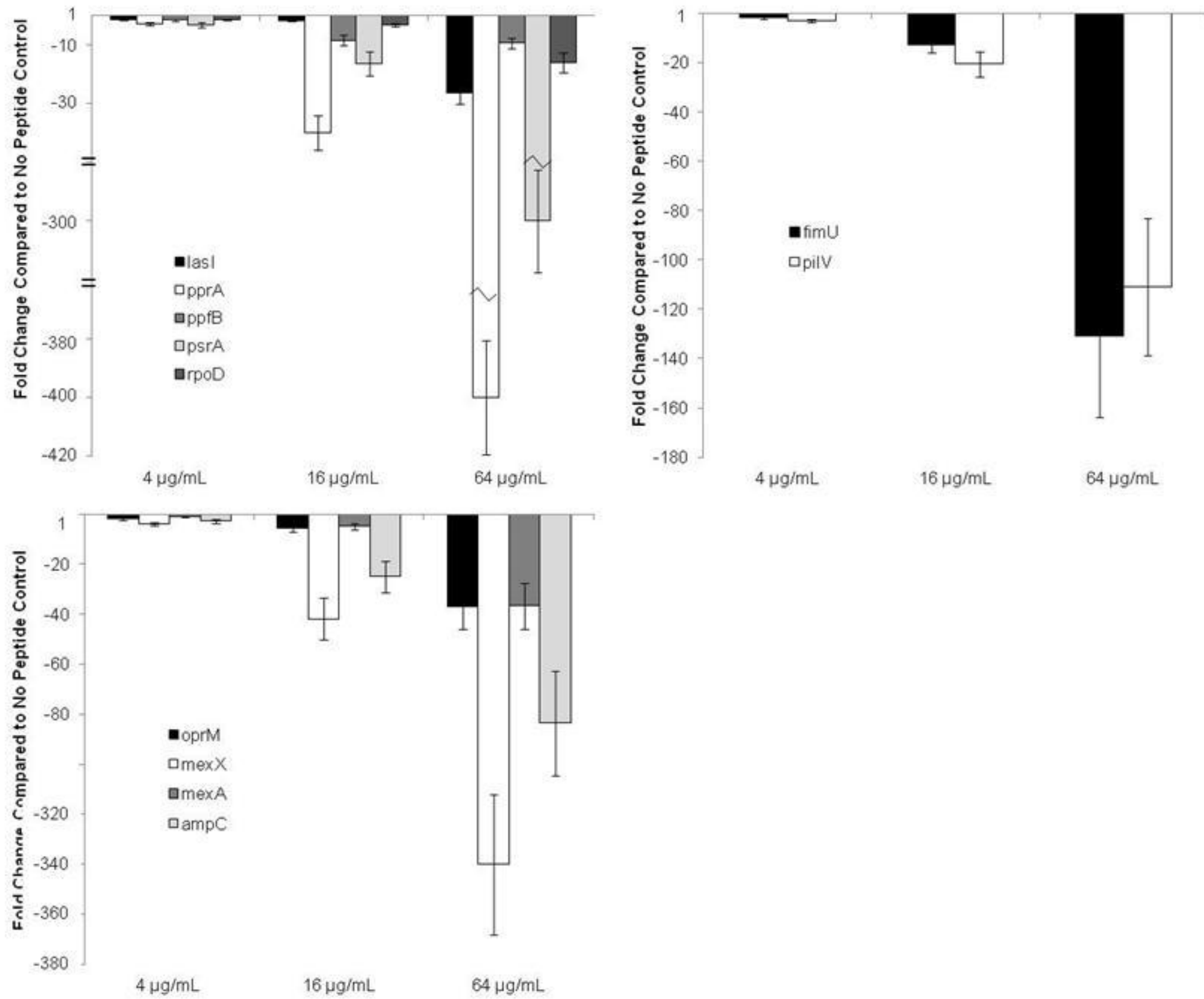


Figure 31. Normalizing the gene expression of biofilms grown in the presence of increasing concentrations of LL-37 for 48 hours to *16S rRNA* results in an increased number of down-regulated genes. Gene expression of regulatory (top left), motility (top right) and antibiotic resistance (bottom) genes from *P. aeruginosa* biofilm following 48 hours of growth in the presence of 4 µg/mL, 16 µg/mL and 64 µg/mL of LL-37. Data are presented as fold changes compared to a control without any peptide present. Genes are normalized using *16S RNA*. Error bars represent the standard error of 3 replicates.

3.5.3 LL-37 pre-treatment has a greater effect on biofilm twitching than on planktonic twitching

Twitching motility has been shown to be very important for the formation of complex biofilm architecture (227). It has been previously shown that inoculating planktonic bacteria into LL-37-containing media causes an increase in bacterial twitching (232); however to my knowledge there have been no studies showing the effect of growing bacteria in the presence of LL-37 and examining the downstream effects on twitching motility. Two conditions of LL-37 treatment were studied: twitching ability in the presence of LL-37 (peptide in the agar); and twitching ability of bacteria grown in the presence of LL-37 (no peptide in the agar). To test the first condition, planktonic bacteria were grown as previously described in the absence of LL-37. Two different twitching plates were prepared, one containing 1% TSA (control), and another with 1% TSA containing 16 µg/mL LL-37. The planktonic bacteria were stab inoculated into both plates. Following 24 hours the twitching zones were measured. The results showed that LL-37 caused an increase in planktonic twitching when the peptide is present in the media compared to control (20 mm vs 14 mm, $p<0.001$) (Figure 32, closed bars). This confirmed the previous findings of Overhage *et al* (232). Next, the planktonic bacteria were grown in the presence or absence of LL-37 (16 µg/mL) for 24 hours and then the peptide was removed for the twitching assay. The control and LL-37 treated planktonic bacteria were then inoculated into plates containing 1% TSA and the twitching zones were measured. There was no significant difference between the control and LL-37-treated planktonic bacteria (14 mm vs 13 mm, $p=0.282$) (Figure 32, open bars). This was expected since the gene expression data showed no difference in motility gene expression with LL-37 treatment for 24 hours. Of course the diameters for the control bacteria are the same between the two experiments since the experimental conditions were the same in both studies.

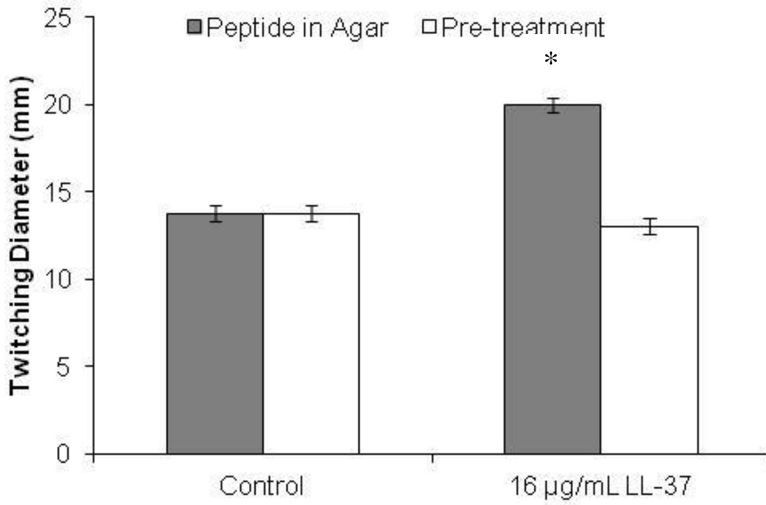


Figure 32. Growing planktonic bacteria in the presence of LL-37 does not alter its downstream twitching ability. Planktonic *P. aeruginosa* were either grown in the presence of absence of LL-37 for 24 hours (pre-treatment, open bars) and then stab inoculated into 1% TSA to determine twitching. Alternatively, planktonic bacteria were inoculated into 1% TSA in the presence of LL-37 (peptide in agar, closed bars) with subsequent twitching determination. Error bars represent standard deviation of 8 replicates and * indicates a significant difference between control and treatment as determined using an unpaired, two-tailed Student's T-test, $p<0.05$

Once a biofilm is formed it is likely that a subset of bacteria maintain the ability to twitch. Moreover, the gene expression studies indicated that 24 hours of exposure to LL-37 stimulated an increase in twitching motility genes. To test the inherent twitching ability of the bacteria within the biofilm a twitching assay was performed. Biofilm and planktonic bacteria were grown in the presence or absence of 16 μg/mL for 24 hours and then rinsed to remove the peptide. The biofilm-containing pegs were then removed and used to stab inoculate the twitching agar. To control for the effects of inoculating planktonic bacteria with a peg versus a loop, the planktonic bacteria were also stab inoculated with a peg and twitching zones were measured. The CFUs were determined to ensure that equal numbers of biofilm and planktonic bacteria were inoculated. There was no significant difference between the twitching ability of the biofilm versus the planktonic bacteria (23 mm vs 26 mm, p=0.067) (Figure 33). It was found that the biofilm grown in the presence of LL-37 increased their twitching zone over the control (25 mm vs 23 mm, p=0.01). This could be due to the up-regulation of the *pilV* gene as seen in Figure 29. This gene encodes a protein that is required for pili assembly and subsequent twitching (4). Contrary to the loop stab inoculation, peg stab inoculation showed that planktonic bacteria grown in the presence of LL-37 were less able to twitch than the control (23 mm vs 26 mm, p=0.02), indicating that the peg inoculation may not be appropriate for determining twitching ability. In summary, pre-treatment with LL-37 caused potential opposing effects in biofilm and planktonic bacteria. Biofilm pre-treated with LL-37 may have a slight increase in their ability to twitch. As mentioned, this may result in biofilms that lose architecture and may result in more bacteria entering the persister phase. If this is the case, it would likely result in a decrease in overall virulence of the biofilm; but could also decrease the effectiveness of antibiotics that target metabolizing bacteria.

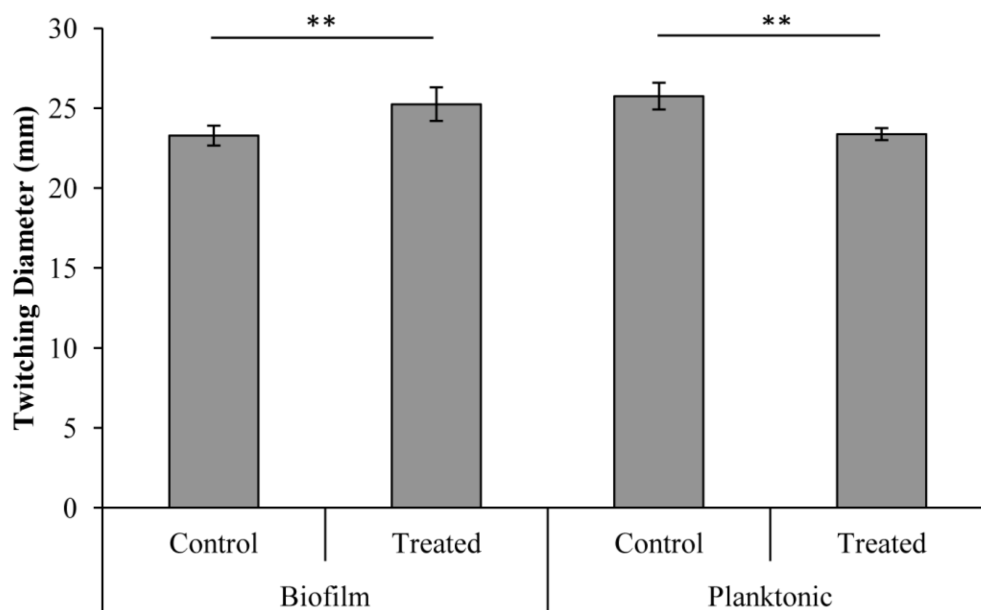


Figure 33. Peg inoculation of twitching media showed opposing effects of LL-37 exposure between biofilm and planktonic bacteria. Biofilm and planktonic bacteria were grown in the presence (16 µg/mL) or absence of LL-37 for 24 hours and then stab inoculated into 1% TSA using a peg from the Calgary Biofilm Device. Twitching zones were then measured. Error bars represent standard deviation of 8 replicates and ** indicates a significant difference using an unpaired, two-tailed Student's T-test, $p<0.05$.

3.5.4 Biofilm and planktonic bacteria grown in the presence of LL-37 have a greater survival when exposed to ciprofloxacin

The results from the gene expression studies revealed that LL-37 causes a dysregulation of antibiotic resistance genes (Section 3.5.2). There was little change in planktonic gene expression following 24 hours of exposure and a universal increase in gene expression in biofilms following exposure to 16 µg/mL and 64 µg/mL LL-37. To determine if the changes in gene expression are reflected in phenotypic changes in antibiotic resistance an antibiotic challenge assay was performed. Planktonic bacteria were grown in the presence or absence of 16 µg/mL LL-37 and biofilm was grown on the Calgary Biofilm Device in the presence or absence of LL-37 for 24 hours. The biofilm and planktonic bacteria were washed and exposed to increasing concentrations of gentamicin, ceftazidime and ciprofloxacin and the MIC, MBC and MBEC was determined as previously described. Of course an MBEC was not determined for the planktonic bacteria. This experiment was repeated 4 times with 2 replicates in each trial. The data indicated that growth in the presence of LL-37 did not alter the susceptibility of biofilm or planktonic bacteria to gentamicin or ceftazidime (Table 8). The bacteria released from the LL-37-treated biofilm were also not any more susceptible to ciprofloxacin; however the MBEC for LL-37-treated biofilm was significantly higher compared to the untreated control biofilm when using an α-value of 0.10 (32-64 µg/mL vs 16-64 µg/mL, $z=1.828$). The MIC and MBC of ciprofloxacin for planktonic bacteria grown in the presence of LL-37 did not change compared to the untreated control. This indicates that growing biofilm in the presence of LL-37 for 24 hours results in more resistance to ciprofloxacin, agreeing with the gene expression data; however planktonic bacteria grown in the presence of LL-37 showed similar survival when exposed to ciprofloxacin.

Table 8. Biofilms grown in the presence of LL-37 are more resistant to ciprofloxacin treatment. Summary of antibiotic challenge assay. Data are given as the range of 12 replicates (median if applicable). Z-scores were calculated with a two-tailed Mann-Whitney rank test. Significance is determined if the absolute value of the z-score is greater than 1.96 ($p<0.05$) or 1.645 ($P<0.10$).

	Gentamicin			**Ceftazidime**			**Ciprofloxacin**		
	MIC	**MBC**	**MBEC**	**MIC**	**MBC**	**MBEC**	**MIC**	**MBC**	**MBEC**
Untreated Biofilm	4-16 (8)	32-256 (64)	256-512 (256)	>2048	>2048	>2048	2-4 (4)	0.25-4 (2)	16-64 (32)
LL-37-treated Biofilm	4-8 (8)	32-128 (64)	256-512 (256)	>2048	>2048	>2048	2-4 (4)	0.25-4 (2)	32-64 (64)
z-score	-0.528	-0.528	0	0	0	0	0	0.692	**1.828**
Untreated Planktonic	2-8 (4)	8-32 (16)		4-32 (16)	64-512 (128)		0.25	0.25-2 (0.25)	
LL-37-treated Planktonic	2-8 (4)	8-32 (32)		4-16 (16)	64-256 (128)		0.25	0.25	
z-score	0.551	0.643		-0.605	-0.752		0	-1.039	

3.5.5 Planktonic bacteria grown in the presence of LL-37 no longer stimulate A549 cell rounding while biofilm grown in the presence of LL-37 are internalized by A549 cells

It is clear from the gene expression data that growing planktonic and biofilm in the presence of LL-37 affects downstream gene expression. There was little change in planktonic gene expression following 24 hours of exposure and a 10-fold increase in selected gene expression in biofilms following exposure to 16 µg/mL and 64 µg/mL LL-37. To determine if growth in the presence of LL-37 alters the interaction with lung epithelia, biofilm and planktonic bacteria were grown in the presence of 16 µg/mL LL-37 for 24 hours and then co-cultured with A549 cells at an MOI of 100 for 1 hour and examined microscopically for morphological changes. As shown in Figure 34 there was no noticeable difference in the A549 morphology following exposure to LL-37-treated biofilm. However growing planktonic bacteria in the presence of LL-37 had a marked effect. Following 1 hour of incubation the planktonic bacteria grown in the presence of LL-37 were unable to cause the previously noted rounding of A549 cells as compared to the control planktonic bacteria. It is possible that exposure to LL-37 decreased the expression of the TTSS in planktonic bacteria. Unfortunately these genes were not included in the gene expression panel.

The data above demonstrated that planktonic bacteria caused cytoskeletal dysregulation of A549 cells (Figure 6). Since LL-37-treated planktonic bacteria were unable to cause the same cell rounding of A549 cells, immunofluorescence studies were done to demonstrate that LL-37-treatment also decreased cytoskeletal dysregulation of A549 cells. Following a 1 hour co-culture of untreated and treated biofilm and planktonic bacteria with A549 epithelia at an MOI of 100 the cells were then stained for DNA, *P. aeruginosa* and Actin. Figure 35 shows that there was less cytoskeletal dysregulation following exposure to LL-37-treated planktonic bacteria

compared to untreated; however some breakdown of Actin filaments was occurring due to the decrease in phalloidin staining. There was no noticeable difference between the Actin staining of biofilm grown in the presence or absence of LL-37. This indicates that planktonic bacteria grown in the presence of LL-37 for 24 hours may have less expression of the TTSS as compared to bacteria grown in the absence of LL-37.

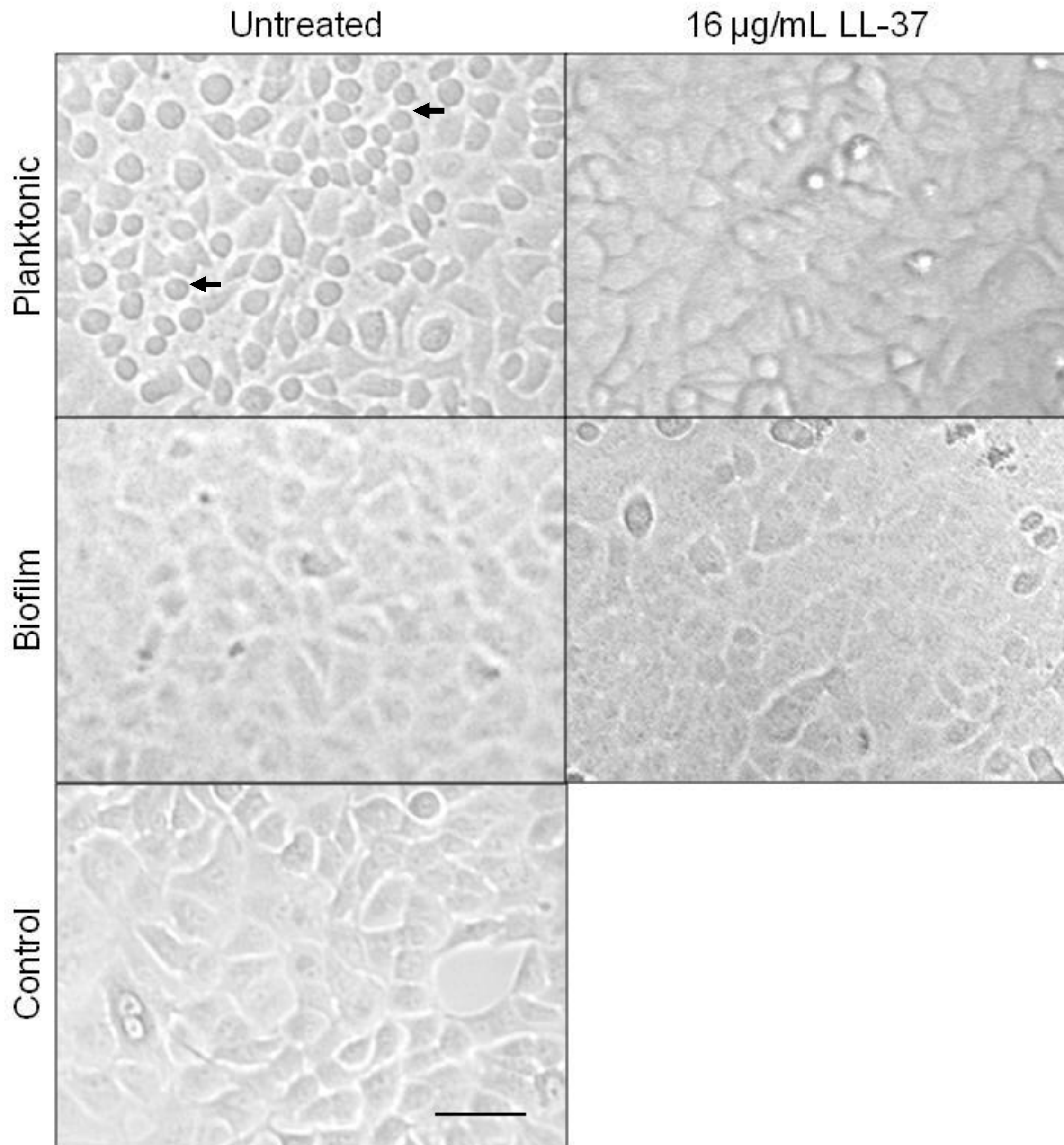


Figure 34. Growing planktonic bacteria in the presence of LL-37 decreases their ability to stimulate A549 cell rounding. A549 cells were exposed to cell culture media (bottom), planktonic bacteria (top), or biofilm (middle) at an MOI of 100 for 1 hour and then viewed under phase microscopy to detect any morphological changes. The bacteria were grown in either TSB (left panel, untreated) or TSB with 16 µg/mL of LL-37 for 24 hours (right panel). Growth in the presence of LL-37 minimal impact on biofilm interactions; however the planktonic bacteria grown in the presence of LL-37 showed significantly less ability to stimulate A549 cell rounding as compared to the untreated control (arrows). Experiment was performed in the 96-well format. Selected images are representations of four replicates. Size bar represents 100 µm.

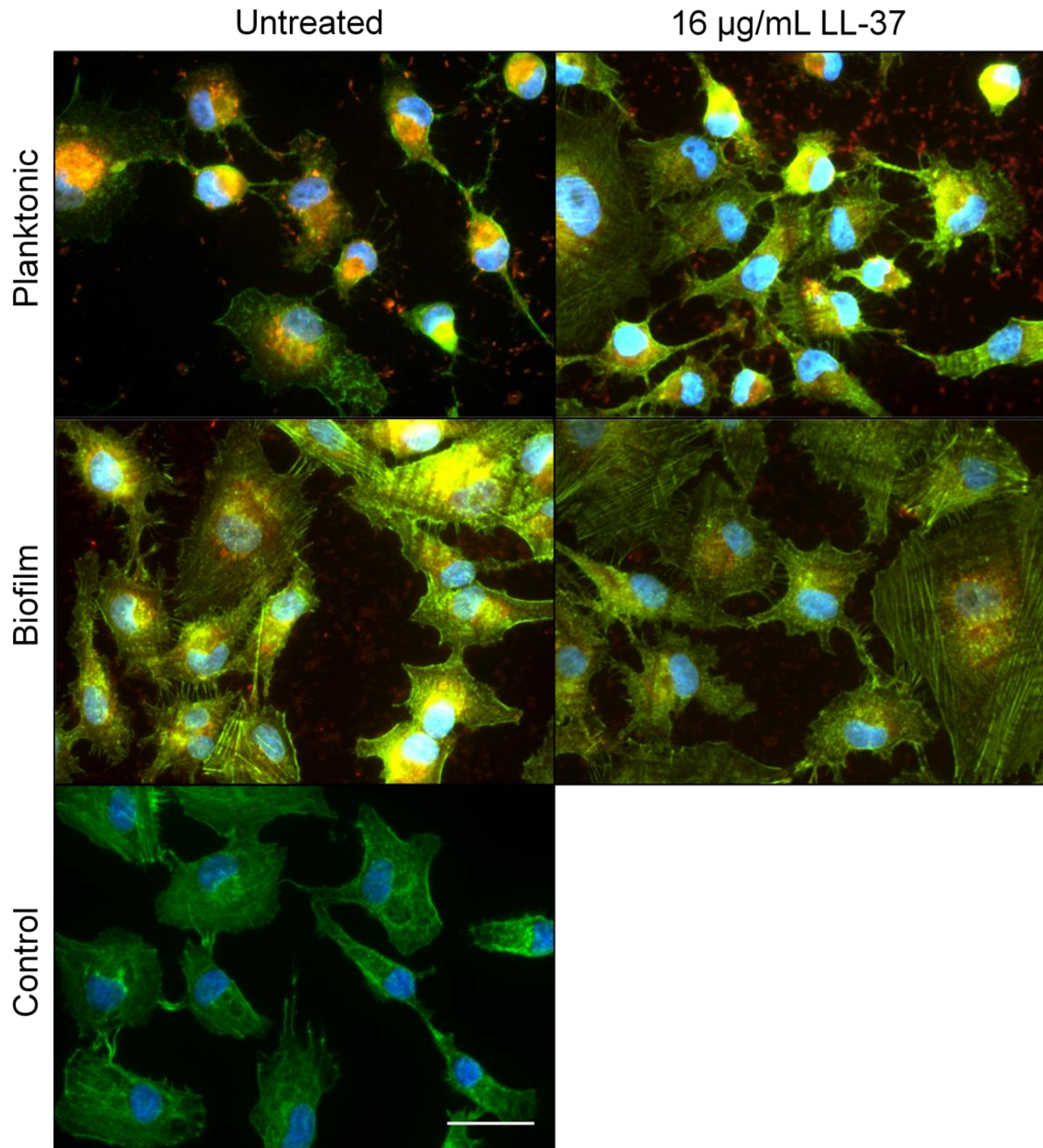


Figure 35. Growth of planktonic bacteria in the presence of LL-37 reduces subsequent A549 cytoskeletal dysregulation. A549 cells were co-cultured with cell media (bottom) in comparison to planktonic bacteria (top), or biofilm (middle) at an MOI of 100 for 1 hour. Following the incubation the cells were washed and fixed with 3% PFA and permeabilized with 0.1% Triton-X-100. After blocking with 1% BSA the cells were stained with phalloidin (green), anti-*Pseudomonas* (red) and DAPI (blue). Slides were imaged using an Olympus BX41 and Retiga 2000R camera. The A549 cells exposed to planktonic bacteria had significant cytoskeletal dysregulation as shown by the rounding and decrease in phalloidin staining of actin filaments; however growing planktonic bacteria in the presence of LL-37 decreased the ability to cause cytoskeletal dysregulation as shown by the stronger actin staining. Selected images are representations of four replicates. Size bar represents 100 μm.

It was possible that the planktonic bacteria grown in the presence of LL-37 were less likely to cause cell rounding due to decreased adherence of the bacteria to the A549 cells. Biofilm and planktonic bacteria were grown in the presence or absence of LL-37 for 24 hours and then co-cultured with A549 cells at an MOI of 100 for 1 hour. The A549 cells were then washed and the CFU of adhered bacteria were determined through spot plate analysis. The percent adherence was calculated by dividing the CFU of the recovered (adhered) bacteria by the CFU in the original inoculum. The results indicate that growing biofilm or planktonic bacteria in the presence of LL-37 does not decrease their ability to adhere to A549 cells compared to the untreated bacteria (planktonic – 11.0% vs 13.1%, p=0.681; biofilm – 11.8% vs 11.2%, p=0.868) (Figure 36A). This indicates that the decreased ability of LL-37-treated planktonic bacteria to cause cell rounding and cytoskeletal dysregulation is due to a decrease in bacterial effects on A549 cell rounding independent of adherence.

Planktonic bacteria are internalized more than biofilm (Figure 7), which may be an important virulence mechanism for either growth form. Since LL-37 treatment decreases the ability of planktonic bacteria to cause A549 cell rounding it was thought that LL-37 treatment may also decrease the internalization of planktonic bacteria by A549 cells. Biofilm and planktonic bacteria were grown in the presence or absence of LL-37 and then co-cultured with A549 cells at an MOI of 100 for 1 hour. Following the co-culture the A549 cells were treated with gentamicin to kill external bacteria and the internalized bacteria were enumerated by spot plate analysis. The percent internalization was calculated by dividing the CFU of the recovered (internal) bacteria by the CFU in the original inoculum. Unlike in the adherence study, there was a significant decrease in the internalization of planktonic bacteria grown in the presence of LL-37 compared to the untreated (0.11% vs 0.19%, p=0.007) (Figure 36B). There was no significant difference in the

internalization of biofilm bacteria grown in the presence of LL-37 compared to control (0.002% vs 0.004%, p=0.092). Because there was less internalization of planktonic bacteria grown in the presence of LL-37, this could indicate that the binding of the bacteria to the A549 cells is altered.

