CALCIUM AFFECTS HOST-PATHOGEN INTERACTIONS OF *PSEUDOMONAS AERUGINOSA* WITH LUNG EPITHELIAL CELLS

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CALCIUM AFFECTS HOST-PATHOGEN INTERACTIONS OF *PSEUDOMONAS AERUGINOSA* WITH LUNG EPITHELIAL CELLS

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DEDICATION

I dedicate this dissertation to my parents, Raj Kumar Luthra and Neelam Luthra, who worked tirelessly to get me to where I am today. I also dedicate this to my siblings, Abhishek and Shweta, and my dear friend in heaven, Harsh Vardhan Pande. It would not have been possible without their constant support, motivation, and strong belief in me.

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Abstract:

Pseudomonas aeruginosa is one of the key bacterial pathogens that cause acute, chronic, and lethal infections in the lungs of patients with cystic fibrosis (CF). *P. aeruginosa* adheres to the mucosal epithelium of the airways to initiate infections, eventually internalizing and establishing a niche. Ca^{2+} is a potent cellular signal has been shown to exist in abundance in pulmonary fluids of CF patients and trigger the expression of virulence factors in *P. aeruginosa*. However, its role in the regulation of host-pathogen interactions is not well understood. In this dissertation, we study the role of Ca^{2+} in adherence, invasion, and intracellular replication of *P. aeruginosa* in lung epithelial cells and its role in the regulation of virulence factors (expression of virulence genes, flagella, and biofilm production) during infection. Two human lung epithelial cell lines, A549 and CuFi-5 (homozygous for the Δ508 mutation in CF) cells infected with PAO1 and FRD1 strains in low and high Ca^{2+} conditions were used for this study. We employ the adhesion assay, immunofluorescence, and scanning electron microscopy (SEM) to study the adhesion of *P. aeruginosa* to lung epithelial cells. The data suggests a significant increase in adherence in high Ca^{2+} with both cell lines. A similar observation was made with the invasion and escape studies. Ca^{2+} binding protein, EfhP, is reported to regulate invasion, intracellular survival, and escape, whereas it plays an insignificant role in adherence. Transcriptome sequencing (RNA-seq) and quantitative RT-PCR analysis also help to understand the importance of Ca^{2+} in the regulation of adhesin transcription (*fliC, pilA* and *lecA*), but this response depends on bacterial strains and the cell line. The Ca^{2+}-dependent upregulation of *fliC* leads to an increased proportion of flagellated bacteria within a population. Ca^{2+} also upregulates other virulence factors of *P. aeruginosa* that could aid biofilm production, biomineralization, and the production of reactive oxygen species. Here, we provide evidence for *P. aeruginosa* host cell escape and Ca^{2+}-mediated activation of P13K and Akt that results in *P aeruginosa* invasion and intracellular survival within lung epithelial cells. Our study provides insight into understanding the regulatory potential of Ca^{2+} and Ca^{2+} binding protein, EfhP, in *P. aeruginosa* infections during CF.

TABLE OF CONTENTS

Chapter Page

1 INTRODUCTION AND LITERATURE REVIEW 1
1.1 Pathophysiology of Cystic Fibrosis (CF) . . . 1
1.2 Microbial diversity in CF . . . 2
1.3 Burden of *Pseudomonas aeruginosa* infections . . . 3
1.4 *P. aeruginosa* virulence factors . . . 4
1.5 Host pathogen interactions of *P. aeruginosa* in cystic fibrosis . . . 7
1.6 Calcium (Ca^{2+}) signaling . . . 9
1.6.1 Ca^{2+} signaling in eukaryotes . . . 9
1.6.2 Ca^{2+} signaling in *P. aeruginosa* . . . 9
1.7 Ca^{2+}-binding proteins (CaBP) . . . 10
1.8 Ca^{2+}-mediated virulence of *P. aeruginosa* . . . 11
1.9 Knowledge Gap and Research Hypothesis . . . 11
1.10 Scientific objective and Significance . . . 12

2 CALCIUM-STIMULATED ADHERENCE OF *PSEUDOMONAS AERUGINOSA* TO LUNG EPITHELIAL CELLS 13
2.1 Introduction . . . 13
2.2 Material and Methods . . . 15
2.2.1 *P. aeruginosa* strains, media, and growth conditions . . . 15
2.2.2 Cell culture . . . 16
2.2.3 Growth curve of *P. aeruginosa strains* . . . 16
2.2.4 Adherence Assay . . . 16
2.2.5 Immunofluorescence Assay . . . 18
2.2.6 Scanning Electron Microscopy . . . 18
2.2.7 RNA sequencing . . . 19
2.2.8 Quantitative Reverse Transcriptase- Polymerase Chain Reaction (RT-qPCR) 20
2.2.9 Statistical analysis . . . 20
2.3 Results . . . 21
2.3.1 Effect of Ca^{2+} on the growth of *P. aeruginosa* strains . . . 21
2.3.2 Presence of Ca^{2+} in cell culture medium . . . 23
2.3.3 Ca^{2+} upregulates adherence of *P. aeruginosa* with A549 cells . . . 24
2.3.4 Ca^{2+} upregulates adherence of *P. aeruginosa* with CuFi-5 cells . . . 26
2.3.5 Visualization of *P. aeruginosa* adherence to epithelial cells . . . 28
2.3.5.1 Immunofluorescence Microscopy . . . 28
2.3.5.2 Scanning electron microscopy (SEM) . . . 33
2.3.6 Effect of Ca^{2+} on the adhesins of *P. aeruginosa* by RNA-seq analysis . . . 35

2.3.7 Ca^{2+} impacts transcription of *P. aeruginosa* genes . . . 35
2.4 Discussion . . . 38

3 VIRULENCE FACTORS OF *PSEUDOMONAS AERUGINOSA* AFFECTED BY CALCIUM 41
3.1 Introduction . . . 41
3.2 Materials and Method . . . 44
3.2.1 *P. aeruginosa* strains, clinical isolates, and cell culture . . . 44
3.2.2 Remel Flagella Staining . . . 44
3.2.3 Reactive Oxygen Species Assay . . . 45
3.2.4 Scanning Electron Microscopy . . . 45
3.2.5 Energy Dispersion X-Ray Spectroscopy (EDS) . . . 46
3.2.6 Crystal Violet Biofilm Assay (CVBA) . . . 46
3.3 Results . . . 47
3.3.1 Ca^{2+} affects the percentage flagellation of *P. aeruginosa* . . . 47
3.3.2 Ca^{2+} influences the production of reactive oxygen species . . . 48
3.3.3 Deposition of Ca^{2+} by *P. aeruginosa* using carbonic anhydrases . . . 50
3.3.4 Increased biofilm production by *P. aeruginosa* clinical isolates in the presence of Ca^{2+} . . . 53
3.4 Discussion . . . 55

4 CALCIUM MEDIATED INVASION, INTRACELLULAR REPLICATION AND ESCAPE OF *PSEUDOMONAS AERUGINOSA* DURING INFECTION WITH HOST CELLS 58
4.1 Introduction . . . 58
4.2 Materials and Methods . . . 60
4.2.1 *P. aeruginosa* strains and cell culture . . . 60
4.2.2 Cytotoxicity Assay . . . 61
4.2.3 Gentamycin protection assay . . . 61
4.2.4 Invasion Assay . . . 62
4.2.5 Escape Assay . . . 63
4.2.6 Western blotting . . . 64
4.2.7 Statistical analysis . . . 64
4.3 Results . . . 65
4.3.1 Cytotoxicity of Ca^{2+} on epithelial cells . . . 65
4.3.2 Effect of Ca^{2+} on the invasion rate of *P. aeruginosa* . . . 66
4.3.3 Intracellular survival and replication of *P. aeruginosa* . . . 67
4.3.4 Evasion of *P. aeruginosa* during infection . . . 68
4.3.5 Invasion and Intracellular replication of (PAO1, Δ*efhP* and complement) . 70
4.3.6 Role of EfhP in Ca^{2+}-mediated escape of *P. aeruginosa* . . . 71
4.3.7 Role of Ca^{2+} in host signaling during infection . . . 73
4.4 Discussion . . . 74

5 CONCLUSIONS AND FUTURE DIRECTIONS **76**
5.1 Conclusions 76
5.2 Limitations 77
5.3 Future Directions 79

REFERENCES **81**

LIST OF TABLES

Table Page

2.1 **Transcriptomic analysis of adhesins of *P. aeruginosa* PAO1 upregulated during growth at 5 mM Ca^{2+}** 37

LIST OF FIGURES

Figure | Page

1.1 **Role of CFTR** in (A) a healthy lung, acts as a chloride channel (B) Cystic Fibrosis lung, chloride ion exchange is disrupted. Image source (Phm, 2018). 2

1.2 **Cystic Fibrosis and *Pseudomonas aeruginosa* infections**. Image adapted from source (Marteyn et al., 2017) 8

2.1 **Adherence Assay of *P. aeruginosa* with lung epithelial cells** 17

2.2 **Growth curve of *P. aeruginosa* strains in BMM**. PAO1 (A), PAO1043 (B) and PAO1043.pMF (C) grown without and with Ca^{2+} (5 mM), samples were collected, and OD_{600} was recorded at regular intervals of 2 and 4 hr for 40 hr in triplicates. Data were plotted using GraphPad Prism, and the standard error of the mean (SEM) was calculated. 22

2.3 **Growth curve of *P. aeruginosa* strains in RPMI.** PAO1 (A), PAO1043 (B) and PAO1043.pMF (C) grown in low (0.54mM) and high (5 mM) Ca^{2+}, samples were collected and OD_{600} was recorded at regular intervals of 2 hr for 8 hr in triplicates. Data was plotted using GraphPad Prism and the standard error of the mean (SEM) was calculated. 23

2.4 **Quantitative calorimetric Ca^{2+} determination.** Using Quantichrom Calcium Assay Kit, the amount of Ca^{2+} in cell culture media was calculated in (A) RPMI, media used to culture A549 cells and (B) AEGM, media used to culture CuFi-5 cells, by following a standard curve method. 24

2.5 **Effect of Ca^{2+} on adherence of PAO1 strains during infection with A549 cells**. CFUs in low and high Ca^{2+} were calculated to calculate (A) the total number of bacteria used for infecting each well, (B) the total number of bacteria 2 hrs post incubation in RPMI, and (C) adherent *P. aeruginosa* per monolayer 2 hpi. Data plotted in the graph with the error bars indicating standard deviation from representative triplicate experiments. Statistical significance using one-way ANOVA and post hoc Newmann-Keuls test is indicated with an asterisk (*$p < 0.05$). ns = not significant. 25

2.6 **Effect of Ca^{2+} on adherence of FRD1 strains during infection with A549 cells**. CFUs in low and high Ca^{2+} were calculated to calculate (A) the total number of bacteria used for infecting each well, (B) the total number of bacteria 2 hrs post incubation in RPMI, and (C) adherent *P. aeruginosa* per monolayer 2 hpi. Data plotted in the graph with the error bars indicating standard deviation from representative triplicate experiments. Statistical significance using one-way ANOVA and post hoc Newmann-Keuls test is indicated with an asterisk (*$p < 0.05$). ns = not significant. 26

2.7 **Effect of Ca^{2+} on adherence of PAO1 strains during infection with CuFi-5 cells**. CFUs in low and high Ca^{2+} were calculated to calculate (A) the total number of bacteria used for infecting each well, (B) the total number of bacteria 2 hrs post incubation in RPMI, and (C) adherent *P. aeruginosa* per monolayer 2 hpi. Data plotted in the graph with the error bars indicating standard deviation from representative triplicate experiments. Statistical significance using one-way ANOVA and post hoc Newmann-Keuls test is indicated with an asterisk (*$p < 0.05$). ns = not significant. 27

2.8 **Effect of Ca^{2+} on adherence of FRD1 strains during infection with CuFi-5 cells**. CFUs in low and high Ca^{2+} were calculated to calculate (A) the total number of bacteria used for infecting each well, (B) the total number of bacteria 2 hrs post incubation in RPMI, and (C) adherent *P. aeruginosa* per monolayer 2 hpi. Data plotted in the graph with the error bars indicating standard deviation from representative triplicate experiments. Statistical significance using one-way ANOVA and post hoc Newmann-Keuls test is indicated with an asterisk (*p <0.05). ns = not significant. 28

2.9 **Elevated Ca^{2+} increases adherence of PAO1 strains to A549 cells**. *P. aeruginosa* was stained green, DNA stained blue (DAPI), and actin stained red. Scale bar, 10 μM. 29

2.10 **Elevated Ca^{2+} increases adherence of FRD1 strains to A549 cells**. *P. aeruginosa* was stained green, DNA stained blue (DAPI), and actin stained red. Scale bar, 10 μM. 30

2.11 **Elevated Ca^{2+} increases adherence of PAO1 strains to CuFi-5 cells**. *P. aeruginosa* was stained green, DNA stained blue (DAPI), and actin stained red. Scale bar, 10 μM. 31

2.12 **Elevated Ca^{2+} increases adherence of FRD1 strains to CuFi-5 cells**. *P. aeruginosa* was stained green, DNA stained blue (DAPI), and actin stained red. Scale bar, 10 μM. 32

2.13 ***P. aeruginosa* adheres to A549 cell surface**. High-power scanning electron micrographs showing attachment of *P. aeruginosa* (A) PAO1 and (B) FRD1 to the A549 cells at magnification of 10,000x in low and high (5mM) Ca^{2+} conditions. (C) PAO1 firmly adheres to the cell surface even after rigorous washes involved in the SEM sample prep, as seen at 20,000x magnification. 34

2.14 ***P. aeruginosa* adheres to CuFi-5 cell surface**. High-power scanning electron micrographs showing attachment of *P. aeruginosa* (A) PAO1 and (B) FRD1 to the CuFi-5 cells at magnification of 10,000x in low and high (5mM) Ca^{2+} conditions. . 34

2.15 **Gene expression of *P. aeruginosa*'s adhesins** *fliC, pilA* and *lecA* during infection with A549 and CuFi-5 cells, quantitated using RT-qPCR. The relative mRNA levels of genes expressed during infection were normalized to rpoD expression by using the 2- ΔΔCT method (n=3) with averages and standard deviation as shown. The data was statistically analyzed using paired Student's t-test with $p < 0.05$ (∗). . 36

3.1 **Modified remel flagella staining to visualize the effect of Ca^{2+}**: *P. aeruginosa* was grown in BMM with 0, 5, and 10 mM Ca^{2+} with standing incubation, followed by Remel staining and light microscopy. Representative images are shown at 1000× magnification, scale bar 10 μm. 47

3.2 **Upregulated Ca^{2+} increases the percentage of flagellated *P. aeruginosa***. The averages and standard deviation of three biological replicates from 15 fields of view are shown. Data were statistically analyzed using one-way analysis of variance (ANOVA), followed by the Newman-Keuls post hoc test, p<0.01(**) and p<0.05 (*). 48

3.3 **Ca^{2+} affects ROS induction by *P. aeruginosa* PAO1 strains at (A) 0.5 OD_{600} and (B) 0.75 OD_{600}.** Averages and standard deviation from three biological replicates are shown. One-way analysis of variance (ANOVA), followed by the Newman-Keuls post hoc test, was performed with p<0.05 (*). 49

3.4 **Ca^{2+} affects ROS induction by *P. aeruginosa* FRD1 strains at (A) 0.5 OD_{600} and (B) 0.75 OD_{600}** Averages and standard deviation from three biological replicates are shown. One-way analysis of variance (ANOVA), followed by the Newman-Keuls post hoc test, was performed with p<0.05(*). Three replicates were performed for this data. 49

3.5 **SEM microscopy of PAO1 infecting CuFi-5 cells.** Mesh-like salt structures observed with CuFi-5 cells during infection at 5 mM Ca^{2+} at a magnification of 10,000x (A), further magnified to 40,000x (B), suggesting the possible development of salt crystals on the cell surface during infection. 50

3.6 **Deposition of Ca^{2+} by *P. aeruginosa* during infection.** The carbonic anhydrase triple mutant *ps*ΔΔΔ*psCa* (A) shows no mesh-like structures at a magnification of 10,000x in high Ca^{2+} (scale bar 5μm). In contrast, with PAO1 (B), the structures were visible at 5000x (scale bar 20μm) using SEM. (C) Energy dispersion X-ray spectroscopy of the samples showed negligible Ca^{2+} deposition by the *ps*ΔΔΔ*psCa*, (0.05 total atomic%), whereas a comparatively higher percentage of Ca^{2+} deposition by PAO1 (0.32% to 0.58%) was observed. 51

3.7 **Energy dispersion X-ray spectroscopy confirming the deposition of Ca^{2+} by PAO1 infecting CuFi-5 cells.** The difference in the Ca^{2+} peaks by spectra generated by PAO1 in low Ca^{2+} (A) and high Ca^{2+} (B) and the mass % deposition (C and D) varying from 0.78% to 1.48% in low and high Ca^{2+} affirming the possible deposition of Ca^{2+} during infection of CuFi-5 cells with PAO1. 52

3.8 **Alginate production by *P. aeruginosa* clinical isolates.** Image source: self-captured. 53

3.9 **Increased biofilm production by *P. aeruginosa* clinical isolates grown in high Ca^{2+}.** PAO1 was used as a control. The data were analyzed using MATLAB, and ANOVA was used to calculate p values with $p<0.05$ (*) and $p<0.01$ (**). 54

4.1 **Determine the role of Ca^{2+} in the invasion, intracellular survival and escape of epithelial cells during infection and the role of Ca^{2+} and Ca^{2+} binding gene, *efhP* in this interaction.** Image source: generated by author using BioRender. . . . 60

4.2 **Gentamycin protection assay.** A549 lung epithelial cells were infected with *P. aeruginosa* to perform invasion and escape (washout assay). 63

4.3 ***P. aeruginosa* induces cytotoxicity during infection of A549 cells.** Attenuation of *P. aeruginosa* cytotoxicity was observed with increasing MOI (x-axis). 65

4.4 **Calcium is cytotoxic to A549 cells at high concentration.** Increasing levels of Ca^{2+}, induces cytotoxicity of epithelial cells in vitro. Additionally, 5 mM Ca^{2+} is substantially cytotoxic to epithelial cells at 4+ hr of incubation (data not shown) . . 66

4.5 **Gentamycin at a concentration of 200 μg/ml can effectively kill *P. aeruginosa* in 30 minutes post-gentamycin treatment.** 67

4.6 **Calcium upregulates *P. aeruginosa* invasion and survival with higher recoverable CFU counts at every hour during infection in high Ca^{2+}** (A) Total CFU/ml of *P. aeruginosa* recovered post-infection, along with planktonic and adhered *P. aeruginosa* as a control. (B) Invasion and intracellular multiplication of *P. aeruginosa*. (C) The abundance of RNAP in total protein obtained after lysis of epithelial cells infected with *P. aeruginosa*. GAPDH was used as an epithelial cell control. . 68

4.7 ***P. aeruginosa* escapes epithelial cells in 30 minutes post gentamycin washout.** CFU quantified from plating supernatant (A) and cell lysates (B) reported higher counts at every hour in high Ca^{2+}. (C) Supernatant obtained from 30-, 60-, and 90- minutes post washout was plated on LB agar. 69

4.8 **Calcium binding protein, EfhP aids in invasion and multiplication of *P. aeruginosa* during infection with A549 lung epithelial cells.** (A) Total CFU/ml of *P. aeruginosa* recovered post-infection, along with planktonic and adhered *P. aeruginosa* as a control. Invasion of P. aeruginosa strains at 1 hr (B), 2hr (C), and 3 hr (D) post gentamycin treatment. 70

4.9 ***P. aeruginosa* escapes A549 lung epithelial cells.** Recoverable CFU/ml of *P. aeruginosa* in supernatant post gentamycin removal, suggesting escape of *P. aeruginosa* strains at 1 hr (A), 2 hr (B), and 3 hr (C) post gentamycin treatment. 71

4.10 **Phosphorylation of PI3K and p-AKT substrates increases during infection in high Ca^{2+} when compared to phosphorylation in low Ca^{2+}** . Total protein was collected from *P. aeruginosa* PAO1 invasion of CuFi-5 cells for 1, 2, and 3 hr. (A) Activation of phospho PI3 Kinase (B) and abundance of total Phospho- AKT substrates increases in the presence of Ca^{2+} 73

5.1 **Summary of research findings.** This research study reports the role of calcium in influencing host-pathogen interactions of *Pseudomonas aeruginosa*, by altering the expression of virulence factors, host signaling and its ability to adhere and invade epithelial cells. 78

Figure Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Pathophysiology of Cystic Fibrosis (CF)

One of the most prevalent disorders in Caucasians, Cystic Fibrosis (CF), is a monogenic disease that affects at least 100,000 children and adults worldwide, with approximately 40,000 individuals in the United States alone (O'Sullivan and Freedman, 2009). It is an autosomal recessive genetic disorder that occurs due to a defect in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which encodes the CFTR protein. More than 75% of people with CF are diagnosed under the age of 2. (Malhotra et al., 2019). CFTR protein in healthy individual acts as a chloride channel, pumping chloride ions into lung secretions and thinning them out (Figure 1.1). Whereas in CF, the lack of functional CFTR results in disruption of chloride and bicarbonate secretions on various epithelial surfaces and impaired mucociliary clearance, leading to obstructive pulmonary disease, morbidity, and mortality (Strausbaugh and Davis, 2007). The early signs of the disorder show microbial infection, inflammation, and excessive pulmonary exacerbation, resulting in respiratory decline (Starner and McCray Jr, 2005). Early identification of symptoms and treatment can prevent respiratory failure, lung transplantation, and even death (Vallières and Elborn, 2014).