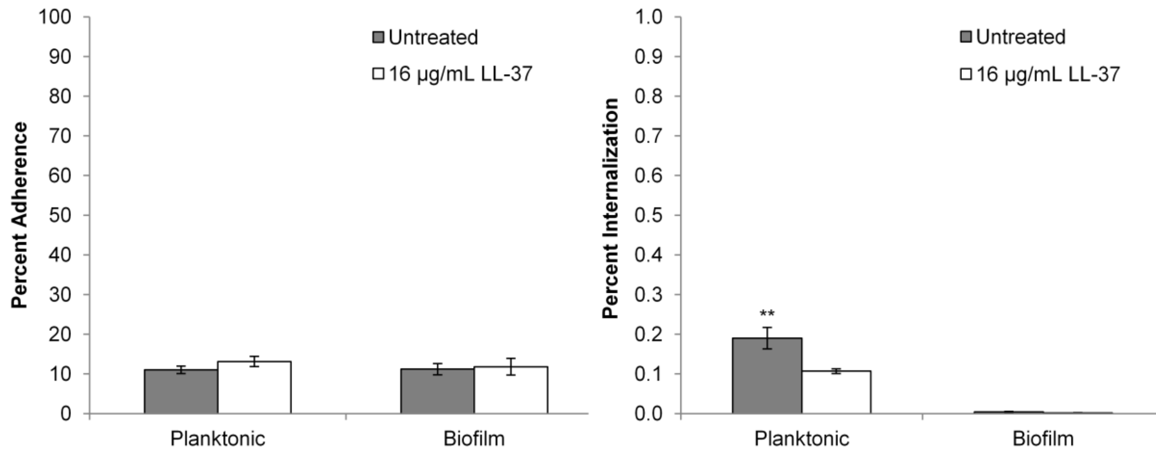


Figure 36. Planktonic bacteria grown in the presence of LL-37 undergo less internalization by A549 cells compared to the untreated control. A549 cells were infected with biofilm and planktonic bacteria at an MOI of 100 for 1 hour. The bacteria were grown in the presence (16 μg/mL) or absence of LL-37 for 24 hours. Following the incubation the media was removed and the A549 cells were rinsed three times with PBS. A) Adherence of bacteria was determined by treating the A549 cells with PBS-T and resuspended with a pipette. The media was then diluted and spot plated. Percent adherence was calculated by dividing the number of recovered bacteria from the number of inoculating bacteria and multiplying by 100. B) Internalization of the bacteria was determined by treating the A549 cells with 200 μg/mL gentamicin for 1 hour. The A549 cells were rinsed three times with PBS then lysed with 1% Triton X 100 for 1 hour. The media was then diluted and spot plated to enumerate the recovered bacteria. Percent internalization was calculated by dividing the number of recovered bacteria by the number of inoculating bacteria and multiplying by 100. Results are from 20 replicates and error bars represent standard deviation. ** indicates a significant difference ($P<0.01$) between the treated and untreated bacteria as determined with an unpaired, two-tailed Student's T-test.

3.5.6 Growth of planktonic bacteria in the presence of LL-37 decreases the ability to cause cell death of A549 cells.

Growing bacteria in the presence of LL-37 clearly has downstream effects on bacterial virulence. There was a time-dependent alteration of selected gene expression and an increase in biofilm twitching with growth in the presence of LL-37 for 24 hours. Moreover, growth of planktonic bacteria in the presence of LL-37 decreased the bacteria's ability to stimulate A549 cell rounding and cytoskeletal dysregulation. Growth of biofilm in the presence of LL-37 decreased the internalization of planktonic bacteria by A549 cells. It is possible that the decreased cytoskeletal dysregulation following planktonic exposure was due to a decreased TTSS in LL-37-exposed planktonic bacteria. The TTSS is also known to mediate A549 cell death following bacterial exposure. A flow cytometric analysis was performed to determine the effects of bacterial LL-37 treatment on downstream cell death or apoptosis of A549 cells. Biofilm and planktonic bacteria were grown in the presence or absence of LL-37 for 24 hours and then co-cultured with A549 cells at an MOI of 100 for 4 hours. Following the incubation the A549 cells were treated with the viability marker Calcein AM and the apoptosis marker Annexin V (binds to translocated phosphatidylserine) and analyzed as previously described. The data showed that the A549 cells exposed to biofilm that had been grown in the presence of LL-37 were undergoing similar rates of cell death and apoptosis as compared to biofilm grown in the absence of peptide (cell death – 25.7% vs 23.2%, p=0.330; apoptosis – 19.1% vs 15.6%, p=0.237) (Figure 37). As expected from the phase contrast data, planktonic bacteria grown in the presence of LL-37 caused less cell death than bacteria that were grown in the absence of peptide (31.9% vs 50.8%, p=0.008) (Figure 37A). There was no statistical difference in the rates of apoptosis between treated and untreated planktonic bacteria (13.2% vs 10.4%, p=0.489) (Figure 37B).

Since planktonic bacteria grown in the presence of LL-37 are causing less cell death than the control planktonic bacteria it is likely that the metabolism of the A549 cells exposed to the LL-37 treated bacteria is also higher. To test this hypothesis biofilm and planktonic bacteria were grown in the presence of LL-37 for 24 hours and co-cultured with A549 cells at an MOI of 100 for 4 hours. Following the incubation the metabolism of the A549 cells was analyzed using the WST-1 assay by measuring the activity of the metabolic enzyme mitochondrial dehydrogenase that is commonly used as a marker of cell viability. As expected, LL-37 treatment of planktonic bacteria resulted in a significantly higher metabolism of the A549 cells compared to the untreated planktonic bacteria (1.527 OD units vs 0.516 OD units, $p<0.001$) (Figure 37C). There was also a significant difference between LL-37 treated and untreated biofilm (1.902 OD units vs 2.229 OD units, $p=0.006$).

From this it can be determined that LL-37 treatment of planktonic bacteria results in decreased virulence as shown with decreased A549 cell death. This decrease in virulence may be attributed to a decrease in the TTSS. It is still unclear whether LL-37 treatment of biofilm affects the downstream virulence of biofilm with A549 cells since cell death and apoptosis were unaltered even though treating biofilm with LL-37 caused less of an increase in A549 metabolism.

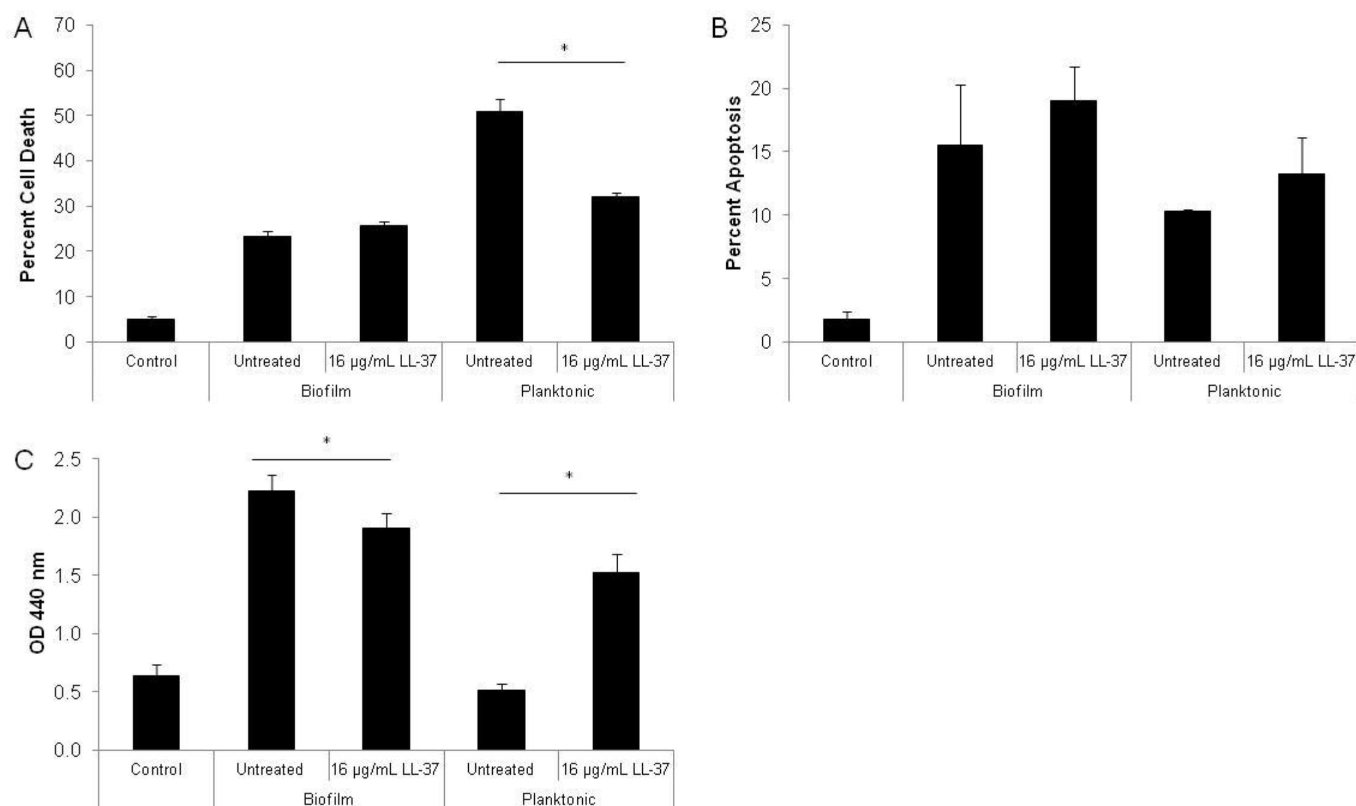


Figure 37. Growing planktonic bacteria in the presence of LL-37 decreases virulence by decreasing A549 cells death. A549 cells were co-cultured with LL-37-treated or untreated biofilm and planktonic bacteria at an MOI of 100 for 4 hours. The supernatant was retained to include detached A549 cells and the adhered cells were treated with trypsin and added to the supernatant portion. The cells were washed and then treated with binding solution containing Calcein AM (live cell indicator) and Annexin V-Alexa Fluor®-647 (apoptosis indicator). The cells were analyzed using the Agilent Cell Chip using the Apoptosis Fast Protocol and were assessed for their a) apoptosis, and b) cell death, n=4. c) Metabolic activity through conversion of WST-1 in A549 cells following a 1 hour incubation with treated and untreated biofilm or planktonic bacteria at an MOI of 100, n=12. Error bars represent standard error. * Indicates a significant difference between treated and untreated as determined with an unpaired, two-tailed Student's T-test, $p<0.05$.

3.5.7 LL-37 pre-treatment of bacteria does not affect IL-8 secretion by A549 cells.

Stimulation of inflammation through IL-8 release is a major virulence mechanism of *P. aeruginosa* biofilms. Since growth of biofilm in the presence of LL-37 for 24 hours caused a significant up-regulation of selected gene expression (Figure 29), it was predicted that stimulation of IL-8 release from A549 cells would also be increased. Biofilm and planktonic bacteria were grown in the presence or absence of LL-37 for 24 hours and then co-cultured with A549 cells at an MOI of 100 for 4 hours, 7 hours and 24 hours. At each time-point the supernatant was removed and IL-8 levels were determined using a sandwich-based IL-8 ELISA. The IL-8 ELISA showed that pretreatment of biofilm and planktonic bacteria with LL-37 did not cause a significant change in their ability to stimulate IL-8 release from A549 cells compared to the untreated bacteria (biofilm: 4 hours – 1007.75 pg/mL vs 923.32 pg/mL, p=0.219; 7 hours – 2039.12 pg/mL vs 1735.78 pg/mL, p=0.196; 24 hours – 415.99 pg/mL vs 193.10 pg/mL, p=0.115; planktonic: 4 hours – 41.79 pg/mL vs 148.424 pg/mL, p=0.005; 7 hours – 0 pg/mL vs 76.35 pg/mL, p=0.074; 24 hours – no IL-8 detected) (Figure 38). This indicates that the increased virulence gene expression with LL-37 treatment does not affect the ability of biofilm or planktonic bacteria to activate A549 cells to produce IL-8.

Biofilm and planktonic bacteria secrete factors that stimulate A549 cells to release IL-8. The CM of biofilm and planktonic bacteria grown in the presence or absence of LL-37 was exposed to A549 cells to determine if LL-37 treatment affected the secretion of factors capable of stimulating IL-8 release. Biofilm and planktonic bacteria were grown in the presence or absence of LL-37 for 24 hours and then CM from treated and untreated biofilm and planktonic bacteria was obtained as previously described. A549 cells were then exposed to the CM for 4 hours, 7

hours, and 24 hours and IL-8 levels in the supernatant were examined. Interestingly, the CM from biofilm grown in the presence of LL-37 for 24 hours caused a significant increase in IL-8 secretion from A549 cells at the 7 hour and 24 hour time-points as compared to the untreated control (4 hours – 165.49 pg/mL vs 98.12 pg/mL, p=0.236; 7 hours – 440.43 pg/mL vs 344.86 pg/mL, p=0.018; 24 hours – 2188.91 pg/mL vs 1647.59 pg/mL, p=0.007) (Figure 39); however in contrast to my previous findings there was no difference between treated and untreated planktonic bacteria (4 hours – 960.76 pg/mL vs 1072.46 pg/mL, p=0.125; 7.5 hours – 1811.95 pg/mL vs 1913.75 pg/mL p=0.441; 24 hours – 4713.28 pg/mL vs 4819.00 pg/mL, p=0.592). This indicates the LL-37 treatment has an effect on planktonic virulence when the bacteria are able to contact the A549 cells; while LL-37 treatment seems to affect the secreted virulence factors of biofilm.

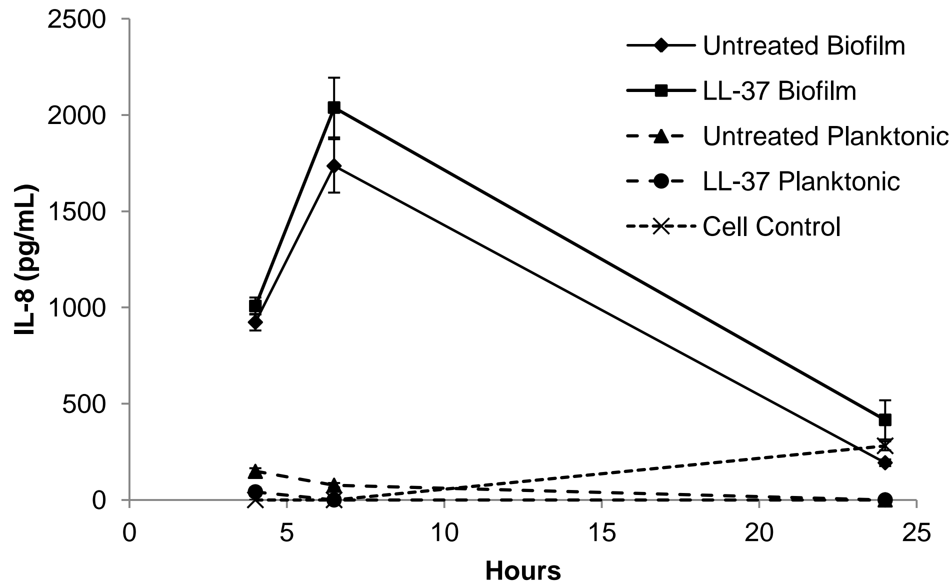


Figure 38. Growth of bacteria in the presence of LL-37 does not affect subsequent IL-8 secretion from A549 cells. A549 cells were exposed to LL-37-treated and untreated biofilm and planktonic *P. aeruginosa* at an MOI of 100 for 4 hours, 7 hours and 24 hours and IL-8 secretion was measured by ELISA. Planktonic bacteria stimulated A549 cell rounding and death at all time-points. After 24 hours of co-culture all A549 cells were dead and lysed. Error bars indicate standard deviation of four biological repeats. Statistical differences were determined using an unpaired, two-tailed Student's T-test

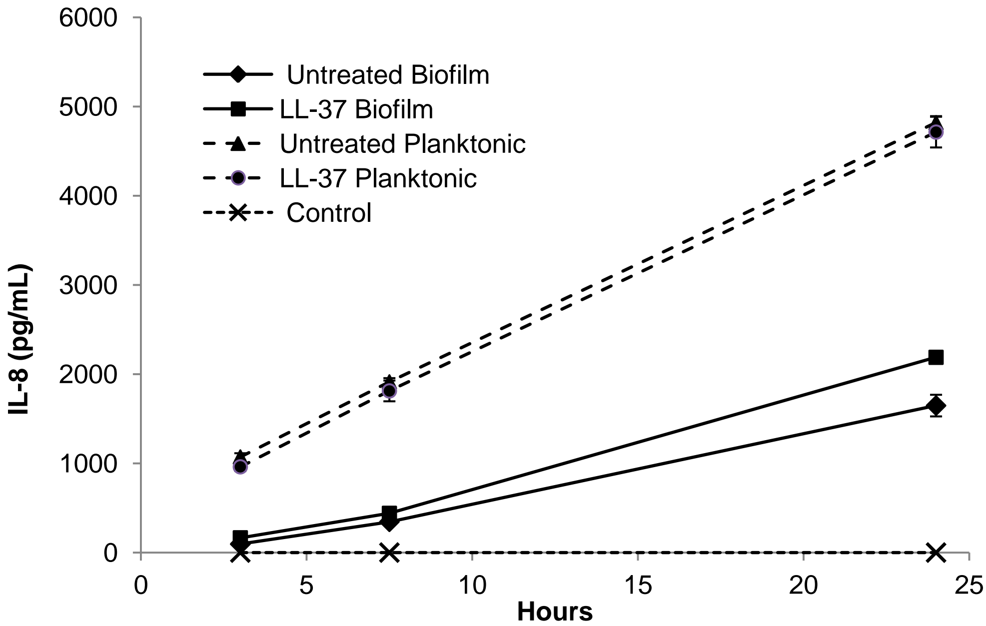


Figure 39. Growth of biofilm in the presence of LL-37 caused an increase stimulation of IL-8 release from A549 cells after 7 and 24 hours. A549 cells were incubated with CM from LL-37-treated and untreated biofilm, planktonic bacteria and control for 24 hours with supernatant collected at 4 hours, 7 hours and 24 hours and IL-8 secretion was measured by ELISA. Error bars indicate standard deviation of four biological repeats. Statistical differences were determined using an unpaired, two-tailed Student's T-test.

3.5.8 LL-37 exposure for 48 hours decreased elastase production from biofilms.

CM from biofilm treated with LL-37 for 24 hours caused a significant increase in IL-8 secretion. This agreed with the previous findings that LL-37 caused a time-dependent regulation of *lasI* gene expression and prompted the investigation into the elastase production following 24 and 48 hours of exposure. A549 cells were exposed to CM from biofilms grown in the presence or absence of 16 μg/mL LL-37 for 24 and 48 hours and the time required for morphological changes to occur was recorded. It was found that CM from biofilm grown in the presence of LL-37 for 24 hours caused morphological changes in 18 hours, only 2 hours faster than the control (20 hours) (Table 9); surprisingly growth in the presence of LL-37 for 48 hours decreased the ability to cause changes, taking 32 hours for changes to occur. This suggests that LL-37 treatment for 24 hours caused a slight increase in elastase secretion from biofilm; while LL-37 treatment for 48 hours caused a decrease in elastase secretion. This disagrees with the gene expression data that showed an increase in *lasI* expression after 24 hours of LL-37 exposure; however there was little change in *lasI* expression after 48 hours of exposure.

Table 9. Growth of biofilm in the presence of LL-37 for 48 hours slows the morphological changes in A549 cells. The time required for morphological changes to occur in A549 cells following incubation with CM from biofilm grown in the presence of 16 μg/mL LL-37 for 24 and 48 hours as determined through phase contrast microscopy.

LL-37 Treatment Time	**Morphological Changes**
Control	20 hours
24 hours	18 hours
48 hours	32 hours

To determine if the rate of morphological changes correlates with amount of elastase the CM was isolated from biofilm grown in the presence or absence of LL-37 for 24 hours and 48 hours and elastase activity was measured with the substrate Elastin Congo Red as previously described. There was a significant decrease between the elastase produced by biofilms grown in the

presence of LL-37 for 48 hours compared to 24 hours (0.003 OD units vs 0.026 OD units, $p<0.001$) (Figure 40). There was a slight increase in elastase production by biofilms grown in the presence of LL-37 for 24 hours compared to the untreated biofilm control; however this difference was not significant (0.026 OD units vs 0.019, $p=0.134$). This was not surprising since the morphological changes only occurred 2 hours earlier with exposure to CM from 24-hour-LL-37-treated biofilm. The fact that there was a significant decrease in elastase activity in the CM from 48-hour-LL-37-treated biofilm confirms the longer incubation time required for morphological change to occur. Since the expression of *lasI* was shown to be unaffected by LL-37 exposure after 48 hours, the decrease in PE secretion indicates that the *lasB* gene is regulated by a separate system, or that LL-37 is able to decrease the secretion of PE through the type 2 secretion system (T2SS).

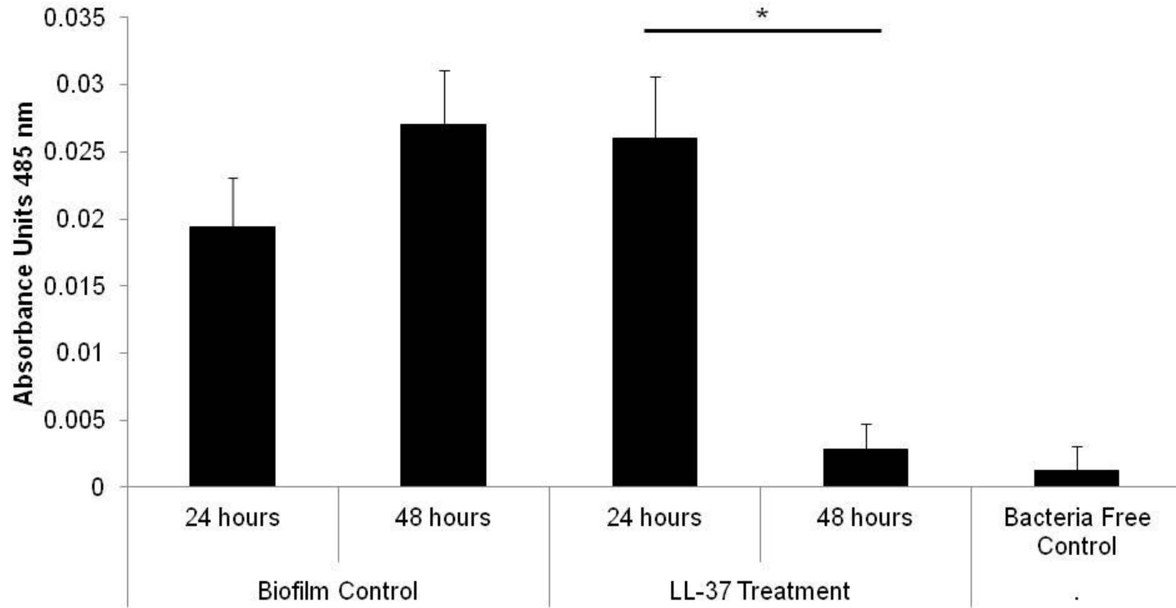


Figure 40. Biofilm grown in the presence of LL-37 for 48 hours had a decrease in PE secretion. Elastase activity in CM from biofilm grown in the presence (16 μg/mL) or absence of LL-37 for 24 or 48 hours as measured by the Elastin Congo Red assay. Error bars represent the standard deviation of 6 biological repeats. * Indicates a significant difference compared to the control as determined with an unpaired, two-tailed Student's T-test, $p<0.05$.

3.5.9 Summary

The effect of LL-37 treatment on downstream virulence of *P. aeruginosa* biofilm grown with the Calgary Biofilm Device and planktonic bacteria was determined. Growth in the presence of LL-37 caused a decrease in biofilm biomass while not significantly decreasing the bacterial numbers within the biofilm. Examination of the effect of growing biofilm and planktonic bacteria in the presence of LL-37 and downstream expression of selected genes indicated that there was little change in planktonic gene expression following 24 hours of exposure and a general decrease in gene expression following 48 hours in the presence of 16 μg/mL and 64 μg/mL LL-37, with the exception of *lasI*, which showed little change, and *mexX*, which showed an increase in expression with 16 μg/mL and 64 μg/mL LL-37. After 24 hours there was a universal increase in gene expression in biofilms following exposure to 16 μg/mL and 64 μg/mL LL-37. After 48 hours this was changed to a decrease in virulence gene expression in all genes examined except for *lasI, pprB, oprM* and *mexA*, which were relatively unaffected. The 48 hour exposure time in biofilm also resulted in a decrease in the logarithmic growth regulatory gene *rpoD* with increasing LL-37 concentration, indicating that more bacteria are potentially in persister phase. This was in contrast to the original hypothesis where I predicted a down-regulation of gene expression after 24 hours exposure and that this would be less pronounced after 48 hours. I predicted that the degradation of LL-37 would allow for the bacteria to recover from any initial effects. Instead, it appears that the early exposure to the peptide, perhaps coupled with nutrient depletion resulted in the majority of the biofilm likely entering persister phase and a resulting cessation of global gene transcription.

Translating the gene expression data to changes in phenotype showed that LL-37 exposure caused an increase in biofilm twitching ability; while growing planktonic bacteria in the presence

of LL-37 caused a slight decrease in twitching ability. This was in agreement with the gene expression results and contrary to my hypothesis where I predicted a universal decrease in twitching by biofilm and planktonic bacteria following growth in the presence of LL-37. Biofilm grown in the presence of LL-37 for 24 hours were also more resistant to ciprofloxacin, agreeing with the expression data. Co-culture studies with the Calgary Biofilm Device co-culture system showed that planktonic bacteria grown in the presence of LL-37 had decreased virulence indicated by a decreased ability to cause A549 cells rounding and cytoskeletal dysregulation, decreased internalization and decreased A549 cell death, as originally hypothesized; however the adherence, apoptosis and ability to stimulate IL-8 release by A549 cells was not changed. Growth of biofilm in the presence of LL-37 did not noticeably affect the virulence of biofilm in direct contact with the A549 cells. There was no change in morphology, cell death and apoptosis or IL-8 secretion by A549 cells. However when the CM of biofilm grown in the presence of LL-37 was examined there was an increase in IL-8 by A549 cells. It was further shown that LL-37 exposure for 48 hours resulted in a significant decrease in the secretion of elastase. Since the expression of *lasI* was shown to be unaffected by LL-37 exposure after 48 hours, the decrease in PE secretion suggests that the *lasB* gene is regulated by a separate system, or that LL-37 is able to decrease the secretion of PE through the T2SS.

4.0 Discussion

4.1 Major findings of thesis

Advances in medical practices in CF have increased the life expectancy of patients. While these practices have helped delay the onset of *P. aeruginosa* infection, there are still no interventions that are able to eliminate this chronic infection. One hindrance in development of treatment for chronic infection stems from an incomplete knowledge on the virulence of the chronic infection within the lung. The goal of this research was to study the interactions of *P. aeruginosa* biofilms with human lung epithelia *in vitro* to aid in understanding the factors contributing to chronic infections *in vivo.* To study biofilms a new co-culture method was developed using the Calgary Biofilm Device for the concurrent study of *P. aeruginosa* PAO1 biofilm and planktonic bacteria with human A549 lung cells. Comparison of biofilm and planktonic bacteria showed that planktonic bacteria caused increased A549 cell rounding and death, while biofilm stimulated significantly increased IL-8 release by A549 cells. Biofilm was shown to secrete significantly more elastase than planktonic, causing A549 morphological changes and loss of tight junctions. Up-regulated proteins secreted from planktonic bacteria were categorized as being mostly for metabolism and growth, with a smaller proportion designated as having virulence and protection functions as compared to proteins up-regulated in biofilm. One third of the secreted proteins unique or up-regulated in biofilms were classified as being for virulence and protection as compared to 12% of planktonic secreted proteins. The antimicrobial peptide LL-37 was shown to differentially affect biofilm and planktonic bacteria. LL-37 caused a decrease in planktonic bacterial virulence factors and exposure to LL-37 for 48 hours resulted in a decrease in PE secretion from biofilms. This decrease in PE secretion did not correlate with a decrease in *lasI*

expression, indicating that LL-37 exposure decreases PE secretion in a quorum-sensing-independent manner. When biofilms were compared with newly adherent bacteria, the newly adherent bacteria were shown to have characteristics similar to both planktonic bacteria and established biofilms.