The severity of CF relies on the extent of the mutation in the CFTR (Veit et al., 2016) and the mutation class genetically influences the disorder. More than 2000 mutations have been reported to occur in the CFTR, classified into classes I-VI, each showing a defect in the production and function of protein (Zielenski and Tsui, 1995). 127 mutations out of more than 2000 mutations have been identified that cause this cystic fibrosis disorder (Veit et al., 2016). The prototypical mutation that renders CFTR dysfunctional occurs in 508th phenylalanine (Δ508). CFTR dysfunction affects vital parts of the body, including the lungs and pancreas, and is responsible for defects

in the immune response. In CF, alterations in immunity augment the susceptibility to bacterial infections, mucus retention, hyper inflammation, neutrophil migration, and lung damage (Elborn, 2016). Neutrophil recruitment is accompanied by impaired transport of antioxidants, antimicrobial proteins, and reactive oxygen species. These compounding effects make the elimination of pathogens by engulfment of neutrophils and macrophages ineffective (Cohen and Prince, 2012).

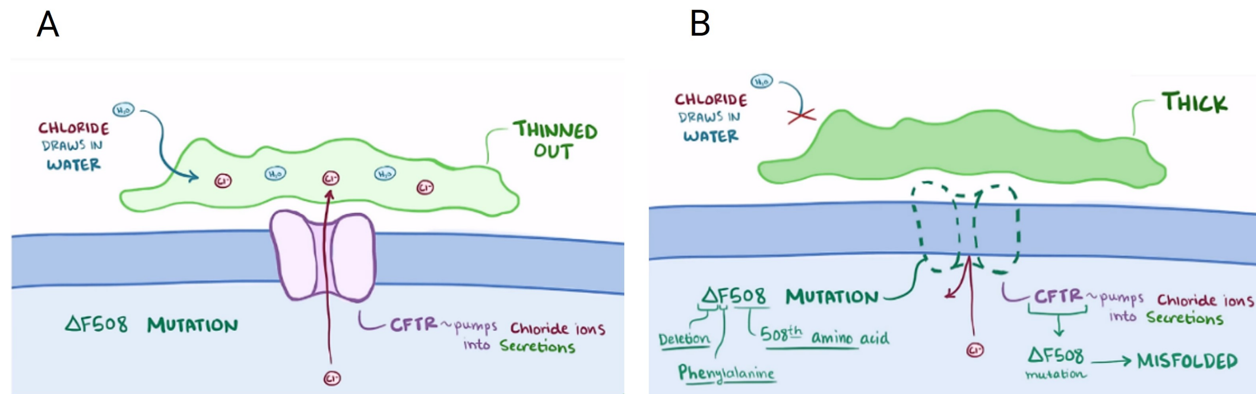


Figure 1.1: **Role of CFTR** in (A) a healthy lung, acts as a chloride channel (B) Cystic Fibrosis lung, chloride ion exchange is disrupted. Image source (Phm, 2018).

1.2 Microbial diversity in CF

A once debated dogma of sterile lower lungs now confirms that lung secretions are always exposed to a concoction of microorganisms through inhalation (Dickson and Huffnagle, 2015). Most of the microbiota is composed of commensals, yet the dysbiosis of the microbiome in a healthy lung contributes to pulmonary and chronic infections (O'Dwyer et al., 2016). Microbes adapt to the lung environment and colonize the oropharynx and upper respiratory tract in abundance (Wilson and Hamilos, 2014). The pathology of CF lung includes various viruses (rhinovirus, influenza, and respiratory syncytial virus) (Huang and LiPuma, 2016; Gilligan, 1991), fungi (*Aspergillus fumigatus* and *Candida spp.*) (Noverr et al., 2004; Lowes et al., 2017) and the most dominant bacterial species *Haemophilus influenza, Pseudomonas aeruginosa, Burkholderia cepacia complex, Staphylococcus aureus* and *epidermidis, Strenotrophomonas maltophilia, Achromobacter xylosoxidans*, and non-tuberculosis *Mycobacterium abscessus* (Surette, 2014). Using metagenomic sequencing,

(Zhao et al., 2012) analyzed the microbiome of CF patient's lung, suggesting the decline in the bacterial community with an increase in the severity of lung abscess. This decrease was related to the presence of chronic *P. aeruginosa* infections, which dominate the lungs due to their high adaptability and increased antibiotic resistance genes (Maughan et al., 2012). *Haemophilus influenzae* is often the early colonizer of the CF lung, followed by methicillin-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The increasing multidrug resistance, hence, the rise in their prevalence highlights the importance of these pathogens for further investigations.

1.3 Burden of *Pseudomonas aeruginosa* infections

Pseudomonas aeruginosa is an opportunistic gram-negative bacterial pathogen that belongs to the genus *Pseudomonas*, the class Gammaproteobacteria and the family Pseudomonadaceae (Garrity, 2007). The term *Pseudomonas* is derived from Greek words, where *pseudo* means 'false' and *monas* means 'unit,' as coined by the German botanist Walter Migula (Araos and D'Agata, 2019). The last name *aeruginosa* is supposedly derived from the Latin word 'verdigris' due to its distinct bluish-green coloration, attributed to the production of pyocyanin (blue) and pyoverdine (green) pigments. Some strains are also known to form pyorubin (red) and pyomelanin (blackish) pigments (Henry, 2012). Otherwise known as an aerobe and non-lactose-fermenting bacteria, it can also thrive under anaerobic conditions by using nitrate as a terminal electron acceptor, which has been observed in heterogeneous lung CF (Schobert and Jahn, 2010). *Pseudomonas*'s versatile genome is $\sim 5.5-7$ megabase pairs (Mbp) long with a rich 65% GC content and adaptable to DNA uptake by horizontal gene transfer (Klockgether et al., 2011).

Pseudomonas aeruginosa is a ubiquitous pathogen commonly found in the soil, water, plants, and surfaces of devices/equipment in hospital settings (Vasil, 1986). This pathogen causes more than 51,000 acute, chronic, and severe infections, including endocarditis, urinary tract infections, bacteremia, respiratory pneumonia, meningitis, ocular, ear, skin, and soft tissue (burn wound) infections (Zhang et al., 2020). A higher percentage of nosocomial infections is caused by *P. aeruginosa*, which targets immunocompromised individuals and intensive care patients that contribute

to the burden of healthcare with a mortality risk between 18-60% (Ammerlaan et al., 2013; Vidal et al., 1996). Reports of the growth of *Pseudomonas* in disinfectants, on biotic and abiotic surfaces in hospitals, are alarming (Kramer et al., 2006; Favero et al., 1971). Furthermore, the increasing challenge of multidrug resistance to effectively treat and eradicate *Pseudomonas* infections persists. It should be noted that often opportunistic pathogenic species of *Pseudomonas* include *P. aeruginosa, P. cepacia, P. mallei, P. maltophilia* (Iglewski, 1996).

In studying the biology of lung infections, *Pseudomonas* has been intrinsically linked to bacterial infections in the lung of CF (Figure 1.2). Abrogation of CFTR impacts early bacterial colonization of the lungs. *P. aeruginosa* form biofilms in the mucosal epithelium of the airways in the lungs of patients with CF (Surette, 2014). The presence of *Pseudomonas* in the lungs has been directly related to lung damage and fatality (Kerem et al., 1990; Pamukcu et al., 1995). At an early age of 6 months, more than 30% infants are diagnosed with *P. aeruginosa* infections and the lungs of CF remain influenced by these infections throughout their life (Li et al., 2005). Thus, early infection is associated with lung decline and irreversible damage, as recorded by chest X-ray imaging (Pillarisetti et al., 2011; Nixon et al., 2002).

1.4 *P. aeruginosa* virulence factors

P. aeruginosa infections in CF are exemplary of typical host-pathogen interactions. The establishment of infection is dependent on the expression of virulence factors to evade immune responses such as neutrophil recruitment and phagocytosis. Some of the most important virulence factors of *P. aeruginosa* include

- **The flagella and Type IV pilus-** *Pseudomonas* species possess single polar flagella and several Type IV pili, necessary for their motility. Flagella constitutes flagellin protein *fliC*, which is responsible for swimming motility (Lillehoj et al., 2002); pili comprising the major pilin protein *pilA* is required for swarming and twitching motility (Burrows, 2012). Swarming motility leads to aggregation and formation of biofilms (Craig et al., 2004). *P. aeruginosa* utilizes flagella and pili to adhere to epithelial cell receptors (asialyated glycolipid

GM1). Upon recognition of bacterial adhesion by immune receptors, NF-κB inflammatory response is initiated, potentially impairing lung functionality (Miao et al., 2007).

- **Type 3 secretion system**- T3SS has been reported to be a major virulence determinant of the pathogenesis of *P. aeruginosa* (Hauser, 2009). T3SS is a needle-shaped injectosome that penetrates the epithelial cell membrane to release virulence-effector proteins into the host cell. Some of the well-characterized effector proteins are ExoU, ExoS, ExoT, and ExoY. ExoU is a phospholipase that hydrolyzes the membrane of the host cell, breaching the junction; ExoS and ExoT are cytotoxins involved in actin cytoskeleton remodeling (Shaver and Hauser, 2004), while ExoY is an adenylate cyclase required for the expression of kruppel factor, KLF6, and induces cytotoxicity in the host cell (Hauser, 2009).

- **Quorum sensing and biofilm production**- Biofilm produced by *P. aeruginosa* is regulated via quorum sensing, a mechanism by which bacterial population communicates for a coordinated adaptation to the environment (Karatan and Watnick, 2009). Quorum signaling allows *P. aeruginosa* to stimulate biofilm production using Las I/R system (Henke and Bassler, 2004). These systems activate autoinducer, including acyl homoserine lactones (AHL) and 3-oxo- dodecanoyl homoserine lactone (3-oc12-HSL), that facilitate coordinated gene expression of the entire bacterial population for the expression of proteins involved in biofilm formation (Deep et al., 2011). Other factors that promote biofilm formation include cyclic di-GMP, sigma factors, and two-component systems (Mikkelsen et al., 2011).

- **Proteases and Lipases**- *P. aeruginosa* secretes enzymes that help break down the host protein and aid in its survival. Alkaline protease 1 leads to the destruction of complement proteins of the immune system and is also involved in the destruction of free flagellin to escape immune detection (Bardoel et al., 2011). A serine protease, LasA is a streptolysin, and the elastase LasB is secreted by the *lasQS* quorum sensing system (Toder et al., 1994). Additionally, extracellular phospholipases C (PlcA, PlcB, PlcH, and PlcN) suppress neutrophil activity by interfering with the PKC pathway (Terada et al., 1999).

- **Pyocyanin and Pyoveridine**- The greenish blue color of *P. aeruginosa* is due to the production of pyocyanin and pyoverdine. Pyocyanin is a redox-active toxin that causes oxidative stress in epithelial cells during infection. It leads to neutrophil apoptosis (Lau et al., 2004). In contrast, pyoverdine is a siderophore that scavenges iron and regulates signaling in *Pseudomonas* to up-regulate ExoA exotoxin, which inhibits the synthesis of host elongation factor (EF-2), leading to cell death (Bonneau et al., 2020; Jimenez et al., 2012). The interaction of pyocyanin with reactive oxygen species accelerates cell death (Manago et al., 2015).

- **Exopolysaccharide**- Bacterial communities secrete extracellular polymeric substances (EPS) to bind bacterial aggregates in an organized manner for the production of biofilms (Donlan, 2002). To replicate within a biofilm matrix using EPS, three polysaccharides are produced by most *P. aeruginosa* strains, namely Alginate, Psl, and Pel. Alg R regulates the overproduction of alginate and mucus, making the lung environment prone to invading bacterial pathogens, and plays a significant role in curbing the host's immune defense mechanisms necessary to eliminate them (Whitchurch et al., 1996). In contrast, Psl extracellular polysaccharides facilitate surface attachment, as seen in *P. aeruginosa* clinical isolates (Colvin et al., 2012) and the production of Pel has been tied to the antibiotic resistance of *P. aeruginosa*, especially aminoglycoside (Colvin et al., 2011). Two of the important *P. aeruginosa* lipopolysaccharides (LPS) are the lipid A and O-polysaccharide (King et al., 2009; Lam et al., 2011). Lipid A binds to epithelial cell receptors to activate signaling cascades, stimulating chemokines and cytokines. Modifications in lipid A by *Pseudomonas* are associated with increased resistance to antibiotics (Gellatly and Hancock, 2013).

- **CFTR inhibitory factors (Cif)**- *Pseudomonas* secretes virulence factors to reduce CFTR expression on the apical membrane. One such factor, Cif, is an epoxide hydrolase, encoded by the PA2934 gene, which inhibits the secretion of chloride ions on the epithelial cell surface (MacEachran et al., 2007; Bomberger et al., 2014). Therefore, inhibition of CFTR by Cif diminishes the bacterial activity of host immune defenses (Ye et al., 2008) and is involved in altering the biology of the CF lung allowing the entry of pathogens via secretion of mucus

and other extracellular virulence factors (Bahl et al., 2010).

1.5 Host pathogen interactions of *P. aeruginosa* in cystic fibrosis

During infection, *P. aeruginosa* faces various forms of environmental stress such as interspecies competition (Figure 1.2), host immunity evasion, hypoxic and variable oxygen gradients due to mucus retention in the lungs, osmotic stress due to reactive oxygen species generated by neutrophil migration, increased influx of antimicrobials; body temperature fluctuations, iron acquisition and disruption of Ca^{2+} homeostasis (Hogardt and Heesemann, 2010). These environmental pressures force *P. aeruginosa* to undergo various phenotypic and genotypic changes that lead to selective mutations for long-term survival in the host. These adaptations facilitate the bacterial growth and severity of lung infections, transitioning from acute to chronic (Dobrindt et al., 2013). To establish a niche, oftentimes some *P. aeruginosa* virulence factors are downregulated (motility, rhamnolipid production, pyocyanin, and pyoverdine), while others are upregulated (biofilm production, exopolysaccharide secretion, alginate, and effector proteins of the secretion system) (Bhagirath et al., 2016). Adaptation to the host environment offers advantages that allow the establishment of chronic irreversible infection (Lovewell, Patankar and Berwin, 2014). The flagellum is crucial for the motility of *P. aeruginosa* because it aids in initial attachment, binds to the host cell receptor using TLR5, and allows microbial recognition by neutrophils and macrophages (Lovewell, Hayes, O'Toole and Berwin, 2014). In addition, down-regulation of flagella-associated genes alters adhesion that helps *Pseudomonas* convert from a motile to a sessile lifestyle and evade immune cell phagocytosis (Cobb et al., 2004).

Similarly, lipopolysaccharide endotoxins undergo mutations in lipid A endotoxin in chronic CF isolates of *P. aeruginosa*. This has been shown to reduce phagocyte movement and lower inflammatory cytokines in mammalian models (Cigana et al., 2009). Compared to planktonic bacteria, *P. aeruginosa* biofilms mitigate the activation of the complement system and restrict neutrophil recruitment (Jesaitis et al., 2003). Mutagenesis of *P. aeruginosa* is attributed to antimicrobials secreted by the host cell. Pathologically adapted *P. aeruginosa* isolated from chronically infected

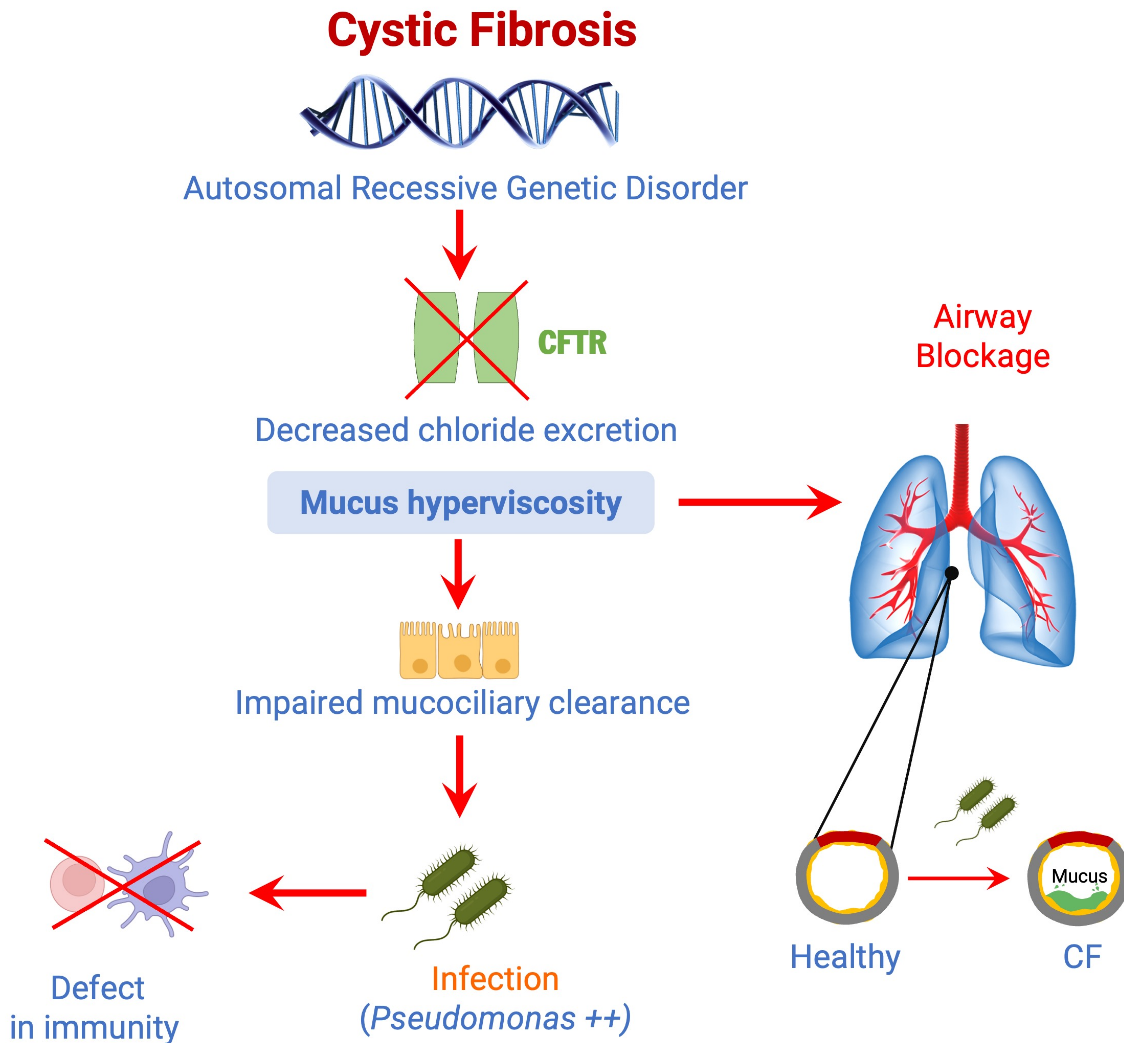


Figure 1.2: **Cystic Fibrosis and *Pseudomonas aeruginosa* infections**. Image adapted from source (Marteyn et al., 2017)

lungs exhibit heterogeneity (Smith et al., 2006). *P. aeruginosa* obtained from the upper lobe of chronic lung infection have comparatively higher antimicrobial resistance and were reported to be more virulent in animal models (Jorth et al., 2015).

1.6 Calcium (Ca^{2+}) signaling

1.6.1 Ca^{2+} signaling in eukaryotes

Ca^{2+} is a versatile secondary messenger that regulates various physiological processes in eukaryotes. The cellular signaling molecule, Ca^{2+} accumulates in the pulmonary fluids of CF patients and alters signaling pathways (Verkhratsky and Parpura, 2014). Ca^{2+} signaling lies at the core of eukaryotic signaling and regulates a wide range of cellular processes such as gene expression, metabolism, cell division, and differentiation (Clapham, 2007). The intracellular concentration of unbound Ca^{2+} ($\sim 10^{-7}$nM) is nearly 10,000 times higher than the extracellular concentration ($\sim 10^{-3}$ nM) (Bagur and Hajnóczky, 2017). Transient changes in intracellular Ca^{2+} concentration disrupt cellular signaling (Zampese and Pizzo, 2012). Furthermore, cell organelles (such as the endoplasmic reticulum and mitochondria, sequester Ca^{2+}, and transport systems) control the translocation of Ca^{2+}, small anions, and metabolites with the cell (Gilabert, 2012). Ca^{2+} ions, although vital for cell function, can be cytotoxic to both microbial and eukaryotic cells (Dominguez, 2004). Abnormalities in Ca^{2+} homeostasis can be detrimental to eukaryotic cells and contribute to diseases and cell death (Case, 1984).

1.6.2 Ca^{2+} signaling in *P. aeruginosa*

Various pathogenic bacterial species, such as *Escherichia coli, Bacillus subtilis, Vibrio cholera, Streptococcus*, and *Pseudomonas* species maintain micromolar intracellular Ca^{2+} levels. The transcriptomic analysis of these pathogens reported an increase in the expression of a number of genes regulated by intracellular Ca^{2+} (Naseem et al., 2009; Oomes et al., 2009; Bilecen and Yildiz, 2009). In response to environmental stress, these bacterial pathogens regulate a transient surge of intracellular Ca^{2+} levels. Recognition of host Ca^{2+} levels by invading microorganisms enables their adaptation and survival under environmental stress. Evidence suggests that Ca^{2+} plays a crucial role in modulating the structural integrity of biofilms (van Loosdrecht et al., 1990). An increasing amount of evidence suggests that Ca^{2+} increases the abundance of proteins required

for iron acquisition, modulating stress responses (Patrauchan et al., 2007), and the expression of virulence genes in *P. aeruginosa* (Guragain et al., 2013). Further investigations suggested that Ca^{2+} enhances the virulence of *P. aeruginosa* by increasing biofilm production (Sarkisova et al., 2005), antibiotic resistance (Khanam et al., 2017), and production of pyocyanin and pyoverdine (Sarkisova et al., 2005)

1.7 Ca^{2+}-binding proteins (CaBP)

Ca^{2+} plays an integral role in various cellular processes. The fluctuations in intracellular concentration of Ca^{2+} are recognized and modulated via Ca^{2+} binding proteins (CaBP) to maintain Ca^{2+} homeostasis (Bagur and Hajnóczky, 2017). These proteins are divided into two major classes: EF-hand and non-EF-hand proteins (Rigden et al., 2003). The EF-hand proteins bind Ca^{2+} in a helix-loop-helix Ca^{2+} motif. The E and F motifs resemble hand-like structures of two α helices while the binding loop is the center; together the cooperate to bind to Ca^{2+} ions. Eukaryotic EF-hand CaBP includes Calmodulin, Calcinurin, and Parvalbumin, whereas the non-EF hand protein constitutes annexins and Calreticulin (Persechini et al., 1989). Calmodulin is a Ca^{2+} sensor that translates signals to epithelial cells, and Parvalbumin is a buffer required by cells to maintain Ca^{2+} concentrations (Yang and Tsai, 2022).

Although bacteria exploit Ca^{2+} signaling pathways as a mechanism to establish infection in the host, prokaryotic Ca^{2+} binding proteins are still very understudied. One of the CaBPs of *P. aeruginosa* is EfhP. It is a 123 amino acid long integral membrane protein (PA4107) whose N-terminal is embedded in the cytoplasm and C-terminal in the cell periplasm. It has been shown to specifically bind to Ca^{2+} and regulate the virulence of *P. aeruginosa* (Kayastha et al., 2022). EfhP contributes to the maintenance of intracellular Ca^{2+} and maintains Ca^{2+} homeostasis. EfhP mutants have been reported to demonstrate reduced biofilm and pyocyanin production, decreased oxidative stress, and infection in a lettuce leaf midrib model (Sarkisova et al., 2014).

1.8 Ca^{2+}-mediated virulence of *P. aeruginosa*

Ca^{2+} triggers lifestyle changes in *P. aeruginosa*. Growth of *P. aeruginosa* in 5 and 10 mM Ca^{2+} up-regulates the expression of virulence factors, such as biofilm and pyocyanin production (Sarkisova et al., 2005), as well as antibiotic resistance to tobramycin (Khanam et al., 2017). The transcription of CarP, a β propeller protein of *P. aeruginosa*, is controlled by Ca^{2+} regulated two-component system of *P. aeruginosa* (King et al., 2021). It contributes to the tolerance of hydrogen peroxide to oxidative stress. Additionally, EfhP (CaBP) modulates stress responses in *P. aeruginosa* under high Ca^{2+} conditions (Sarkisova et al., 2014). *P. aeruginosa* is shown to deposit extracellular Ca^{2+} in the form of calcium carbonate under high Ca^{2+} conditions, which are directly linked to biomineralization in bodily organs leading to diseases such as atherosclerosis (Lotlikar et al., 2019).

Furthermore, after testing the virulence of *P. aeruginosa* in high Ca^{2+} using the lettuce leaf mid rib and *Galleria mellonella* worm model, it was reported that Ca^{2+} significantly enhances the virulence of *P. aeruginosa*. EfhP mutants of PAO1 and FRD1 showed not only a reduction in virulence but also in alginate production by FRD1. Additionally, a preliminary study conducted by Dr. Aya Kubo (not published) directed toward the possible role of EfhP in the invasion, as EfhP gene expression was higher in epithelial cell lysates obtained from invasion.

1.9 Knowledge Gap and Research Hypothesis

As described in §1.8, elevated Ca^{2+} levels lead to increased virulence in *P. aeruginosa* (Kayastha et al., 2022; Sarkisova et al., 2014), but little is known about the regulation and expression of virulence genes by Ca^{2+} and EfhP during infection with human lung epithelial cells.

Based on the existing knowledge in the literature, we anticipate that the presence of Ca^{2+} is crucial in the regulation of pathogenic interactions between *P. aeruginosa* and the host cell. We hypothesize that Ca^{2+} may not only serve as one of the host determinants to trigger colonization (adherence), but also plays a key role in invasion, intracellular survival, and escape of *P. aeruginosa* from epithelial cells or transmigrate, enhancing its virulence capability. This evoked a need to study

host-pathogen interactions of *P. aeruginosa* at a cellular level.

1.10 Scientific objective and Significance

Using human cell lung epithelial cell lines, i.e., A549 and CuFi5 (homozygous for the Δ508 mutation in CFTR), and various strains of *P. aeruginosa* including lab strains and clinical isolates, I aim to establish a better understanding of the role of the Ca^{2+} and Ca^{2+}-binding protein, EfhP, in mediating adhesion, invasion, and intracellular survival of *P. aeruginosa* in lung epithelial cells. Here, I will quantify the expression of virulence genes and flagellation in *P. aeruginosa*, biofilm production, biomineralization, and activation of host signaling pathways by *P. aeruginosa* to reveal the contribution of Ca^{2+} to up-regulate the virulence of *P. aeruginosa*. The key to understanding chronic *P. aeruginosa* pathogenesis in cystic fibrosis lies in deciphering the interactions between host and pathogen at a cellular and molecular level.

An in-depth understanding of Ca^{2+} in *P. aeruginosa* infections will pave the path to develop novel strategies to prevent and combat these infections, in turn, helping extend the life span of patients with CF.

CHAPTER 2

CALCIUM-STIMULATED ADHERENCE OF *PSEUDOMONAS AERUGINOSA* TO LUNG EPITHELIAL CELLS

This chapter is reproduced with modifications from the manuscript drafted:

Deepali Luthra, Ty Lutze, Sharmily Khanam, Brian Couger, Aya Kubo, Marianna Patrauchan, Erika I. Lutter, "**Calcium-Stimulated Host Adherence of *Pseudomonas aeruginosa* during infection**".

All the experiments resulting in data generation for this chapter were conducted by DL under the supervision of EL and MP, apart from the RNA-seq (conducted by SK and BC).

2.1 Introduction

Cystic fibrosis (CF) is a genetic disorder that occurs because of mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene (Lukacs et al., 1993), which functions as a chloride ion channel. The deletion of phenylalanine at residue 508 (ΔF508) in CFTR protein is the most common mutation, leading to misfolding of the CFTR protein affecting its function and localization. As a result, there are thick secretions in the lungs caused by altered chloride ion kinetics that, in turn, predispose to bacterial colonization (Shteinberg et al., 2021).

Pseudomonas aeruginosa is one of the most prevalent bacterial pathogens of chronic CF lung infections in the United States and worldwide (Davies, 2002) and forms biofilms in airway mucosal epithelium in the lungs of CF patients, thus making infections difficult to treat (Bhagirath et al., 2016). Severe pneumonia, permanent lung damage, and mortality are some long-term consequences of *P. aeruginosa* infections (Kang et al., 2003), constituting a significant burden on the economy and human health (Coutinho et al., 2008).

P. aeruginosa possesses the ability to adhere to and interact with lung epithelial cells

(Plotkowski et al., 1989, 1991), including cells deficient in the CFTR protein (Ramphal and Pier, 1985; Sheth et al., 1994; Yamaguchi and Yamada, 1991). Adherence to alveolar and bronchial epithelial cells is facilitated using alginate, mucoid exopolysaccharide, LPS, and respiratory mucin (Prince, 1992; Carnoy et al., 1994). Apart from the lung conditions in CF, it is understood that *P. aeruginosa* is responsible for the excessive production of mucus in the CF lungs (Yan et al., 2008). The CF tracheobronchial mucosa is commonly exposed to infections and has abnormal levels of Ca^{2+} and other divalent cations (Schöni et al., 1987; Murphy et al., 1988).

Ca^{2+}, a well-known cellular signaling ion and a secondary messenger, regulates various cell functions, including metabolism, motility, secretion, gene expression, cell wall synthesis, and survival (Berridge, 2012). It acts as a trigger for the expression of virulence factors and is involved in regulating the host responses to bacterial infections (Domínguez et al., 2015). Abnormalities in Ca^{2+} signaling can lead to abnormal gene expression, cell death, increased susceptibility to bacterial infections, and other deleterious events.

Smith et al. (2014) reports that Ca^{2+} is associated with a possibility of influencing virulence of bacteria in CF by accumulating in the pulmonary fluids of CF patients. It has been shown that elevated Ca^{2+} levels lead to the secretion of virulence in *P. aeruginosa*, such as increased swarming motility, production of biofilm, alginate, pyoverdine and pyocyanin (Sarkisova et al., 2014; King et al., 2021; Sarkisova et al., 2005), as well as the ability to cause infection in lettuce leaf plant and galleria mellonella worm model (Kayastha et al., 2022). A calcium binding protein of *P. aeruginosa*, EfhP (PA4107), is shown to bind to Ca^{2+} selectively, and mediate responses for expression of virulence factors (Kayastha et al., 2022). However, little is known about how Ca^{2+} regulates virulence during infection with human lung epithelial cells.

Virulence factors of *P. aeruginosa* are involved in initial colonization, or adherence using flagella and pili (Feldman et al., 1998; Bucior et al., 2012), and in causing damage to the epithelial cells with pyocyanin, pyoverdine, rhamnolipids and proteases (Sarkisova et al., 2005; Lau et al., 2004). Our objective is to establish the role of Ca^{2+} in the host-pathogen interactions of *P. aeruginosa* during infection. We anticipate that this study will provide significant insights about the

importance of Ca^{2+} in regulating pathogenic interactions between *P. aeruginosa* and the host cell. Additionally, we hypothesize that Ca^{2+} serves as one of the host determinants that trigger colonization and expression of virulence genes of *P. aeruginosa* during infection, the knowledge of which is important to understand the enhanced virulence of *P. aeruginosa* in CF.

2.2 Material and Methods

2.2.1 *P. aeruginosa* strains, media, and growth conditions

P. aeruginosa strains used for this study included PAO1 (laboratory strain isolated from a wound infection) (Stover et al., 2000); FRD1 (mucA22) (mucoid pulmonary isolate from a CF patient) (Ohman and Chakrabarty, 1981); Δ*efhp* mutant (PAO1043, FRD1043) and complement (PAO1043.pMF 470.1, FRD143.pMF 470.1), generated in PAO1 and FRD1 background. (Kayastha et al., 2022) (Sarkisova et al., 2014). Cultures were routinely grown in Biofilm Minimal Media (BMM) (Sarkisova et al., 2005), which contains (per liter) 9.0 mM sodium glutamate, 145 mM NaCl, 50 mM glycerol, 0.34 mM K_2HPO_4, 0.15 mM NaH_2PO_4, 0.02 mM $MgSO_4$, 1 ml of vitamin solution and 200 µl of trace metals. The vitamin solution (per liter) contains 0.5 g of thiamine and 1 mg of biotin. The trace metal solution (per liter) consisted of 5.0 g of $CuSO_4.5H_2O$, 5.0 g of $ZnSO_4 \cdot 7H_20$, 5.0 g of $FeSO_4 \cdot 7H_20$ and 2.0 g of $MnCl_2 \cdot 4H_20$. Ca^{2+} levels were tested using the QuantiChrom^{TM} calcium assay kit. When required, 1 M of calcium chloride solution ($CaCl_2 \cdot 2H_20$) (Sigma Aldrich, St. Louis, MO) was added to obtain a final concentration of 5 mM or 10 mM, which will be considered a high Ca^{2+} condition for all the experiments. Bacterial cells were grown at 37°C, 220 rpm until reaching a mid-log growth phase (∼8 hr), cultures were normalized to 0.2 OD_{600}, and 0.1% of the culture was used for sub-culturing in BMM (Patrauchan et al., 2007). The subcultures were again incubated to reach the mid-logarithmic phase and normalized to 0.2 OD_{600} in BMM for infection studies. *P. aeruginosa* were plated using Luria- Bertani (LB) agar medium, and the bacterial colonies were counted to calculate the respective colony forming units (CFU/ml) of *P. aeruginosa*.

2.2.2 Cell culture

Two different human lung epithelial cell lines were used for this study: A549 adenocarcinoma alveolar basal epithelial cells (ATCC® CCL-185) (Giard et al., 1973), and CF bronchial CuFi-5 (ATCC® CRL-4016) bronchus lung epithelial cells which are homozygous for the ΔF508 mutation of the CFTR protein in CF (Molina et al., 2015). For CuFi-5 cells, the tissue culture flasks and plates were coated with 100 µg/ml of PureCol Type I bovine collagen solution (Advanced BioMatrix, Carlsbad, CA). A549 cells were grown in Roswell Park Memorial Institute (RPMI 1640, Gibco) medium supplemented with 5% fetal bovine serum (FBS), and CuFi-5 cells were grown in Airway Epithelial Growth Media (AEGM), including its supplement mix (Promega®, C-39165). Cells were passaged using tissue culture-treated flasks (Falcon, Corning) and maintained at 37°C with 5% (v/v) in a CO_2 incubator (NuAire, MN). The concentration of Ca^{2+} in cell culture media was measured using QuantiChrom™ Calcium Assay Kit (BioAssay Systems, Hayward, CA). The media was adjusted to 5 mM Ca^{2+} and quantified by following the manufacturer's guidelines.

2.2.3 Growth curve of *P. aeruginosa strains*

For quantitative growth curves, *P. aeruginosa* primary cultures were grown to the mid-log phase and diluted to an optical density of 0.1 OD_{600} , and 0.1% of the culture was used to start a secondary culture in triplicates. Growth data were obtained in BMM for 40 hr and in RPMI (supplemented with 5% FBS) for 8 hr, with 0 and 5 mM Ca^{2+}. Samples were collected at regular intervals, and OD_{600} was recorded using a Bio-Spectrophotometer (Hamburg, Germany). This was followed by serially diluting the samples in Phosphate Buffered Saline (PBS) and plating on LB agar to calculate the CFU/ml of *P. aeruginosa* at each optical density.

2.2.4 Adherence Assay

A549 and CuFi-5 cells were seeded in tissue culture-treated 24 well plates (CellTreat Scientific, Pepperell, MA) to obtain confluency of $\sim 0.2\times10^6$ cells after 48 hr of incubation. Infection with different *P. aeruginosa* strains were performed at a multiplicity of infection (MOI) of ∼50 in the

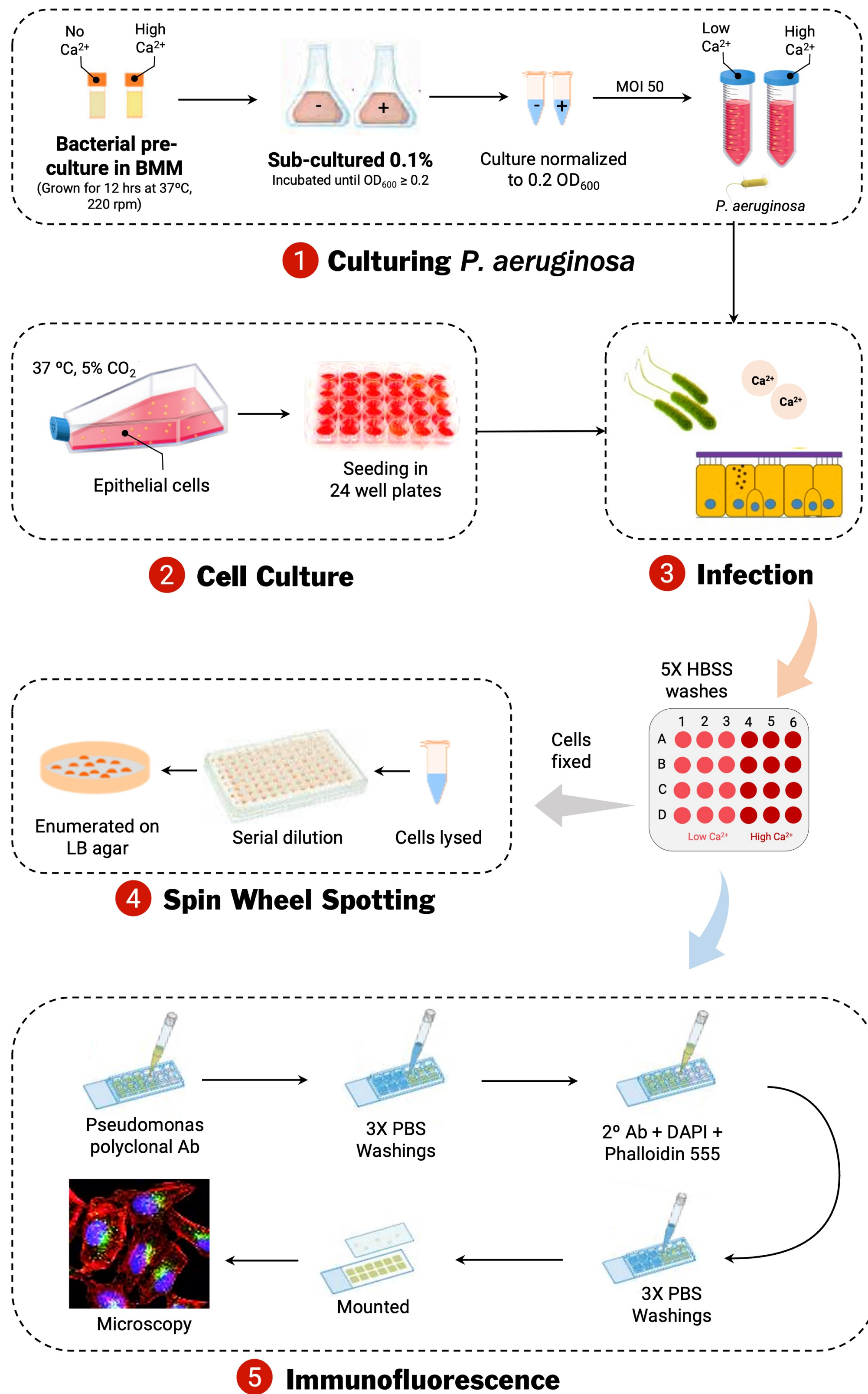


Figure 2.1: **Adherence Assay of *P. aeruginosa* with lung epithelial cells**

respective medium. This was followed by centrifugation for 10 mins at 500g to synchronize infection and incubated for 2 hr at 37°C with 5% CO_2. Two hr post-infection, cell monolayers were gently washed (5x) with Hank's Balanced Salt Solution (HBSS, pH 7.5) (Cytiva, Marlborough, MI) with 5 mins of incubation in between washes to remove non-adherent and loosely adhered bacterial cells. Cells were subsequently lysed with sterile water and scraped with a cell scraper (Biologix Research Co, Lenexa, KS). Cell lysates were serially diluted to quantify adherent bacteria in 1×PBS (pH 7.5) and plated onto LB agar plates for the CFU enumeration, as mentioned in 2.1, confirming the infection dose.