4.2 The importance of studying established biofilm

The use of planktonic bacteria to study the interaction between pathogen and lung epithelia does not always reflect the complexity of the infection pathology *in vivo* where it has been shown that chronic infections involve the biofilm growth form. This includes the examination of CF sputum indicating the presence of bacteria in clusters surrounded by extracellular matrix (266). Moreover the transition to a chronic infection is accompanied by the emergence of antibacterial resistance that is not reflective of MIC values for the bacterial isolates (134). The disease symptoms and pathologies clearly indicate that biofilm and planktonic *P. aeruginosa* interact very differently with the host epithelia. Cystic fibrosis patients chronically infected with *P. aeruginosa* are slow to develop symptoms however they suffer periodic acute highly symptomatic exacerbations (65,310). The symptoms of acute exacerbations are relieved with the use of antibiotics, however the chronic infection remains (41,191). These acute exacerbations are likely caused by the release and growth of planktonic bacteria from the biofilm reservoir (298). There are two separate bacterial populations, biofilm and planktonic, that interact very differently with the host. Chronically infected CF patients often suffer from chronic inflammation within the lung, leading to tissue damage and lung fibrosis (22,264,317). The nature of CF severely impairs the innate and adaptive immune systems in the lung. A mutated CFTR causes dehydrated mucous and impaired airway clearance making these patients

vulnerable to infections such as *P. aeruginosa* and *S. aureus* infections (256). *P. aeruginosa* is an important pathogen in CF disease and the pathogenicity is partly due to the biofilm infections in the lungs of CF patients. There is still little known about the interaction of established *P. aeruginosa* biofilms with the host lung epithelia and all current co-culture systems are not sufficient to fully examine this relationship (32,58,136,159,208,209). Even though it is clear that biofilm and planktonic bacteria have two very different virulence mechanisms within the lung, there are very few models that have studied the interactions of established biofilms with lung epithelia. In my thesis research, I developed a novel co-culture system that aims to differentiate the interactions of established biofilm and planktonic bacteria with lung A549 cells.

4.3 The Calgary Biofilm Device co-culture model

The Calgary Biofilm Device co-culture model differs from other biofilm systems because it allows a biofilm to form prior to exposure to the mammalian cells (49,208). Moreover, the use of a 96-peg lid allows for formation of multiple uniform biofilms allowing for replicate analysis and internal controls. This system facilitates *P. aeruginosa* biofilms to form as matrix-encased communities which can then be used to inoculate human lung epithelia.

The Calgary Biofilm Device co-culture method has several limitations that must be taken into consideration when interpreting the results. The growth of biofilm on the abiotic surface of the pegs may not result in equivalent biofilms as those grown on biotic surfaces (8,207). Moreover, as in all static systems there is a depletion of nutrients and accumulation of waste products in the media that cannot be controlled for as in the flow cell model. Since the biofilms are formed on pegs it is questioned whether or not the biofilm would contact and directly interact with the mammalian cells seeded at the bottom of the wells. Importantly, it is unlikely that the biofilms

formed on the Calgary Biofilm Device obtain the mushroom-cap architecture that many flow cell biofilms develop (126), even though this architecture has been seen with other static systems (39). In fact, Müsken *et al.* (216) showed that after 24 hours biofilms formed in a static model produced a flat lawn of biofilm which continued to develop over time. The final maturation of these static biofilms did occur at a shorter time-frame than with flow cell biofilms. The importance of this architecture in the interaction with host epithelial cells is not thoroughly understood and some have speculated whether or not this architecture occurs *in vivo* (30,158). Kirketerp-Møller *et al*. (158) attempted to replicate the mushroom-cap architecture with an *in vivo* model and were unable to view this structure in an animal model. Moreover, when biofilms grown on the Calgary Biofilm Device were exposed to heavy metal cations they showed killing of exterior bacteria and the presence of persister internal bacteria (125,285). The presence of persister cells in biofilms grown on the Calgary Biofilm Device was confirmed by Spoering and Lewis (275).

The strain of *P. aeruginosa* used in these studies was PAO1. While this lab strain may not fully reflect the virulence range of *P. aeruginosa*, it is a convenient strain to use for initial characterization because of the availability of gene and protein expression data for this isolate. Using this strain also allowed for a better comparison with previous co-culture studies, which also used PAO1 (47,49,208,232).

For the development of this model the human lung cell line A549 was used. This immortal epithelial line is often used for the study of bacterial interactions with lung epithelia (47,49,54,127). A549 cells are a cancerous cell line derived from type II alveolar pneumocytes (109). Interestingly, thorough characterization of this cell line has suggested that rather than representing type II alveolar epithelia, this cell line is more characteristic of type I alveolar

epithelia (284). Type I alveolar epithelia constitute approximately 95% of the alveolar surface area while representing approximately 40% of the alveolar epithelial cells (50). Immunofluorescent microscopy shows that the cells formed a confluent monolayer with the formation of cell-to-cell junctions as evident by the production of Occludin and E-Cadherin. The basal level of apoptosis and cell death was low in the absence of stimulation despite the serum starvation. Further, the cells did not secrete IL-8 basally in the absence of stimulation. These results indicate that the A549 cells were behaving as expected and served as a suitable mammalian cell model.

4.4 ***Characterization of the biofilm grown on the Calgary Biofilm Device***

Previous studies have characterized the maturation of *P. aeruginosa* biofilms using the flow cell method by following gene expression of developing biofilms (259). The Calgary Biofilm Device grows biofilm in a static system and therefore it was expected that the maturation of the biofilm would occur in a shorter time period. To determine the length of incubation required to form a biofilm the gene expression of four genes in *P. aeruginosa* biofilms grown on the Calgary Biofilm Device and planktonic bacteria were compared to determine if they were different populations. I found that biofilms grown with the device for 24-hours were distinct from the planktonic population and biofilms grown for 48-hours were more similar to the planktonic population. This conclusion was made because after 24 hours the gene expression of the motility gene *fimU* and the quorum sensing synthase *lasI* were similar between planktonic and biofilm bacteria, while the expression of the outer membrane protein *mexX* was down-regulated in biofilms. This gene expression pattern was comparable to the gene expression results found for mature biofilms grown in a flow cell for 5 days (315) as well as to biofilms grown on a drip-flow

reactor for 3 days (279). In the study by Whitley *et al*. (315) there was no difference in the expression of global response regulator *psrA* between biofilm and planktonic; however the biofilm grown on the Calgary Biofilm Device for 24 hours showed a slight down-regulation compared to the planktonic bacteria. Since three of the four genes showed similar expression previously determined for biofilm, I concluded that a 24-hour biofilm grown on the Calgary Biofilm Device was representative of a biofilm. The differential expression of *psrA* between biofilms grown with a flow cell versus the Calgary Biofilm Device highlights the fact that static biofilms may behave differently than flow cell biofilms. It is surprising that *psrA* was down-regulated in the static biofilm because the corresponding protein promotes bacteria to enter stationary phase. Since there is likely more nutrient limitation in a static biofilm it was expected that *psrA* would be elevated.

Even though a 24-hour Calgary Biofilm Device biofilm is likely representative of a fully formed biofilm, I wanted to ensure that a longer incubation period didn't produce a biofilm that was even more representative of a mature biofilm. When the incubation time on the Calgary Biofilm Device was extended to 48 hours the gene expression profile of the biofilm changed. There was only 1 gene that showed a greater than 2-fold difference between biofilm and planktonic, *mexX*, and its expression was higher in biofilm compared to the planktonic bacteria. According to protein expression profiles of flow cell biofilms reported by Sauer *et al.* (259) the 48-hour Calgary Biofilm Device biofilms most represent late biofilms, but not at the dispersion phase. After 48 hours the expression of *lasI* decreased in both biofilm and planktonic bacteria compared to the 24-hour time-point. This agrees with previous data that shows a down-regulation of the quorum-sensing operon over time (74). This data is important for the understanding of the co-culture studies and confirms that the interpretation of 24-hour biofilms grown on the Calgary

Biofilm Device represent fully formed biofilms. This agrees with other studies of static biofilms where gene expression of a 24-hour biofilm was considered mature and 48 hours was late mature (89,304). The genes that were analyzed here were selected based on their importance in host-pathogen interactions and not to specifically differentiate biofilm and planktonic bacteria. Therefore, a more thorough characterization of the gene expression changes between biofilms grown on the Calgary Biofilm Device and planktonic bacteria should be performed. Ideally, expression analysis of biofilm versus planktonic bacteria using a microarray would allow the direct comparison of these biofilms with the previously reported global gene expression of flow cell biofilms (305). This would allow the biofilm maturation of these static biofilms to be aligned with the maturation of biofilms grown on the flow cells. Also, previous gene expression analysis of static biofilms has shown that expression of the exopolysaccharide *pel* was uniquely up-regulated in 24-hour biofilms (89). Unfortunately there seem to be very few universal genetic indicators of biofilm formation, with expression being dependent on growth conditions. However Sauer *et al.* (259) showed that two phosphoproteins – BmfR and MifR – were consistent markers of the maturation-1 and maturation-2 stages of biofilm growth. However to my knowledge this has not been tested in static biofilms.

The 24-hour *P. aeruginosa* biofilms were then used in co-culture with human A549 lung epithelia to determine if the virulence mechanisms of established biofilms and planktonic bacteria differ, and if the differences may reflect the differences in *in vivo* pathology of chronic and acute infections.

4.5 Planktonic P. aeruginosa and biofilm display different virulence mechanisms when co-cultured with human A549 epithelial cells as well as a differential secretion of virulence factors

Using the Calgary Biofilm Device co-culture system to compare the interactions of planktonic and biofilm *P. aeruginosa* with A549 lung epithelia identified important differences in virulence factors between the two bacterial growth forms. The A549 epithelia exposed to planktonic bacteria became rounded and lost their cytoskeletal organization. This agrees with published data showing the rounding of epithelial cells exposed to planktonic *P. aeruginosa* (49,54,61,177). However, the results were completely different when the epithelia were exposed to biofilm. The epithelial cells did not appear to change in morphology or lose their cytoskeletal organization. Gellatly *et al.* (107) have shown that the adherence of *P aeruginosa* to lung HBE cells activates the PhoPQ operon leading to the production of proteases and lipases that are cytotoxic to the epithelia. This, along with toxins secreted by the TTSS could be responsible for the cell rounding following exposure to the planktonic bacteria. In fact, CHO cell rounding by planktonic *P. aeruginosa* was shown to be mediated by its TTSS and that clinical isolates from late infections lost the ability to stimulate rounding through TTSS (177). The fact that biofilm did not cause the same cell rounding could indicate that these systems were not activated upon bacterial exposure. This could be the result of a separate adherence strategy by the biofilm or it could indicate that these virulence mechanisms are down-regulated in the biofilm bacteria. Interestingly the protein secretomes from biofilm showed the presence of Exotoxin A, indicating that components of the TTSS are likely expressed in 24-hour biofilms grown on the Calgary Biofilm Device. The presence and role of TTSS in biofilm formation is not completely understood (141,202,292). This indicates that *P. aeruginosa* biofilms may cause less tissue

damage and I speculate that this may be a contributing factor to chronic infections that are less likely to further invade the tissues to cause septicemia.

The TTSS of *P. aeruginosa* requires direct bacterial contact and the effector proteins are directly injected into the host cell. An examination of the secreted factors of biofilm and planktonic bacteria further highlights the indirect virulence of the two growth forms. When the bacterial cells were removed from the system and effects of secreted biofilm and planktonic factors were examined, very different results were obtained. The secreted factors from planktonic bacteria had no noticeable effect on A549 morphology; this contrasted to the biofilm secreted factors that caused A549 cells to lose their tight junctions and spread out, eventually lifting off the plate. This was a very interesting difference in virulence between *P. aeruginosa* biofilm and planktonic bacteria and will be discussed in more detail later. The inability of planktonic CM to stimulate the same A549 cell rounding as direct bacterial contact further suggests that the cytoskeletal dysregulation may be mediated by the TTSS.

It is possible that the difference in the interaction between the two growth forms was due to reduced adherence of the biofilms bacteria to the A549 cells compared to the planktonic bacteria. Bacterial adherence was tested using the Calgary Biofilm Device co-culture method, which allowed the concurrent examination of biofilms and planktonic bacteria. The use of the Calgary Biofilm Device allows control over the infection numbers of both planktonic bacteria and biofilm, which is necessary in order to study bacterial adherence. With the Calgary Biofilm Device multiple pegs can be used as controls within the same assay to ensure consistent biofilm formation. The amount of biofilm formed on each peg can then be used to determine the planktonic inoculum in order to infect with similar MOIs. The percent adherence obtained for planktonic bacteria was 11%, similar to what has been shown previously (47,49,54), while,

interestingly the adherence of biofilm to the lung epithelia was equivalent to the planktonic bacteria. Since the biofilm is attached to the pegs of the device it was predicted that less bacteria would be in contact with the A549 lung epithelial cells. In fact, the biofilm was constantly sloughing off aggregates of bacteria as well as releasing planktonic bacteria. This sloughing of biofilm aggregates is a known dispersion technique (65,105). Since biofilm were also less able to stimulate A549 cell rounding it is likely that the two growth forms are using separate adherence mechanisms.

It has been shown that planktonic *P. aeruginosa* is able to survive inside airway epithelia for up to 7 hours, leading to host cell death and bacterial release (246). The uptake of *P. aeruginosa* by airway epithelia is thought to be beneficial to the bacteria rather than the host (246); however this is not known and the role that internalization of *P. aeruginosa* plays in pathogenesis and infection kinetics is not clear. The internalization of *P. aeruginosa* by epithelia is mediated by LPS binding to CFTR and subsequent tyrosine kinase signaling and uptake of bacteria (94). Since the LPS of *P. aeruginosa* biofilms differs from planktonic bacteria it was predicted that the internalization of the biofilm bacteria would differ between the two growth forms. I found that the planktonic bacteria were internalized 15-fold more than the biofilm. As will be discussed later, biofilms were found to secrete significantly more Pseudomonal Elastase, and this protein has been shown to degrade epithelial surfactant proteins and prevent phagocytosis (169). This was not surprising since the biofilm may not require internalization to evade the host's immune system; while the planktonic bacteria are more susceptible to immune killing and therefore promote their internalization into epithelial cells in order to evade the host's immune response.

Epithelial cells are the first line of defense against invading bacteria and help direct the host immune response. Epithelial cells become activated once they encounter pathogens and this

results in an increase in cellular metabolism. The WST-1 assay is typically used to assess cell death through the measurement of cell metabolism (219) and was used in this co-culture system to evaluate the effect of planktonic and biofilm bacterial on A549 cells. Prior to exposure to bacteria the A549 cells were incubated with serum-free media for 24 hours, bringing them to a quiescent state (293). For the A549 cells exposed to planktonic bacteria, after 1 and 4 hours of exposure the level of metabolic activity was lower than the control. However after 1 hour of exposure to biofilm the metabolic activity of the A549 cells had increased. Increasing the incubation time in the presence of biofilm to 4 hours resulted in a lowering of A549 cell metabolism, likely due to the onset of epithelial cell death. This indicates that this co-culture system cannot be used to study host-biofilm interactions beyond a 4-hour time-point.

The integrity of the epithelial barrier is crucial for preventing the dissemination of bacteria throughout the body. A mechanism that *P. aeruginosa* uses to disrupt the epithelial barrier is to stimulate death of epithelial cells through necrosis or apoptosis. The Calgary Biofilm Device co-culture system was used to compare the cell death and apoptosis of A549 cells following exposure to established biofilm and planktonic *P. aeruginosa.*. The selection of an apoptosis marker is important since the co-culture incubation time is very short. Here, the use of an early indicator of apoptosis, Annexin V binding to translocated phosphatidylserine, is necessary because prolonged co-culture with biofilm will result in an over-growth of the released planktonic bacteria from the biofilm, which is a limitation of this system and all biofilm co-culture systems. There was no significant difference in the number of apoptotic A549 cells following biofilm or planktonic bacteria exposure for 4 hours; however planktonic exposure caused approximately twice as much A549 cell death than biofilm, which was expected based on the WST-1 results. When the bacteria were removed from the system and the bacterial secreted

factors were examined through conditioned media, it was clear that the planktonic bacteria require direct contact to cause cell death. This is most likely due to the TTSS as well as the activation of proteases and lipases upon bacterial adherence, described previously (107). While not significant, it is interesting that there was a trend to increased cell death following 48 hours of exposure to biofilm secreted factors. This was not surprising since most of the A549 cells were no longer adherent. It was surprising that the level of apoptosis in these cells did not mirror the cell death. It was predicted that these cells would be undergoing anoikis – cell apoptosis initiated by detachment – however the A549 cell line is derived from a cancer as an immortalized cell line that is protected from anoikis (183).

Chronic lung infections in CF patients are characterized by constitutively high levels of IL-8 and subsequent inflammation. The direct comparison between the ability of planktonic bacteria and established biofilm to stimulate IL-8 release has not been shown in a controlled *in vitro* setting. I found that biofilm, not planktonic bacteria, cause an increase in IL-8 secretion by A549 cells. It is likely that the A549 cells exposed to planktonic bacteria were not releasing IL-8 because they were shown to be undergoing cell death. However, after 4 hours of exposure the planktonic bacteria stimulated twice as much A549 cell death, while biofilm stimulated 6-times more IL-8 release. This indicates that the increased IL-8 secretion stimulated by biofilms is due to phenotypic differences between the two growth forms and not just differences in A549 cell death.

Exposure to biofilm stimulates an immune response in the A549 cells, evident by the increase in IL-8 secretion; while the planktonic bacteria are quick to cause cell death. It is interesting that CM from biofilm was able to stimulate equivalent levels of IL-8 release from A549 cells as with direct bacterial contact. This is potentially reflective of the *in vivo* pathology where in CF

patients the transition to a chronic infection correlates to a movement of the bacteria off of the lung epithelia and into the lumen (23); therefore it follows that different immunogenic factors of biofilm would be secreted compared to planktonic. There are several bacterial secreted factors that are known to stimulate IL-8 production from lung epithelia, including LPS (119), flagellin (254) and quorum-sensing lactones (49,86). During chronic infection the IL-8 secretion and subsequent inflammation is perpetuated by the constant release of the quorum-sensing lactones (86,269). The LPS of *P. aeruginosa* biofilms is modified compared to planktonic bacteria. Moreover, the expression of flagellin is supposedly lost in biofilms; however, as will be discussed later the CM from biofilms contained a significant amount of Flagellin B. Aong with suspected presence of lactones, PE in biofilm CM also likely contributed to IL-8 stimulation (92). These results confirm the hypothesis that established *P. aeruginosa* biofilms stimulate more IL-8 release from A549 lung cells, as seen *in vivo* in chronic infections. The stimulation of IL-8 release by A549 cells by biofilms relies mostly in the secreted factors. Planktonic bacteria also secrete factors that stimulate IL-8 release from A549 epithelia; however the presence of the bacteria also leads to significant epithelial cell death rather.

4.6 4-hour and 24-hour biofilms differ in their β-lactamase and PE secretion but interact similarly with A549 epithelial cells

The transition from an acute infection to a chronic infection likely involves a transition from planktonic bacteria to adherent planktonic and then finally to biofilms. It is still unknown if the virulence of the bacteria changes at the onset of biofilm formation or only when a full biofilm is formed. Comparing 4-hour and 24-hour biofilms allows us to delineate infection characteristics over time and determine if newly adhered bacteria behave more similarly to planktonic bacteria or to biofilms. The Calgary Biofilm Device was used to compare virulence mechanisms of 4-

hour and 24-hour biofilms. I was able to control for differences in bacterial numbers and compare to equivalent planktonic MOIs; however the MOIs of the 4-hour and 24-hour biofilms will not be equal simply due to the different kinetics of the biofilm growth curve. A key limitation to studying the kinetics of infection using this model is the lack of a biological surface. The virulence of bacteria adhered to pegs for 4-hours will almost certainly be different than the virulence of bacteria adhered to an epithelial layer for 4-hours (107). The initial characterization of the 4-hour and 24-hour biofilms showed that both have similar tolerances to antibiotics. Biofilms, both forming and established, are consistently releasing planktonic bacteria into the environment. This differs from the dispersion phase of biofilms, where most of the bacteria undergo a phenotypic shift towards planktonic growth. The planktonic bacteria that are continuously released from the biofilm likely resemble a phenotype similar to the bacteria that stay within the biofilm. Planktonic bacteria released from the 24-hour biofilm were slightly more resistant to gentamicin and ciprofloxacin as compared to the planktonic bacteria released from the 4-hour biofilms. Biofilms have been shown previously to secrete proteases upon exposure to ciprofloxacin, allowing for their survival (229). 24-hour biofilms were shown to be more resistant than 4-hour biofilms to ceftazidime and agrees with the observation that chronic *P. aeruginosa* biofilm infections become less susceptible to ceftazidime treatment over time (7). Since new, acute infections involve planktonic bacteria or a newly forming biofilms these results suggest that ceftazidime treatment would be more effective for treatment of these bacterial forms; however chronic infections, especially with *P. aeruginosa* are primarily caused by fully established, mature biofilms. It has been shown that biofilms likely cycle through phases of releasing planktonic bacteria during ideal conditions in the absence of antibiotic treatments, resulting in recurring disease symptoms (10). I demonstrated that 24-hour biofilms are the major

producers of β-lactamase after 5 hours of high ceftazidime exposure. Because the established biofilms secrete high levels of β-lactamase the concentration of antibiotics would be lowered by enzymatic hydrolysis allowing the growth of the released planktonic bacteria. It has been shown that ceftazidime stimulates β-lactamase production in the outer layers of *P. aeruginosa* biofilms and this production peaks after 4 to 5 hours of induction and is non-existent after 10 hours (17,18). This was not the case with 24-hour biofilms grown on the Calgary Biofilm Device, which maintained β-lactamase production after 24 hours exposure to both high and low concentrations of ceftazidime. At a 24 hour time-point I found that the biofilm maintains β-lactamase production when exposed to ceftazidime; however it is unable to maintain high levels against meropenem and no released planktonic bacteria are able to survive. This implies that biofilms secrete factors able to lower the concentration of β-lactams, thus allowing their survival and the survival of planktonic bacteria, as well as other opportunistic bacteria within the lung (3). These results give insight into the observation that repeated treatment with ceftazidime in CF patients decreases its ability to eliminate symptoms while not resulting in selection of hyper-resistant *P. aeruginosa* mutants (15).

When co-cultured with human A549 lung cells I found that newly adherent 4-hour biofilms have a phenotype along a spectrum from planktonic to established biofilms. 4-hour biofilms secrete proteins similar to planktonic bacteria, with 24-hour biofilms secreting more β-lactamase and PE than 4-hour biofilms. Since β-lactamase and PE are both secreted through the T2SS, these results suggest that this secretion system is preferentially activated in established biofilms compared to the newly adherent 4-hour biofilms. However, 4-hour biofilms stimulated similar IL-8 release from A549 lung cells while not causing a significant increase in metabolic activity, as originally hypothesized. This data agrees with protein expression profiles of developing biofilms, where

younger biofilms express proteins characteristic of both planktonic bacteria, as well as mature biofilms (259,273). These results provide insight into the infection kinetics of planktonic bacteria to formed biofilms. However, perhaps studying the virulence of newly forming biofilms is best done with the co-culture model of Moreau-Marquis (208) where biofilms are formed directly onto the epithelial layer. The Calgary Biofilm Device co-culture model can help provide insight into the interactions of established biofilms with epithelia; however the differences in growth conditions must be considered. Comparing 4-hour and 24-hour biofilms within the same system provides uniformity of growth conditions, while not necessarily reflecting the *in vivo* growth environment.

4.7 ***Growth of biofilm and planktonic bacteria in the presence of LL-37 has time-dependent effects on downstream virulence***

Previous studies have examined the interaction of the cathelicidin LL-37 with *P. aeruginosa* biofilms formed using the flow-cell and static microtitre methods (232). However, to my knowledge the downstream effect of LL-37 exposure on host-biofilm interactions has not been assessed with an *in vitro* co-culture model.

Consistent with previous studies, I have shown that LL-37 caused a decrease in biomass of 24-hour biofilms (232). The decrease in biomass after 24 hours could be attributed to reduced production of extracellular matrices in the presence of LL-37 since I showed that the bacterial numbers within the biofilms were not significantly changed regardless of LL-37 exposure. The *P. aeruginosa* extracellular matrix consists of polysaccharides, extracellular DNA, proteins and debris from dead bacteria (259,311,314). This is important since the extracellular matrix is crucial for the structural integrity and protection of the biofilm. The presence of LL-37 delays the

formation of biofilm during the first hour of incubation. This delay could be due to the initial reduced adherence of the bacteria to the surface in the presence of LL-37 (232). However the bacteria quickly overcome the delay since the bacterial numbers were the same after only 1.5 hours regardless of LL-37 exposure. This effect is unique to static systems where the peptide is likely degraded by the bacteria. In flow systems with constant media replenishment LL-37 has been shown to successfully inhibit the formation of bacterial biofilms (6,248). Overhage *et al.* (232) have shown that treatment with LL-37 during biofilm formation causes the biofilm to flatten and lose its typical mushroom architecture. Losing this architecture could alter the components in its extracellular matrix, thereby decreasing the biofilm biomass. My data confirms the decrease in biomass while showing that this decrease is not attributed to a decrease in bacterial numbers. This is in agreement with very recent data from other (82,149,217). I noted a 55% decrease in crystal violet staining with growth in the presence of 16 μg/mL of LL-37 while this concentration of peptide resulted in less than 1 log reduction in biofilm CFUs. This has been seen by others indicating that the extracellular matrix may bind to crystal violet to a greater extent than the bacteria (290). It has also been shown by others that growth in the presence of LL-37 significantly decreases the thickness of *P. aeruginosa* biofilms when grown with the flow cell system (232). However biofilm thickness does not necessarily reflect bacterial numbers (72,250). Davies *et al.* (72) showed that biofilms deficient in quorum sensing produced flat biofilms that were more densely packed with bacteria. The decrease in biofilm thickness was not correlated with a decrease in bacterial numbers. Therefore, the data here does not contradict the findings of Overhage *et al.* (232) since two different mutually exclusive characteristics were being examined. However other synthetic cationic peptides, such as IDR-1018 have been shown to stimulate cell death within biofilms (79,80).

Previous studies have examined the effects growing biofilm in a flow cell exposed to 4 μg/mL of LL-37 on changes in bacterial gene expression. In the flow cell there is a constant replenishment of peptide. However a therapeutic scenario would likely involve periodic pulses of LL-37 followed by a phase of decreased exposure. I used the Calgary Biofilm Device to examine the downstream gene expression changes after growing a biofilm in a static system with no replenishment of peptide. After 24 hours of biofilm exposure to LL-37 there was a universal increase in expression of all the genes examined with exposure to 16 μg/mL and 64 μg/mL of LL-37, but not 4 μg/mL. At lower concentrations it is possible that *P. aeruginosa* is able to avoid the activity of LL-37 through the activation of the PmrAB regulatory system and through degradation of the peptide (211). This system is activated by the presence of cationic antimicrobial compounds and mediates structural changes in the lipid A moiety of the LPS, decreasing the binding capability of LL-37. The local concentration of LL-37 in the lungs of adults and neonates is estimated to be 2 μg/mL and 5 μg/mL respectively (21,261). This increases to approximately 6 μg/mL and 15 μg/mL during infection (20,53,261). However other infection sites have reported local concentrations as high as 1500 μg/mL (230). Therefore the higher concentration of 64 μg/mL used could be considered physiologically relevant. In *P. aeruginosa* an early event following LL-37 exposure is phosphorylation of PhoP, the sensor kinase in the PhoP/PhoQ two-component signaling system (16). This system is responsible for the gene transcription of many virulence genes in *P. aeruginosa*. This system has also been shown to respond to many cationic peptides such as polymyxin B and CP28 (188). The results suggest that the phosphorylation of PhoP following LL-37 exposure may occur in biofilm as indicated by the concentration-dependent increase in expression of genes following 24 hours. Another anti-biofilm cationic peptide, IDR-1018, has been shown to bind to, and stimulate

degradation of the second messenger ppGpp (80). This molecule plays an important role in *P. aeruginosa* biofilm maintenance. In particular it has been linked to the formation of the persister population within biofilms. Decreasing this persister population would potentially increase the overall metabolism of the biofilm. However it would be expected that an overall increase in biofilm metabolism would be reflected in an altering of the *rpoD/16S rRNA* ratio, which was not observed here. The alteration of the persister population by LL-37 clearly requires further analysis.

Interestingly, after 24 hours the planktonic bacteria did not show any changes in gene expression between the control and LL-37 exposed bacteria. Previous studies have examined the gene expression changes of planktonic bacteria exposed to LL-37 for only 2 hours (282). While they showed that several genes were differentially regulated, none of the genes examined in my panel were shown to be altered.

Increasing the incubation time to 48 hours shows that several genes were moderately regulated in response to LL-37 exposure in planktonic bacteria. This longer time-point also caused a down-regulation of genes in the biofilm involved in regulatory pathways, motility and antibiotic resistance with the exception of *lasI, oprM* and *mexA*. It is important to consider the stability of LL-37 when incubated with *P. aeruginosa* when comparing results obtained with static systems versus flow cell systems. Previous studies have shown that *P. aeruginosa* secretes proteases that completely degrade LL-37 (262). Since LL-37 is only added to the medium at the beginning of the experiment – rather than continuously, as in a flow-cell model – the effects seen here may represent downstream events from an initial exposure. Within the CF lung the secretion of LL-37 is limited due to the thick, viscous mucus. The static model represents a therapeutic scenario where a slightly higher concentration of peptide is introduced into the lung. This may represent

the clinical scenario where therapeutic DNase treatment may liberate high concentrations of LL-37 in intermittent doses.

The regulatory genes of *P. aeruginosa*, including quorum sensing and two-component systems, are crucial for the bacteria to respond to changes in the environment. The quorum sensing gene *lasI* has been shown to be required for biofilm maturation. There are also several genes whose importance in biofilm formation and virulence are unknown. These include the two-component regulatory genes *pprA* and *pprB*. The gene *pprB* has been shown to be down-regulated in biofilm, while the sensor gene *pprA* has not been shown to be decreased. This suggests that it may be important for biofilm virulence. The characterization of the gene expression profiles of 24-hour biofilms revealed that the global gene regulator *psrA* was shown to be down-regulated in biofilm, which is interesting since this regulatory protein promotes the bacteria to enter stationary phase. Dysregulation of these regulatory genes could affect the biofilm formation capability, survival and virulence of *P. aeruginosa.* For planktonic bacteria there was an approximately 3-fold increase in the expression of *lasI* with exposure to 4 µg/mL of LL-37; but at 16 µg/mL and 64 µg/mL of LL-37 the increase dropped to less than 1.5-fold. In biofilms the fold change in *lasI* expression was negligible at all three peptide concentrations. In the study by Overhage *et al.*(232) the *lasI* gene was shown to be down-regulated 2.3-fold. The global regulatory gene *psrA* was decreased approximately 2-fold with 16 µg/mL and 64 µg/mL peptide exposure in planktonic bacteria; in biofilms the expression decreased to approximately 4-fold and 9-fold with these two peptide concentrations. In flow-cell biofilms *psrA* was shown to be down-regulated 1.6-fold (232). In planktonic bacteria the change in *pprA* was negligible at all three peptide concentrations; while in biofilm its expression was decreased 9-fold at the two highest concentrations of peptide. The other half of this two-component regulatory system, *pprB*

showed the opposite trend: the change in its expression was negligible in biofilm, while in planktonic it was down-regulated 2.5-fold with exposure to 64 μg/mL peptide exposure. Overhage *et al.* (232) showed that LL-37 exposure for 4 days did not affect the expression of *pprA* or *pprB*. The *pprA* and *pprB* genes are part of the pprA/B two-component regulatory system. Since they work in concert with each other, the down-regulation of one gene results in the down-regulation of the entire system. The data presented here is novel to demonstrate that the regulation of the *pprA/B* system in biofilm seems to involve the control of the sensor kinase; whereas in planktonic bacteria control seems to lie in regulating the response regulator. The PprA/PprB system is involved in a global up-regulation of regulatory systems, including the regulation of pili synthesis and membrane permeability regulation (28,309).

Bacterial motility is an important virulence and survival mechanism. In particular, twitching been shown to be necessary for adhesion and cytotoxicity (62,200). Twitching motility uses the Type IV pili system. Moreover, twitching has also been shown to be necessary for complex biofilm structure formation in flow cell systems (226). Control of the pili genes is critical for achieving the mushroom-cap architecture *in vitro*. Since previous studies have noted that biofilm growth in the presence of LL-37 results in a flattened biofilm, it is possible that LL-37 causes a dysregulation in the motility genes. These include the motility genes *fimU* and *pilV*, which are required for proper pilus function. Therefore, the expression of these two genes was determined in the presence of increasing concentrations of LL-37. After 48 hours the two motility genes, *fimU* and *pilV* were simultaneously repressed with increasing peptide concentration in both planktonic bacteria and biofilm. However, at the lowest peptide concentration there was a slight increase in the expression of these two genes in planktonic bacteria; while there was little change in biofilm. The down-regulation of both of these genes at 16 μg/mL and 64 μg/mL was more

pronounced in the biofilm. This is in contrast to the data from after 24 hours of exposure where both of these two genes were up-regulated in biofilm following exposure to 16 μg/mL and 64 μg/mL of LL-37; while there was no change in planktonic gene expression. Moreover, exposure to continuous levels of LL-37 for 4 days resulted in a 1.8- and 2.6-fold increase in *pilV* and *fimU* expression respectively (232). The *fimU* gene and the *pilV* gene are part of the same operon responsible for pilus assembly, therefore it was not surprising that the expression of these two genes were similar (26). The up-regulation of *fimU* and *pilV* in *P. aeruginosa* following 24 hours of LL-37 exposure indicates that this operon is activated and there is likely an increase in pilus assembly; this functional outcome is confirmed by the increase in twitching following a 24 hour exposure to LL-37. In the biofilm there was a dysregulation in pilus gene expression following 24 hours and 48 hours. This suggests that the flattened biofilm appearance following LL-37 exposure may be due to dysregulation of twitching motility.

To confirm the gene expression results, the twitching ability following exposure to LL-37 was assessed. Using the peg of the Calgary Biofilm Device the twitching difference between planktonic and biofilm could be tested. My results show that biofilm bacteria were slightly less able to twitch compared to planktonic; however the reliability of these results may be questioned due to the use of the peg for inoculation. However, these findings are interesting as the bacteria within the biofilm have been previously shown to be highly motile with their pili (4,161). Previous studies have shown that adding LL-37 into the twitching media results in an increase in twitching of planktonic bacteria and these results were confirmed here (232). The Calgary Biofilm Device was used to grow biofilms and planktonic bacteria in the presence of LL-37, then removing the peptide and determining the differences in twitching capabilities in the presence or absence of LL-37. I found that LL-37 had an opposing effect on biofilm and planktonic bacteria

with LL-37 exposure causing an increase in twitching in biofilms and a decrease in twitching in planktonic bacteria. The increase in twitching of biofilms exposed to LL-37 for 24 hours was not surprising since the expression of *fimU* and *pilV* were increased with 24 hour LL-37 exposure. The decrease in twitching of the planktonic bacteria was surprising since the gene expression data showed no changes in the expression of the motility genes.

The expression of antibiotic resistance genes, such as *oprM, mexX, mexA,* and *ampC,* within *P. aeruginosa* biofilms is of interest because of increased drug resistance of *P. aeruginosa* biofilm. Therapeutics that can decrease the expression of these genes without directly killing the bacteria would be of interest to work synergistically with antibiotics to restore the sensitivity of the bacteria. After 24 hours of exposure to LL-37 there was no change in expression of the antibiotic resistance genes in planktonic bacteria. This is similar to previous results showing LL-37 did not alter the expression of these genes in planktonic *P. aeruginosa* (282). However there was a universal increase in the expression of all 4 genes in biofilm. To confirm these findings biofilm and planktonic bacteria were grown in the presence of 16 μg/mL LL-37 for 24 hours and then exposed to gentamicin, ceftazidime and ciprofloxacin. Neither biofilm nor planktonic bacteria had their resistance to gentamicin, ciprofloxacin or ceftazidime altered by LL-37. Interestingly, Strempel *et al.* (282) found that a short pre-incubation (2 hours) of planktonic bacteria with LL-37 resulted in an increase in bacterial resistance to ciprofloxacin and gentamicin. This indicates that the effects of LL-37 on planktonic phenotypes may be short-lived since the longer pre-incubation of 24 hours, shown here, had no effect on bacterial resistance. Biofilms grown in the absence of LL-37 are already completely resistant to ceftazidime, so any increase in β-lactamase would not have an effect. It has been shown previously that *P. aeruginosa* mediates much of its resistance to ciprofloxacin through the MexCD-OprJ and

MexEF-OprN pumps (148,185). Moreover, biofilms in particular were shown to not require any efflux pumps to mediate resistance to antibiotics (279). Therefore the increase in expression of the *mexA/oprM* and *mexX/oprM* that was seen following 24-hour LL-37 exposure would be unlikely to affect the resistance to ciprofloxacin.

When biofilm and planktonic bacteria were grown in the presence of LL-37 for 48 hours there were significant changes in the gene expression of antibiotic resistance genes. This is in contrast to previous studies using flow-cell biofilms and continuous exposure to LL-37 for 4 days, where there was no change in the gene expression of any of the selected resistance genes (232). Following 48 hours of LL-37 exposure the significant transport protein *oprM* was unchanged in biofilm and was only slightly down-regulated in planktonic bacteria. The efflux gene *mexA* (whose protein associates with oprM to form an efflux pump) was down-regulated in planktonic bacteria with increasing peptide concentration; but it remained unchanged in biofilm. It is interesting that both components of the efflux pump were down-regulated in planktonic bacteria since this major pump is a component to antibiotic resistance in *P. aeruginosa.* The down-regulation shown here correlates with previous studies where there was a decrease in antibiotic resistance of *P. aeruginosa* when incubated with LL-37 (324). This indicates that this efflux pump may be more involved in antibiotic resistance than the mexXY/oprM pump, since the expression of *mexX* was actually increased in planktonic bacteria. This latter pump has been shown to be involved in resistance as well, but to a lesser degree (214). The change in expression of *mexX* was increased in planktonic bacteria and decreased in biofilm. It is important to remember that the normal expression of *mexX* is significantly lower in biofilms compared to planktonic bacteria and, unlike planktonic bacteria, the *mexX* system is not crucial for the antibiotic resistance of biofilms (77). In fact, recent studies have shown that *P. aeruginosa*

biofilms do not require any efflux pumps to mediate antibiotic resistance (279). The decrease in *ampC* expression in biofilms is interesting since I showed earlier that β-lactamase production is generally increased in biofilms. The decrease in expression of *ampC* may lead to biofilm susceptibility to β-lactam antibiotics. From this it is possible that co-administration of LL-37 with antibiotics may increase the susceptibility of previously resistant bacteria. This may not be true for biofilms since the primary efflux pump mexA/oprM remained relatively unchanged. Moreover, *P. aeruginosa* biofilms employ other biofilm-specific strategies to enable their resistance to antibiotics, including decreased antibiotic penetration through the extracellular matrix and the formation of a persister cell population.

One of the many intriguing findings of my gene expression data was dysregulation of the housekeeping gene *rpoD* where LL-37 exposure for 48 hours stimulated a biofilm-wide decrease in *rpoD* expression. *P. aeruginosa* biofilms are heterogeneous populations with the active metabolizing bacteria on the exterior and dormant persister population in the interior. Unfortunately it is difficult to identify a suitable housekeeping gene for the expression analysis in biofilms. Normalizing gene expression to the sigma factor *rpoD* is not ideal since it is differentially expressed between growth phases (101,232); however this gene has been used by others to compare gene expression changes between biofilms (43,328). The expression of the *16S rRNA* shows an approximately equal distribution throughout biofilms therefore it can be used to confirm that *rpoD* expression remains unchanged between growth conditions (180,238,316). The use of gene microarrays may circumvent the issue of identifying housekeeping genes since this system uses a signal intensity function to normalize the data.

When determining the difference in virulence of a biofilm it is often practical to discriminate towards only examining changes in expression among the metabolizing bacteria. In this case, the

logarithmic growing phase gene *rpoD* is a suitable normalizing gene. However changes in the distribution of bacteria between the metabolizing and dormant populations are also an important aspect to consider. These changes would be reflected in the ratio of *rpoD/16S rRNA* between the control and peptide biofilms. As expected, when I examined the *rpoD/16S rRNA* ratio I found that this ratio did not change with peptide exposure to planktonic bacteria, since none of the bacteria were expected to be dormant. Further, biofilm grown in the presence of LL-37 for 24 hours did not have alterations in their ratio either; however when this was extended to 48 hours the ratio decreased with increasing peptide concentration. This indicates that more of the bacteria in the biofilm were potentially in the dormant phase, as opposed to actively metabolizing. This would decrease the release of virulence factors by the biofilms; however it would also result in a larger population of bacteria that would be resistant to antibiotics, which target actively metabolizing bacteria. Future investigations of biofilms grown on the Calgary Biofilm Device in the presence of LL-37 for 48 hours could include the examination of specific persister markers. Previous studies have found specific genes are involved in the formation of the persister population, including *spoT, relA* and *dksA* (300). The genes *spoT* and *relA* are associated with the production of guanosine tetra-phosphate (ppGpp), which has been shown to mediate persister formation in *P. aeruginosa* (322). The increase in the persister population would therefore likely be indirectly linked to LL-37 exposure rather than a direct action.

Despite the fact that *rpoD* was shown to be down-regulated it is still important to normalize the data to *rpoD* because it allows us to examine changes within that small sub-set of metabolizing bacteria. When the genes are normalized to *16S rRNA* it results in an exaggerated down-regulation of all gene expression. This is simply a reflection of the changes in metabolic activity throughout the entire biofilm and not the changes in virulence gene expression among the

actively metabolizing bacteria. A more reliable method of discriminating these two populations is with laser capture microdissection, where the bacteria on the exterior of the biofilm are physically separated from the interior prior to gene expression analysis (180,238,316). However, as the distribution of these two populations changes, even this method becomes more problematic. When *rpoD* was used to normalize in 48 hour biofilms there was a trend towards a decrease in expression most genes with increasing peptide concentration. It is possible that the metabolic activity of these bacteria is decreasing and they may be entering dormancy as well. However the expression of *lasI, pprB, oprM,* and *mexA* remained stable with increasing LL-37 concentration.

The Calgary Biofilm Device was used to grow biofilms and planktonic bacteria in the presence of 16µg/mL LL-37 for 24 hours and then expose the bacteria to A549 lung cells. With direct bacterial contact the changes in virulence following LL-37 exposure were more pronounced in planktonic bacteria. The ability of the planktonic bacteria to cause A549 epithelial cell rounding was significantly delayed when the bacteria were grown in the presence of LL-37 for 24 hours, despite the fact that the planktonic bacteria were equally adherent regardless of LL-37 treatment. Since it has been shown previously that the TTSS of *P. aeruginosa* is required for cell rounding, this suggests that this secretion system may be affected by 24 hour exposure to LL-37. Strempel *et al.* (282) found that a short incubation (2 hours) of planktonic *P. aeruginosa* with LL-37 resulted in a down-regulation of Exotoxin S of the TTSS. This protein has been shown to stimulate A549 cell rounding (61). Once again, biofilm did not stimulate A549 cell rounding, even following 24 hours of LL-37 exposure. This was surprising since growth of biofilm in the presence of LL-37 for 24 hours caused a uniform increase in all genes examined; however genes of the TTSS were not analyzed in my panel. Overhage *et al. (232)* showed that continuous

exposure to LL-37 in a flow cell for 4 days resulted in a down-regulation of a genes involved in the TTSS apparatus. Growing planktonic bacteria in the presence of LL-37 for 24 hours also decreased the ability of the bacteria to stimulate A549 cell death, also suggesting a decrease in TTSS following LL-37 exposure. LL-37 treatment of biofilm did not affect A549 cell death or metabolism either. The changes in biofilm phenotype were apparent following CM treatment where significantly less IL-8 was released by A549 epithelial cells following exposure to biofilm CM that had been grown in the presence of LL-37 compared to the control. The secretion of elastase by biofilms was also affected by LL-37 exposure. Since the *lasI* gene was significantly up-regulated following 24 hours of exposure to LL-37, it was expected that PE (encoded by *lasB*) would also be up-regulated. Contrary to this hypothesis, there was no significant change in PE activity in biofilm CM following 24-hours of LL-37 exposure. Interestingly, increasing the LL-37 incubation time to 48 hours caused a significant decrease in PE activity that was correlated with a longer CM incubation time required to stimulate morphological changes in A549 cells. The decrease following 48 hours of exposure was also of interest since the expression of *lasI* did not decrease significantly with increasing LL-37 concentration. Since PE is mostly under the transcriptional control of quorum sensing it was expected that exposure to LL-37 for 48 hours would not cause a significant decrease in PE secretion. This indicates that either the expression of *lasB* is under the control of a separate regulatory system; or, more likely, the secretion of PE is down-regulated by LL-37 exposure. While the expression of T2SS components were not examined in this study, Overhage *et al.* (232) found that there was a significant down-regulation of several T2SS components following biofilm exposure to LL-37.

4.8 ***Biofilm secretes more PE than planktonic bacteria and this acts as an important virulence factor***

Comparing the secreted factors of biofilm and planktonic bacteria is often challenging because it is difficult to control for bacterial numbers between the two growth forms. Because of this, the characterization between biofilm and planktonic bacteria is often limited to simply examining unique proteins. The Calgary Biofilm Device provides an opportunity to compare overall quantities of proteins secreted by biofilm and planktonic bacteria using controlled bacterial numbers. The modification of the Calgary Biofilm Device for use in 8-well plates allows for the control of bacterial numbers while using a functional volume of supernatant. An unbiased LC/MS/MS shotgun proteomic approach is often used for identifying and comparing quantities of proteins (33,318). To compare the secretomes of biofilm and planktonic bacteria CM was isolated from each growth form and tryptic digests were examined through LC/MS/MS. When the identified proteins were categorized based on function it was clear that planktonic bacteria produce a larger proportion of proteins related to metabolism and growth. This was in accordance with previous studies that highlighted the population heterogeneity of biofilms includes a population of bacteria with suppressed metabolism (238,279,316). I show that there was a significantly increased secretion of PE by biofilms compared to planktonic cultures. PE is encoded by the gene *lasB* and is under the control of quorum sensing of the LasI system (224). The role PE plays in bacterial virulence is complex with previous studies showing that this protease is required for swarming motility, while not affecting swimming or twitching motility (231). Moreover, planktonic bacteria have been shown to quickly up-regulate PE production upon adhering to HBE cells (107). Recently this has been confirmed using a nitrocellulose static model for biofilm growth (329). In this model a nitrocellulose filter is placed on agar to mediate biofilm growth. This study found that PE secretion was up-regulated in biofilms correlated to the

late maturation phase. When the kinetics of *lasB* RNA expression were compared to the protein secretion they found that increased expression occurred during early biofilm development in contrast to the late biofilm development stage in which the PE protein was released. Potentially the propeptide of this protease is maintained within the cell until a timed release and processing to its active form. Tielen *et al.* (288) used a *lasB*-overexpressing mutant to show that this protease plays a role in establishing the composition of the extracellular matrix. They also found that the overexpression of *lasB* led to significantly decreased biofilm formation. Moreover, in another study *lasB* mutants were shown to be defective in forming biofilms (231). These mutants had otherwise fully functioning LasI quorum-sensing systems.

The LL-37 studies showed that growth of biofilm in the presence of LL-37 for 48 hours did not alter the expression of *lasI.* Since PE is regulated by this quorum-sensing system it was expected that LL-37 exposure for 48 hours would not impact PE secretion. Contrary to this, it was found that biofilms grown in the presence of LL-37 for 48 hours had significantly decreased PE secretion. This points to the possibility that either *lasB* can be regulated by a system other than the LasI quorum-sensing system, or that perhaps the secretion system was impacted by LL-37 exposure. PE is initially translated as a propeptide that undergoes cleavage to the active form within the periplasm (38). It is then transported through the outer membrane through the T2SS (38,150). The T2SS system is related to the Type IV pilus and even involves the PilD protein that is transcribed by the pilin operon. Perhaps the down-regulation of the *pilV/fimU* genes following 48 hour LL-37 exposure is related to the decreased secretion of PE by biofilms. Interestingly, Overhage *et al.* (232) found an increase in *pilD* expression following 4-days of continuous exposure to LL-37; however several probable T2SS proteins were down-regulated. Unfortunately the enzymatic activity of PE secreted by these flow-cell biofilms was not reported.

However, Strempel *et al.* (282) found that treatment of planktonic *P. aeruginosa* with LL-37 showed an increase in both transcription and enzymatic activity of PE. This indicates that the control over PE secretion may differ between biofilm and planktonic *P. aeruginosa*. Moreover the T2SS has been shown to be regulated by the quorum-sensing-independent system PUMA3 (also known as Vre) (184). This system has been previously shown to respond to changes in nutrient availability, such as phosphate through the Pho system. Since it is possible that the biofilm exposed to LL-37 for 48 hours were in nutrient-depleted environment compared to the control, it is possible that the T2SS was down-regulated through the PUMA3 control pathway.

While the increased secretion of PE by biofilms is a novel discovery; the interaction of PE with lung epithelia has previously been reported (2,11-13,15,24,51,92,165,175,179,190,271). It should be noted that secretion of PE by stationary phase planktonic *P. aeruginosa* was greatly affected by growth medium, with nutrient depleted media resulting in significantly less PE secretion (263). However others have shown that the genetic expression of *lasB* did not change in artificial sputum or artificial CF media for exponentially or stationary phase *P. aeruginosa* (103). Therefore further analysis of the increased secretion of PE by *P. aeruginosa* biofilms should be performed using more clinically relevant media as well as with clinical isolates. PE has been shown to be important for virulence within the lung and negatively affects patient outcomes (13,14,165). This increase in secretion could act as a biomarker to indicate the formation of a biofilm and subsequent chronic infection. Moreover, by comparing the CM of 4-hour and 24-hour biofilms, I showed that the secretion of significant amount of PE is limited to 24-hour biofilms. This was not surprising since the *lasI* operon is known to be activated during the biofilm maturation stage and is not required for initial biofilm formation (72,75). This supports the use of PE as a biomarker of the transition to a biofilm infection. However a recent study has

shown that late isolates from CF patients have lost the ability to produce PE through a mutation in the *lasR* gene (170). They showed that the loss of PE results in an over-active and neutrophil-dominated immune response associated with a decrease in lung function. This is mediated through the reduced cytokine degradation following the loss of PE. This shows the complex dynamics of *P. aeruginosa* infection where the production of PE may mediate early biofilm virulence while loss of PE may allow for late biofilm persistence. Once again, PE may serve as an appropriate biomarker to signal the onset of a chronic *P. aeruginosa* infection as well as a transition to a late, hyper-inflammatory state.

4.8.1 PE as a potential biomarker of transition to a chronic infection

Thus far there are very few methods to diagnose and classify a CF patient as having a chronic infection, highlighting the need of identifying a surrogate biomarker for biofilm infection. One biomarker that has been proposed to indicate a chronic infection is phospholipase C (88,139). However, when patients have a secondary infection with a different bacteria, particularly *S. aureus* significant phospholipase C secretion by *P. aeruginosa* planktonic bacteria also occurs (139). Since dual infection of *P. aeruginosa* and *S. aureus* is extremely common, the suitability of phospholipase C as a marker for chronic *P. aeruginosa* infection may be limited. Moreover, this previous study showed phospholipase C to be secreted by both planktonic and biofilm, with levels slightly higher in planktonic CM. A second biomarker that has been proposed for diagnosing a chronic infection is the isolation of *P. aeruginosa* isolates with a mucoid phenotype. However, Döring and Høiby (88) found that, while biopsies of chronic lung infections showed mucoid *P. aeruginosa*, isolating and culturing the bacteria often resulted in a loss of the mucoid phenotype. It is likely that the difference lies in the planktonic/biofilm phenotype, rather than a permanent genetic change (88). Since an ideal biomarker should not

require obtaining patient biopsies, using the mucoid phenotype may not be ideal as a biomarker for chronic infection. Caution should be used, however, since previous studies have shown late CF isolates evolve loss-of-function mutations in many virulence genes, including *lasB* (124,170,267). Interestingly, comparison of isogenic strains from acute and chronic infections shows an increase in virulence gene expression by the late isolate (218). This highlights the complexity of bacterial evolution dynamics within the CF lung ecosystem. Future studies to correlate the PE concentration in patient sputum with biopsies indicating chronic infection may indicate whether PE would serve as a suitable biomarker.

4.8.2 PE is an important virulence factor in vivo

In the early 1980s the importance of proteases to bacterial virulence was highlighted and studied in the lungs of CF patients (146). An analysis of serum antibody levels against PE showed an increase in PE-specific antibodies in patients who were chronically infected with *P. aeruginosa* (139,147,162,280). It was found that serum antibody levels were also higher in patients whose *P. aeruginosa* strain were a mucoid phenotype, which has been linked to high biofilm-forming ability (147,182). This indicates that the *in vivo* expression of PE likely increases with the switch to the biofilm phenotype. It has been shown that there was also a differential PE expression pattern across isolates from CF patients (146). However this *in vitro* expression pattern was determined from planktonic bacteria (277). It is interesting that many isolates from chronically infected patients showed low PE levels when grown as planktonic bacteria; it would have been interesting to grow these isolates with the Calgary Biofilm Device to see if the secretion of elastase increases significantly. The authors also observed that when two different isolates were obtained from a patient experiencing an exacerbation of infection, one isolate secreted significantly more elastase than the other (146).

Epithelial permeability has been shown to be disrupted by PE (15). It was shown that this effect was specific to PE and porcine pancreatic elastase did not have the same effect. Moreover, the same study showed that the PE degraded Occludin as shown through immunofluorescent microscopy. Occludin is an important protein in the tight junctions of epithelial cells and its degradation leads to epithelial permeability through the disrupted barrier. Azghani *et al.* (15) also show that PE did not affect E-Cadherin in the epithelia. In this study, CM isolated from biofilms contained the ability to degrade Occludin, while not affecting E-Cadherin expression. This data indicates that the activity may be due to the high PE secreted by the biofilm. PE has been shown to up-regulate the production of IL-8 by epithelial cells (12). This activity was mirrored in my study using CM from *P. aeruginosa* biofilm where biofilm CM – containing high levels of PE – stimulated A549 cells to secrete a significant amount of IL-8. Leidal *et al.* (179) also showed the degradation of MCP-1 by PE and LaFayette *et al.* (170) showed degradation of IL-8 and IL-6 by PE. My studies found that this occurred, but to a much lesser extent as compared to RANTES. This indicates that the immune activation results I obtained using biofilm CM were possibly due to the secretion of PE by biofilms. The significance of this activity *in vivo* is that there is an increase in inflammation but with the recruitment of less effector cells. It has been shown in a rat model that PE caused a significant increase in IL-8 and an influx of neutrophils (165); however PE has also been associated with a dampened immune response and the eventual loss of PE production by biofilms is associated with an increased neutrophil response (170). Interestingly, neutrophils have been shown to be effective in killing planktonic bacteria; but effectiveness is lost once the bacteria switch over to the biofilm mode of growth (326). The timing of PE release by biofilms and neutrophil recruitment seems to take advantage of this capability. Moreover,

neutrophils have been shown to promote biofilm formation through the formation of neutrophil extracellular traps (NETs) (140,287).

The importance of PE as a virulence factor *in vivo* has also been shown in numerous immunological studies (51,271). In fact, rats immunized against PE peptides had a significant decrease in the number of polymorphonuclear cells in bronchoalveolar lavage (BAL) fluid and subsequent decrease in lung pathology. The immunization had no effect on bacterial numbers, highlighting the importance of PE in stimulating an over-active immune response. In a study examining the serum antibodies to alkaline phosphatase and PE, the titres against PE were generally less than against alkaline phosphatase; however the actual levels of produced by each strain were consistently higher than alkaline phosphatase (88). Once again, this was measured *in vitro* from the planktonic growth form of the bacteria. To my knowledge there has never been a longitudinal measurement of PE from the sputum or BAL of CF patients. This indicates that PE is not necessary for biofilm growth within the lung; however it likely plays a key role in the pathogenesis of *P. aeruginosa* chronic infections and has a deleterious effect on lung function.

4.9 ***Summary/Conclusions***

4.9.1 Major findings

The work described here examines the interactions of lung epithelial cells with established biofilm and planktonic *P. aeruginosa*. Comparison of biofilm and planktonic bacteria showed that planktonic bacteria caused more A549 cell rounding and death, while biofilm stimulated more IL-8 release by A549 cells, an indicator of inflammation. The ability of planktonic bacteria to stimulate these morphological changes and cell death required direct bacterial contact; whereas the majority of the effects caused by biofilms were through the secretion of virulence

factors. Biofilm was shown to secrete significantly more PE than planktonic bacteria, causing epithelial morphological changes and loss of tight junctions. The majority of the proteins secreted by planktonic bacteria were for metabolism and growth, while biofilm secreted more proteins involved in virulence and protection. When 24-hour biofilms were compared with newly-adherent 4-hour biofilms, newly adherent biofilms were shown to have characteristics similar to both planktonic bacteria and fully formed biofilms. The decreased secretion of β-lactamase and PE by newly adherent biofilms may indicates that the T2SS system is not yet fully developed as compared to fully formed biofilms. Biofilm and planktonic bacteria were grown in the presence of the antimicrobial peptide LL-37 and then downstream effects of the peptide were assessed. LL-37 was shown to differentially affect biofilm and planktonic bacteria. LL-37 caused a decrease in twitching of planktonic bacteria and exposure to LL-37 48 hours resulted in a decrease in PE secretion. This was contrary to the gene expression studies where *lasI* remained unchanged, indicating that perhaps the T2SS of biofilms is down-regulated by LL-37 exposure. Treatment with LL-37 for 48 hours may result in more bacteria within the biofilm to enter the persister phase and maintain less metabolic activity. These results were obtained using the novel Calgary Biofilm Device co-culture system. Previous work to study the co-culture of lung epithelia with *P. aeruginosa* biofilms has been limited to early infections. The model described here allows the exposure of lung epithelia to established biofilms. This model system could be easily manipulated to better reflect the *in vivo* environment.