2.2.5 Immunofluorescence Assay

As described in § 2.2.4, A549 and CuFi-5 cells were seeded in 24 well plates with round glass coverslips. After infection, cells were fixed with 4% paraformaldehyde in PBS, rinsed in PBS, and blocked with 1% bovine serum albumin (BSA). Coverslips were incubated with *P. aeruginosa* primary polyclonal antibody (ThermoFisher Scientific) in 1% BSA and incubated at 4°C overnight. After the PBS washes, Alexa Fluor® 488 AffiniPure Goat Anti-Rabbit IgG secondary antibody (Jackson ImmunoResearch Laboratories, Pennsylvania, USA), DAPI (ThermoFisher Scientific) and Alexa FluorTM Phalloidin 555 (ThermoFisher Scientific) were added and incubated for 1 hr at room temperature. Using Dako Mounting Medium (Agilent Technologies, Santa Clara, CA), glass coverslips were mounted to glass slides and observed with the Leica DM16000B (Leica Microsystems, Buffalo Grove, IL).

2.2.6 Scanning Electron Microscopy

A549 and CuFi-5 cells were seeded in 24 well tissue culture treated plates onto round glass coverslips in triplicates and grown until ∼95% confluency. Cells were infected with *P. aeruginosa* at an MOI ∼50, incubated for 2 hr, followed by five washes in HBSS and fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer with 0.035 M $CaCl_2$. Coverslips were washed three times with buffered wash (0.2 M cacodylate buffer with 0.26 M of sucrose) along with 15 min incubation at

room temperature between each wash, fixed for 1 hr in 1% aqueous osmium tetraoxide and washed three times with the buffered wash. Dehydration was obtained using three washes with 50%, 70%, 90%, 95%, and 100% alcohol for 15 mins each. The coverslips were dried using hexamethyl-disilazane (HMDS), mounted on aluminum stubs, and sputter coated with gold palladium. The samples were visualized by FEI Quanta 600 field-emission gun Environmental Scanning Electron Microscope (SEM).

2.2.7 RNA sequencing

P. aeruginosa was grown to the mid-log growth phase (defined by OD_{600}-monitored growth curves) in BMM without and with 5 mM Ca^{2+}. RNA was isolated from the collected bacterial cells as described in (Lotlikar et al., 2019). Briefly, total RNA was isolated using RNeasy Protect Bacteria Mini kit (Qiagen) or ZR Fungal/ Bacterial RNA MiniPrep TM (Zymo Research) from three replicates for each growth condition. The purified RNA was checked for its quality using a Bioanalyzer 2100 (Agilent) and 1% agarose gel electrophoresis. RNA-Seq analysis was performed by Vertis Biotechnology AG (Germany) as explained in (Conway et al., 2014). Before library preparation, ribosomal RNA was removed from the total RNA samples using a bacterial Ribo-Zero rRNA Removal Kit (Illumina). The RNA library was amplified and sequenced on an Illumina NextSeq 500 system using 75 bp read length (NCBI accession number- PRJNA703087). The Illumina reads were aligned to the reference *Pseudomonas* PAO1 genome (downloaded from the Pseudomonas Genome Database) with 95-97% alignment rate by using the short read alignment program Bowtie 2 (Langmead and Salzberg, 2012). Quantification of transcripts was achieved by using the RSEM (RNA-seq by Expectation-Maximization) program (Li and Dewey, 2011). Statistical analysis for differential gene expression was conducted using the RSEM count matrixes in EdgeR (Robinson et al., 2010). Differentially expressed genes were selected based on a $\log_2$ fold change $\geq$ 1.0 and false discovery rate (adjusted P value $\leq$ 0.05). Homology searches using NCBI and UniProt databases were used for additional gene annotation.

2.2.8 Quantitative Reverse Transcriptase- Polymerase Chain Reaction (RT-qPCR)

Reverse transcription RT-qPCR was performed to test the expression of *P. aeruginosa* adhesins genes (fliC, pilA and lecA). Using the purified RNA, template cDNA was synthesized from the adherence assay samples of PAO1 and FRD1 with both A549 and CuFi-5 cell lines in low and high Ca^{2+} conditions. For each sample, the infected cell monolayers were washed with HBSS and lysed in RNA Later (ThermoFisher Scientific) to preserve the integrity of the RNA. RNA was isolated using RNAeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. RNA was treated with DNase I (Roche Diagnostics, Basel, Switzerland), and its quality was tested by agarose gel electrophoresis. Primers and expected transcript sizes are shown in Table S1. For positive control, 16S-1100-R / 16S-533-F primers were used to amplify the 16S rRNA gene. Purified RNA samples were tested for the absence of genomic DNA contamination using respective primer sets before cDNA synthesis. Primer specificity was tested using the DreamTaq master mix (ThermoFisher Scientific) using PAO1 and FRD1 genomic DNA as the template. The PCR reactions for the 16s rRNA gene were carried out using the following protocol: 95°C for 2 min, and 30 cycles of 95°C for 30 sec, 56°C for 30 sec, and 72°C for 40 sec, followed by 72°C for 2 min. Using gel electrophoresis, PCR-amplified samples were cross-checked for the absence of genomic DNA prior to cDNA synthesis. cDNA was synthesized using the ThermoFisher Maxima First-strand cDNA Synthesis Kit (ThermoFisher Scientific). All the RNA samples were adjusted to 1 µg. RT-qPCR was performed using 1 µl of cDNA per reaction with SuperScript^{TM} III Platinum^{TM} SYBR^{TM} Green one-step Kit (ThermoFisher Scientific). The relative mRNA levels of genes expressed during infection were normalized to rpoD. RT-qPCR was performed in a Roche Lightcycler 480 Instrument II (Roche Diagnostics, Basel, Switzerland) and the $2^{-\Delta\Delta CT}$ method was used to analyze the relative fold change in gene expression levels.

2.2.9 Statistical analysis

Statistical analysis was performed using Microsoft MS Excel and GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). One-way ANOVA with the post-hoc Newmann-Keuls Test was per-

formed for the comparison of total *P. aeruginosa* vs adhered *P. aeruginosa*, and the Student's *t*-test was performed to analyze all other datasets.

2.3 Results

2.3.1 Effect of Ca^{2+} on the growth of *P. aeruginosa* strains

To study the effect of Ca^{2+} on the growth of *P. aeruginosa*, the most widely used laboratory strain PAO1, along with PAO1043 (Δ*efhP* mutant) and PAO1043.pMF (Δ*efhP* complemented strain) were used. It is known that Ca^{2+} levels are continuously increasing in the CF lung, which aid in the survival and pathogenesis of bacteria. Based on the disease severity and the patient's condition, Ca^{2+} levels fluctuate between 0.5 to 6.9 mM . Studies suggest that *P. aeruginosa* possesses the ability to establish microniches with elevated Ca^{2+}, thus, 5 mM Ca^{2+} was chosen to represent disease state and study *P. aeruginosa* infections in context of CF. Strains were grown without (No Ca^{2+}) and with 5 mM Ca^{2+} for 40 hours in Biofilm Minimal Media (BMM) and for 8 hrs in Roswell Park Memorial Institute, RPMI (growth medium for culturing A549 cells) to test for differences between their growth patterns.

In BMM (Figure 2.2), the strains were grown in triplicates at 37°C under shaking conditions (220 rpm) and optical density of OD_{600} was recorded at regular intervals at 0, 4, 6, 8, 10, 13, 16, 20, 24, 28, 30, 36, and 40 hr. The lower growth was observed with the PAO1043 (Figure 2.2 B) compared to PAO1 and POA1043.pMF after 20 hr at 0mM Ca^{2+}, whereas a slight increase in the growth rate was observed at 5mM Ca^{2+} post 16 hr. These slight differences in growth pattern do not signify a growth defect suggesting Δ*efhP* mutation does not affect growth rate, and possibly that the effect of mutation is not likely to cause any polar mutations downstream.

In RPMI (Figure 2.3), the growth rates of PAO1 WT, PAO1043, and PAO1043.pMF had fairly similar growth patterns at both Ca^{2+} conditions. Interestingly, PAO1 showed the lowest OD_{600} at 5 hr in low Ca^{2+} condition, which possibly was due to human error, as at 7 hr, the OD_{600} of all three strains was very similar.

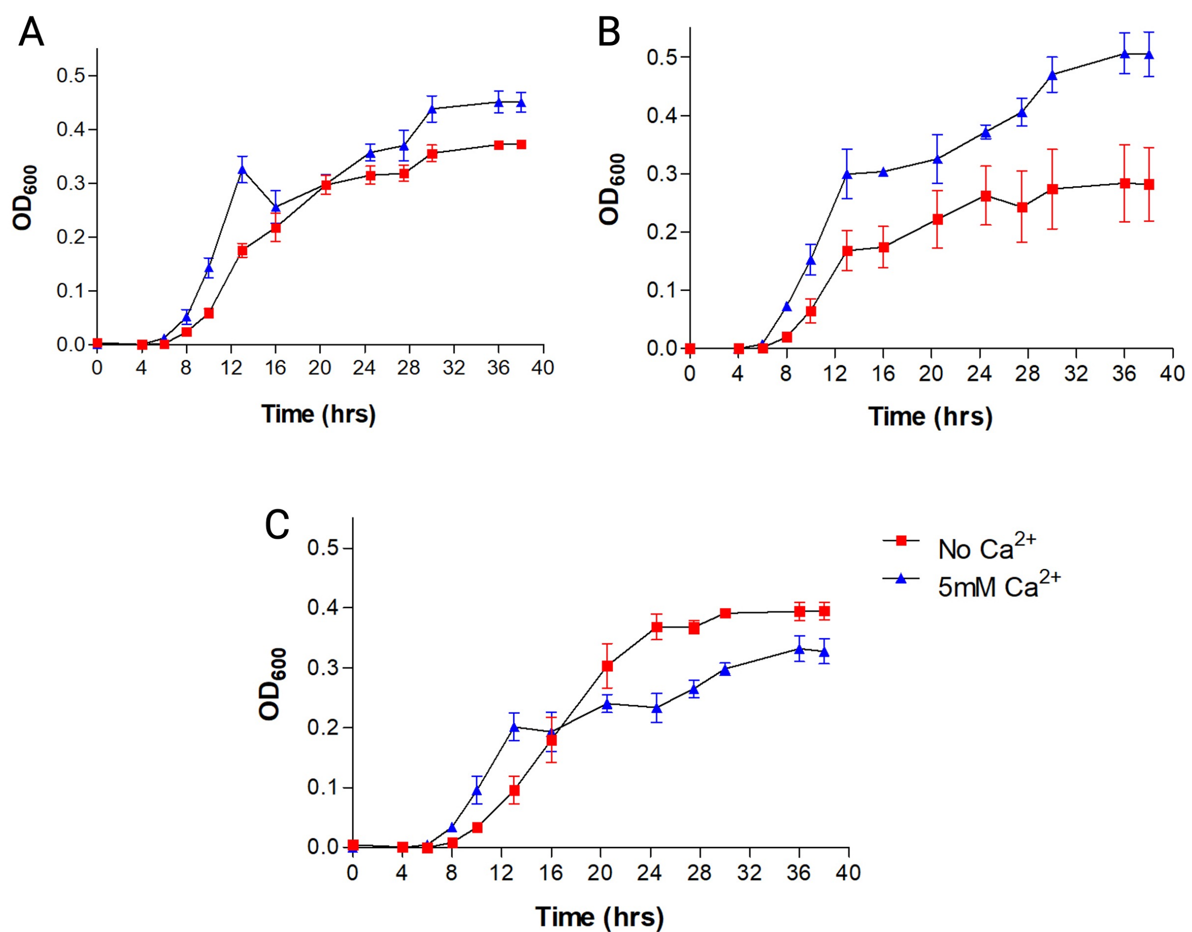


Figure 2.2: **Growth curve of *P. aeruginosa* strains in BMM**. PAO1 (A), PAO1043 (B) and PAO1043.pMF (C) grown without and with Ca^{2+} (5 mM), samples were collected, and OD_{600} was recorded at regular intervals of 2 and 4 hr for 40 hr in triplicates. Data were plotted using GraphPad Prism, and the standard error of the mean (SEM) was calculated.

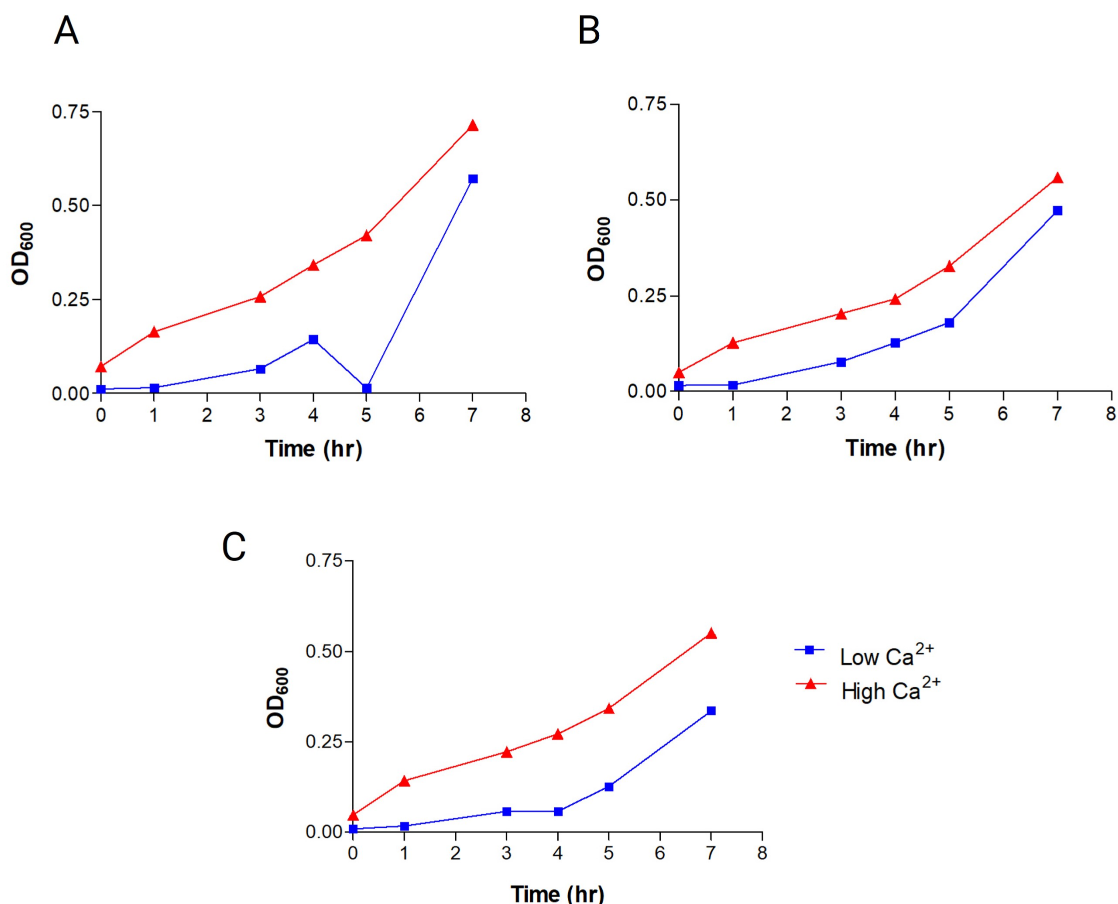


Figure 2.3: **Growth curve of *P. aeruginosa* strains in RPMI.** PAO1 (A), PAO1043 (B) and PAO1043.pMF (C) grown in low (0.54mM) and high (5 mM) Ca^{2+}, samples were collected and OD_{600} was recorded at regular intervals of 2 hr for 8 hr in triplicates. Data was plotted using GraphPad Prism and the standard error of the mean (SEM) was calculated.

2.3.2 Presence of Ca^{2+} in cell culture medium

The cell culture media RPMI for A549 cells and AEGM for CuFi-5 cells contain traces of Ca^{2+}. In order to quantitate the amount of Ca^{2+} present in the medium, Quantichrom Ca^{2+} assay kit was used. Using the standards provided in the kit, a standard curve was generated (Figure 2.4). Data suggests that RPMI contains approximately 0.54-0.6 mM of Ca^{2+}, which varies between every batch of RPMI, whereas, commercially made AEGM contains 0.101 mM of Ca^{2+}. These concentrations were used as the low Ca^{2+} condition for the following experiments. When needed, the media were spiked with 1 M $CaCl_2$ to generate high Ca^{2+} conditions of 5 mM Ca^{2+}.

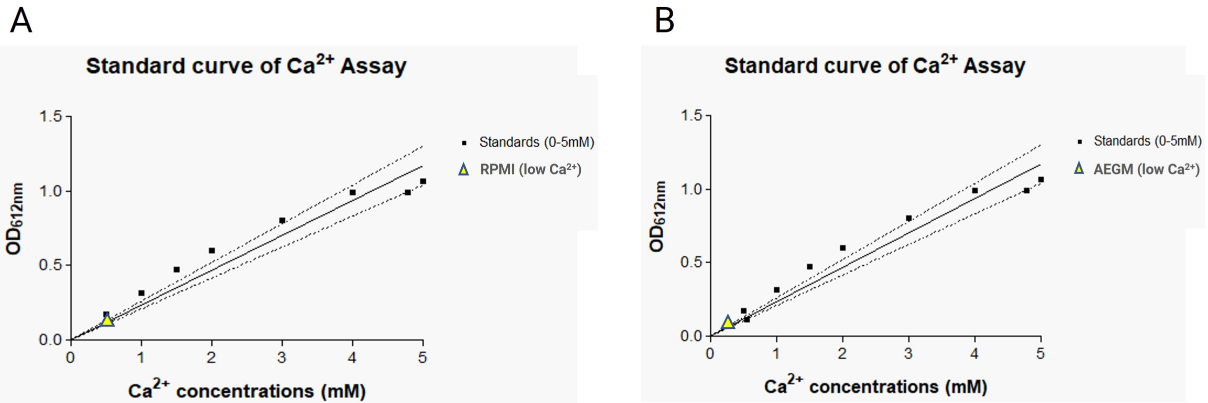


Figure 2.4: **Quantitative calorimetric Ca^{2+} determination.** Using Quantichrom Calcium Assay Kit, the amount of Ca^{2+} in cell culture media was calculated in (A) RPMI, media used to culture A549 cells and (B) AEGM, media used to culture CuFi-5 cells, by following a standard curve method.

2.3.3 Ca^{2+} upregulates adherence of *P. aeruginosa* with A549 cells

For each adherence assay with A549 cells, *P. aeruginosa* strains, were normalized to an OD_{600} of 0.2 in RPMI and plated on LB agar. The CFUs were counted and back-calculated to confirm the approximated multiplicity of infection (MOI) of 50 bacteria/ cell confirming the infection dose. No significant difference between the CFU/ml of PAO1 and FRD1 strains suggests the consistency of the initial inoculum used for infection (Figure 2.5 A and 2.6 A).

Some of the preliminary adherence studies suggested that *P. aeruginosa* grows well in the nutrient-rich cell culture media. In order to monitor the differences in growth of *P. aeruginosa*, the bacterial strains were grown in low and high Ca^{2+} RPMI (with 5% FBS) for 2 hours without epithelial cells. Bacterial cells were subsequently collected, pelletized (5000 rpm, 10 mins), resuspended, and serially diluted in PBS, followed by plating and CFU enumeration to calculate the total number of *P. aeruginosa* cells post-incubation in cell culture. No significant differences between both PAO1 and FRD1 strains were observed (Figure 2.5 B and 2.6 B), eliminating the possible adherence difference due to the growth of bacterial strains in different Ca^{2+} conditions.