4.9.2 Future directions

The Calgary Biofilm Device co-culture model provides an excellent opportunity to compare the virulence of various clinical isolates of *P. aeruginosa*. Thus far, comparison of the virulence of these isolates has been limited to planktonic growth or early biofilms. It is not surprising that the

virulence characteristics of isolates from chronically infected patients have been difficult to replicate *in vitro* (146,147,272). These isolates have adapted as biofilms over the course of infection and they have altered sensitivities to quorum-sensing molecules resulting in a differential expression of regulatory genes (272,277). Using these isolates in the Calgary Biofilm Device co-culture method may uncover new mechanisms of virulence of isolates from chronic infections. Moreover, the secretomes of these isolates growing as biofilms may be more representative of proteins found in sputum (272,277).

This work describes the co-culture method using a simple monolayer of A549 lung cells. This cell line has been ideal for the development of new methods because it is cost effective and easily maintained with no specialty equipment of media required. Moreover it quickly forms confluent monolayers to be used in the assays. Further it forms a uniform cell population that is ideal for method development because there is little variation between each well. It would be interesting to use a primary cell line isolated from a CF patient that lacks a functional CFTR since this protein has been shown to play a role in *P. aeruginosa* pathogenesis (207,244,245). Adapting these cell lines to become differentiated, polarized cells can also be done with this system. The cells can be polarized by growth on a collagen matrix (96,246). Even more representative of the physiological conditions would be the use of *ex vivo* tissue samples. Since these methods have already been established for planktonic co-culture there would be very little optimization required to adapt these new Calgary Biofilm Device methods.

In this study the A549 cells were grown with simple DMEM media. Ideally the co-culture would involve a more physiologically relevant environment. Adaptation of the model could include the use of artificial sputum instead of DMEM. Artificial sputum has been manufactured to better represent the characteristics of sputum within the CF lung (157,276). This includes a higher salt

content, mucus, and higher amino acid and DNA concentrations. Moreover, growing the biofilm in this similar physiological media could alter the gene expression of the growing biofilm. This may help uncover new virulence mechanisms of the biofilm that was grown in an environment more similar to the host lung. Further, coating the pegs of the Calgary Biofilm Device with mucins may better reflect the *in vivo* biofilm condition and could impact the phenotype of the biofilm.

I have established that the release of high levels of PE seems to occur once *P. aeruginosa* biofilms reach maturity. Since 4-hour biofilms produce similar amounts of PE as planktonic bacteria, the appearance of PE in sputum could represent the establishment of a fully formed biofilm. Longitudinal examination of CF patient sputum for the appearance of PE would help corroborate these results. If the appearance of PE correlates with the establishment of a chronic infection then this would indicate that PE may be a valuable biomarker. Previous studies have also examined the potential of targeting PE as a therapeutic approach in CF patients (139,162,271). Immunization with a peptide derived from PE was shown to decrease the pathology of chronic *P. aeruginosa* lung infections. Even though there was no effect on bacterial numbers, this treatment may improve the lung function of CF patients.

4.9.3 Conclusion

In conclusion, this study provides the foundation for the examination of the host-pathogen interactions of mammalian epithelia with mature *P. aeruginosa* biofilms. There is a need for therapeutic interventions that help prevent and eliminate chronic *P. aeruginosa* infections in CF patients. Studying the virulence and host-pathogen interactions of human lung epithelia with mature biofilms may uncover therapeutic strategies that would target chronic infections. I found

key differences in the pathogenesis of mature biofilm and planktonic *P. aeruginosa*. Planktonic bacteria are able to disrupt the cell-to-cell interactions of A549 cells and stimulate cell death, while the biofilm has minimal effect on epithelial morphology. Biofilm stimulates the release of the inflammatory cytokine IL-8 and also secretes significantly more PE than planktonic bacteria. The Calgary Biofilm Device co-culture system has proven valuable for allowing the growth and maturation of the biofilm prior to host cell exposure. The future adaptations of this model will better represent the *in vivo* characteristics of chronic lung infection to delineate ongoing virulence mechanisms of the bacteria causing host cell stimulation and damage.

5.0 Reference List

1. Ahlgren, H. G., A. Benedetti, J. S. Landry, J. Bernier, E. Matouk, D. Radzioch, L. C. Lands, S. Rousseau, and D. Nguyen. 2015. Clinical outcomes associated with *Staphylococcus aureus* and *Pseudomonas aeruginosa* airway infections in adult cystic fibrosis patients. BMC pulmonary medicine 15:67.

2. Alcorn, J. F. and J. R. Wright. 2004. Degradation of pulmonary surfactant Protein D by *Pseudomonas aeruginosa* elastase abrogates innate immune function. Journal of Biological Chemistry 279:30871-30879.

3. Ali, L., F. Khambaty, and G. Diachenko. 2006. Investigating the suitability of the Calgary Biofilm Device for assessing the antimicrobial efficacy of new agents. Bioresource Technology 97:1887-1893.

4. Alm, R. A. and J. S. Mattick. 1995. Identification of a gene, pilV, required for type 4 fimbrial biogenesis in *Pseudomonas aeruginosa*, whose product possesses a pre-pilin-like leader sequence. Molecular Microbiology 16:485-496.

5. Alshalchi, S. A. and G. G. Anderson. 2014. Involvement of stress-related genes polB and PA14_46880 in biofilm formation of *Pseudomonas aeruginosa*. Infection and Immunity 82:4746-4757.

6. Amer, L. S., B. M. Bishop, and M. L. van Hoek. 2010. Antimicrobial and antibiofilm activity of cathelicidins and short, synthetic peptides against *Francisella*. Biochemical and Biophysical Research Communications 396:246-251.

7. Anderson, G. G., T. F. Kenney, D. L. MacLeod, N. R. Henig, and G. A. O'Toole. 2013. Eradication of *Pseudomonas aeruginosa* biofilms on cultured airway cells by a fosfomycin/tobramycin antibiotic combination. Pathogens and Disease 67:39-45.

8. Anderson, G. G., S. Moreau-Marquis, B. A. Stanton, and G. A. O'Toole. 2008. *In vitro* analysis of tobramycin-treated *Pseudomonas aeruginosa* biofilms on cystic fibrosis-derived airway epithelial cells. Infection and Immunity 76:1423-1433.

9. Anderson, M. J., P. J. Parks, and M. L. Peterson. 2013. A mucosal model to study microbial biofilm development and anti-biofilm therapeutics. Journal of Microbiological Methods 92:201-208.

10. Arens, R., D. Gozal, K. J. Omlin, J. Vega, K. P. Boyd, T. G. Keens, and M. S. Woo. 1994. Comparison of high frequency chest compression and conventional chest physiotherapy in hospitalized patients with cystic fibrosis. American Journal of Respiratory and Critical Care Medicine 150:1154-1157.

11. Azghani, A. O. 1996. *Pseudomonas aeruginosa* and epithelial permeability: role of virulence factors elastase and exotoxin A. American Journal of Respiratory Cell and Molecular Biology 15:132-140.

12. Azghani, A. O., J. W. Baker, S. Shetty, E. J. Miller, and G. J. Bhat. 2002. *Pseudomonas aeruginosa* elastase stimulates ERK signaling pathway and enhances IL- 8 production by alveolar epithelial cells in culture. Inflammation Research 51:506-510.

13. Azghani, A. O., T. Bedinghaus, and R. Klein. 2000. Detection of elastase from *Pseudomonas aeruginosa* in sputum and its potential role in epithelial cell permeability. Lung 178:181-189.

14. Azghani, A. O., J. C. Connelly, B. T. Peterson, L. D. Gray, M. L. Collins, and A. R. Johnson. 1990. Effects of *Pseudomonas aeruginosa* elastase on alveolar epithelial permeability in guinea pigs. Infection and Immunity 58:433-438.

15. Azghani, A. O., L. D. Gray, and A. R. Johnson. 1993. A bacterial protease perturbs the paracellular barrier function of transporting epithelial monolayers in culture. Infection and Immunity 61:2681-2686.

16. Bader, M. W., S. Sanowar, M. E. Daley, A. R. Schneider, U. Cho, W. Xu, R. E. Klevit, H. Le Moual, and S. I. Miller. 2005. Recognition of antimicrobial peptides by a bacterial sensor kinase. Cell 122:461-472.

17. Bagge, N., M. Hentzer, J. B. Andersen, O. Ciofu, M. Givskov, and N. Høiby. 2004. Dynamics and spatial distribution of β-lactamase expression in *Pseudomonas aeruginosa* biofilms. Antimicrobial Agents and Chemotherapy 48:1168-1174.

18. Bagge, N., M. Schuster, M. Hentzer, O. Ciofu, M. Givskov, E. P. Greenberg, and E. T. Jensen. 2004. *Pseudomonas aeruginosa* biofilms exposed to imipenem exhibit changes in global gene expression and [beta]-lactamase and alginate production. Antimicrobial Agents and Chemotherapy 48:1175-1187.

19. Bals, R., D. Hubert, and B. Tümmler. 2011. Antibiotic treatment of CF lung disease: from bench to bedside. Journal of Cystic Fibrosis 10, Supplement 2:S146-S151.

20. Bals, R., X. Wang, M. Zasloff, and J. M. Wilson. 1998. The peptide antibiotic LL-37/hCAP-18 is expressed in epithelia of the human lung where it has broad antimicrobial activity at the airway surface. Proceedings of the National Academy of Sciences 95:9541-9546.

21. Bals, R., D. J. Weiner, R. L. Meegalla, and J. M. Wilson. 1999. Transfer of a cathelicidin peptide antibiotic gene restores bacterial killing in a cystic fibrosis xenograft model. The Journal of Clinical Investigation 103:1113-1117.

22. Bals, R., D. J. Weiner, and J. M. Wilson. 1999. The innate immune system in cystic fibrosis lung disease. The Journal of Clinical Investigation 103:303-307.

23. Baltimore, R. S., C. D. C. Christie, and G. J. W. Smith. 1989. Immunohistopathologic localization of *Pseudomonas aeruginosa* in lungs from patients with cystic fibrosis: implications for the pathogenesis of progressive lung deterioration. American Review of Respiratory Disease 140:1650-1661.

24. Beaufort, N., E. Corvazier, A. Hervieu, C. Choqueux, M. Dussiot, L. Louedec, A. Cady, S. de Bentzmann, J. B. Michel, and D. Pidard. 2011. The thermolysin-like metalloproteinase and virulence factor LasB from pathogenic *Pseudomonas aeruginosa* induces anoikis of human vascular cells. Cellular Microbiology 13:1149-1167.

25. Beaumont, P. E., B. McHugh, E. Gwyer Findlay, A. Mackellar, K. J. Mackenzie, R. L. Gallo, J. R. W. Govan, A. J. Simpson, and D. J. Davidson. 2014. Cathelicidin host defence peptide augments clearance of pulmonary *Pseudomonas aeruginosa* infection by its influence on neutrophil function *in vivo*. PLoS ONE 9:e99029.

26. Belete, B., H. Lu, and D. J. Wozniak. 2008. *Pseudomonas aeruginosa* AlgR regulates Type IV pilus biosynthesis by activating transcription of the fimU-pilVWXY1Y2E operon. Journal of Bacteriology 190:2023-2030.

27. Beloin, C. and J. M. Ghigo. 2005. Finding gene-expression patterns in bacterial biofilms. Trends in Microbiology 13:16-19.

28. Bernard, C. S., C. Bordi, E. Termine, A. Filloux, and S. de Bentzmann. 2009. Organization and PprB-dependent control of the *Pseudomonas aeruginosa* tad locus, involved in Flp pilus biology. Journal of Bacteriology 191:1961-1973.

29. Bever, R. A. and B. H. Iglewski. 1988. Molecular characterization and nucleotide sequence of the *Pseudomonas aeruginosa* elastase structural gene. Journal of Bacteriology 170:4309-4314.

30. Bjarnsholt, T., M. Alhede, M. Alhede, S. R. Eickhardt-Sørensen, C. Moser, M. Kühl, P. Ø. Jensen, and N. Høiby. 2013. The *in vivo* biofilm. Trends in Microbiology 21:466-474.

31. Bjarnsholt, T. and M. Givskov. 2007. The role of quorum sensing in the pathogenicity of the cunning aggressor *Pseudomonas aeruginosa*. Analytical and Bioanalytical Chemistry 387:409-414.

32. Bjarnsholt, T., P. Ø. Jensen, M. J. Fiandaca, J. Pedersen, C. R. Hansen, C. B. Andersen, T. Pressler, M. Givskov, and N. Høiby. 2009. *Pseudomonas aeruginosa* biofilms in the respiratory tract of cystic fibrosis patients. Pediatric Pulmonology 44:547-558.

33. Blanchard, A. A., P. Ezzati, D. Shamshurin, A. C. Nistor, E. Leygue, J. A. Wilkins, and Y. Myal. 2015. Towards further defining the proteome of mouse saliva. Proteome Science 13:10.

34. Bollinger, N., D. J. Hassett, B. H. Iglewski, J. W. Costerton, and T. R. McDermott. 2001. Gene expression in *Pseudomonas aeruginosa*: evidence of iron override effects on quorum sensing and biofilm-specific gene regulation. Journal of Bacteriology 183:1990-1996.

35. Bonfield, T. L., J. R. Panuska, M. W. Konstan, K. A. Hilliard, J. B. Hilliard, H. Ghnaim, and M. Berger. 1995. Inflammatory cytokines in cystic fibrosis lungs. American Journal of Respiratory and Critical Care Medicine 152:2111-2118.

36. Bowler, L. L., T. B. Ball, and L. L. Saward. 2014. A *novel in vitro* co-culture system allows the concurrent analysis of mature biofilm and planktonic bacteria with human lung epithelia. Journal of Microbiological Methods 101:49-55.

37. Bowler, L. L., G. G. Zhanel, T. B. Ball, and L. L. Saward. 2012. Mature *Pseudomonas aeruginosa* biofilms prevail compared to young biofilms in the presence of ceftazidime. Antimicrobial Agents and Chemotherapy 56:4976-4979.

38. Braun, P., A. de Groot, W. Bitter, and J. Tommassen. 1998. Secretion of elastinolytic enzymes and their propeptides by *Pseudomonas aeruginosa*. Journal of Bacteriology 180:3467-3469.

39. Bridier, A., F. Dubois-Brissonnet, A. Boubetra, V. Thomas, and R. Briandet. 2010. The biofilm architecture of sixty opportunistic pathogens deciphered using a high throughput CLSM method. Journal of Microbiological Methods 82:64-70.

40. Brint, J. M. and D. E. Ohman. 1995. Synthesis of multiple exoproducts in *Pseudomonas aeruginosa* is under the control of RhlR-RhlI, another set of regulators in strain PAO1 with homology to the autoinducer-responsive LuxR-LuxI family. Journal of Bacteriology 177:7155-7163.

41. Brown, M. R. W., D. G. Allison, and P. E. Gilbert. 1988. Resistance of bacterial biofilms to antibiotics a growth-rate related effect? Journal of Antimicrobial Chemotherapy 22:777-780.

42. Brözel, V. S., G. M. Strydom, and T. E. Cloete. 1995. A method for the study of *de novo* protein synthesis in *Pseudomonas aeruginosa* after attachment. Biofouling 8:195-201.

43. Bruchmann, J., S. Kirchen, and T. Schwartz. 2013. Sub-inhibitory concentrations of antibiotics and wastewater influencing biofilm formation and gene expression of multi-resistant *Pseudomonas aeruginosa* wastewater isolates. Environmental science and pollution research international 20:3539-3549.

44. Bucior, I., J. Abbott, Y. Song, M. A. Matthay, and J. N. Engel. 2013. Sugar administration is an effective adjunctive therapy in the treatment of *Pseudomonas aeruginosa* pneumonia. American Journal of Physiology - Lung Cellular and Molecular Physiology 305:L352-L363.

45. Bucki, R., F. J. Byfield, and P. A. Janmey. 2007. Release of the antimicrobial peptide LL-37 from DNA/F-actin bundles in cystic fibrosis sputum. European Respiratory Journal 29:624-632.

46. Burns, J. L., R. L. Gibson, S. McNamara, D. Yim, J. Emerson, M. Rosenfeld, P. Hiatt, K. McCoy, R. Castile, A. L. Smith, and B. W. Ramsey. 2001. Longitudinal assessment of *Pseudomonas aeruginosa* in young children with cystic fibrosis. Journal of Infectious Diseases 183:444-452.

47. Byrd, M. S., B. Pang, M. Mishra, W. E. Swords, and D. J. Wozniak. 2010. The *Pseudomonas aeruginosa* exopolysaccharide Psl facilitates surface adherence and NF-KB Activation in A549 cells. mBio 1:e00140-10 .

48. Cabral, D. A., B. A. Loh, and D. P. Speert. 1987. Mucoid *Pseudomonas aeruginosa* resists nonopsonic phagocytosis by human neutrophils and macrophages. Pediatric Research 22:429-431.

49. Carterson, A. J., K. Honer zu Bentrup, C. M. Ott, M. S. Clarke, D. L. Pierson, C. R. Vanderburg, K. L. Buchanan, C. A. Nickerson, and M. J. Schurr. 2005. A549 lung epithelial cells grown as three-dimensional aggregates: alternative tissue culture model for *Pseudomonas aeruginosa* pathogenesis. Infection and Immunity 73:1129-1140.

50. Castranova, V., J. Rabovsky, J. H. Tucker, and P. R. Miles. 1988. The alveolar type II epithelial cell: A multifunctional pneumocyte. Toxicology and Applied Pharmacology 93:472-483.

51. Cathcart, G. R. A., D. Quinn, B. Greer, P. Harriott, J. F. Lynas, B. F. Gilmore, and B. Walker. 2011. Novel inhibitors of the *Pseudomonas aeruginosa* virulence factor LasB: a potential therapeutic approach for the attenuation of virulence mechanisms in Pseudomonal infection. Antimicrobial Agents and Chemotherapy 55:2670-2678.

52. Ceri, H., M. E. Olson, C. Stremick, R. R. Read, and D. Morck. 1999. The Calgary Biofilm Device: New technology for rapid determination of antibiotic susceptibilities. Journal of clinical microbiology 37:1771-1776.

53. Chen, C. I. U., S. Schaller-Bals, K. P. Paul, U. Wahn, and R. Bals. 2004. β-defensins and LL-37 in bronchoalveolar lavage fluid of patients with cystic fibrosis. Journal of Cystic Fibrosis 3:45-50.

54. Chi, E., T. Mehl, D. Nunn, and S. Lory. 1991. Interaction of *Pseudomonas aeruginosa* with A549 pneumocyte cells. Infection and Immunity 59:822-828.

55. Chiang, P. and L. L. Burrows. 2003. Biofilm formation by hyperpiliated mutants of *Pseudomonas aeruginosa*. Journal of Bacteriology 185:2374-2378.

56. Chun, C. K., E. A. Ozer, M. J. Welsh, J. Zabner, and E. P. Greenberg. 2004. Inactivation of a *Pseudomonas aeruginosa* quorum-sensing signal by human airway epithelia. Proceedings of the National Academy of Sciences of the United States of America 101:3587-3590.

57. Ciofu, O., B. Giwercman, N. Höiby, and S. Pedersen. 1994. Development of antibiotic resistance in *Pseudomonas aeruginosa* during two decades of antipseudomonal treatment at the Danish CF Center. APMIS 102:674-680.

58. Ciofu, O., L. F. Mandsberg, H. Wang, and N. Hoiby. 2012. Phenotypes selected during chronic lung infection in cystic fibrosis patients: implications for the treatment of *Pseudomonas aeruginosa* biofilm infections. FEMS Immunology & Medical Microbiology 65:215-225.

59. Ciornei, C. D., A. Novikov, C. Beloin, C. Fitting, M. Caroff, J. M. Ghigo, J. M. Cavaillon, and M. Adib-Conquy. 2010. Biofilm-forming *Pseudomonas aeruginosa* bacteria undergo lipopolysaccharide structural modifications and induce enhanced inflammatory cytokine response in human monocytes. Innate Immunity 16:288-301.

60. Clair, G., S. Roussi, J. Armengaud, and C. Duport. 2010. Expanding the known repertoire of virulence factors produced by *Bacillus cereus* through early secretome profiling in three redox conditions. Molecular & Cellular Proteomics 9:1486-1498.

61. Coburn, J. and D. W. Frank. 1999. Macrophages and epithelial cells respond differently to the *Pseudomonas aeruginosa* Type III secretion system. Infection and Immunity 67:3151-3154.

62. Comolli, J. C., A. R. Hauser, L. Waite, C. B. Whitchurch, J. S. Mattick, and J. N. Engel. 1999. *Pseudomonas aeruginosa* gene products *pilT* and *pilU* are required for cytotoxicity *in vitro* and virulence in a mouse model of acute pneumonia. Infection and Immunity 67:3625-3630.

63. Conrad, J., M. Gibiansky, F. Jin, V. Gordon, D. Motto, M. Mathewson, W. Stopka, D. Zelasko, J. Shrout, and G. Wong. 2011. Flagella and pili-mediated near-surface single-cell motility mechanisms in *P. aeruginosa*. Biophysical Journal 100:1608-1616.

64. Cory, T. J., S. E. Birket, B. S. Murphy, C. Mattingly, J. M. Breslow-Deckman, and D. J. Feola. 2013. Azithromycin increases in vitro fibronectin production through interactions between macrophages and fibroblasts stimulated with *Pseudomonas aeruginosa*. Journal of Antimicrobial Chemotherapy 68:840-851.

65. Costerton, J. W., P. S. Stewart, and E. P. Greenberg. 1999. Bacterial biofilms: A common cause of persistent infections. Science 284:1318-1322.

66. Cramer, N., L. Wiehlmann, O. Ciofu, S. Tamm, N. Høiby, and B. Tümmler. 2012. Molecular epidemiology of chronic *Pseudomonas aeruginosa* airway infections in cystic fibrosis. PLoS ONE 7:e50731.

67. Cystic Fibrosis Canada. Canadian CF Patient Data Registry - 2010 Report. 2012. Toronto, Ontario.

68. Cystic Fibrosis Canada. Canadian Cystic Fibrosis Registry - 2011 Annual Report. 2013. Toronto, ON, Cystic Fibrosis Canada.

69. D'Arezzo, S., S. Lanini, V. Puro, G. Ippolito, and P. Visca. 2012. High-level tolerance to triclosan may play a role in *Pseudomonas aeruginosa* antibiotic resistance in immunocompromised hosts: evidence from outbreak investigation. BMC Research Notes 5:43.

70. Dasgupta, P., W. Rizwani, S. Pillai, R. Kinkade, M. Kovacs, S. Rastogi, S. Banerjee, M. Carless, E. Kim, D. Coppola, E. Haura, and S. Chellappan. 2009. Nicotine induces cell proliferation, invasion and epithelial-mesenchymal transition in a variety of human cancer cell lines. International Journal of Cancer 124:36-45.

71. Davies, D. G., A. M. Chakrabarty, and G. G. Geesey. 1993. Exopolysaccharide production in biofilms: substratum activation of alginate gene expression by *Pseudomonas aeruginosa*. Applied and Environmental Microbiology 59:1181-1186.

72. Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science 280:295-298.

73. De Groote, V. N., N. Verstraeten, M. Fauvart, C. I. Kint, A. M. Verbeeck, S. Beullens, P. Cornelis, and J. Michiels. 2009. Novel persistence genes in *Pseudomonas aeruginosa* identified by high-throughput screening. FEMS Microbiology Letters 297:73-79.

74. De Kievit, T., e, Patrick C., Nez, J. on, Pas, L. dor, Igl, and B. H. ski. 1999. RsaL, a novel repressor of virulence gene expression in *Pseudomonas aeruginosa*. Journal of Bacteriology 181:2175-2184.

75. De Kievit, T. R., R. Gillis, S. Marx, C. Brown, and B. H. Iglewski. 2001. Quorum-sensing genes in *Pseudomonas aeruginosa* biofilms: Their role and expression patterns. Applied and Environmental Microbiology 67:1865-1873.

76. De Kievit, T. R. and B. H. Iglewski. 2000. Bacterial quorum sensing in pathogenic relationships. Infection and Immunity 68:4839-4849.

77. De Kievit, T. R., M. D. Parkins, R. J. Gillis, R. Srikumar, H. Ceri, K. Poole, B. H. Iglewski, and D. G. Storey. 2001. Multidrug efflux pumps: expression patterns and contribution to antibiotic resistance in *Pseudomonas aeruginosa* biofilms. Antimicrobial Agents and Chemotherapy 45:1761-1770.

78. de la Fuente-Nunez, C., V. Korolik, M. Bains, U. Nguyen, E. B. M. Breidenstein, S. Horsman, S. Lewenza, L. Burrows, and R. E. W. Hancock. 2012. Inhibition of bacterial biofilm formation and swarming motility by a small synthetic cationic peptide. Antimicrobial Agents and Chemotherapy 56:2696-2704.

79. de la Fuente-Núñez, C., S. C. Mansour, Z. Wang, L. Jiang, E. B. M. Breidenstein, M. Elliott, F. Reffuveille, D. P. Speert, S. L. Reckseidler-Zenteno, Y. Shen, M. Haapasalo, and R. E. W. Hancock. 2014. Anti-biofilm and immunomodulatory activities of peptides that inhibit

biofilms formed by pathogens isolated from cystic fibrosis patients. Antibiotics (Basel, Switzerland) 3:509-526.

80. **de la Fuente-Núñez, C., F. Reffuveille, E. F. Haney, S. K. Straus, and R. E. W. Hancock. 2014. Broad-spectrum anti-biofilm peptide that targets a cellular stress response. PLoS Pathogens 10:e1004152.**

81. **De, Y., Q. Chen, A. P. Schmidt, G. M. Anderson, J. M. Wang, J. Wooters, J. J. Oppenheim, and O. Chertov. 2000. LI-37, the neutrophil granule–and epithelial cell–derived cathelicidin, utilizes formyl peptide receptor–like 1 (Fprl1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. The Journal of Experimental Medicine 192:1069-1074.**

82. **Dean, S. N., B. M. Bishop, and M. L. van Hoek. 2011. Susceptibility of *Pseudomonas aeruginosa* biofilm to α-helical peptides: D-enantiomer of LL-37. Frontiers in Microbiology 2:128.**

83. **Demko, C. A., P. J. Byard, and P. B. Davis. 1995. Gender differences in cystic fibrosis: *Pseudomonas aeruginosa* infection. Journal of Clinical Epidemiology 48:1041-1049.**

84. **Denning, G. M., S. S. Iyer, K. J. Reszka, Y. Malley, G. T. Rasmussen, and B. E. Britigan. 2003. Phenazine-1-carboxylic acid, a secondary metabolite of *Pseudomonas aeruginosa*, alters expression of immunomodulatory proteins by human airway epithelial cells. American Journal of Physiology - Lung Cellular and Molecular Physiology 285:L584-L592.**

85. **Diamond, G., D. Legarda, and L. K. Ryan. 2000. The innate immune response of the respiratory epithelium. Immunological Reviews 173:27-38.**

86. **DiMango, E., H. J. Zar, R. Bryan, and A. Prince. 1995. Diverse *Pseudomonas aeruginosa* gene products stimulate respiratory epithelial cells to produce interleukin-8. The Journal of Clinical Investigation 96:2204-2210.**

87. **Dong, Y. H., X. F. Zhang, H. M. L. Soo, E. P. Greenberg, and L. H. Zhang. 2005. The two-component response regulator PprB modulates quorum-sensing signal production and global gene expression in *Pseudomonas aeruginosa*. Molecular Microbiology 56:1287-1301.**

88. **Döring, G. and N. Höiby. 1983. Longitudinal study of immune response to *Pseudomonas aeruginosa* antigens in cystic fibrosis. Infection and Immunity 42:197-201.**

89. **Dötsch, A., D. Eckweiler, M. Schniederjans, A. Zimmermann, V. Jensen, M. Scharfe, R. Geffers, and S. Häussler. 2012. The *Pseudomonas aeruginosa* transcriptome in planktonic cultures and static biofilms using RNA sequencing. PLoS ONE 7:e31092.**

90. **Drumm, M. L., A. G. Ziady, and P. B. Davis. 2012. Genetic variation and clinical heterogeneity in cystic fibrosis. Annual Review of Pathology: Mechanisms of Disease 7:267-282.**

91. **Dudley, M. N., J. Loutit, and D. C. Griffith. 2008. Aerosol antibiotics: considerations in pharmacological and clinical evaluation. Current Opinion in Biotechnology 19:637-643.**

92. **Dulon, S., D. Leduc, G. S. Cottrell, J. D'Alayer, K. K. Hansen, N. W. Bunnett, M. D. Hollenberg, D. Pidard, and M. Chignard. 2005. *Pseudomonas aeruginosa* elastase disables Proteinase-Activated Receptor 2 in respiratory epithelial cells. American Journal of Respiratory Cell and Molecular Biology 32:411-419.**

93. Engel, J. and Y. Eran. 2011. Subversion of mucosal barrier polarity by *Pseudomonas aeruginosa*. Frontiers in Microbiology 2:114.

94. Esen, M., H. Grassme, J. Riethmuller, A. Riehle, K. Fassbender, and E. Gulbins. 2001. Invasion of human epithelial cells by *Pseudomonas aeruginosa* involves Src-like tyrosine kinases p60Src and p59Fyn. Infection and Immunity 69:281-287.

95. Fernández, L., W. J. Gooderham, M. Bains, J. B. McPhee, I. Wiegand, and R. E. W. Hancock. 2010. Adaptive resistance to the "last hope" antibiotics polymyxin B and colistin in *Pseudomonas aeruginosa* is mediated by the novel two-component regulatory system ParR-ParS. Antimicrobial Agents and Chemotherapy 54:3372-3382.

96. Fleiszig, S. M., D. J. Evans, N. Do, V. Vallas, S. Shin, and K. E. Mostov. 1997. Epithelial cell polarity affects susceptibility to Pseudomonas aeruginosa invasion and cytotoxicity. Infection and Immunity 65:2861-2867.

97. Fleiszig, S. M., T. S. Zaidi, M. J. Preston, M. Grout, D. J. Evans, and G. B. Pier. 1996. Relationship between cytotoxicity and corneal epithelial cell invasion by clinical isolates of *Pseudomonas aeruginosa*. Infection and Immunity 64:2288-2294.

98. Flume, P. A., P. J. Mogayzel, K. A. Robinson, C. H. Goss, R. L. Rosenblatt, R. J. Kuhn, and B. C. Marshall. 2009. Cystic Fibrosis Pulmonary Guidelines. American Journal of Respiratory and Critical Care Medicine 180:802-808.

99. Folsom, J., L. Richards, B. Pitts, F. Roe, G. Ehrlich, A. Parker, A. I. Mazurie, and P. Stewart. 2010. Physiology of *Pseudomonas aeruginosa* in biofilms as revealed by transcriptome analysis. BMC Microbiology 10:1-17.

100. Frisch, S. M. and H. Francis. 1994. Disruption of epithelial cell-matrix interactions induces apoptosis. The Journal of Cell Biology 124:619-626.

101. Fujita, M., K. Tanaka, H. Takahashi, and A. Amemural. 1994. Transcription of the principal sigma-factor genes, rpoD and rpoS, in *Pseudomonas aeruginosa* is controlled according to the growth phase. Molecular Microbiology 13:1071-1077.

102. Fujitani, S., H. Y. Sun, V. L. Yu, and J. A. Weingarten. 2011. Pneumonia due to *Pseudomonas aeruginosa*. Chest 139:909-919.

103. Fung, C., S. Naughton, L. Turnbull, P. Tingpej, B. Rose, J. Arthur, H. Hu, C. Harmer, C. Harbour, and D. J. Hassett. 2010. Gene expression of *Pseudomonas aeruginosa* in a mucin-containing synthetic growth medium mimicking cystic fibrosis lung sputum. Journal of Medical Microbiology 59.

104. Furukawa, S., S. L. Kuchma, and G. A. O'Toole. 2006. Keeping their options open: acute versus persistent infections. Journal of Bacteriology 188:1211-1217.

105. Fux, C. A., J. W. Costerton, P. S. Stewart, and P. Stoodley. 2005. Survival strategies of infectious biofilms. Trends in Microbiology 13:34-40.

106. Galarneau, A., M. Primeau, L. E. Trudeau, and S. W. Michnick. 2002. β-Lactamase protein fragment complementation assays as *in vivo* and *in vitro* sensors of protein-protein interactions. Nature Biotechnology 20:619-622.

107. Gellatly, S. L., B. Needham, L. Madera, M. S. Trent, and R. E. W. Hancock. 2012. The *Pseudomonas aeruginosa* PhoP-PhoQ two-component regulatory system is induced upon

interaction with epithelial cells and controls cytotoxicity and inflammation. Infection and Immunity 80:3122-3131.

108. Geller, D. E. 2009. Aerosol antibiotics in cystic fibrosis. Respiratory Care 54:658-670.

109. Giard, D. J., S. A. Aaronson, G. J. Todaro, P. Arnstein, J. H. Kersey, H. Dosik, and W. P. Parks. 1973. *In vitro* cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. Journal of the National Cancer Institute 51:1417-1423.

110. Gilois, N., N. Ramarao, L. Bouillaut, S. Perchat, S. Aymerich, C. Nielsen-LeRoux, D. Lereclus, and M. Gohar. 2007. Growth-related variations in the *Bacillus cereus* secretome. PROTEOMICS 7:1719-1728.

111. Girón Moreno, R. M., A. Salcedo Posadas, and R. Mar Gómez-Punter. 2011. Inhaled antibiotic therapy in cystic fibrosis. Archivos de Bronconeumología 47, Supplement 6:14-18.

112. Giwercman, B., C. Meyer, P. A. Lambert, C. Reinert, and N. Høiby. 1992. High-level β-lactamase activity in sputum samples from cystic fibrosis patients during antipseudomonal treatment. Antimicrobial Agents and Chemotherapy 36:71-76.

113. Gooderham, W. J., M. Bains, J. B. McPhee, I. Wiegand, and R. E. W. Hancock. 2008. Induction by cationic antimicrobial peptides and involvement in intrinsic polymyxin and antimicrobial peptide resistance, biofilm formation, and swarming motility of PsrA in *Pseudomonas aeruginosa*. Journal of Bacteriology 190:5624-5634.

114. Gooderham, W. J. and R. E. W. Hancock. 2009. Regulation of virulence and antibiotic resistance by two-component regulatory systems in *Pseudomonas aeruginosa*. FEMS Microbiology Reviews 33:279-294.

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

116. Govan, J. R. W. and J. W. Nelson. 1992. Microbiology of lung infection in cystic fibrosis. British Medical Bulletin 48:912-930.

117. Grubb, S. E. W., C. Murdoch, P. E. Sudbery, S. P. Saville, J. L. Lopez-Ribot, and M. H. Thornhill. 2009. Adhesion of *Candida albicans* to endothelial cells under physiological conditions of flow. Infection and Immunity 77:3872-3878.

118. Guggenheim, B., R. Gmør, J. C. Galicia, P. G. Stathopoulou, M. R. Benakanakere, A. Meier, T. Thurnheer, and D. F. Kinane. 2009. *In vitro* modeling of host-parasite interactions: the 'subgingival' biofilm challenge of primary human epithelial cells. BMC Microbiology 9:280.

119. Guillot, L., S. Medjane, K. Le-Barillec, V. Balloy, C. Danel, M. Chignard, and M. Si-Tahar. 2004. Response of human pulmonary epithelial cells to lipopolysaccharide involves Toll-like Receptor 4 (TLR4)-dependent signaling pathways: evidence for an intracellular compartmentalization of TLR4. Journal of Biological Chemistry 279:2712-2718.

120. Hagimoto, N., K. Kuwano, M. Kawasaki, M. Yoshimi, Y. Kaneko, R. Kunitake, T. Maeyama, T. Tanaka, and N. Hara. 1999. Induction of interleukin-8 secretion and apoptosis in bronchiolar epithelial cells by Fas ligation. American journal of respiratory cell and molecular biology 21:436-445.

121. Hajjar, A. M., R. K. Ernst, J. H. Tsai, C. B. Wilson, and S. I. Miller. 2002. Human Toll-like receptor 4 recognizes host-specific LPS modifications. Nature immunology 3:354-359.

122. Hancock, R. E., L. M. Mutharia, L. Chan, R. P. Darveau, D. P. Speert, and G. B. Pier. 1983. *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis: a class of serum-sensitive, nontypable strains deficient in lipopolysaccharide O side chains. Infection and Immunity 42:170-177.

123. Hancock, R. E. W. and D. P. Speert. 2000. Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and impact on treatment. Drug resistance updates : reviews and commentaries in antimicrobial and anticancer chemotherapy 3:247-255.

124. Hare, N. J., N. Solis, C. Harmer, N. B. Marzook, B. Rose, C. Harbour, B. Crossett, J. Manos, and S. J. Cordwell. 2012. Proteomic profiling of *Pseudomonas aeruginosa* AES-1R, PAO1 and PA14 reveals potential virulence determinants associated with a transmissible cystic fibrosis-associated strain. BMC Microbiology 12:1-14.

125. Harrison, J. J., R. J. Turner, and H. Ceri. 2005. Persister cells, the biofilm matrix and tolerance to metal cations in biofilm and planktonic *Pseudomonas aeruginosa*. Environmental Microbiology 7:981-994.

126. Harrison, J., H. Ceri, J. Yerly, C. Stremick, Y. Hu, R. Martinuzzi, and R. Turner. 2006. The use of microscopy and three-dimensional visualization to evaluate the structure of microbial biofilms cultivated in the calgary biofilm device. Biological Procedures Online 8:194-215.

127. Hawdon, N. A., P. S. Aval, R. J. Barnes, S. K. Gravelle, J. Rosengren, S. Khan, O. Ciofu, H. K. Johansen, N. Hoiby, and M. Ulanova. 2010. Cellular responses of A549 alveolar epithelial cells to serially collected *Pseudomonas aeruginosa* from cystic fibrosis patients at different stages of pulmonary infection. FEMS Immunology & Medical Microbiology 59:207-220.

128. Hayashi, N., M. Matsukawa, Y. Horinishi, K. Nakai, A. Shoji, Y. Yoneko, N. Yoshida, S. Minagawa, and N. Gotoh. 2013. Interplay of flagellar motility and mucin degradation stimulates the association of *Pseudomonas aeruginosa* with human epithelial colorectal adenocarcinoma (Caco-2) cells. Journal of Infection and Chemotherapy 19:305-315.

129. Heilborn, J. D., M. F. Nilsson, G. Kratz, G. Weber, O. Sørensen, N. Borregaard, and M. Ståhle-Bäckdahl. 2003. The cathelicidin anti-microbial peptide LL-37 is involved in re-epithelialization of human skin wounds and is lacking in chronic ulcer epithelium. Journal of Investigative Dermatology 120:379-389.

130. Henry, R. L., C. M. Mellis, and L. Petrovic. 1992. Mucoid *Pseudomonas aeruginosa* is a marker of poor survival in cystic fibrosis. Pediatric pulmonology 12:158-161.

131. Heydorn, A., A. T. Nielsen, M. Hentzer, C. Sternberg, M. Givskov, B. K. Ersbøll, and S. Molin. 2000. Quantification of biofilm structures by the novel computer program COMSTAT. Microbiology 146 (Pt 10):2395-2407.

132. Heydorn, A., B. Ersbøll, J. Kato, M. Hentzer, M. R. Parsek, T. Tolker-Nielsen, M. Givskov, and S. Molin. 2002. Statistical analysis of *Pseudomonas aeruginosa* biofilm development: impact of mutations in genes involved in twitching motility, cell-to-cell signaling, and stationary-phase sigma factor expression. Applied and Environmental Microbiology 68:2008-2017.

133. Hoffmann, N., B. Lee, M. Hentzer, T. B. Rasmussen, Z. Song, H. K. Johansen, M. Givskov, and N. Høiby. 2007. Azithromycin blocks quorum sensing and alginate polymer formation and increases the sensitivity to serum and stationary-growth-phase killing of *Pseudomonas aeruginosa* and attenuates chronic *P. aeruginosa* lung infection in Cftr–/– mice. Antimicrobial Agents and Chemotherapy 51:3677-3687.

134. Høiby, N. 1993. Antibiotic therapy for chronic infection of *Pseudomonas* in the lung. Annual Review of Medicine 44:1-10.

135. Höiby, N., O. Ciofu, and T. Bjarnsholt. 2010. *Pseudomonas aeruginosa* biofilms in cystic fibrosis. Future Microbiology 5:1663-1674.

136. Høiby, N., H. Krogh Johansen, C. Moser, Z. Song, O. Ciofu, and A. Kharazmi. 2001. *Pseudomonas aeruginosa* and the *in vitro* and *in vivo* biofilm mode of growth. Microbes and Infection 3:23-35.

137. Høiby, N. and S. Olling. 1977. *Pseudomonas aeruginosa* infection in cystic fibrosis. Acta Pathologica Microbiologica Scandinavica Section C Immunology 85C:107-114.

138. Holloway, B. W., V. Krishnapillai, and A. F. Morgan. 1979. Chromosomal genetics of *Pseudomonas.* Microbiological Reviews 43:73-102.

139. Hollsing, A. E., M. Granström, M. L. Vasil, B. Wretlind, and B. Strandvik. 1987. Prospective study of serum antibodies to *Pseudomonas aeruginosa* exoproteins in cystic fibrosis. Journal of Clinical Microbiology 25:1868-1874.

140. Hong, W., R. A. Juneau, B. Pang, and W. E. Swords. 2009. Survival of bacterial biofilms within neutrophil extracellular traps promotes nontypeable *Haemophilus influenzae* persistence in the chinchilla model for otitis media. Journal of Innate Immunity 1:215-224.

141. Horsman, S. R., R. A. Moore, and S. Lewenza. 2012. Calcium chelation by alginate activates the Type III Secretion System in mucoid *Pseudomonas aeruginosa* biofilms. PLoS ONE 7:e46826.

142. Ibrahim, B. M., M. D. Tsifansky, Y. Yang, and Y. Yeo. 2011. Challenges and advances in the development of inhalable drug formulations for cystic fibrosis lung disease. Expert Opinion on Drug Delivery 8:451-466.

143. Iimura, M., R. L. Gallo, K. Hase, Y. Miyamoto, L. Eckmann, and M. F. Kagnoff. 2005. Cathelicidin mediates innate intestinal defense against colonization with epithelial adherent bacterial pathogens. The Journal of Immunology 174:4901-4907.

144. Jackson, K. D., M. Starkey, S. Kremer, M. R. Parsek, and D. J. Wozniak. 2004. Identification of psl, a locus encoding a potential exopolysaccharide that is essential for *Pseudomonas aeruginosa* PAO1 biofilm formation. Journal of Bacteriology 186:4466-4475.

145. Jacobsen, F., C. Fisahn, M. Sorkin, I. Thiele, T. Hirsch, I. Stricker, T. Klaasen, A. Roemer, B. Fugmann, and L. Steinstraesser. 2011. Efficacy of topical delivered Moxifloxacin against MRSA and *Pseudomonas aeruginosa* wound infection. Antimicrobial Agents and Chemotherapy.

146. Jagger, K. S., D. R. Bahner, and R. L. Warren. 1983. Protease phenotypes of *Pseudomonas aeruginosa* isolated from patients with cystic fibrosis. Journal of Clinical Microbiology 17:55-59.

147. Jagger, K. S., D. L. Robinson, M. N. Franz, and R. L. Warren. 1982. Detection by enzyme-linked immunosorbent assays of antibody specific for *Pseudomonas* proteases and exotoxin A in sera from cystic fibrosis patients. Journal of Clinical Microbiology 15:1054-1058.

148. Jalal, S., O. Ciofu, N. Høiby, N. Gotoh, and B. Wretlind. 2000. Molecular mechanisms of fluoroquinolone resistance in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. Antimicrobial Agents and Chemotherapy 44.

149. Johansson, J., G. H. Gudmundsson, M. E. Rottenberg, K. D. Berndt, and B. Agerberth. 1998. Conformation-dependent antibacterial activity of the naturally occurring human peptide LL-37. Journal of Biological Chemistry 273:3718-3724.

150. Jyot, J., V. Balloy, G. Jouvion, A. Verma, L. Touqui, M. Huerre, M. Chignard, and R. Ramphal. 2011. Type II Secretion System of *Pseudomonas aeruginosa*: *in vivo* evidence of a significant role in death due to lung infection. Journal of Infectious Diseases 203:1369-1377.

151. Kalluri, R. and E. G. Neilson. 2003. Epithelial-mesenchymal transition and its implications for fibrosis. The Journal of Clinical Investigation 112:1776-1784.

152. Kanthakumar, K., G. W. Taylor, D. R. Cundell, R. B. Dowling, M. Johnson, P. J. Cole, and R. Wilson. 1996. The effect of bacterial toxins on levels of intracellular adenosine nucleotides and human ciliary beat frequency. Pulmonary Pharmacology 9:223-230.

153. Kasai, H., J. Allen, R. Mason, T. Kamimura, and Z. Zhang. 2005. TGF-β1 induces human alveolar epithelial to mesenchymal cell transition (EMT). Respiratory Research 6:56.

154. Kim, J., M. Hegde, and A. Jayaraman. 2010. Co-culture of epithelial cells and bacteria for investigating host-pathogen interactions. Lab on a Chip 10:43-50.

155. King, A., K. Shannon, and I. Phillips. 1980. *In vitro* antibacterial activity and susceptibility of cefsulodin, an antipseudomonas cephalosporin, to β-lactamases. Antimicrobial Agents and Chemotherapy 17.

156. Kipnis, E., T. Sawa, and J. Wiener-Kronish. 2006. Targeting mechanisms of *Pseudomonas aeruginosa* pathogenesis. Medecine et maladies infectieuses 36:78-91.

157. Kirchner, S., J. L. Fothergill, E. A. Wright, C. E. James, E. Mowat, and C. Winstanley. 2012. Use of artificial sputum medium to test antibiotic efficacy against *Pseudomonas aeruginosa* in conditions more relevant to the cystic fibrosis lung. Journal of Visualized Experimentse3857.

158. Kirketerp-Møller, K., P. Jensen, M. Fazli, K. G. Madsen, J. Pedersen, C. Moser, T. Tolker-Nielsen, N. Høiby, M. Givskov, and T. Bjarnsholt. 2008. Distribution, organization, and ecology of bacteria in chronic wounds. Journal of clinical microbiology 46:2717-2722.

159. Kirov, S. M., J. S. Webb, C. Y. O'May, D. W. Reid, J. K. K. Woo, S. A. Rice, and S. Kjelleberg. 2007. Biofilm differentiation and dispersal in mucoid *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. Microbiology 153:3264-3274.

160. Klausen, M., A. Aaes-Jorgensen, S. Molin, and T. Tolker-Nielsen. 2003. Involvement of bacterial migration in the development of complex multicellular structures in *Pseudomonas aeruginosa* biofilms. Molecular Microbiology 50:61-68.

161. **Klausen, M., A. Heydorn, P. Ragas, L. Lambertsen, A. Aaes-Jorgensen, S. Molin, and T. Tolker-Nielsen. 2003. Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. Molecular Microbiology 48:1511-1524.**

162. **Klinger, J. D., D. C. Straus, C. B. Hilton, and J. A. Bass. 1978. Antibodies to proteases and Exotoxin A of *Pseudomonas aeruginosa* in patients withcystic fibrosis: demonstration by radioimmunoassay. Journal of Infectious Diseases 138:49-58.**

163. **Kojic, M., C. Aguilar, and V. Venturi. 2002. TetR family member PsrA directly binds the *Pseudomonas* rpoS and psrA promoters. Journal of Bacteriology 184:2324-2330.**

164. **Kojic, M., B. Jovcic, A. Vindigni, F. Odreman, and V. Venturi. 2005. Novel target genes of PsrA transcriptional regulator of *Pseudomonas aeruginosa*. FEMS Microbiology Letters 246:175-181.**

165. **Kon, Y., H. Tsukada, T. Hasegawa, K. Igarashi, K. Wada, E. Suzuki, M. Arakawa, and F. Gejyo. 1999. The role of *Pseudomonas aeruginosa* elastase as a potent inflammatory factor in a rat air pouch inflammation model. FEMS Immunology & Medical Microbiology 25:313-321.**

166. **Koyama, S., E. Sato, H. Nomura, K. Kubo, M. Miura, T. Yamashita, S. Nagai, and T. Izumi. 1999. The potential of various lipopolysaccharides to release monocyte chemotactic activity from lung epithelial cells and fibroblasts. European Respiratory Journal 14:545-552.**

167. **Krieg, D. P., R. J. Helmke, V. F. German, and J. A. Mangos. 1988. Resistance of mucoid *Pseudomonas aeruginosa* to nonopsonic phagocytosis by alveolar macrophages *in vitro*. Infection and Immunity 56:3173-3179.**

168. **Kronborg, G., M. B. Hansen, M. Svenson, A. Fomsgaard, N. Hsiby, and K. Bendtzen. 1993. Cytokines in sputum and serum from patients with cystic fibrosis and chronic *Pseudomonas aeruginosa* infection as markers of destructive inflammation in the lungs. Pediatric pulmonology 15:292-297.**

169. **Kuang, Z., Y. Hao, B. E. Walling, J. L. Jeffries, D. E. Ohman, and G. W. Lau. 2011. *Pseudomonas aeruginosa* elastase provides an escape from phagocytosis by degrading the ulmonary Surfactant Protein-A. PLoS ONE 6:e27091.**

170. **LaFayette, S. L., D. Houle, T. Beaudoin, G. Wojewodka, D. Radzioch, L. R. Hoffman, J. L. Burns, A. A. Dandekar, N. E. Smalley, J. R. Chandler, J. E. Zlosnik, D. P. Speert, J. Bernier, E. Matouk, E. Brochiero, S. Rousseau, and D. Nguyen. 2015. Cystic fibrosis–adapted *Pseudomonas aeruginosa q*uorum sensing lasR mutants cause hyperinflammatory responses. Science Advances 1.**

171. **Lamont, I. L., P. A. Beare, U. Ochsner, A. I. Vasil, and M. L. Vasil. 2002. Siderophore-mediated signaling regulates virulence factor production in *Pseudomonas aeruginosa*. Proceedings of the National Academy of Sciences 99:7072-7077.**

172. **Lau, G. W., D. J. Hassett, H. Ran, and F. Kong. 2004. The role of pyocyanin in *Pseudomonas aeruginosa* infection. Trends in Molecular Medicine 10:599-606.**

173. **Lawrence, J. R., D. R. Korber, B. D. Hoyle, J. W. Costerton, and D. E. Caldwell. 1991. Optical sectioning of microbial biofilms. Journal of Bacteriology 173:6558-6567.**

174. Lazazzera, B. A. 2005. Lessons from DNA microarray analysis: the gene expression profile of biofilms. Current Opinion in Microbiology 8:222-227.

175. Leduc, D., N. Beaufort, S. de Bentzmann, J. C. Rousselle, A. Namane, M. Chignard, and D. Pidard. 2007. The *Pseudomonas aeruginosa* LasB metalloproteinase regulates the human urokinase-type plasminogen activator receptor through domain-specific endoproteolysis. Infection and Immunity 75:3848-3858.

176. Lee, T. W. R., K. G. Brownlee, S. P. Conway, M. Denton, and J. M. Littlewood. 2003. Evaluation of a new definition for chronic *Pseudomonas aeruginosa* infection in cystic fibrosis patients. Journal of Cystic Fibrosis 2:29-34.

177. Lee, V. T., R. S. Smith, B. Tümmler, and S. Lory. 2005. Activities of *Pseudomonas aeruginosa* effectors secreted by the Type III Secretion System *in vitro* and during infection. Infection and Immunity 73:1695-1705.

178. Leid, J. G., C. J. Willson, M. E. Shirtliff, D. J. Hassett, M. R. Parsek, and A. K. Jeffers. 2005. The exopolysaccharide alginate protects *Pseudomonas aeruginosa* biofilm bacteria from IFN-γ-mediated macrophage killing. The Journal of Immunology 175:7512-7518.

179. Leidal, K. G., K. L. Munson, M. C. Johnson, and G. M. Denning. 2003. Metalloproteases from *Pseudomonas aeruginosa* Degrade Human RANTES, MCP-1, and ENA-78. Journal of Interferon & Cytokine Research 23:307-318.

180. Lenz, A. P., K. S. Williamson, B. Pitts, P. S. Stewart, and M. J. Franklin. 2008. Localized gene expression in *Pseudomonas aeruginosa* biofilms. Applied and Environmental Microbiology 74:4463-4471.

181. Lewenza, S. 2013. Extracellular DNA-induced antimicrobial peptide resistance mechanisms in *Pseudomonas aeruginosa*. Frontiers in microbiology 4:21.

182. Li, Z. 2005. Longitudinal development of mucoid *Pseudomonas aeruginosa* infection and lung disease progression in children with cystic fibrosis. JAMA 293:581-588.

183. Liu, G., X. Meng, Y. Jin, J. Bai, Y. Zhao, X. Cui, F. Chen, and S. Fu. 2008. Inhibitory role of focal adhesion kinase on anoikis in the lung cancer cell A549. Cell Biology International 32:663-670.

184. Llamas, M. a. A., A. van der Sar, B. C. H. Chu, M. Sparrius, H. J. Vogel, and W. Bitter. 2009. A novel extracytoplasmic function (ECF) sigma factor regulates virulence in *Pseudomonas aeruginosa*. PLoS Pathogens 5:e1000572.

185. Lomovskaya, O., M. S. Warren, A. Lee, J. Galazzo, R. Fronko, M. Lee, J. Blais, D. Cho, S. Chamberland, T. Renau, R. Leger, S. Hecker, W. Watkins, K. Hoshino, H. Ishida, and V. J. Lee. 2001. Identification and characterization of inhibitors of multidrug resistance efflux pumps in *Pseudomonas aeruginosa*: novel agents for combination therapy. Antimicrobial Agents and Chemotherapy 45:105-116.

186. Ma, L., M. Conover, H. Lu, M. R. Parsek, K. Bayles, and D. J. Wozniak. 2009. Assembly and development of the *Pseudomonas aeruginosa* biofilm matrix. PLoS Pathogens 5:e1000354.

187. MacDonald, K., K. McKenzie, and P. Zeitlin. 2007. Cystic fibrosis transmembrane regulator protein mutations. Pediatric-Drugs 9:1-10.

188. Macfarlane, E. L. A., A. Kwasnicka, and R. E. W. Hancock. 2000. Role of *Pseudomonas aeruginosa* PhoP-PhoQ in resistance to antimicrobial cationic peptides and aminoglycosides. Microbiology 146:2543-2554.

189. Marchiando, A. M., W. V. Graham, and J. R. Turner. 2010. Epithelial barriers in homeostasis and disease. Annual Review of Pathology: Mechanisms of Disease 5:119-144.

190. Mariencheck, W. I., J. F. Alcorn, S. M. Palmer, and J. R. Wright. 2003. P*seudomonas aeruginosa* elastase degrades surfactant Proteins A and D. American Journal of Respiratory Cell and Molecular Biology 28:528-537.

191. Marrie, T. J., J. Nelligan, and J. W. Costerton. 1982. A scanning and transmission electron microscopic study of an infected endocardial pacemaker lead. Circulation 66:1339-1341.

192. Martin, C., G. Thévenot, S. Danel, J. Chapron, A. Tazi, J. Macey, D. J. Dusser, I. Fajac, and P. R. Burgel. 2011. *Pseudomonas aeruginosa* induces vascular endothelial growth factor synthesis in airway epithelium *in vitro* and *in vivo*. European Respiratory Journal 38:939-946.

193. Martin, D. W., M. J. Schurr, M. H. Mudd, J. R. Govan, B. W. Holloway, and V. Deretic. 1993. Mechanism of conversion to mucoidy in *Pseudomonas aeruginosa* infecting cystic fibrosis patients. Proceedings of the National Academy of Sciences 90:8377-8381.

194. Mascher, T., J. D. Helmann, and G. Unden. 2006. Stimulus perception in bacterial signal-transducing histidine kinases. Microbiology and Molecular Biology Reviews 70:910-938.

195. Masuda, N. and S. Ohya. 1992. Cross-resistance to meropenem, cephems, and quinolones in *Pseudomonas aeruginosa*. Antimicrobial Agents and Chemotherapy 36:1847-1851.

196. Masuda, N., E. Sakagawa, S. Ohya, N. Gotoh, H. Tsujimoto, and T. Nishino. 2000. Contribution of the MexX-MexY-OprM efflux system to intrinsic resistance in *Pseudomonas aeruginosa*. Antimicrobial Agents and Chemotherapy 44:2242-2246.

197. McIver, K., E. Kessler, and D. E. Ohman. 1991. Substitution of active-site His-223 in *Pseudomonas aeruginosa* elastase and expression of the mutated lasB alleles in *Escherichia coli* show evidence for autoproteolytic processing of proelastase. Journal of Bacteriology 173:7781-7789.

198. McIver, K. S., E. Kessler, and D. E. Ohman. 2004. Identification of residues in the *Pseudomonas aeruginosa* elastase propeptide required for chaperone and secretion activities. Microbiology 150:3969-3977.

199. McLean, R. J. C., M. Whiteley, D. J. Stickler, and W. C. Fuqua. 1997. Evidence of autoinducer activity in naturally occurring biofilms. FEMS Microbiology Letters 154:259-263.

200. Merz, A. J., M. So, and M. P. Sheetz. 2000. Pilus retraction powers bacterial twitching motility. Nature 407:98-102.