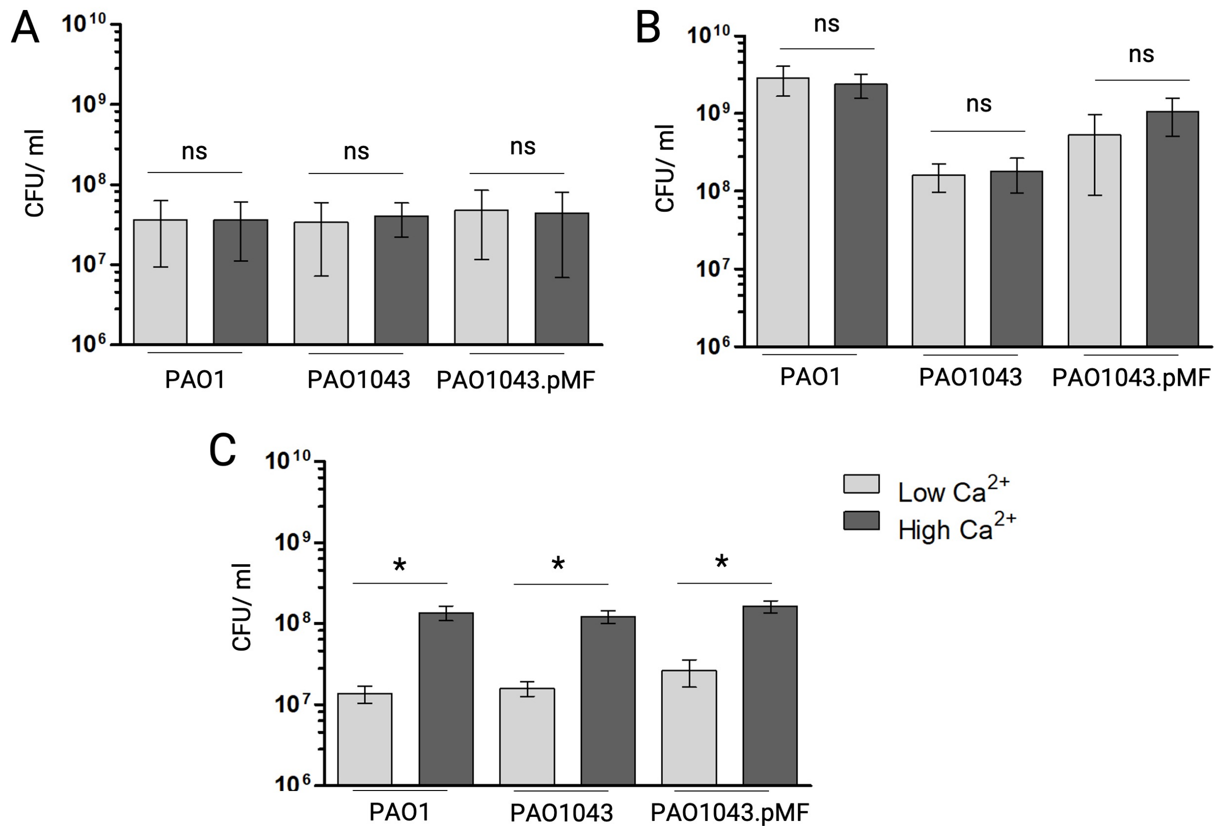


Figure 2.5: **Effect of Ca^{2+} on adherence of PAO1 strains during infection with A549 cells**. CFUs in low and high Ca^{2+} were calculated to calculate (A) the total number of bacteria used for infecting each well, (B) the total number of bacteria 2 hrs post incubation in RPMI, and (C) adherent *P. aeruginosa* per monolayer 2 hpi. Data plotted in the graph with the error bars indicating standard deviation from representative triplicate experiments. Statistical significance using one-way ANOVA and post hoc Newmann-Keuls test is indicated with an asterisk (*p <0.05). ns = not significant.

When each strain was compared amongst itself in different Ca^{2+} conditions, there was a significant difference in adherence upon infection (Figure 2.5 C and 2.6 C). Approximately ∼1-1.5 log greater adherence was recorded in 5 mM Ca^{2+} compared to 0.54- 0.6 mM Ca^{2+} ($p < 0.05$), suggesting the importance of Ca^{2+} concentration in upregulating adherence of PAO1 and FRD1 strains. On the contrary, no significant differences in adherence were observed upon a comparison of PAO1 (WT) with PAO1043 (Δ*efhp*) and PAO1043.pMF (Δ*efhp*:Δ*efhp*) in low and high Ca^{2+} condition. This suggests the absence of the possible role of Ca^{2+} binding protein EfhP in adhesion of *P. aeruginosa*. Similar observations were made in adherence of FRD1 (WT), FRD1043 (Δ*efhp*)

and FRD1043.pMF (Δ*efhp*:Δ*efhp*) in low and high Ca^{2+}.

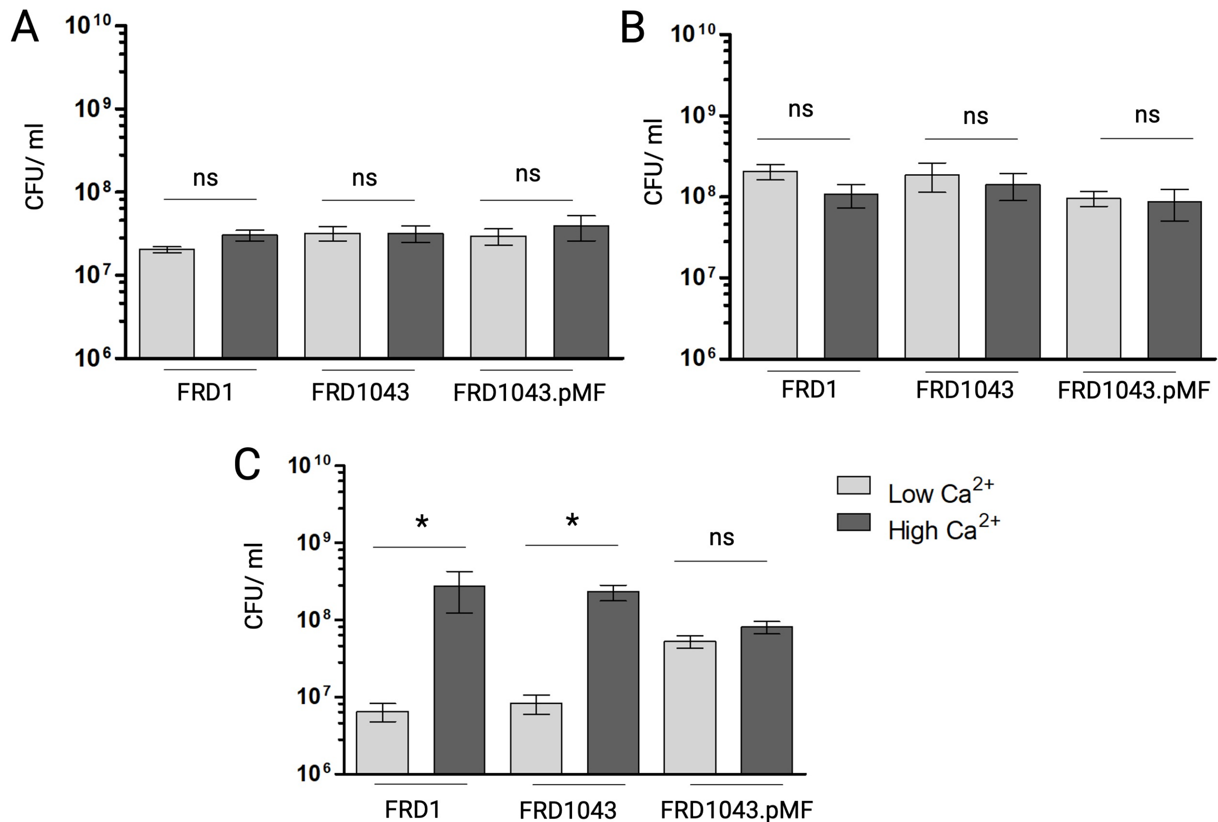


Figure 2.6: **Effect of Ca^{2+} on adherence of FRD1 strains during infection with A549 cells**. CFUs in low and high Ca^{2+} were calculated to calculate (A) the total number of bacteria used for infecting each well, (B) the total number of bacteria 2 hrs post incubation in RPMI, and (C) adherent *P. aeruginosa* per monolayer 2 hpi. Data plotted in the graph with the error bars indicating standard deviation from representative triplicate experiments. Statistical significance using one-way ANOVA and post hoc Newmann-Keuls test is indicated with an asterisk (*p <0.05). ns = not significant.

2.3.4 Ca^{2+} upregulates adherence of *P. aeruginosa* with CuFi-5 cells

Approximately 70-75% CF patients have at least one copy of cystic fibrosis transmembrane conductance regulator (CFTR) protein, mutated at the 508th amino acid, commonly known as F508ΔCFTR. In order to test for the adherence of *P. aeruginosa* in the context of cystic fibrosis lung infections, CuFi-5 bronchus airway epithelial cell line having the same Δ508 mutation in CFTR (CuFi-5 $^{\Delta F508/F508}$) were used. For infection, flasks and plates were coated with collagen

solution and cells were seeded to obtain a monolayer. Infection was performed using 0.1 mM and 5 mM $Ca^{2}+$ as low and high conditions at the MOI of ~50 bacteria/ cell, respectively. Similar to the results obtained with A549 cells, both PAO1 and FRD1 strains, along with their Δ*efhP* mutant and complement, showed increased adherence in high Ca^{2+} ($p < 0.05$) (Figure 2.7 C and 2.8 C). The increased CFU/ml was not due to initial inoculum (Figure 2.7 A and 2.8A) nor bacterial growth in the different Ca^{2+} conditions, except FRD1 WT (Figure 2.7 B and 2.8 B), as explained in §§ 2.3.3. Additionally, a mutation in EfhP does not affect the adherence of *P. aeruginosa*, which thereby confirms the absence of a significant role of EfhP in adherence during infection with CuFi-5 cells.

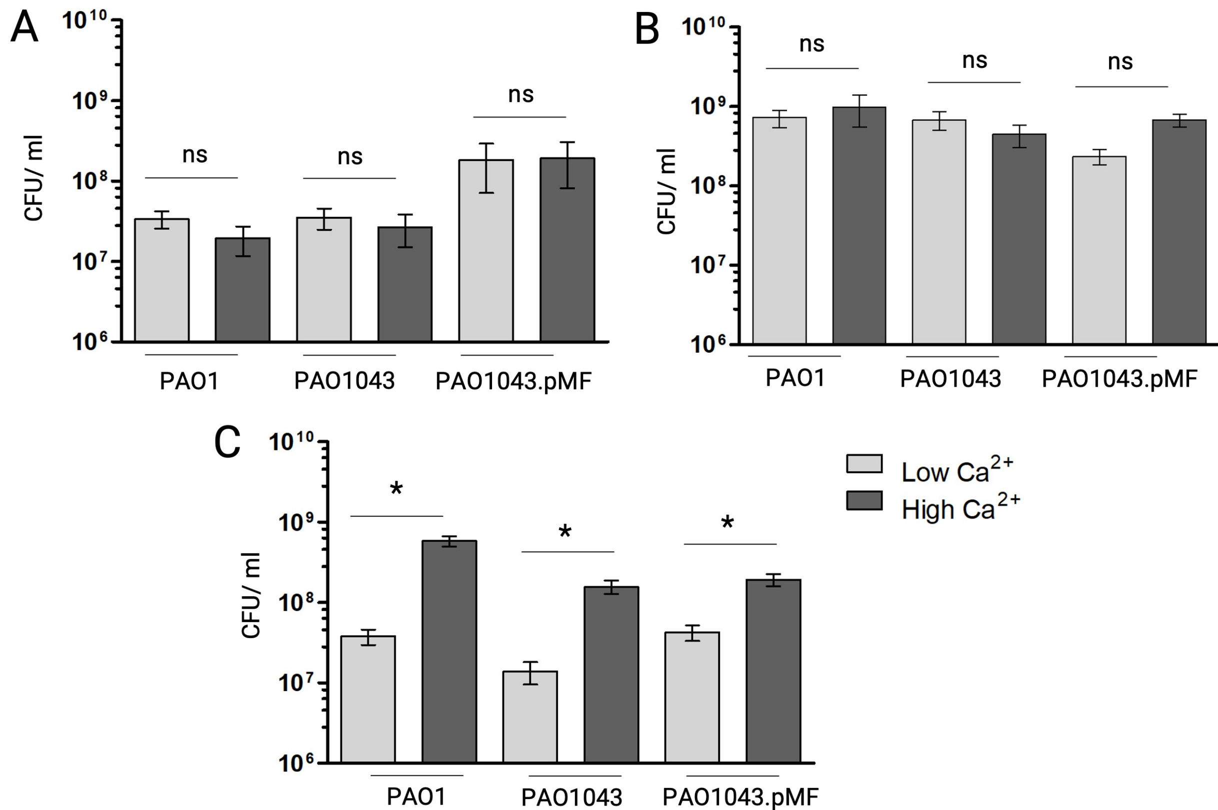


Figure 2.7: **Effect of Ca^{2+} on adherence of PAO1 strains during infection with CuFi-5 cells**. CFUs in low and high Ca^{2+} were calculated to calculate (A) the total number of bacteria used for infecting each well, (B) the total number of bacteria 2 hrs post incubation in RPMI, and (C) adherent *P. aeruginosa* per monolayer 2 hpi. Data plotted in the graph with the error bars indicating standard deviation from representative triplicate experiments. Statistical significance using one-way ANOVA and post hoc Newmann-Keuls test is indicated with an asterisk (*p <0.05). ns = not significant.

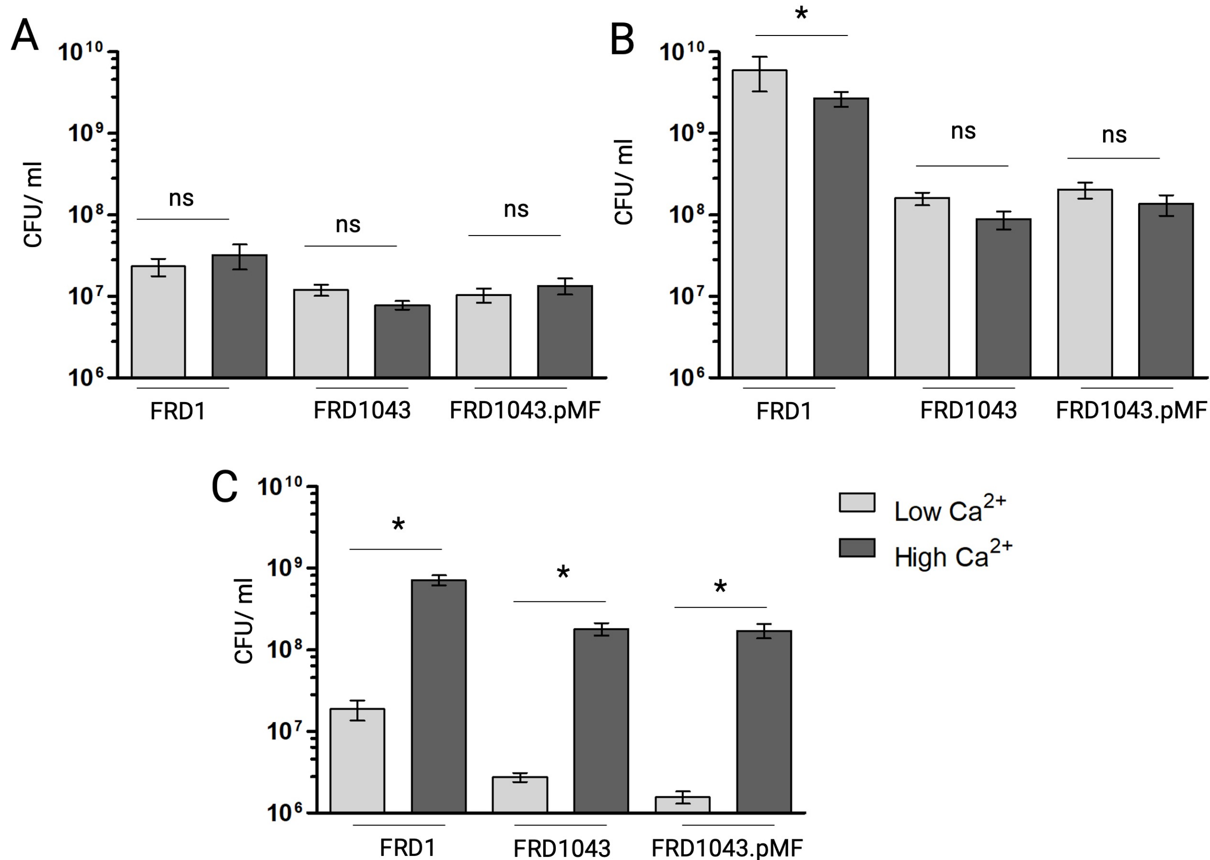


Figure 2.8: **Effect of Ca^{2+} on adherence of FRD1 strains during infection with CuFi-5 cells**. CFUs in low and high Ca^{2+} were calculated to calculate (A) the total number of bacteria used for infecting each well, (B) the total number of bacteria 2 hrs post incubation in RPMI, and (C) adherent *P. aeruginosa* per monolayer 2 hpi. Data plotted in the graph with the error bars indicating standard deviation from representative triplicate experiments. Statistical significance using one-way ANOVA and post hoc Newmann-Keuls test is indicated with an asterisk (*$p < 0.05$). ns = not significant.

2.3.5 Visualization of *P. aeruginosa* adherence to epithelial cells

To further understand the adherence of *P. aeruginosa* with epithelial cells, and investigate the morphological details of the interactions, adherent *P. aeruginosa* were visualized using immunofluorescence microscopy and scanning electron microscopy.

2.3.5.1 Immunofluorescence Microscopy

During infection with A549 and CuFi-5 cells, comparatively more *P. aeruginosa* adhered to the epithelial cell surface in high Ca^{2+}, compared to low Ca^{2+} as shown in Figure 2.9, 2.10, 2.11, 2.12.

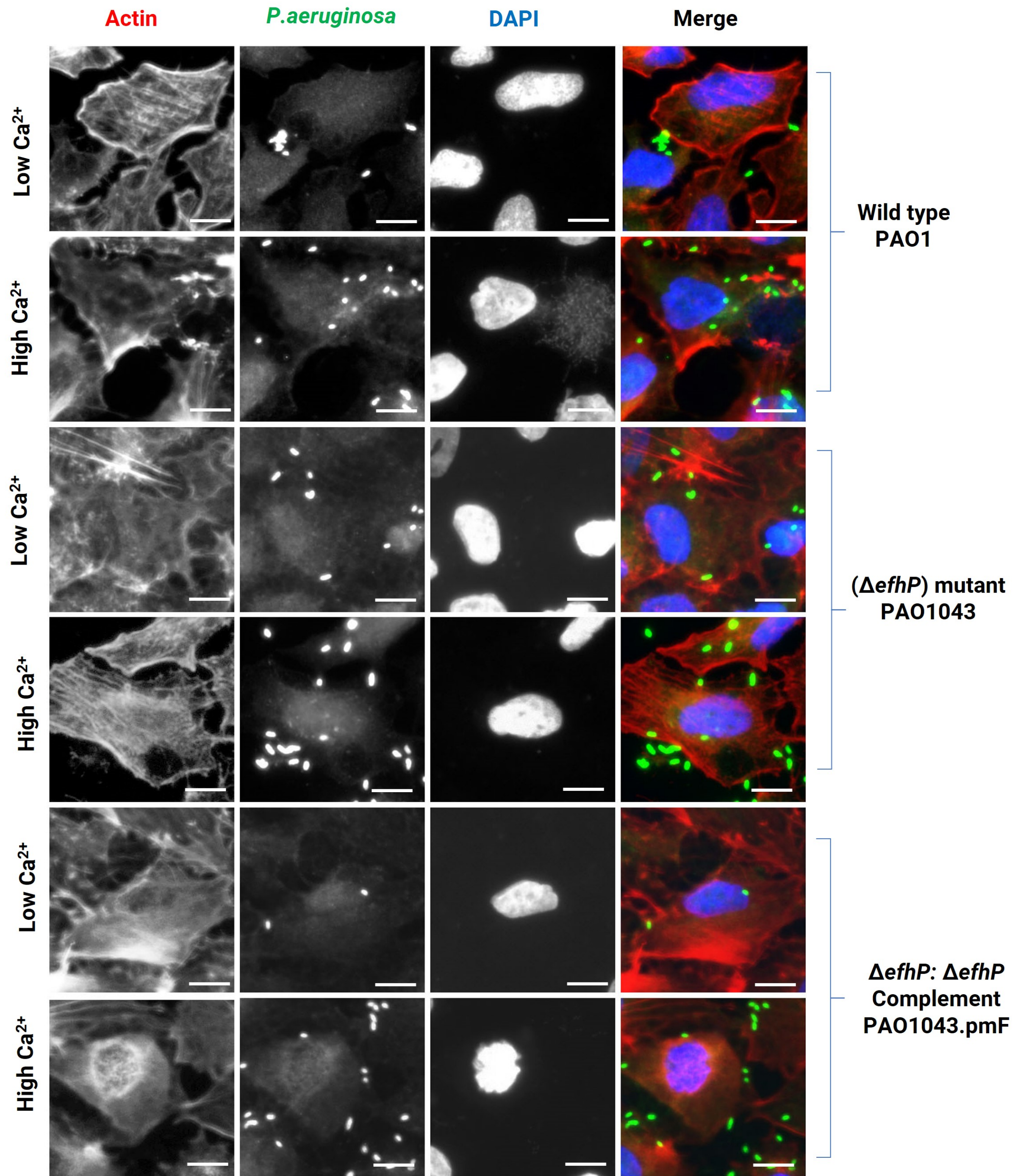


Figure 2.9: **Elevated Ca^{2+} increases adherence of PAO1 strains to A549 cells**. *P. aeruginosa* was stained green, DNA stained blue (DAPI), and actin stained red. Scale bar, 10 μM.