201. Meshulam, T., N. Obedeanu, D. Merzbach, and J. D. Sobel. 1984. Phagocytosis of mucoid and nonmucoid strains of *Pseudomonas aeruginosa*. Clinical Immunology and Immunopathology 32:151-165.

202. Mikkelsen, H., N. J. Bond, M. E. Skindersoe, M. Givskov, K. S. Lilley, and M. Welch. 2009. Biofilms and type III secretion are not mutually exclusive in *Pseudomonas aeruginosa*. Microbiology 155:687-698.

203. Mikkelsen, H., Z. Duck, K. S. Lilley, and M. Welch. 2007. Interrelationships between colonies, biofilms, and planktonic Cells of *Pseudomonas aeruginosa*. Journal of Bacteriology 189:2411-2416.

204. Mikkelsen, H., M. Sivaneson, and A. Filloux. 2011. Key two-component regulatory systems that control biofilm formation in *Pseudomonas aeruginosa*. Environmental Microbiology 13:1666-1681.

205. Möker, N., C. R. Dean, and J. Tao. 2010. *Pseudomonas aeruginosa* increases formation of multidrug-tolerant persister cells in response to quorum-sensing signaling molecules. Journal of Bacteriology 192:1946-1955.

206. Mookherjee, N., K. L. Brown, D. M. E. Bowdish, S. Doria, R. Falsafi, K. Hokamp, F. M. Roche, R. Mu, G. H. Doho, J. Pistolic, J. P. Powers, J. Bryan, F. S. L. Brinkman, and R. E. W. Hancock. 2006. Modulation of the TLR-mediated inflammatory response by the endogenous human host defense peptide LL-37. The Journal of Immunology 176:2455-2464.

207. Moreau-Marquis, S., J. M. Bomberger, G. G. Anderson, A. Swiatecka-Urban, S. Ye, G. A. O'Toole, and B. A. Stanton. 2008. The ΔF508-CFTR mutation results in increased biofilm formation by *Pseudomonas aeruginosa* by increasing iron availability. American Journal of Physiology - Lung Cellular and Molecular Physiology 295:L25-L37.

208. Moreau-Marquis, S., C. V. Redelman, B. A. Stanton, and G. G. Anderson. 2010. Co-culture models of *Pseudomonas aeruginosa* biofilms grown on live human airway cells. Journal of Visualized Experimentse2186.

209. Moreau-Marquis, S., B. A. Stanton, and G. A. O'Toole. 2008. *Pseudomonas aeruginosa* biofilm formation in the cystic fibrosis airway. Pulmonary Pharmacology & Therapeutics 21:595-599.

210. Morgan, W. J., S. M. Butler, C. A. Johnson, A. A. Colin, S. C. FitzSimmons, D. E. Geller, M. W. Konstan, M. J. Light, H. R. Rabin, W. E. Regelmann, D. V. Schidlow, D. C. Stokes, M. E. Wohl, H. Kaplowitz, and M. M. Wyatt. 1999. Epidemiologic study of cystic fibrosis: Design and implementation of a prospective, multicenter, observational study of patients with cystic fibrosis in the U.S. and Canada. Pediatric Pulmonology 28:231-241.

211. Moskowitz, S. M., R. K. Ernst, and S. I. Miller. 2004. PmrAB, a two-component regulatory system of *Pseudomonas aeruginosa* that modulates resistance to cationic antimicrobial peptides and addition of aminoarabinose to Lipid A. Journal of Bacteriology 186:575-579.

212. Muhlebach, M. S., W. Reed, and T. L. Noah. 2004. Quantitative cytokine gene expression in CF airway. Pediatric pulmonology 37:393-399.

213. Mulcahy, H., L. Charron-Mazenod, and S. Lewenza. 2008. Extracellular DNA chelates cations and induces antibiotic resistance in *Pseudomonas aeruginosa* biofilms. PLoS Pathogen 4:e1000213.

214. Muller, C., P. Plésiat, and K. Jeannot. 2011. A two-component regulatory system interconnects resistance to polymyxins, aminoglycosides, fluoroquinolones, and β-

Lactams in *Pseudomonas aeruginosa*. Antimicrobial Agents and Chemotherapy 55:1211-1221.

215. Murakami, K., T. Ono, D. Viducic, S. Kayama, M. Mori, K. Hirota, K. Nemoto, and Y. Miyake. 2005. Role for rpoS gene of *Pseudomonas aeruginosa* in antibiotic tolerance. FEMS Microbiology Letters 242:161-167.

216. Müsken, M., S. Di Fiore, A. Dötsch, R. Fischer, and S. Häussler. 2010. Genetic determinants of *Pseudomonas aeruginosa* biofilm establishment. Microbiology 156:431-441.

217. Nagant, C., B. Pitts, K. Nazmi, M. Vandenbranden, J. G. Bolscher, P. S. Stewart, and J. P. Dehaye. 2012. Identification of peptides derived from the human antimicrobial peptide LL-37 active against biofilms formed by *Pseudomonas aeruginosa* using a library of truncated fragments. Antimicrobial Agents and Chemotherapy 56:5698-5708.

218. Naughton, S., D. Parker, T. Seemann, T. Thomas, L. Turnbull, B. Rose, P. Bye, S. Cordwell, C. Whitchurch, and J. Manos. 2011. *Pseudomonas aeruginosa* AES-1 exhibits increased virulence gene expression during chronic infection. PLoS ONE 6.

219. Ngamwongsatit, P., P. P. Banada, W. Panbangred, and A. K. Bhunia. 2008. WST-1-based cell cytotoxicity assay as a substitute for MTT-based assay for rapid detection of toxigenic *Bacillus* species using CHO cell line. Journal of Microbiological Methods 73:211-215.

220. Nichols, W. W., S. M. Dorrington, M. P. Slack, and H. L. Walmsley. 1988. Inhibition of tobramycin diffusion by binding to alginate. Antimicrobial Agents and Chemotherapy 32:518-523.

221. Nivens, D. E., D. E. Ohman, J. Williams, and M. J. Franklin. 2001. Role of alginate and Its O acetylation in formation of *Pseudomonas aeruginosa* microcolonies and biofilms. Journal of Bacteriology 183:1047-1057.

222. Nizet, V., T. Ohtake, X. Lauth, J. Trowbridge, J. Rudisill, R. A. Dorschner, V. Pestonjamasp, J. Piraino, K. Huttner, and R. L. Gallo. 2001. Innate antimicrobial peptide protects the skin from invasive bacterial infection. Nature 414:454-457.

223. Nouda, H., E. T. Harabe, Y. Sumita, T. Okuda, and M. Fukasawa. 1992. β-lactamase stability and inhibitory activity of meropenem combined with a potent antibacterial activity. Chemotherapy 38:218-224.

224. Nouwens, A. S., S. A. Beatson, C. B. Whitchurch, B. J. Walsh, H. P. Schweizer, J. S. Mattick, and S. J. Cordwell. 2003. Proteome analysis of extracellular proteins regulated by the las and rhl quorum sensing systems in *Pseudomonas aeruginosa* PAO1. Microbiology 149:1311-1322.

225. Nouwens, A. S., M. D. P. Willcox, B. J. Walsh, and S. J. Cordwell. 2002. Proteomic comparison of membrane and extracellular proteins from invasive (PAO1) and cytotoxic (6206) strains of *Pseudomonas aeruginosa*. PROTEOMICS 2:1325-1346.

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

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

228. Oermann, C. M., G. Z. Retsch-Bogart, A. L. Quittner, R. L. Gibson, K. S. McCoy, A. B. Montgomery, and P. J. Cooper. 2010. An 18-month study of the safety and efficacy of repeated courses of inhaled aztreonam lysine in cystic fibrosis. Pediatric pulmonology 45:1121-1134.

229. Oldak, E. and E. A. Trafny. 2005. Secretion of proteases by *Pseudomonas aeruginosa* biofilms exposed to ciprofloxacin. Antimicrobial Agents and Chemotherapy 49:3281-3288.

230. Ong, P. Y., T. Ohtake, C. Brandt, I. Strickland, M. Boguniewicz, T. Ganz, R. L. Gallo, and D. Y. M. Leung. 2002. Endogenous antimicrobial peptides and skin infections in atopic dermatitis. New England Journal of Medicine 347:1151-1160.

231. Overhage, J., M. Bains, M. D. Brazas, and R. E. W. Hancock. 2008. Swarming of *Pseudomonas aeruginosa* is a complex adaptation leading to increased production of virulence factors and antibiotic resistance. Journal of Bacteriology 190:2671-2679.

232. Overhage, J., A. Campisano, M. Bains, E. C. W. Torfs, B. H. A. Rehm, and R. E. W. Hancock. 2008. Human host defense peptide LL-37 prevents bacterial biofilm formation. Infection and Immunity 76:4176-4182.

233. Ozer, E. A., A. Pezzulo, D. M. Shih, C. Chun, C. Furlong, A. J. Lusis, E. P. Greenberg, and J. Zabner. 2005. Human and murine paraoxonase 1 are host modulators of *Pseudomonas aeruginosa* quorum-sensing. FEMS Microbiology Letters 253:29-37.

234. Park, P. W., G. B. Pier, M. T. Hinkes, and M. Bernfield. 2001. Exploitation of syndecan-1 shedding by *Pseudomonas aeruginosa* enhances virulence. Nature 411:98-102.

235. Parkins, M. D., H. Ceri, and D. G. Storey. 2001. *Pseudomonas aeruginosa* GacA, a factor in multihost virulence, is also essential for biofilm formation. Molecular Microbiology 40:1215-1226.

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

237. Pedersen, S., A. Kharazmi, F. Espersen, and N. Høiby. 1990. *Pseudomonas aeruginosa* alginate in cystic fibrosis sputum and the inflammatory response. Infection and Immunity 58:3363-3368.

238. Pérez-Osorio, A. C., K. S. Williamson, and M. J. Franklin. 2010. Heterogeneous rpoS and rhlR mRNA Levels and 16S rRNA/rDNA (rRNA gene) Ratios within *Pseudomonas aeruginosa* biofilms, sampled by laser capture microdissection. Journal of Bacteriology 192:2991-3000.

239. Pesci, E. C., J. P. Pearson, P. C. Seed, and B. H. Iglewski. 1997. Regulation of las and rhl quorum sensing in *Pseudomonas aeruginosa*. Journal of Bacteriology 179:3127-3132.

240. Petrova, O. E. and K. Sauer. 2009. A novel signaling network essential for regulating *Pseudomonas aeruginosa* biofilm development. PLoS Pathogens 5:e1000668.

241. Petrova, O. E. and K. Sauer. 2010. The novel two-component regulatory system BfiSR regulates biofilm development by controlling the small RNA rsmZ through CafA. Journal of Bacteriology 192:5275-5288.

242. Pier, G. B. 2007. *Pseudomonas aeruginosa* lipopolysaccharide: A major virulence factor, initiator of inflammation and target for effective immunity. International Journal of Medical Microbiology 297:277-295.

243. Pier, G. B., M. Grout, and T. S. Zaidi. 1997. Cystic fibrosis transmembrane conductance regulator is an epithelial cell receptor for clearance of *Pseudomonas aeruginosa* from the CF lung. Proceedings of the National Academy of Sciences 94:12088-12093.

244. Pier, G. B., M. Grout, T. S. Zaidi, and J. B. Goldberg. 1996. How mutant CFTR may contribute to *Pseudomonas aeruginosa* infection in cystic fibrosis. American Journal of Respiratory and Critical Care Medicine 154:S175-S182.

245. Pier, G. B., M. Grout, T. S. Zaidi, J. C. Olsen, L. G. Johnson, J. R. Yankaskas, and J. B. Goldberg. 1996. Role of mutant CFTR in hypersusceptibility of cystic fibrosis patients to lung infections. Science 271:64-67.

246. Plotkowski, M. C., S. de Bentzmann, S. H. Pereira, J. M. Zahm, O. Bajolet-Laudinat, P. Roger, and E. Puchelle. 1999. *Pseudomonas aeruginosa* internalization by human epithelial respiratory cells depends on cell differentiation, polarity, and junctional complex integrity. American journal of respiratory cell and molecular biology 20:880-890.

247. Plotkowski, M. C., H. C. C. Póvoa, J. M. Zahm, G. Lizard, G. M. B. Pereira, J. M. Tournier, and E. Puchelle. 2002. Early mitochondrial dysfunction, superoxide anion production, and DNA degradation are associated with non-apoptotic death of human airway epithelial cells induced by *Pseudomonas aeruginosa* Exotoxin A. American Journal of Respiratory Cell and Molecular Biology 26:617-626.

248. Pompilio, A., M. Scocchi, S. Pomponio, F. Guida, A. Di Primio, E. Fiscarelli, R. Gennaro, and G. Di Bonaventura. 2011. Antibacterial and anti-biofilm effects of cathelicidin peptides against pathogens isolated from cystic fibrosis patients. Peptides 32:1807-1814.

249. Prince, A. 2006. Flagellar activation of epithelial signaling. American journal of respiratory cell and molecular biology 34:548-551.

250. Purevdorj, B., J. W. Costerton, and P. Stoodley. 2002. Influence of hydrodynamics and cell signaling on the structure and behavior of *Pseudomonas aeruginosa* biofilms. Applied and Environmental Microbiology 68:4457-4464.

251. Pütsep, K., G. Carlsson, H. G. Boman, and M. Andersson. 2002. Deficiency of antibacterial peptides in patients with morbus Kostmann: an observation study. The Lancet 360:1144-1149.

252. Quinton, P. M. 2010. Role of epithelial $HCO3^{-}$ transport in mucin secretion: lessons from cystic fibrosis. American Journal of Physiology - Cell Physiology 299:C1222-C1233.

253. Rajan, S., G. Cacalano, R. Bryan, A. J. Ratner, C. U. Sontich, A. van Heerckeren, P. Davis, and A. Prince. 2000. *Pseudomonas aeruginosa* induction of apoptosis in respiratory epithelial cells. American Journal of Respiratory Cell and Molecular Biology 23:304-312.

254. Ramos, H. C., M. Rumbo, and J. C. Sirard. 2004. Bacterial flagellins: mediators of pathogenicity and host immune responses in mucosa. Trends in Microbiology 12:509-517.

255. Raoust, E., V. Balloy, I. Garcia-Verdugo, L. Touqui, R. Ramphal, and M. Chignard. 2009. *Pseudomonas aeruginosa* LPS or Flagellin are sufficient to activate TLR-dependent signaling in murine alveolar macrophages and airway epithelial cells. PLoS ONE 4:e7259.

256. Ratjen, F. 2006. Restoring airway surface liquid in cystic fibrosis. New England Journal of Medicine 354:291-293.

257. Rodrigue, A., Y. Quentin, A. Lazdunski, V. Méjean, and M. Foglino. 2000. Cell signalling by oligosaccharides. Two-component systems in *Pseudomonas aeruginosa*: why so many? Trends in Microbiology 8:498-504.

258. Rogers, G. B., L. R. Hoffman, and G. Döring. 2011. Novel concepts in evaluating antimicrobial therapy for bacterial lung infections in patients with cystic fibrosis. Journal of Cystic Fibrosis 10:387-400.

259. Sauer, K., A. K. Camper, G. D. Ehrlich, J. W. Costerton, and D. G. Davies. 2002. *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. Journal of Bacteriology 184:1140-1154.

260. Sawicki, G. S., J. E. Signorovitch, J. Zhang, D. Latremouille-Viau, M. von Wartburg, E. Q. Wu, and L. Shi. 2012. Reduced mortality in cystic fibrosis patients treated with tobramycin inhalation solution. Pediatric pulmonology 47:44-52.

261. Schaller-Bals, S., A. Schulze, and R. Bals. 2002. Increased levels of antimicrobial peptides in tracheal aspirates of newborn infants during infection. American Journal of Respiratory and Critical Care Medicine 165:992-995.

262. Schmidtchen, A., I. M. Frick, E. Andersson, H. Tapper, and L. Björck. 2002. Proteinases of common pathogenic bacteria degrade and inactivate the antibacterial peptide LL-37. Molecular Microbiology 46:157-168.

263. Scott, N. E., N. J. Hare, M. Y. White, J. Manos, and S. J. Cordwell. 2013. Secretome of transmissible *Pseudomonas aeruginosa* AES-1R grown in a cystic fibrosis lung-like environment. Journal of proteome research.

264. Selman, M., T. E. King, and A. Pardo. 2001. Idiopathic pulmonary fibrosis: prevailing and evolving hypotheses about its pathogenesis and implications for therapy. Annals of Internal Medicine 134:136-151.

265. Shornick, L. P., A. G. Wells, Y. Zhang, A. C. Patel, G. Huang, K. Takami, M. Sosa, N. A. Shukla, E. Agapov, and M. J. Holtzman. 2008. Airway epithelial versus immune cell Stat1 function for innate defense against respiratory viral infection. The Journal of Immunology 180:3319-3328.

266. Singh, P. K., A. L. Schaefer, M. R. Parsek, T. O. Moninger, M. J. Welsh, and E. P. Greenberg. 2000. Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. Nature 407:762-764.

267. Smith, E. E., D. G. Buckley, Z. Wu, C. Saenphimmachak, L. R. Hoffman, D. A. D'Argenio, S. I. Miller, B. W. Ramsey, D. P. Speert, S. M. Moskowitz, J. L. Burns, R. Kaul, and M. V. Olson. 2006. Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. Proceedings of the National Academy of Sciences 103:8487-8492.

268. Smith, J. J., S. M. Travis, E. P. Greenberg, and M. J. Welsh. 1996. Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid. Cell 85:229-236.

269. Smith, R. S., E. R. Fedyk, T. A. Springer, N. Mukaida, B. H. Iglewski, and R. P. Phipps. 2001. IL-8 production in human lung fibroblasts and epithelial cells activated by the

Pseudomonas autoinducer N-3-Oxododecanoyl homoserine lactone is transcriptionally regulated by NF-κB and Activator Protein-2. Journal of Immunology 167:366-374.

270. Smith, R. S. and B. H. Iglewski. 2003. *P. aeruginosa* quorum-sensing systems and virulence. Current Opinion in Microbiology 6:56-60.

271. Sokol, P. A., C. Kooi, R. S. Hodges, P. Cachia, and D. E. Woods. 2000. Immunization with a *Pseudomonas aeruginosa* elastase peptide reduces severity of experimental lung infections due to *P. aeruginosa* or *Burkholderia cepacia*. Journal of Infectious Diseases 181:1682-1692.

272. Son, M. S., W. J. Matthews, Y. Kang, D. T. Nguyen, and T. T. Hoang. 2007. *In vivo* evidence of *Pseudomonas aeruginosa* nutrient acquisition and pathogenesis in the lungs of cystic fibrosis patients. Infection and Immunity 75:5313-5324.

273. Southey-Pillig, C. J., D. G. Davies, and K. Sauer. 2005. Characterization of temporal protein production in *Pseudomonas aeruginosa* biofilms. Journal of Bacteriology 187:8114-8126.

274. Speert, D. P., M. E. Campbell, D. A. Henry, R. Milner, F. Taha, A. Gravelle, A. G. Davidson, L. T. K. Wong, and E. Mahenthiralingam. 2002. Epidemiology of *Pseudomonas aeruginosa* in cystic fibrosis in British Columbia, Canada. American Journal of Respiratory and Critical Care Medicine 166:988-993.

275. Spoering, A. L. and K. Lewis. 2001. Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. Journal of Bacteriology 183:6746-6751.

276. Sriramulu, D. D., H. Lünsdorf, J. S. Lam, and U. Römling. 2005. Microcolony formation: a novel biofilm model of *Pseudomonas aeruginosa* for the cystic fibrosis lung. Journal of Medical Microbiology 54:667-676.

277. Sriramulu, D. D., M. Nimtz, and U. Romling. 2005. Proteome analysis reveals adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis lung environment. Proteomics 5:3712-3721.

278. Stewart, P. S. 1996. Theoretical aspects of antibiotic diffusion into microbial biofilms. Antimicrobial Agents and Chemotherapy 40:2517-2522.

279. Stewart, P. S., M. J. Franklin, K. S. Williamson, J. P. Folsom, L. Boegli, and G. A. James. 2015. Contribution of stress responses to antibiotic tolerance in *Pseudomonas aeruginosa* biofilms. Antimicrobial Agents and Chemotherapy 59:3838-3847.

280. Storey, D. G., E. E. Ujack, H. R. Rabin, and I. Mitchell. 1998. Pseudomonas aeruginosa lasRTranscription correlates with the transcription of lasA, lasB, and toxA in chronic lung infections associated with cystic fibrosis. Infection and Immunity 66:2521-2528.

281. Stover, C. K., X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrener, M. J. Hickey, F. S. L. Brinkman, W. O. Hufnagle, D. J. Kowalik, M. Lagrou, R. L. Garber, L. Goltry, E. Tolentino, S. Westbrock-Wadman, Y. Yuan, L. L. Brody, S. N. Coulter, K. R. Folger, A. Kas, K. Larbig, R. Lim, K. Smith, D. Spencer, G. K. S. Wong, Z. Wu, I. T. Paulsen, J. Reizer, M. H. Saier, R. E. W. Hancock, S. Lory, and M. V. Olson. 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature 406:959-964.

282. Strempel, N., A. Neidig, M. Nusser, R. Geffers, J. Vieillard, O. Lesouhaitier, G. Brenner-Weiss, and J. Overhage. 2013. Human host defense peptide LL-37 stimulates virulence

factor production and adaptive resistance in *Pseudomonas aeruginosa*. PLoS ONE 8:e82240.

283. Suh, S. J., L. Silo-Suh, D. E. Woods, D. J. Hassett, S. E. H. West, and D. E. Ohman. 1999. Effect of rpoS mutation on the stress response and expression of virulence factors in *Pseudomonas aeruginosa*. Journal of Bacteriology 181:3890-3897.

284. Swain, R. J., S. J. Kemp, P. Goldstraw, T. D. Tetley, and M. M. Stevens. 2010. Assessment of cell line models of primary human cells by Raman spectral phenotyping. Biophysical Journal 98:1703-1711.

285. Teitzel, G. M. and M. R. Parsek. 2003. Heavy metal resistance of biofilm and planktonic *Pseudomonas aeruginosa*. Applied and Environmental Microbiology 69:2313-2320.

286. Thomas, J., D. J. Cook, and D. Brooks. 1995. Chest physical therapy management of patients with cystic fibrosis: a meta-analysis. American Journal of Respiratory and Critical Care Medicine 151:846-850.

287. Thornton, R. B., S. P. Wiertsema, L. A. Kirkham, P. J. Rigby, S. Vijayasekaran, H. L. Coates, and P. C. Richmond. 2013. Neutrophil extracellular traps and bacterial biofilms in middle ear effusion of children with recurrent acute otitis media – a potential treatment target. PLoS ONE 8:e53837.

288. Tielen, P., F. Rosenau, S. Wilhelm, K. E. Jaeger, H. C. Flemming, and J. Wingender. 2010. Extracellular enzymes affect biofilm formation of mucoid *Pseudomonas aeruginosa*. Microbiology 156:2239-2252.

289. Tolker-Nielsen, T., U. C. Brinch, P. C. Ragas, J. B. Andersen, C. S. Jacobsen, and S. Molin. 2000. Development and dynamics of *Pseudomonas sp.* biofilms. Journal of Bacteriology 182:6482-6489.

290. Tomlin, K. L., R. J. Malott, G. Ramage, D. G. Storey, P. A. Sokol, and H. Ceri. 2005. Quorum-sensing mutations affect attachment and stability of *Burkholderia cenocepacia* biofilms. Applied and Environmental Microbiology 71:5208-5218.

291. Tramper-Stranders, G. A., T. F. W. Wolfs, S. van Haren Noman, W. M. C. van Aalderen, A. F. Nagelkerke, M. Nuijsink, J. L. L. Kimpen, and C. K. van der Ent. 2010. Controlled trial of cycled antibiotic prophylaxis to prevent initial *Pseudomonas aeruginosa* infection in children with cystic fibrosis. Thorax 65:915-920.

292. Tran, C. S., S. M. Rangel, H. Almblad, A. Kierbel, M. Givskov, T. Tolker-Nielsen, A. R. Hauser, and J. N. Engel. 2014. The *Pseudomonas aeruginosa* type III translocon is required for biofilm formation at the epithelial barrier. PLoS Pathogen 10:e1004479.

293. Tzen, C. Y., R. E. Scott, and F. D. Robinson. 1997. Serum deprivation induces SV40 early promoter activity. Cell Proliferation 30:53-60.

294. Ulrich, M., J. Berger, J. G. Möller, and G. Döring. 2005. Moxifloxacin and ciprofloxacin protect human respiratory epithelial cells against *Streptococcus pneumoniae, Staphylococcus aureus, Pseudomonas aeruginosa*, and *Haemophilus influenzae in vitro*. Infection 33:50-54.

295. Ulrich, M. and G. Döring. 2004. Three-dimensional human airway epithelial cell cultures. Journal of Cystic Fibrosis 3, Supplement 2:55-57.

296. van Westreenen, M. and H. Tiddens. 2010. New antimicrobial strategies in cystic fibrosis. Pediatric-Drugs 12:343-352.

297. Vance, R. E., A. Rietsch, and J. J. Mekalanos. 2005. Role of the type III secreted exoenzymes S, T, and Y in systemic spread of *Pseudomonas aeruginosa* PAO1 *in vivo*. Infection and Immunity 73:1706-1713.

298. VanDevanter, D. R. and J. M. Van Dalfsen. 2005. How much do *Pseudomonas* biofilms contribute to symptoms of pulmonary exacerbation in cystic fibrosis? Pediatric pulmonology 39:504-506.

299. Venier, A. G., T. Lavigne, P. Jarno, F. L–heriteau, B. Coignard, A. Savey, and A. M. Rogues. 2012. Nosocomial urinary tract infection in the intensive care unit: when should *Pseudomonas aeruginosa* be suspected? Experience of the French national surveillance of nosocomial infections in the intensive care unit, Rea-Raisin. Clinical Microbiology and Infection 18:E13-E15.

300. Viducic, D., T. Ono, K. Murakami, H. Susilowati, S. Kayama, K. Hirota, and Y. Miyake. 2006. Functional analysis of *spoT, relA* and *dksA* genes on quinolone tolerance in *Pseudomonas aeruginosa* under nongrowing condition. Microbiology and Immunology 50:349-357.

301. Vilain, S., P. Cosette, M. Hubert, C. Lange, G. A. Junter, and T. Jouenne. 2004. Comparative proteomic analysis of planktonic and immobilized *Pseudomonas aeruginosa* cells: a multivariate statistical approach. Analytical Biochemistry 329:120-130.

302. von Götz, F., S. Häussler, D. Jordan, S. S. Saravanamuthu, D. Wehmhöner, A. Strüßmann, J. Lauber, I. Attree, J. Buer, B. Tümmler, and I. Steinmetz. 2004. Expression analysis of a highly adherent and cytotoxic small colony variant of *Pseudomonas aeruginosa* isolated from a lung of a patient with cystic fibrosis. Journal of Bacteriology 186:3837-3847.

303. Wagner, V. E., D. Bushnell, L. Passador, A. I. Brooks, and B. H. Iglewski. 2003. Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: Effects of growth phase and environment. Journal of Bacteriology 185:2080-2095.

304. Waite, R. D., A. Papakonstantinopoulou, E. Littler, and M. A. Curtis. 2005. Transcriptome analysis of *Pseudomonas aeruginosa* growth: Comparison of gene expression in planktonic cultures and developing and mature biofilms. Journal of Bacteriology 187:6571-6576.

305. Waite, R. D., A. Papakonstantinopoulou, E. Littler, and M. A. Curtis. 2005. Transcriptome analysis of *Pseudomonas aeruginosa* growth: Comparison of gene expression in planktonic cultures and developing and mature biofilms. Journal of Bacteriology 187:6571-6576.

306. Waite, R., A. Paccanaro, A. Papakonstantinopoulou, J. Hurst, M. Saqi, E. Littler, and M. Curtis. 2006. Clustering of *Pseudomonas aeruginosa* transcriptomes from planktonic cultures, developing and mature biofilms reveals distinct expression profiles. BMC Genomics 7:1-14.

307. Walters, M. C., F. Roe, A. Bugnicourt, M. J. Franklin, and P. S. Stewart. 2003. Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. Antimicrobial Agents and Chemotherapy 47:317-323.

308. **Wan, M., A. M. van der Does, X. Tang, L. Lindbom, B. Agerberth, and J. Z. Haeggström. 2014. Antimicrobial peptide LL-37 promotes bacterial phagocytosis by human macrophages. Journal of Leukocyte Biology 95:971-981.**

309. **Wang, Y., U. Ha, L. Zeng, and S. Jin. 2003. Regulation of membrane permeability by a two-component regulatory system in *Pseudomonas aeruginosa*. Antimicrobial Agents and Chemotherapy 47:95-101.**

310. **Ward, K. H., M. E. Olson, K. Lam, and J. W. Costerton. 1992. Mechanism of persistent infection associated with peritoneal implants. Journal of Medical Microbiology 36:406-413.**

311. **Webb, J. S., L. S. Thompson, S. James, T. Charlton, T. Tolker-Nielsen, B. Koch, M. Givskov, and S. Kjelleberg. 2003. Cell death in *Pseudomonas aeruginosa* biofilm development. Journal of Bacteriology 185:4585-4592.**

312. **Wehmhoner, D., S. Haussler, B. Tummler, L. Jansch, F. Bredenbruch, J. Wehland, and I. Steinmetz. 2003. Inter- and intraclonal diversity of the *Pseudomonas aeruginosa* proteome manifests within the secretome. Journal of Bacteriology 185:5807-5814.**

313. **Whitchurch, C. B., R. A. Alm, and J. S. Mattick. 1996. The alginate regulator AlgR and an associated sensor FimS are required for twitching motility in *Pseudomonas aeruginosa*. Proceedings of the National Academy of Sciences 93:9839-9843.**

314. **Whitchurch, C. B., T. Tolker-Nielsen, P. C. Ragas, and J. S. Mattick. 2002. Extracellular DNA required for bacterial biofilm formation. Science 295:1487.**

315. **Whiteley, M., M. G. Bangera, R. E. Bumgarner, M. R. Parsek, G. M. Teitzel, S. Lory, and E. P. Greenberg. 2001. Gene expression in *Pseudomonas aeruginosa* biofilms. Nature 413:860-864.**

316. **Williamson, K. S., L. A. Richards, A. C. Perez-Osorio, B. Pitts, K. McInnerney, P. S. Stewart, and M. J. Franklin. 2012. Heterogeneity in *Pseudomonas aeruginosa* biofilms includes expression of ribosome hibernation factors in the antibiotic-tolerant subpopulation and hypoxia-induced stress response in the metabolically active population. Journal of Bacteriology 194:2062-2073.**

317. **Wilson, M. S. and T. A. Wynn. 2009. Pulmonary fibrosis: pathogenesis, etiology and regulation. Mucosal Immunology 2:103-121.**

318. **Wisniewski, J. R., A. Zougman, N. Nagaraj, and M. Mann. 2009. Universal sample preparation method for proteome analysis. Nature Methods 6:359-362.**

319. **Wnorowska, U., K. Niemirowicz, M. Myint, S. L. Diamond, M. Wröblewska, P. B. Savage, P. A. Janmey, and R. Bucki. 2015. Bactericidal activities of cathelicidin LL-37 and select cationic lipids against the hypervirulent *Pseudomonas aeruginosa* strain LESB58. Antimicrobial Agents and Chemotherapy 59:3808-3815.**

320. **Woodworth, B. A., E. Tamashiro, G. Bhargave, N. A. Cohen, and J. N. Palmer. 2008. An *in vitro* model of *Pseudomonas aeruginosa* biofilms on viable airway epithelial cell monolayers. American journal of rhinology 22:235-238.**

321. **Wu, P. J., K. Shannon, and I. Phillips. 1994. Effect of hyperproduction of TEM-1 β-lactamase on in vitro susceptibility of Escherichia coli to β-lactam antibiotics. Antimicrobial Agents and Chemotherapy 38:494-498.**

322. Xiao, H., M. Kalman, K. Ikehara, S. Zemel, G. Glaser, and M. Cashel. 1991. Residual guanosine 3',5'-bispyrophosphate synthetic activity of *relA* null mutants can be eliminated by *spoT* null mutations. Journal of Biological Chemistry 266:5980-5990.

323. Xu, K. D., M. J. Franklin, C. H. Park, G. A. McFeters, and P. S. Stewart. 2001. Gene expression and protein levels of the stationary phase sigma factor, RpoS, in continuously-fed *Pseudomonas aeruginosa* biofilms. FEMS Microbiology Letters 199:67-71.

324. Yan, H. and R. E. W. Hancock. 2001. Synergistic interactions between mammalian antimicrobial defense peptides. Antimicrobial Agents and Chemotherapy 45:1558-1560.

325. Yang, Y., A. V. Bazhin, J. Werner, and S. Karakhanova. 2013. Reactive oxygen species in the immune system. International Reviews of Immunology 32:249-270.

326. Young, R. L., K. C. Malcolm, J. E. Kret, S. M. Caceres, K. R. Poch, D. P. Nichols, J. L. Taylor-Cousar, M. T. Saavedra, S. H. Randell, M. L. Vasil, J. L. Burns, S. M. Moskowitz, and J. A. Nick. 2011. Neutrophil extracellular trap (NET)-mediated killing of *Pseudomonas aeruginosa*: evidence of acquired resistance within the CF airway, independent of CFTR. PLoS ONE 6:e23637.

327. Yu, Q., E. F. Griffin, S. Moreau-Marquis, J. D. Schwartzman, B. A. Stanton, and G. A. O'Toole. 2012. *In vitro* evaluation of tobramycin and aztreonam versus *Pseudomonas aeruginosa* biofilms on cystic fibrosis-derived human airway epithelial cells. Journal of Antimicrobial Chemotherapy 67:2673-2681.

328. Zhang, L., A. J. Hinz, J. P. Nadeau, and T. F. Mah. 2011. *Pseudomonas aeruginosa* tssC1 Links Type VI Secretion and biofilm-specific antibiotic resistance. Journal of Bacteriology 193:5510-5513.

329. Zhang, W., J. Sun, W. Ding, J. Lin, R. Tian, L. Lu, X. Liu, X. Shen, and P. Y. Qian. 2015. Extracellular matrix-associated proteins form an integral and dynamic system during *Pseudomonas aeruginosa* biofilm development. Frontiers in Cellular and Infection Microbiology 5.

330. Zobell, J. T., C. D. Waters, D. C. Young, C. Stockmann, K. Ampofo, C. M. T. Sherwin, and M. G. Spigarelli. 2013. Optimization of anti-pseudomonal antibiotics for cystic fibrosis pulmonary exacerbations: II. cephalosporins and penicillins. Pediatric pulmonology 48:107-122.

331. Zuker, M., D. H. Mathews, and D. H. Turner. 1999. Algorithms and thermodynamics for RNA secondary structure prediction: a practical guide, p. 11-43. *In*: J. Barciszewski and B. Clark (eds.), RNA Biochemistry and Biotechnology. 70 ed. Springer Netherlands.

Appendix

Appendix 1. Table of secreted proteins identified in biofilm CM and planktonic CM that were similarly expressed between the two forms.

Accession	Description	Accession	Description
gi|15595200|	DNA polymerase III subunit beta [Pseudomonas aeruginosa PAO1].	gi|15599126|	hypothetical observed protein PA3931 [Pseudomonas aeruginosa PAO1].
gi|15595218|	hypothetical observed protein PA0020 [Pseudomonas aeruginosa PAO1].	gi|15599226|	inorganic pyrophosphatase [Pseudomonas aeruginosa PAO1].
gi|15595337|	alkyl hydroperoxide reductase [Pseudomonas aeruginosa PAO1].	gi|15599370|	endopeptidase IV [Pseudomonas aeruginosa PAO1].
gi|15595463|	4-aminobutyrate aminotransferase [Pseudomonas aeruginosa PAO1].	gi|15599428|	single-stranded DNA-binding protein [Pseudomonas aeruginosa PAO1].
gi|15595511|	L-cysteine ABC transporter protein FliY [Pseudomonas aeruginosa PAO1].	gi|15599433|	50S ribosomal protein L17 [Pseudomonas aeruginosa PAO1].
gi|15595512|	hypothetical observed protein PA0315 [Pseudomonas aeruginosa PAO1].	gi|15599436|	30S ribosomal protein S11 [Pseudomonas aeruginosa PAO1].
gi|15595620|	hypothetical observed protein PA0423 [Pseudomonas aeruginosa PAO1].	gi|15599437|	30S ribosomal protein S13 [Pseudomonas aeruginosa PAO1].
gi|15595629|	S-adenosyl-L-homocysteine hydrolase [Pseudomonas aeruginosa PAO1].	gi|15599440|	50S ribosomal protein L15 [Pseudomonas aeruginosa PAO1].
gi|15595745|	transketolase [Pseudomonas aeruginosa PAO1].	gi|15599442|	30S ribosomal protein S5 [Pseudomonas aeruginosa PAO1].
gi|15595749|	phosphoglycerate kinase [Pseudomonas aeruginosa PAO1].	gi|15599444|	50S ribosomal protein L6 [Pseudomonas aeruginosa PAO1].
gi|15595769|	hypothetical observed protein PA0572 [Pseudomonas aeruginosa PAO1].	gi|15599445|	30S ribosomal protein S8 [Pseudomonas aeruginosa PAO1].
gi|15595776|	30S ribosomal protein S21 [Pseudomonas aeruginosa PAO1].	gi|15599447|	50S ribosomal protein L5 [Pseudomonas aeruginosa PAO1].
gi|15595791|	peptidyl-prolyl cis-trans isomerase SurA [Pseudomonas aeruginosa PAO1].	gi|15599448|	50S ribosomal protein L24 [Pseudomonas aeruginosa PAO1].
gi|15596068|	pterin-4-alpha-carbinolamine dehydratase [Pseudomonas aeruginosa PAO1].	gi|15599452|	50S ribosomal protein L16 [Pseudomonas aeruginosa PAO1].
gi|15596092|	bifunctional N-succinyldiaminopimelate-aminotransferase/acetylornithine transaminase protein [Pseudomonas aeruginosa PAO ...	gi|15599455|	30S ribosomal protein S19 [Pseudomonas aeruginosa PAO1].
gi|15596153|	prolyl-tRNA synthetase [Pseudomonas aeruginosa PAO1].	gi|15599456|	50S ribosomal protein L2 [Pseudomonas aeruginosa PAO1].
gi|15596159|	dna-binding stress protein [Pseudomonas aeruginosa PAO1].	gi|15599457|	50S ribosomal protein L23 [Pseudomonas aeruginosa PAO1].
gi|15596169|	translocation protein TolB [Pseudomonas aeruginosa PAO1].	gi|15599459|	50S ribosomal protein L3 [Pseudomonas aeruginosa PAO1].
gi|15596207|	dihydrodipicolinate synthase [Pseudomonas aeruginosa PAO1].	gi|15599460|	30S ribosomal protein S10 [Pseudomonas aeruginosa PAO1].
gi|15596271|	branched-chain amino acid ABC transporter [Pseudomonas aeruginosa PAO1].	gi|15599461|	elongation factor Tu [Pseudomonas aeruginosa PAO1].
gi|15596284|	flagellar hook-associated protein FlgL [Pseudomonas aeruginosa PAO1].	gi|15599462|	elongation factor G [Pseudomonas aeruginosa PAO1].
gi|15596290|	hypothetical observed protein PA1093 [Pseudomonas aeruginosa PAO1].	gi|15599464|	30S ribosomal protein S12 [Pseudomonas aeruginosa PAO1].

gi|15596291|	flagellar capping protein FliD [Pseudomonas aeruginosa PAO1].	gi|15599465|	DNA-directed RNA polymerase subunit beta' [Pseudomonas aeruginosa PAO1].
gi|15596345|	exotoxin A [Pseudomonas aeruginosa PAO1].	gi|15599466|	DNA-directed RNA polymerase subunit beta [Pseudomonas aeruginosa PAO1].
gi|15596539|	ABC transporter [Pseudomonas aeruginosa PAO1].	gi|15599467|	50S ribosomal protein L7/L12 [Pseudomonas aeruginosa PAO1].
gi|15596690|	sulfate ABC transporter substrate-binding protein [Pseudomonas aeruginosa PAO1].	gi|15599468|	50S ribosomal protein L10 [Pseudomonas aeruginosa PAO1].
gi|15596776|	hypothetical observed protein PA1579 [Pseudomonas aeruginosa PAO1].	gi|15599469|	50S ribosomal protein L1 [Pseudomonas aeruginosa PAO1].
gi|15596783|	dihydrolipoamide succinyltransferase [Pseudomonas aeruginosa PAO1].	gi|15599471|	transcription antitermination protein NusG [Pseudomonas aeruginosa PAO1].
gi|15596793|	heat shock protein 90 [Pseudomonas aeruginosa PAO1].	gi|15599529|	fumarase [Pseudomonas aeruginosa PAO1].
gi|15596806|	3-oxoacyl-ACP synthase [Pseudomonas aeruginosa PAO1].	gi|15599562|	superoxide dismutase [Pseudomonas aeruginosa PAO1].
gi|15596965|	hypothetical observed protein PA1768 [Pseudomonas aeruginosa PAO1].	gi|15599581|	molecular chaperone GroEL [Pseudomonas aeruginosa PAO1].
gi|15596967|	phosphoenolpyruvate synthase [Pseudomonas aeruginosa PAO1].	gi|15599582|	co-chaperonin GroES [Pseudomonas aeruginosa PAO1].
gi|15596984|	bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase [Pseudomonas aeruginosa PAO1].	gi|15599649|	hypothetical observed protein PA4453 [Pseudomonas aeruginosa PAO1].
gi|15596990|	peptidyl-prolyl cis-trans isomerase B [Pseudomonas aeruginosa PAO1].	gi|15599656|	hypothetical observed protein PA4460 [Pseudomonas aeruginosa PAO1].
gi|15596991|	glutaminyl-tRNA synthetase [Pseudomonas aeruginosa PAO1].	gi|15599721|	type 4 fimbrial PilA [Pseudomonas aeruginosa PAO1].
gi|15596992|	cysteinyl-tRNA synthetase [Pseudomonas aeruginosa PAO1].	gi|15599751|	type 4 fimbrial biogenesis protein PilY2 [Pseudomonas aeruginosa PAO1].
gi|15596997|	trigger factor [Pseudomonas aeruginosa PAO1].	gi|15599880|	hypothetical observed protein PA4685 [Pseudomonas aeruginosa PAO1].
gi|15597001|	DNA-binding protein HU [Pseudomonas aeruginosa PAO1].	gi|15599881|	hypothetical observed protein PA4686 [Pseudomonas aeruginosa PAO1].
gi|15597044|	hypothetical observed protein PA1847 [Pseudomonas aeruginosa PAO1].	gi|15599882|	ferric iron-binding periplasmic protein HitA [Pseudomonas aeruginosa PAO1].
gi|15597728|	thiol peroxidase [Pseudomonas aeruginosa PAO1].	gi|15599917|	suppressor protein DksA [Pseudomonas aeruginosa PAO1].
gi|15597819|	isocitrate dehydrogenase [Pseudomonas aeruginosa PAO1].	gi|15599933|	hypothetical observed protein PA4739 [Pseudomonas aeruginosa PAO1].
gi|15597820|	isocitrate dehydrogenase [Pseudomonas aeruginosa PAO1].	gi|15599934|	polynucleotide phosphorylase [Pseudomonas aeruginosa PAO1].
gi|15597855|	hypothetical observed protein PA2659 [Pseudomonas aeruginosa PAO1].	gi|15599955|	molecular chaperone DnaK [Pseudomonas aeruginosa PAO1].
gi|15597939|	translation initiation factor IF-3 [Pseudomonas aeruginosa PAO1].	gi|15599956|	heat shock protein GrpE [Pseudomonas aeruginosa PAO1].
gi|15598047|	elongation factor P [Pseudomonas aeruginosa PAO1].	gi|15600040|	acetyl-CoA carboxylase biotin carboxyl carrier protein subunit [Pseudomonas aeruginosa PAO1].
gi|15598049|	outer membrane lipoprotein OprI [Pseudomonas aeruginosa PAO1].	gi|15600041|	acetyl-CoA carboxylase biotin carboxylase subunit [Pseudomonas aeruginosa PAO1].
gi|15598147|	electron transfer flavoprotein subunit alpha [Pseudomonas aeruginosa PAO1].	gi|15600115|	azurin [Pseudomonas aeruginosa PAO1].
gi|15598162|	acyl carrier protein [Pseudomonas aeruginosa PAO1].	gi|15600128|	30S ribosomal protein S6 [Pseudomonas aeruginosa PAO1].
gi|15598358|	30S ribosomal protein S1 [Pseudomonas aeruginosa PAO1].	gi|15600208|	pyruvate dehydrogenase subunit E1 [Pseudomonas aeruginosa PAO1].
gi|15598423|	peptidyl-prolyl cis-trans isomerase A [Pseudomonas aeruginosa PAO1].	gi|15600209|	dihydrolipoamide acetyltransferase [Pseudomonas aeruginosa PAO1].
gi|15598509|	hypothetical observed protein PA3313 [Pseudomonas aeruginosa PAO1].	gi|15600364|	arginine deiminase [Pseudomonas aeruginosa PAO1].
gi|15598593|	ferredoxin-NADP reductase [Pseudomonas aeruginosa PAO1].	gi|15600367|	beta-ketoacyl synthase [Pseudomonas aeruginosa PAO1].

gi|15598725|	peroxidase [Pseudomonas aeruginosa PAO1].	gi|15600385|	phosphoenolpyruvate carboxykinase [Pseudomonas aeruginosa PAO1].
gi|15598729|	hypothetical observed protein PA3533 [Pseudomonas aeruginosa PAO1].	gi|15600433|	thioredoxin [Pseudomonas aeruginosa PAO1].
gi|15598833|	CTP synthetase [Pseudomonas aeruginosa PAO1].	gi|15600478|	hypothetical observed protein PA5285 [Pseudomonas aeruginosa PAO1].
gi|15598852|	30S ribosomal protein S2 [Pseudomonas aeruginosa PAO1].	gi|15600493|	cytochrome C5 [Pseudomonas aeruginosa PAO1].
gi|15598937|	50S ribosomal protein L19 [Pseudomonas aeruginosa PAO1].	gi|15600509|	50S ribosomal protein L28 [Pseudomonas aeruginosa PAO1].
gi|15598940|	30S ribosomal protein S16 [Pseudomonas aeruginosa PAO1].	gi|15600523|	hypothetical observed protein PA5330 [Pseudomonas aeruginosa PAO1].
gi|15598958|	phosphoribosylformylglycinamidine synthase [Pseudomonas aeruginosa PAO1].	gi|15600562|	phosphate ABC transporter substrate-binding protein [Pseudomonas aeruginosa PAO1].
gi|15598964|	GMP synthase [Pseudomonas aeruginosa PAO1].	gi|15600691|	adhesin [Pseudomonas aeruginosa PAO1].
gi|15599002|	nucleoside diphosphate kinase [Pseudomonas aeruginosa PAO1].	gi|15600747|	ATP synthase F0F1 subunit beta [Pseudomonas aeruginosa PAO1].
gi|15599031|	hypothetical observed protein PA3836 [Pseudomonas aeruginosa PAO1].	gi|15600749|	ATP synthase F0F1 subunit alpha [Pseudomonas aeruginosa PAO1].

Appendix 2. Table of secreted proteins identified in both biofilm CM and planktonic CM but significantly higher in biofilm CM.

Functional Class	Accession	Description
Flagella and Pili	gi|15598547|	protein FlgM [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15599888|	ketol-acid reductoisomerase [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15599630|	oxidoreductase [Pseudomonas aeruginosa PAO1].
Other	gi|15598980|	hypothetical observed protein PA3785 [Pseudomonas aeruginosa PAO1].
Other	gi|15599117|	hypothetical observed protein PA3922 [Pseudomonas aeruginosa PAO1].
Other	gi|15600226|	hypothetical observed protein PA5033 [Pseudomonas aeruginosa PAO1].
Virulence and Protection	gi|15596049|	chitin-binding protein CbpD [Pseudomonas aeruginosa PAO1].
Virulence and Protection	gi|15598135|	aminopeptidase [Pseudomonas aeruginosa PAO1].
Virulence and Protection	gi|15598919|	elastase LasB [Pseudomonas aeruginosa PAO1].
Virulence and Protection	gi|15597068|	LasA protease [Pseudomonas aeruginosa PAO1].

Appendix 3. Table of secreted proteins identified in both biofilm CM and planktonic CM but significantly higher in planktonic CM.

Functional Class	Accession	Description	Functional Class	Accession	Description
Flagella and Pili	gi|15596289|	flagellin type B [Pseudomonas aeruginosa PAO1].	Metabolism and Growth	gi|15600131|	adenylosuccinate synthetase [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15595743|	S-adenosylmethionine synthetase [Pseudomonas aeruginosa PAO1].	Metabolism and Growth	gi|15598965|	inosine 5'-monophosphate dehydrogenase [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15598335|	aromatic amino acid aminotransferase [Pseudomonas aeruginosa PAO1].	Metabolism and Growth	gi|15598813|	recombinase A [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15596535|	gamma-glutamyltranspeptidase [Pseudomonas aeruginosa PAO1].	Metabolism and Growth	gi|15599949|	transcription elongation factor GreA [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15596067|	aromatic amino acid aminotransferase [Pseudomonas aeruginosa PAO1].	Metabolism and Growth	gi|15599511|	transcriptional regulator MvaT [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15599202|	gamma-glutamyl phosphate reductase [Pseudomonas aeruginosa PAO1].	other	gi|15595865|	tyrosyl-tRNA synthetase [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15599950|	carbamoyl phosphate synthase large subunit [Pseudomonas aeruginosa PAO1].	other	gi|15596100|	alanyl-tRNA synthetase [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15599952|	carbamoyl phosphate synthase small subunit [Pseudomonas aeruginosa PAO1].	other	gi|15596160|	aspartyl-tRNA synthetase [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15600365|	ornithine carbamoyltransferase [Pseudomonas aeruginosa PAO1].	other	gi|15598849|	ribosome recycling factor [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15596085|	arginine/ornithine binding protein AotJ [Pseudomonas aeruginosa PAO1].	other	gi|15598851|	elongation factor Ts [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15598163|	3-ketoacyl-ACP reductase [Pseudomonas aeruginosa PAO1].	other	gi|15600360|	c4-dicarboxylate-binding protein [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15598164|	malonyl CoA-ACP transacylase [Pseudomonas aeruginosa PAO1].	other	gi|15595585|	hypothetical observed protein PA0388 [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15595703|	acyl-CoA dehydrogenase [Pseudomonas aeruginosa PAO1].	other	gi|15595739|	hypothetical observed protein PA0542 [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15595715|	cytochrome C-551 [Pseudomonas aeruginosa PAO1].	other	gi|15597658|	hypothetical observed protein PA2462 [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15596784|	dihydrolipoamide dehydrogenase [Pseudomonas aeruginosa PAO1].	other	gi|15598871|	hypothetical observed protein PA3675 [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15596785|	succinyl-CoA synthetase subunit beta [Pseudomonas aeruginosa PAO1].	other	gi|15600687|	hypothetical observed protein PA5494 [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15596786|	succinyl-CoA synthetase subunit alpha [Pseudomonas aeruginosa PAO1].	virulence and protection	gi|15596484|	glutathione peroxidase [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15598148|	electron transfer flavoprotein subunit beta [Pseudomonas aeruginosa PAO1].	virulence and protection	gi|15598505|	hypothetical observed protein PA3309 [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15598197|	glyceraldehyde-3-phosphate dehydrogenase [Pseudomonas aeruginosa PAO1].	virulence and protection	gi|15600410|	iron ABC transporter [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15598729|	hypothetical observed protein PA3533 [Pseudomonas aeruginosa PAO1].	virulence and protection	gi|15597400|	ABC transporter [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15598817|	ferredoxin I [Pseudomonas aeruginosa PAO1].	virulence and protection	gi|15596906|	translocator outer membrane protein PopD [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15599783|	cytochrome C551 peroxidase [Pseudomonas aeruginosa PAO1].	virulence and protection	gi|15600310|	regulatory protein TypA [Pseudomonas aeruginosa PAO1].

Appendix 4. Table of secreted proteins identified only in biofilm CM.

Functional Class	Accession	Description
Virulence and Protection	gi|15599566|	Insulin-cleaving metalloproteinase outer membrane protein [Pseudomonas aeruginosa PAO1].
Virulence and Protection	gi|15597895|	hypothetical observed protein PA2699 [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15597648|	hypothetical observed protein PA2452, partial [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15595992|	methylcitrate synthase [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15597221|	glutathione reductase [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15598377|	keto-hydroxyglutarate-aldolase/keto-deoxy-phosphogluconate aldolase [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15597498|	protein AmbE [Pseudomonas aeruginosa PAO1].
Flagella and Pili	gi|15596277|	flagellar hook protein FlgE [Pseudomonas aeruginosa PAO1].
Flagella and Pili	gi|15596279|	flagellar basal body rod protein FlgG [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15599104|	extracelullar DNA degradation protein EddB [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15595599|	aspartate carbamoyltransferase [Pseudomonas aeruginosa PAO1].
Virulence and Protection	gi|15595320|	hypothetical observed protein PA0122 [Pseudomonas aeruginosa PAO1].
Virulence and Protection	gi|15595460|	secreted protein Hcp [Pseudomonas aeruginosa PAO1].
Other	gi|15597647|	hypothetical observed protein PA2451 [Pseudomonas aeruginosa PAO1].
Other	gi|15599821|	hypothetical observed protein PA4625 [Pseudomonas aeruginosa PAO1].

Appendix 5. Table of secreted proteins identified only in planktonic CM.

Functional Class	Accession	Description	Functional Class	Accession	Description
Flagella and Pili	gi|15595606|	twitching motility protein PilH [Pseudomonas aeruginosa PAO1].	Metabolism and Growth	gi|15600048|	phosphoribosylamine--glycine ligase [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15599603|	cell division protein FtsZ [Pseudomonas aeruginosa PAO1].	Metabolism and Growth	gi|15596489|	3-mercaptopyruvate sulfurtransferase [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15595862|	iron-sulfur cluster insertion protein ErpA [Pseudomonas aeruginosa PAO1].	Metabolism and Growth	gi|15600481|	nitrogen regulatory protein P-II 2 [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15596062|	4-hydroxyphenylpyruvate dioxygenase [Pseudomonas aeruginosa PAO1].	Other	gi|15597808|	seryl-tRNA synthetase [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15600468|	frataxin-like protein [Pseudomonas aeruginosa PAO1].	Other	gi|15597937|	50S ribosomal protein L20 [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15599602|	UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase [Pseudomonas aeruginosa PAO1].	Other	gi|15600125|	50S ribosomal protein L9 [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15595679|	malate synthase G [Pseudomonas aeruginosa PAO1].	Other	gi|15600321|	preprotein translocase subunit SecB [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15595979|	bifunctional proline dehydrogenase/pyrroline-5-carboxylate dehydrogenase [Pseudomonas aeruginosa PAO1].	Other	gi|15598807|	hypothetical observed protein PA3611 [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15596084|	acetyl-CoA synthetase [Pseudomonas aeruginosa PAO1].	Other	gi|15595498|	polyamine transporter [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15596093|	arginine/ornithine succinyltransferase AI subunit [Pseudomonas aeruginosa PAO1].	Other	gi|15600665|	hypothetical observed protein PA5472 [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15596094|	arginine/ornithine succinyltransferase AII subunit [Pseudomonas aeruginosa PAO1].	Other	gi|15595517|	hypothetical observed protein PA0320 [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15596095|	succinylglutamic semialdehyde dehydrogenase [Pseudomonas aeruginosa PAO1].	Other	gi|15595702|	hypothetical observed protein PA0505 [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15596352|	ribonucleotide-diphosphate reductase subunit beta [Pseudomonas aeruginosa PAO1].	Other	gi|15596053|	hypothetical observed protein PA0856 [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15596353|	ribonucleotide-diphosphate reductase subunit alpha [Pseudomonas aeruginosa PAO1].	Other	gi|15596836|	hypothetical observed protein PA1639 [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15597180|	NAD+ dependent aldehyde dehydrogenase ExaC [Pseudomonas aeruginosa PAO1].	Other	gi|15598398|	hypothetical observed protein PA3202 [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15597678|	cytochrome C [Pseudomonas aeruginosa PAO1].	Other	gi|15598843|	hypothetical observed protein PA3647 [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15598146|	trans-2-enoyl-CoA reductase [Pseudomonas aeruginosa PAO1].	Virulence and Protection	gi|15596999|	ATP-dependent protease ATP-binding subunit ClpX [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15600366|	carbamate kinase [Pseudomonas aeruginosa PAO1].	Virulence and Protection	gi|15597951|	ecotin [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15600558|	phosphate uptake regulatory protein PhoU [Pseudomonas aeruginosa PAO1].	Virulence and Protection	gi|15599097|	hypothetical observed protein PA3902 [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15600751|	ATP synthase F0F1 subunit B [Pseudomonas aeruginosa PAO1].	Virulence and Protection	gi|15595716|	nitrite reductase [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15596142|	phosphoribosylaminoimidazole synthetase [Pseudomonas aeruginosa PAO1].	Virulence and Protection	gi|15596986|	hypothetical observed protein PA1789 [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi|15597825|	adenylosuccinate lyase [Pseudomonas aeruginosa PAO1].	Virulence and Protection	gi|15595920|	coat protein B of bacteriophage Pf1 [Pseudomonas aeruginosa PAO1].