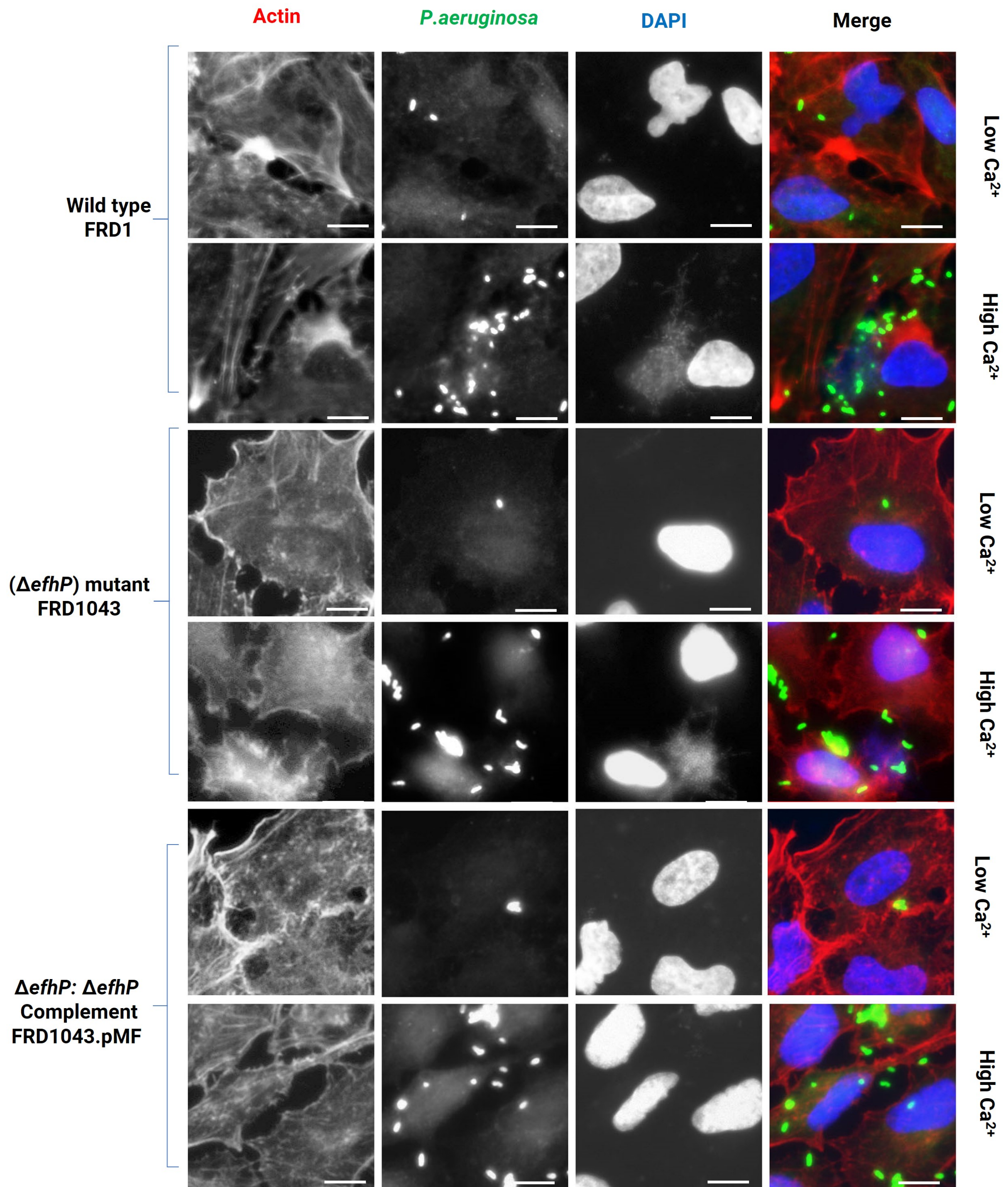


Figure 2.10: **Elevated Ca^{2+} increases adherence of FRD1 strains to A549 cells**. *P. aeruginosa* was stained green, DNA stained blue (DAPI), and actin stained red. Scale bar, 10 μM.

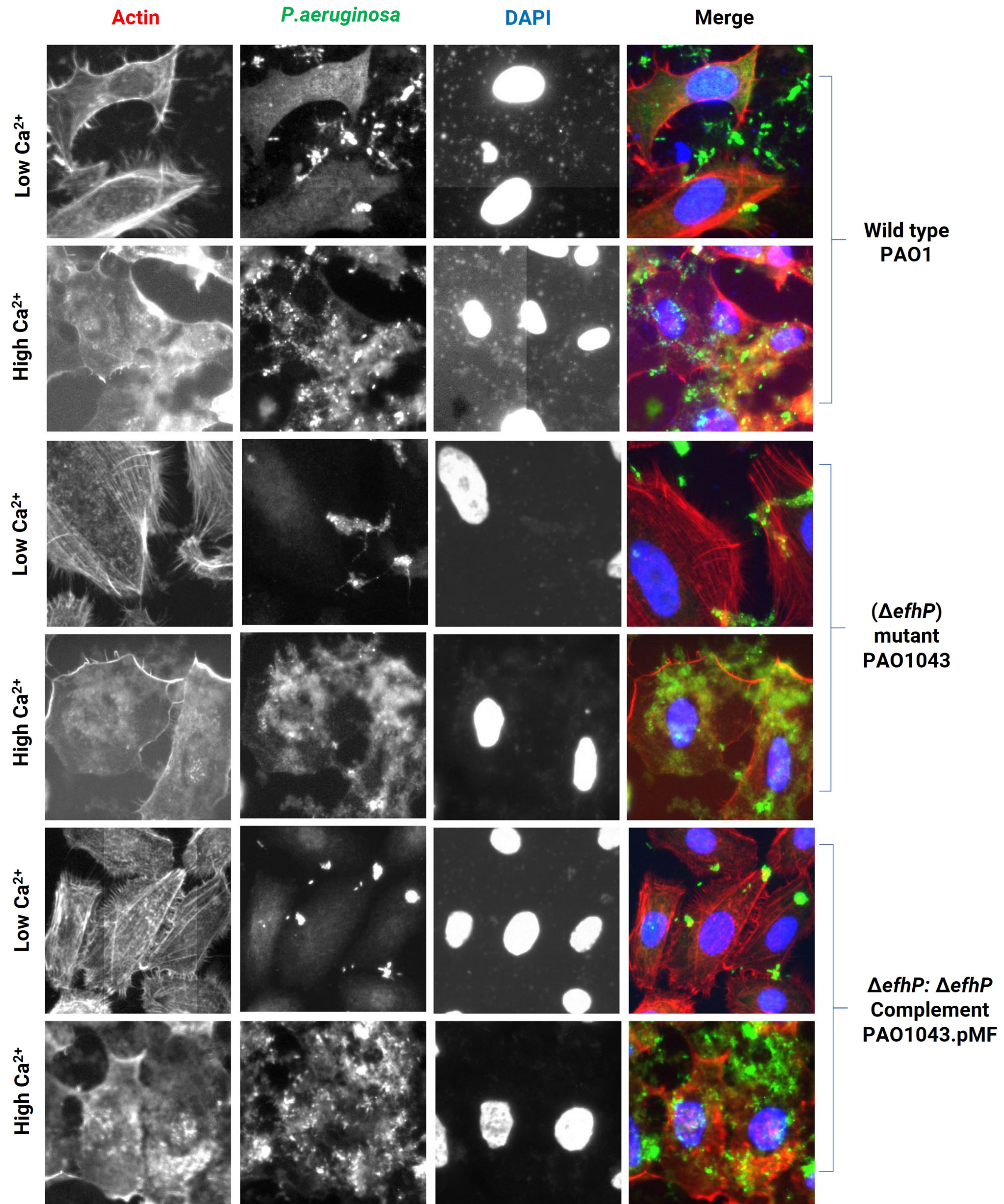


Figure 2.11: **Elevated Ca^{2+} increases adherence of PAO1 strains to CuFi-5 cells**. *P. aeruginosa* was stained green, DNA stained blue (DAPI), and actin stained red. Scale bar, 10 μM.

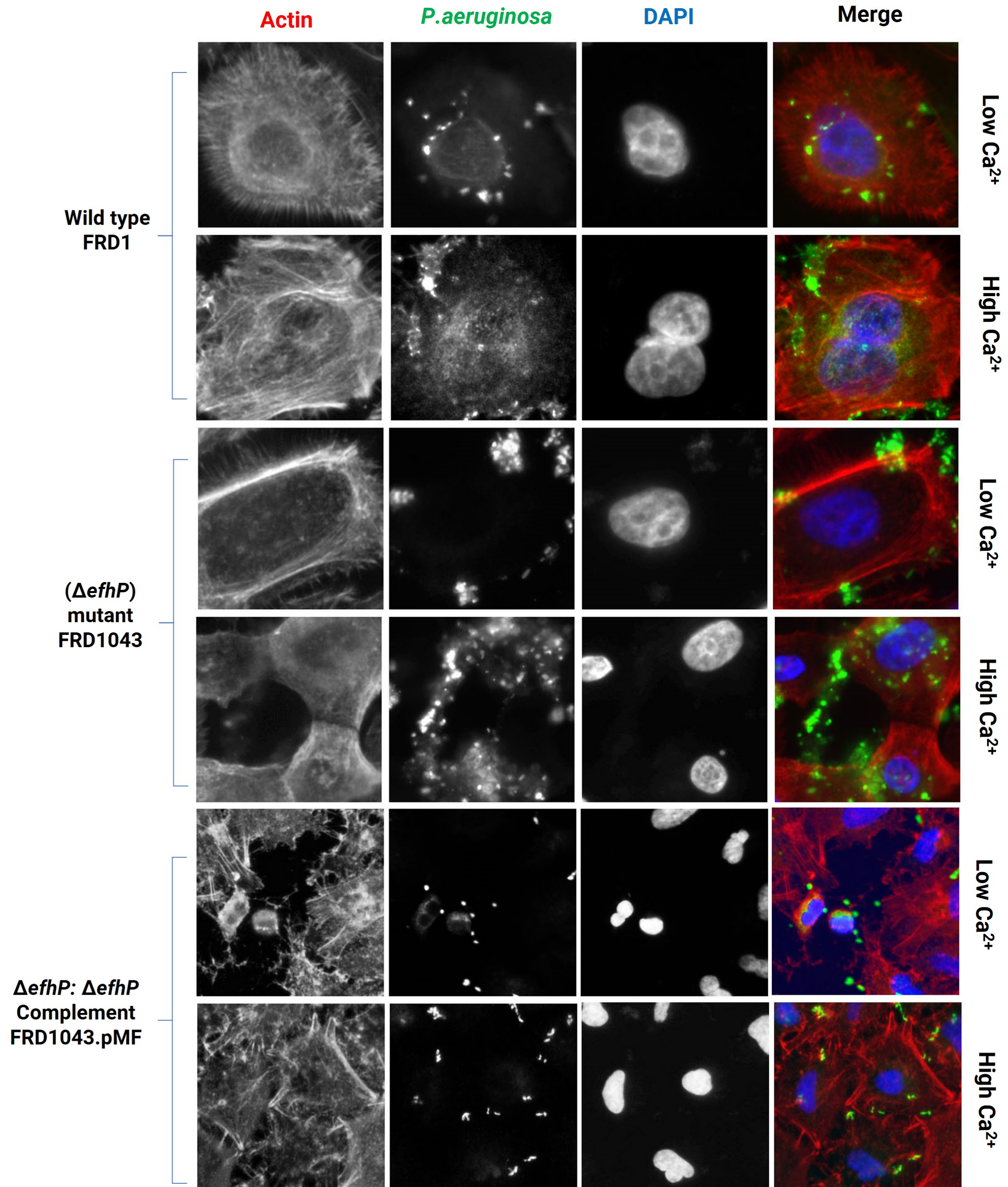


Figure 2.12: **Elevated Ca^{2+} increases adherence of FRD1 strains to CuFi-5 cells**. *P. aeruginosa* was stained green, DNA stained blue (DAPI), and actin stained red. Scale bar, 10 μM.

The Δ*efhP* mutants, PAO1043 and FRD1043 also showed increased adherence in high Ca^{2+}, validating the results obtained by CFU enumeration in §§ 2.3.3 and §§ 2.3.4. As FRD1 is an

alginate-producing CF pulmonary isolate of *P. aeruginosa*, clusters of *P. aeruginosa* were observed to form on the cell surface as expected, in less than 2 hpi in A549 and CuFi-5 cells (Figure 2.10, 2.12). Contrastingly, these aggregates on the epithelial cell surfaces were not seen with PAO1 infecting A549 cells (Figure 2.9), but initiation of biofilm was observed with CuFi-5 cells (Figure 2.10.

Studies suggest CFTR protein is a cellular receptor for *P. aeruginosa* binding, endocytosis, and clearing bacteria to maintain normal lung functioning. The mutation in CFTR makes epithelial cells susceptible to bacterial infections. Upon comparison of adherence with A549 vs CuFi-5 cells, visible clustering of bacteria was observed with CuFi-5 cells. These observations strengthen the hypothesis of enhanced adherence with ΔCFTR cells, indicating a direct correlation between CFTR Δ508 mutation and clinical manifestations of CF.

2.3.5.2 Scanning electron microscopy (SEM)

To better understand the clusters of *P. aeruginosa* on the cell monolayer, as observed by fluorescence microscopy, it was imperative to study the interaction closely. High-resolution scanning electron microscopy provides insights into understanding bacterial surface structures and morphology. Glass coverslips with epithelial cell monolayer infected by *P. aeruginosa* were processed for SEM. Samples were visualized at 5000x, 10000x, and 20000x magnification. Data was collected in triplicates and analyzed for adherence.

Adherent *P. aeruginosa* were observed attaching on the surface monolayers of both A549 (Figure 2.13) and CuFi-5 cells (Figure 2.14) with greater numbers of adherent bacteria in high Ca^{2+}. Notably, the pattern of adherence from single randomly distributed bacteria to clusters and aggregates of bacteria is consistent with the addition of Ca^{2+}. The tight binding of *P. aeruginosa* to epithelial cell surface is also confirmed upon magnification of 20,000x (Figure 2.13 C). As expected, these clusters and aggregates of *P. aeruginosa* were in greater abundance on the surface of CuFi-5 cells (Figure 2.14) compared to A549 cells . This aligns with the knowledge of enhanced colonization of *P. aeruginosa* in the CF lung, to establish infection.

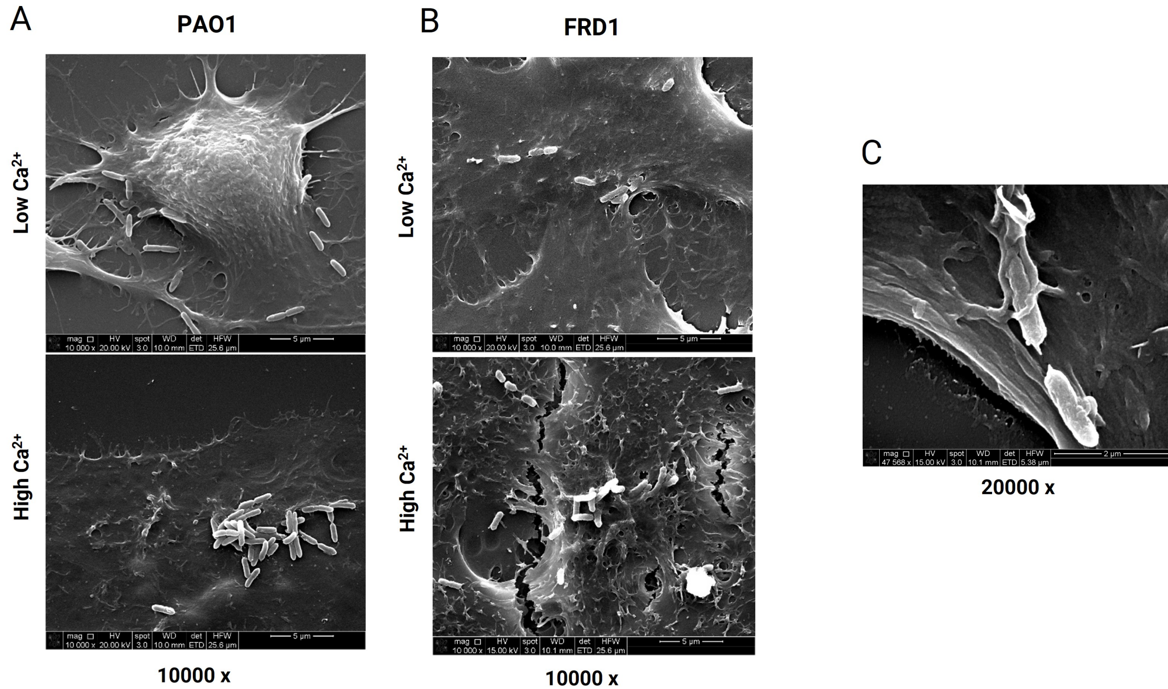


Figure 2.13: ***P. aeruginosa* adheres to A549 cell surface**. High-power scanning electron micrographs showing attachment of *P. aeruginosa* (A) PAO1 and (B) FRD1 to the A549 cells at magnification of 10,000x in low and high (5mM) Ca^{2+} conditions. (C) PAO1 firmly adheres to the cell surface even after rigorous washes involved in the SEM sample prep, as seen at 20,000x magnification.

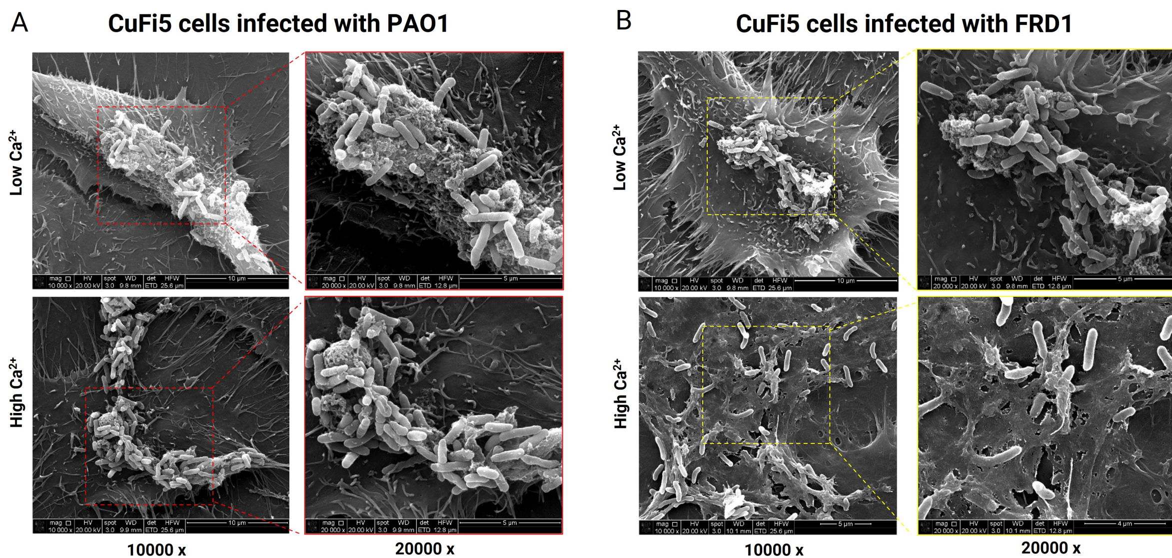


Figure 2.14: ***P. aeruginosa* adheres to CuFi-5 cell surface**. High-power scanning electron micrographs showing attachment of *P. aeruginosa* (A) PAO1 and (B) FRD1 to the CuFi-5 cells at magnification of 10,000x in low and high (5mM) Ca^{2+} conditions.

2.3.6 Effect of Ca^{2+} on the adhesins of *P. aeruginosa* by RNA-seq analysis

To identify the Ca^{2+} regulated genes involved in *P. aeruginosa* adhesion, a genome-wide transcriptional (RNA-seq) approach was used to examine gene expression of PAO1 grown in the absence (0 mM) and presence (5 mM) of Ca^{2+}. Simultaneously, a thorough literature search was conducted to identify the adhesins of *P. aeruginosa* which are reportedly known to contribute to adherence (Table 1). The adhesins were divided into categories based on their functions, such as genes/ proteins associated with flagellar apparatus, Type VIa pili, Tad pili, and the ability to produce alginate and exopolysaccharide.

RNA-seq analysis identified transcriptional changes in multiple *P. aeruginosa* genes involved in adherence. The data suggest that Ca^{2+} regulates the transcription of several important adhesins, including flagella, pili, and lipopolysaccharide. Of the adhesins identified, three in particular, *fliC, pilA*, and *lecA*, almost doubled in expression in the presence of Ca^{2+} with a 1.9-, 1.7- and 1.7-fold increase, respectively.

2.3.7 Ca^{2+} impacts transcription of *P. aeruginosa* genes

To further verify the role of Ca^{2+} in regulating the transcription of *fliC, pilA*, and *lecA* during infection, RT-qPCR was conducted using RNA extracted from adhered and invaded *P. aeruginosa* 2 hpi in A549 and CuFi-5 cells. The gene expression of *fliC* was 12.6- fold and 3.1-fold higher in high Ca^{2+} during infection of WT PAO1 and FRD1 with A549 cells, whereas the expression was reduced to 0.5- fold and 1.5- fold during infection in CuFi-5 cells, respectively (Figure 2.15). Additionally, the increased expression of *pilA* during infection with A549 and CuFi-5 cells by both PAO1 and FRD1 in high Ca^{2+} suggests the possible role of Ca^{2+} in adhesion through Type VI pili of *P. aeruginosa* during infection. Upon comparison with low Ca^{2+}, a fold change of 2.1- and 4.5- in the transcription of *pilA* by PAO1 and FRD1 in A549 cells, respectively, and 1.6- and 3.5- during infection with CuFi-5 cells was recorded. Contrastingly, there was a 26.4- fold increase in *lecA* expression by PAO1 but a 2.8- fold decrease with FRD1 during infection in high Ca^{2+} with A549 cells. Similarly, with CuFi-5 cells, a significant reduction in gene expression

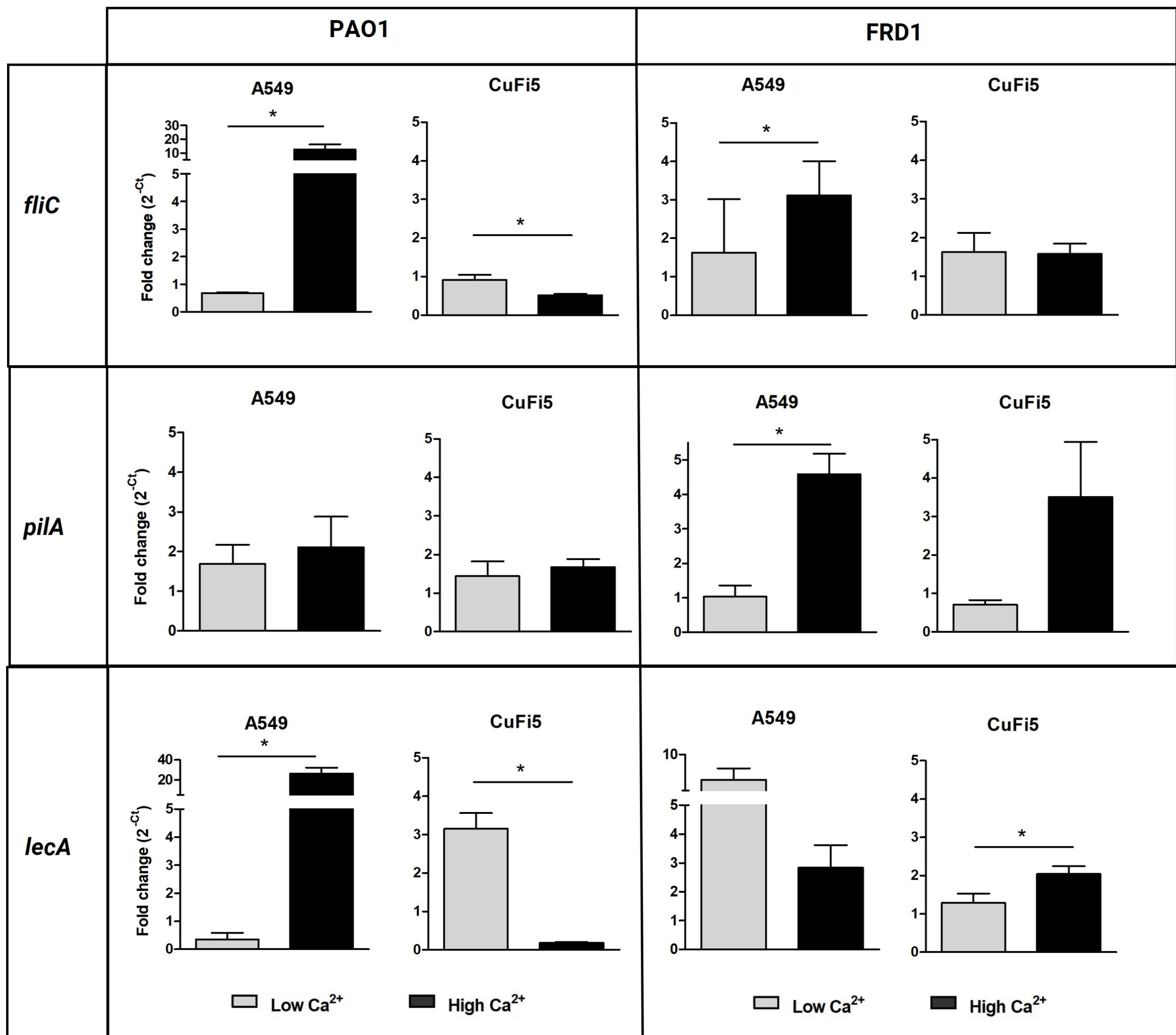


Figure 2.15: **Gene expression of *P. aeruginosa*'s adhesins** *fliC, pilA* and *lecA* during infection with A549 and CuFi-5 cells, quantitated using RT-qPCR. The relative mRNA levels of genes expressed during infection were normalized to rpoD expression by using the 2- ΔΔCT method (n=3) with averages and standard deviation as shown. The data was statistically analyzed using paired Student's t-test with $p < 0.05$ ($*$).

of *lecA* by PAO1 was contrasted by the increase in gene expression by FRD1. As FRD1 is a mucoid CF pulmonary isolate, it was hypothesized to have a significantly higher expression of *lecA* than its non-mucoid counterpart, PAO1, but the data suggests otherwise. Taken together, the transcriptomics and transcriptional analysis provides a trend of increased gene expression of adhesins (*fliC, pilA,* and *lecA*) during adherence with A549 and CuFi-5 cells in most conditions on

the addition of Ca^{2+} suggesting the importance of Ca^{2+} in adhering to *P. aeruginosa*.

Table 2.1: **Transcriptomic analysis of adhesins of *P. aeruginosa* PAO1 upregulated during growth at 5 mM Ca^{2+}**

Category	PA name	Protein name	Properties	Contribution to adhesion	Response to 5 mM Ca^{2+} (Fold induction)	References
Flagellar apparatus	PA1092	FliC	Main Flagellin protein	Adhesin responsible for binding to Muc1 mucin on the epithelial cell surface. Flagellin deficient (PAK/fliC) bacteria were no more adherent to CHO-Muc1.	1.9	Lillehoj et al. (2002)
	PA1094	FliD	flagellar capping protein FliD	Mucin adhesion	2.0	Arora et al. (1998)
	PA1097	FleQ	transcriptional regulator FleQ	Mucin adhesion, FleQ, that can potentially work with RpoN to regulate flagellar expression and mucin adhesion	2.1	Arora et al. (1997) Ritchings et al. (1995)
	PA1099	FleR	Response regulator protein	Deletion of this gene leads to poor adherence to mucin	2.1	Ritchings et al. (1995)
	PA1098	FleS	Response regulator protein	Involved in adhesion to mucin and regulating motility	1.7	Ritchings et al. (1995)
	PA1080	FlgE	flagellar hook protein FlgE	Part of flagellar apparatus, indirect role in adhesion	2.0	Ramphal et al. (1996)
Type VIa pili	PA4525	PilA	Major pilin protein involved in adhesion	Fimbrial adhesin to biotic and abiotic surfaces and involved in biofilm formation	1.7	Burrows (2012)
T4 Pili other proteins	PAO395	PilT	Nucleotide-binding protein	Required for in vitro adherence and cytotoxicity to epithelial cells.	1.3	Comolli et al. (1999)
Tad pili	PA4300, PA4299, PA4297, PA4295, PA4305,	TadC, TadD, TadG, FppA, RcpC	Tad genes encode a transport system that is required for the biogenesis of Flp.	Minor genes involved in the functioning of Flp Tad pili that helps in attachment of *P. aeruginosa* to epithelial cells.	1.1 1.1 1.1 1.0 1.6	Tomich et al. (2007) Bernard et al. (2009) Berne et al. (2015)
Alginate	PA0762	AlgU	Sigma factor	Adhesion to A549 lung epithelial cells	1.4	Damron et al. (2009)
	PA3692	LptF	Lipotoxin F	Adhesion to epithelial cells, and survival *P. aeruginosa* survival in CF lung colonization upregulated expression in CF isolates	1.1	Damron et al. (2009)
Exo-polysaccharide (EPS)	PA2231	PslA	The expression of pslA gene, restored the biofilm-forming phenotype of the wild type, indicating that PslA is required for biofilm formation by nonmucoid *P. aeruginosa*	Anchored to epithelial cells provides attachment to biotic and abiotic surfaces, promotes cell-cell interactions and assembly of the matrix, to hold the bacteria in the biofilm	1.0	Periasamy et al. (2015) Overhage et al. (2005)
	PA2570	LecA	Lectin that binds to exopolysaccharides	LecA possesses affinities towards sugars found in matrix EPS and mediate adherence of *P. aeruginosa* to target host cells	1.7	Passos da Silva et al. (2019)

2.4 Discussion

Bacterial adhesion to mucosal surfaces is an important etiological factor during infection. Adherence leads to bacterial colonization and adds to the ability of bacteria to initiate many types of infections, including pulmonary infections. CF patients are colonized by *P. aeruginosa*, which forms biofilm on the cell surface epithelia in the lungs (Moreau-Marquis et al., 2008). When studied in vitro, *P. aeruginosa* adheres to the buccal cells of CF patients in far greater numbers than to other epithelial cells, indicating the adhesion of *P. aeruginosa* to the upper respiratory epithelium of CF patients (Lingner et al., 2017). This adherence is mediated by both host and bacterial virulence factors in CF lungs (Bhagirath et al., 2016).

The host produces a wide range of factors to provide defense against bacterial pathogens in the form of osmotic stress, hypoxia, and secretion of various host proteins (Hickey et al., 2018), whereas *P. aeruginosa* factors are cell-mediated or secreted during infection, providing evasion from host immune responses to establish a bacterial infection successfully. Cell-mediated factors of *P. aeruginosa*, such as flagella, pili, and lipopolysaccharide, contribute towards attachment to host epithelial surfaces and motility. In contrast, secreted virulence factors such as extracellular proteases, phospholipases, rhamnolipids, toxins and pyocyanin contribute towards damage of host cells and in building resistance to host defenses (Ballok and O'Toole, 2013). Studies suggest that mucoid exopolysaccharide producing *P. aeruginosa* show enhanced adherence to injured mouse trachea in comparison to laboratory strains (Ramphal and Pier, 1985), implying the mucoid exopolysaccharide is the adhesin for *P. aeruginosa* helping in initial colonization. This confirms the importance of both mucoid and non mucoid strains in studying colonization. Thus, we used PAO1 and FRD1 strains for this study.

Abnormal Ca^{2+} homeostasis leads to an increase in the Ca^{2+} levels in the lung fluids of CF patients and aids pathogens like *P. aeruginosa* to establish chronic infections (Zhivotovsky and Orrenius, 2011; Ribeiro, 2006). Elevated Ca^{2+} levels are characteristic of nasal and lung fluids in patients with CF, where it triggers host immune responses against invading pathogens (Lorin et al., 1976; Gewirtz et al., 2000). Various studies have shown that *P. aeruginosa* uses elevated Ca^{2+}

to enhance the virulence factors such as biofilm production, swarming motility, and production of pyocyanin, proteases and alginate (Sarkisova et al., 2005, 2014; Broder et al., 2016). On the other hand, Ca^{2+} triggers signaling in eukaryotes and is an important component that responds to infections with pathogens such as *P. aeruginosa*. Previously, it has been shown *P. aeruginosa* strains PAO1 and FRD1 have increased virulence in high Ca^{2+} in the lettuce infection model (Sarkisova et al., 2014). However, the role of Ca^{2+} during infection of *P. aeruginosa* with lung epithelial cells was not studied. From this study, we understood that *P. aeruginosa* possesses the capability to adhere to A549 and CuFi-5 cells, and this adherence is enhanced in the presence of high Ca^{2+} ion concentration. Additionally, the calcium binding protein EfhP does not seem to play an important role in intial colonization. This was confirmed by immunofluorescence microscopy and SEM, which showed clusters of *P. aeruginosa* adhering to the surface of epithelial cells 2 hpi, yet there are no differences between the wild type and mutant strains. Since CF lungs have abnormally high levels of Ca^{2+} (Lorin et al., 1976), the enhanced adherence of *P. aeruginosa* in the presence of increased Ca^{2+} compared to the controls implies that there is some alteration in the surface adhesion of *P. aeruginosa*.

As previously studied, the *fliC* and *pilA*mutants of *P. aeruginosa* show significantly impaired epithelial cell binding, with a 95% reduction in the number of adherent organisms, and contributes to virulence in the murine model (Feldman et al., 1998). *P. aeruginosa* flagellar proteins are regulated by Type III secretion injectosome system. Contrary to these earlier findings, our study suggests the upregulation of these genes in the presence of high Ca^{2+}during adherence with A549 cells but a reduced gene expression with CuFi-5 cells.

High levels of FliC was detected in cellular and supernatant fractions in *P. aeruginosa* strains irrespective of the growth conditions, as reported previously (Ince et al., 2015). Our study suggested that *fliC* gene expression was enhanced in the presence of Ca^{2+} during the infection with A549 but was reduced during infection with CuFi-5 cells. The reduction of *fliC* expression in *P. aeruginosa* infecting CuFi-5 cells can be correlated to the reduced expression of flagellin as seen in *P. aeruginosa* grown in CF respiratory fluid as previously studied (Wolfgang et al., 2004).

Further, the Type IV pili of *P. aeruginosa* is composed of thousands of subunits of the major pilin protein. The transcription of pilA is regulated by the PilS-PilR two-component regulatory system (Boyd et al., 1994), pilA in *Burkholderia pseudomallei* (Essex-Lopresti et al., 2005) and pilin structural protein subunit in PAK strain of *P. aeruginosa* contains an epithelial cell binding domain that contributes towards adherence to human epithelial cells and virulence in a murine model (Irvin et al., 1989). During infection with *P. aeruginosa* strains, PAO1 and FRD1, upregulation of *pilA* gene expressions in high Ca^{2+} strengthens the importance of type VI pilus in adhesion and Ca^{2+} as a driving factor for increased expression.

Lectin-mediated bacterial adhesion is an important part of *P. aeruginosa* pathogenesis. Studies suggest the soluble lectin and a virulence factor of *P. aeruginosa*, LecA mediates its adherence to A549 cells (Diggle et al., 2006). Lectins lecA and lecB produced by *P. aeruginosa* are associated with virulence factors (Gilboa-Garber, 1982) and are regulated by sigma factor RpoS and quorum sensing (Winzer et al., 2000), which have shown to play a significant role in *P. aeruginosa* virulence by exopolysaccharide production. Since FRD1 produces alginate, the increase in transcription of lecA was anticipated. During infection of A549 cells with PAO1 in high Ca^{2+}, a significant increase in lecA expression was observed, yet attenuated expression with CuFi-5 cells remains unknown.

Overall, this study suggests the importance of Ca^{2+} ion concentration in the initial attachment of *P. aeruginosa* with lung epithelial cells and how it regulates the transcription of virulence genes of *P. aeruginosa*. This knowledge will help bridge the gap between bacterial adaptation to the host during CF making *P. aeruginosa* an important multidrug-resistant pathogen from an opportunistic human pathogen.

CHAPTER 3

VIRULENCE FACTORS OF *PSEUDOMONAS AERUGINOSA* AFFECTED BY CALCIUM

This chapter is reproduced with modifications from sections of the manuscript drafted:

Deepali Luthra, Ty Lutze, Sharmily Khanam, Brian Couger, Aya Kubo, Marianna Patrauchan, Erika I. Lutter, "**Calcium Stimulated Host Adherence of *Pseudomonas aeruginosa* during infection**".

The experiments resulting in data generation for this chapter were conducted by DL and undergrad mentees Ty Lutze (flagella work) and Clay Deal (biofilm assays).

3.1 Introduction

Pseudomonas aeruginosa, an opportunistic human pathogen, colonizes almost 70% of the lungs of patients with cystic fibrosis (CF). In addition to being a dominant pathogen in CF, it is also a major cause of infections in healthcare settings due to its multidrug resistance. Therefore, *P. aeruginosa* is a critical pathogen for studying antibiotic resistance and chronic infections (Tacconelli et al., 2018). *P. aeruginosa* possesses an arsenal of virulence factors that contribute to its pathogenicity. These include its ability to produce pyocyanin and pyoverdine, effectors of type III and type VI secretion systems, rhamnolipids, proteases, use of flagella and type IV pili for motility, exopolysaccharide secretion, alginate, and biofilm production to form a bacterial niche (Jurado-Martín et al., 2021). The extensive repertoire of virulence determinants allows *P. aeruginosa* to undergo genetic mutations and selection, making it more adaptive to environmental conditions in CF-affected lungs.

Calcium (Ca^{2+}) homeostasis plays a crucial role in cellular pathophysiology, and Ca^{2+} levels in CF-affected lung fluids increase depending on the severity of the disease and metabolic abnor-

malities (Roomans, 1986). Any fluctuation in actual Ca^{2+} levels leads to the disruption of Ca^{2+} signaling in both prokaryotic and eukaryotic cells, which invokes innate immune responses during infection. Several studies suggest that Ca^{2+} leads to an increase in virulence of *P. aeruginosa* that contributes to biofilm overproduction, pyocyanin, pyoverdine, increased swarm motility, and induction of hydrogen peroxide ions (Sarkisova et al., 2005, 2014; Patrauchan et al., 2007; King et al., 2021; Guragain et al., 2013, 2016).

Flagella, one of the virulence factors, aids in the binding of ligands to the surfaces of eukaryotic cells, thus initiating infection. *P. aeruginosa* possesses single polar monotrichous flagella that provide motility, help in the acquisition of essential nutrients, and aid in colonization (Feldman et al., 1998; Arora et al., 1997; Ritchings et al., 1995; Ramphal and Pier, 1985). Several flagella-associated genes of *P. aeruginosa* facilitate respiratory tract clearance by binding to respiratory mucin, while *fliC*, a major flagellin protein, helps adhere and colonize biotic and abiotic surfaces (Song and Yoon, 2014; Lillehoj et al., 2002). Several studies suggest that flagellar motility in *P. aeruginosa* is directly related to respiratory and wound infections, leading to morbidity and mortality (Feldman et al., 1998; Moradali et al., 2017). These points highlight the importance of understanding the role of Ca^{2+} ion on the flagellation of *P. aeruginosa*.

Extracellular Ca^{2+} cells interact with various signaling pathways and systems, including one associated with the generation of reactive oxygen species (ROS) (Görlach et al., 2015). ROS generation is lethal to DNA, lipids, and proteins (Cabiscol Català et al., 2000) and indicates an early response of innate immunity to microbial invasion. Elevated levels of Ca^{2+} activate ROS-generating enzymes and free radical formation, which are essential in many pathophysiological conditions (Chinopoulos and Adam-Vizi, 2006; Raturi et al., 2014). Certain opportunistic pathogens, including *P. aeruginosa* have adapted to alter intracellular ROS production, adding to its virulence and persistence (Spooner and Yilmaz, 2011). *P. aeruginosa* promotes host mucin secretion through the production of lipopolysaccharides (LPS), a key component of the bacterial cell wall that up-regulates the expression of the MUC2 and MUC5AC genes. These contribute to mucin production and obstruction of the airways, also observed in CF-affected lungs. This is known to be associ-

ated with ROS induction via PKC-NADPH oxidase signaling in eukaryotic cells (Li et al., 2013). Furthermore, studies suggest that Ca^{2+} significantly affects the production of a toxic secondary metabolite, pyocyanin, in *P. aeruginosa* that is known to induce ROS in mitochondria (Manago et al., 2015). The addition of pyocyanin resulted in an immediate increase in superoxide anion, a precursor for most ROS (Wilson et al., 1988). Pyocyanin production also affects neutrophil phagocytosis, thus manipulating apoptosis during infection in CF (Bianchi et al., 2008). Furthermore, antibiotics also induce ROS to kill bacteria (Dong et al., 2015) effectively. However, it is suspected that microbial species such as *Pseudomonas* have developed mechanisms to compete with antibiotic activity by overcoming the effect of ROS, thus supporting persistence in a microbial niche. Although the role of Ca^{2+} in pyocyanin overproduction is known, its importance in the induction or suppression of ROS by *P. aeruginosa* remains unclear.

Certain *P. aeruginosa* virulence factors are up-regulated in the presence of high Ca^{2+} that can disrupt Ca^{2+} homeostasis in lungs affected by CF (Hall et al., 2016; Rada and Leto, 2011). It contributes to the imbalance of Ca^{2+} leading to health conditions such as tissue and cardiovascular calcification (Gamble, 2006; Hutcheson et al., 2015), and accumulation in the CF lungs (Von Ruecker et al., 1984). Tissue calcification has also been reported to cause bacterial endocarditis (Minardi et al., 2009). *P. aeruginosa*'s ability to deposit Ca^{2+} in the form of calcium carbonate was initially studied by Li et al. (2015). Molecular investigations suggested the ability of *P. aeruginosa* to deposit Ca^{2+} using β -carbonic anhydrase, psCA1, adding to its virulence capacity (Lotlikar et al., 2019), yet the deposition of Ca^{2+} during infection of *P. aeruginosa* in vitro remains unexplored.

Calcium carbonate mineralization has also been shown to be essential in the formation of biofilms in CF lung infections (Cohen-Cymberknoh et al., 2022). A complex biofilm matrix formed by microbial communities is often seen as an indicator of chronic bacterial infections. *P. aeruginosa* forms a biofilm in the mucosal epithelium of the respiratory airways, leading to a severe decline in lung function, morbidity, and mortality. *P. aeruginosa* clinical isolates from CF lungs overproduce exopolysaccharide, alginate, and mucus and possess a distinct phenotype. Thick biofilms formed by *P. aeruginosa* in CF lungs are often difficult to eradicate once established

(Cohen-Cymberknoh et al., 2016). Cohen-Cymberknoh et al. (2022) showed how biological systems regulate the mineral scaffolding intrinsic to biofilm formation by *P. aeruginosa* and calcite mineralization obtained from sputum samples from CF patients. Additional evidence suggested the presence of minerals in the biofilm of *P. aeruginosa* as tested by calcein staining and confocal microscopy (Li et al., 2015).

In this study, we investigate the role of Ca^{2+} in the virulence of *P. aeruginosa* in particular, in flagellation, ROS induction, Ca^{2+} deposition during infection, and in biofilm formation. The results of this study establish the importance of Ca^{2+} in the up-regulation of *P. aeruginosa* virulence factors and strengthen the relationship between Ca^{2+} deposition and biofilm formation. Overall, this work not only advances scientific knowledge but also provides an in-depth understanding of the role of calcium in the virulence of *P. aeruginosa* in the lungs affected by cystic fibrosis.

3.2 Materials and Method

3.2.1 *P. aeruginosa* strains, clinical isolates, and cell culture

P. aeruginosa strains used in this study included laboratory strains (Wild Type, WT: PAO1 and FRD1), Δ*efhp* mutant (M: PAO1043, FRD1043) and complement (C: PAO1043.pMF 470.1, FRD143.pMF 470.1), generated in the PAO1 and FRD1 background (Kayastha et al., 2022); triple mutant of β-carbonic anhydrases of *P. aeruginosa*, psCA1 (PA0102), psCA2 (PA2053), psCA3 (PA4676), also known as $\Delta\Delta\Delta$*psCa*. CF clinical isolates were obtained from the Pulmonary and Cystic Fibrosis Clinic of the Oklahoma Children's Hospital, Oklahoma City, in the year 2014. *P. aeruginosa* was cultured as mentioned in §§2.2.1. CuFi-5 cells were grown in AEGM and incubated at 37°C, with 5% CO_2 with 95% humidified air.

3.2.2 Remel Flagella Staining

P. aeruginosa strains were grown with and without 5 mM and 10 mM Ca^{2+} in BMM at 37 ° C without agitation. A loopful of bacterial culture was gently transferred onto a drop of sterile water placed on a clean microscope glass slide using the inoculating loop. The slide was tilted to an

angle of 30° to allow the water drop to flow with the suspension covered by a glass coverslip. A drop of Remel Flagella Stain (Remel R40041, ThermoFisher Scientific) was placed at one end of the coverslip, allowing the stain to flow under it. The slides were examined under a Leica DM500 microscope (Leica Microsystems, Buffalo Grove, IL). The number of flagellated and total *P. aeruginosa* were counted for 15 fields of view, followed by statistical analysis using Student's *t* test.

3.2.3 Reactive Oxygen Species Assay

P. aeruginosa strains were cultured as previously described in §§3.2.1 and normalized to an OD_{600} of 0.5 and 0.75. Bacterial strains (100 μl each) were added to 96 well microtiter plates under low (0 mM) and high (5 mM) Ca^{2+} conditions, followed by centrifugation at 500 g for 10 min. The plates were incubated at 37 °C for 2 hr. A stock of 20 μM oxidative stress indicatory dye (100 μl, $CMH_2DCF(DA)$, ThermoFisher Scientific) (Gomes et al., 2005; Kalyanaraman et al., 2012) was added to obtain a final concentration of 10 μM per well, followed by 30 minutes of incubation at 37 °C. The buffer containing dye was then transferred onto a flat bottom 96 well tissue culture-treated plate. Light intensity was measured using a microplate reader (Synergy HTX multimode plate reader, Biotek Instruments, Winooski, VT) with an excitation/emission frequency of ∼485/528 nm. Dimethylsulfoxide (DMSO) was used as a negative control. Data were statistically analyzed (unpaired student's *t* test) and plotted in GraphPad Prism 5.

3.2.4 Scanning Electron Microscopy

CuFi-5 cells were seeded in 24 well-tissue culture-treated plates on round glass coverslips and grown until ∼95% confluency. Cells were infected with *P. aeruginosa* at MOI ∼ 50, incubated for 2 hours, followed by five washes in Hank's Balanced Salt Solution (HBSS) and fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer with 0.035 M $CaCl_2$. The coverslips were washed three times with buffered wash (0.2 M cacodylate buffer with 0.26 M sucrose) along with 15 minutes of incubation at room temperature between each wash. The coverslips were then fixed

for 1 hour in 1% aqueous osmium tetraoxide and washed three times with buffered wash. The samples were then dehydrated using three washes with 50%, 70%, 90%, 95%, and 100% alcohol for 15 min each. The coverslips were dried with hexamethyldisilazane (HMDS), mounted on aluminum stubs, and sputter coated with gold palladium. The samples were then visualized using the scanning electron microscope (SEM Quanta 600 FEG, FEI, The Netherlands).

3.2.5 Energy Dispersion X-Ray Spectroscopy (EDS)

To quantify elements in low and high Ca^{2+} SEM samples obtained from PAO1 infection and ΔΔΔ*psCa* with CuFi-5 cells, elemental analysis was performed using energy dispersion X-ray spectroscopy (EDS). An X-ray was generated by an electron beam attached to the SEM apparatus, leading to the emission of electrons at various wavelengths. Bruker Quantax CFlash SDD EDS system was used to record the total atomic percentage of Ca^{2+} and other elements, followed by the generation of atomic spectra of all of these elements.

3.2.6 Crystal Violet Biofilm Assay (CVBA)

Isolated *P. aeruginosa* PAO1 and CF clinical isolates were grown in 5 ml tubes, with and without 5 mM Ca^{2+} in BMM at 37°C, shaking at 220 rpm for 12 hours. Cultures were normalized at 0.1 OD_{600} and subcultured in 5 ml of BMM. 300 μl of the subcultures were used to seed a 96-well, round bottom untreated microtitre plate. The plates were then sealed with AlumaSeal® 96 film (Millipore Sigma, Sigma Aldrich, St. Louis, MO) and incubated at 37°C, stationary for 24 hr. Planktonic bacteria were removed and transferred to an optically clear 96-well flat bottom plate. The wells of the original plate were rinsed with 220 μL of 1× PBS. Subsequently, 205 μl of 0.1% crystal violet biofilm solution in 33% acetic acid was added to each well and incubated at room temperature to allow biofilm staining. After 10 minutes, the wells were rinsed with DI water until they ran clear. 200 μL of solubilizing solution (30% acetic acid, 70% ethanol) was added to each well, incubated for 15 min at room temperature (RT) and the contents were transferred to an optically clear 96-well plate. Plates were read using a Synergy HTX multimode plate reader

(Biotek Instruments, Winooski, VT) at 600 nm. An uninoculated plate with BMM was used as a negative control. The one-way ANOVA test was performed to statistically analyze the data (in triplicate) with significance levels of $p < 0.05$ (*) and $p < 0.01$ (**).

3.3 Results

3.3.1 Ca^{2+} affects the percentage flagellation of *P. aeruginosa*

P. aeruginosa has a single polar flagellum that plays a key role in motility and adhesion, thus contributing to overall bacterial virulence. The transcriptional data suggest the importance of *fliC* in the adhesion of *P. aeruginosa* in response to elevated Ca^{2+} (see §§2.3.7). However, the effect of Ca^{2+} on the flagellation of *P. aeruginosa* yet remains unknown. To determine the Ca^{2+} stimulated flagellar response, *P. aeruginosa* was cultured at 0, 5, and 10 mM Ca^{2+}, stained (modified Remel staining), and visualized by light microscopy. Phenotypical differences existed between flagella of *P. aeruginosa* cultured under different Ca^{2+} conditions. As the level of Ca^{2+} increases, the length of the flagella also appears to increase (Figure 3.1).

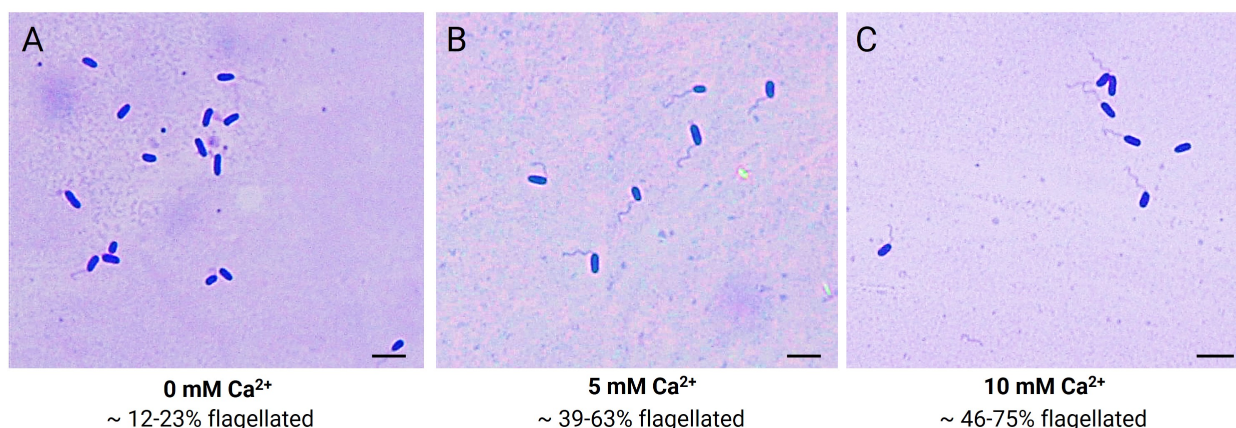


Figure 3.1: **Modified remel flagella staining to visualize the effect of Ca^{2+}**: *P. aeruginosa* was grown in BMM with 0, 5, and 10 mM Ca^{2+} with standing incubation, followed by Remel staining and light microscopy. Representative images are shown at 1000× magnification, scale bar 10 μm.

Bacterial subpopulations were counted on coverslips (n=3 with 15 fields of view on each coverslip) to determine the percentage population of flagellated *P. aeruginosa*. In the absence of Ca^{2+}, approximately 20% of the population were flagellated; while, in the presence of 5 and 10 mM

Ca^{2+}, flagellated bacteria population increased to 51% and 60.5% ($p<0.05$), respectively (Figure 3.2). In the presence of Ca^{2+}, overexpression of *fliC* could be a possible reason for more flagellation. The results presented in figure 3.2 indicate that Ca^{2+} significantly increases the number of flagellated *P. aeruginosa*, which may contribute to its motility and adhesion to host cells and overall virulence.

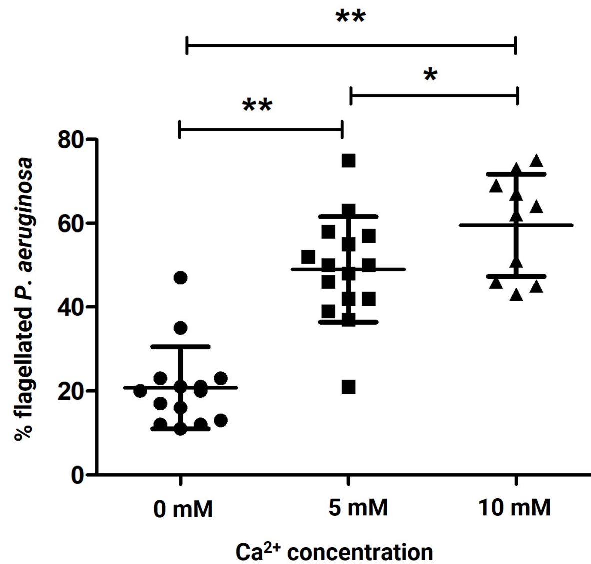


Figure 3.2: **Upregulated Ca^{2+} increases the percentage of flagellated *P. aeruginosa***. The averages and standard deviation of three biological replicates from 15 fields of view are shown. Data were statistically analyzed using one-way analysis of variance (ANOVA), followed by the Newman-Keuls post hoc test, $p<0.01$(**) and $p<0.05$ (*).

3.3.2 Ca^{2+} influences the production of reactive oxygen species

Evidence suggests that increasing levels of Ca^{2+} leads to the activation of ROS-generating enzymes in CF (Raturi et al., 2014). ROS is the component of the killing response of immune cells on a microbial attack. In CF, ROS induction by *P. aeruginosa* is modulated by LPS production via the PKC-NADPH pathway. Furthermore, *P. aeruginosa* pyocyanin induces ROS in mitochondria. Therefore, we hypothesize that Ca^{2+} acts as the signal for ROS production by *P. aeruginosa*, in turn adding to its virulence capability. To test this, cultured bacterial cells were exposed to an oxidative stress-detecting fluorescent probe, CM-H_2DCFDA (5-(and 6)-chloromethyl-2'7'- dichlorodihydrofluorescein diacetate), which detects a wide range of ROS (hydroxyl radical,

hydrogen peroxide, oxygen, hydroxyl ion and superoxide radical). It tests the levels of cellular oxygen by permeating the plasma membrane of *P. aeruginosa* and leading to the formation of fluorescent dichlorofluorescein (DCF) (Gomes et al., 2005; Kalyanaraman et al., 2012).

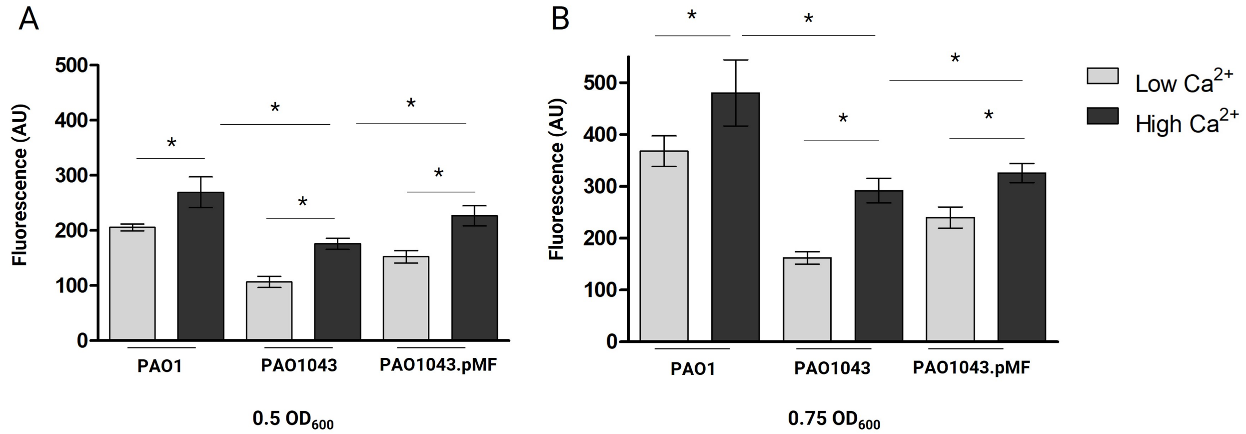


Figure 3.3: **Ca^{2+} affects ROS induction by *P. aeruginosa* PAO1 strains at (A) 0.5 OD_{600} and (B) 0.75 OD_{600}.** Averages and standard deviation from three biological replicates are shown. One-way analysis of variance (ANOVA), followed by the Newman-Keuls post hoc test, was performed with $p<0.05$ (*).

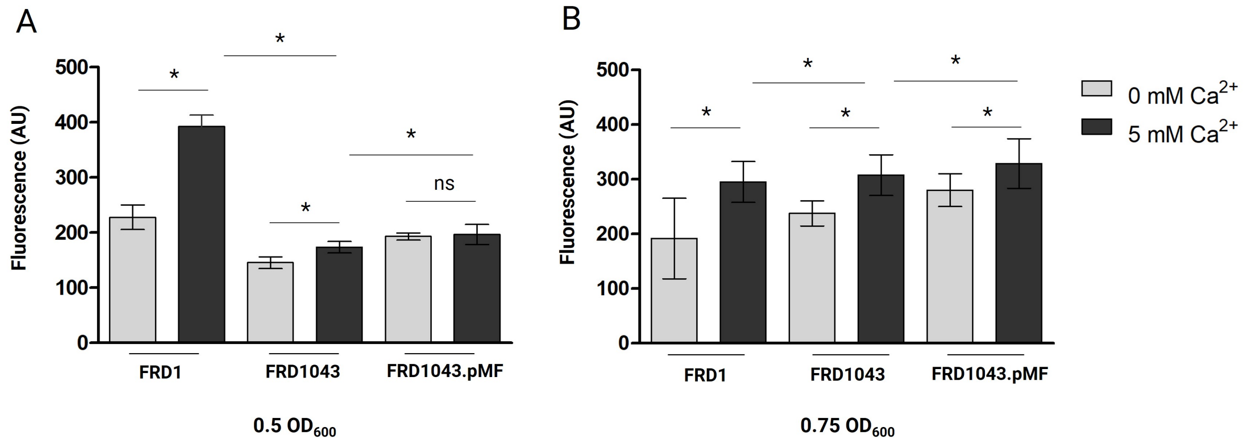


Figure 3.4: **Ca^{2+} affects ROS induction by *P. aeruginosa* FRD1 strains at (A) 0.5 OD_{600} and (B) 0.75 OD_{600}** Averages and standard deviation from three biological replicates are shown. One-way analysis of variance (ANOVA), followed by the Newman-Keuls post hoc test, was performed with $p<0.05$(*). Three replicates were performed for this data.

Upon exposure of the dye to bacterial cells, the exposure time (30 min) was optimized to ensure the complete survival of *P. aeruginosa*. At different OD_{600}, both PAO1 and FRD1 strains recorded a significant increase in ROS generation in high Ca^{2+} compared to low Ca^{2+}. Figure 3.3 shows

that ROS production by PAO1 strains increases significantly from 0.5 (Figure 3.3 A) to 0.75 OD_{600} (Figure 3.3 B), with the addition of 5 mM Ca^{2+}. Furthermore, a reduction in ROS induction was recorded with PAO1043 (M), suggesting that the EfhP (Ca^{2+} binding protein of *P. aeruginosa*) plays a vital role in ROS generation. Similar results were observed with FRD1 strains. However, a more pronounced effect of Ca^{2+} was observed at 0.5 OD_{600} (Figure 3.4 A), compared to 0.75 (Figure 3.4 B). These results support that *P. aeruginosa* induces ROS in the presence of high Ca^{2+} and high cell count.

3.3.3 Deposition of Ca^{2+} by *P. aeruginosa* using carbonic anhydrases

SEM analysis of infected cell monolayers showed the formation of a mesh-like salt structure on the surface of the epithelial cell during PAO1 infection with CuFi-5 cells (Figure 3.5). We hypothesized that these mesh-like formations might contain Ca^{2+} salts such as calcium carbonate or pyrophosphate. To test this hypothesis, EDS was performed for elemental analysis of PAO1-infected cell monolayers at 5 mM Ca2+ using energy dispersal X-ray spectroscopy (see Figure 3.7 C and D).

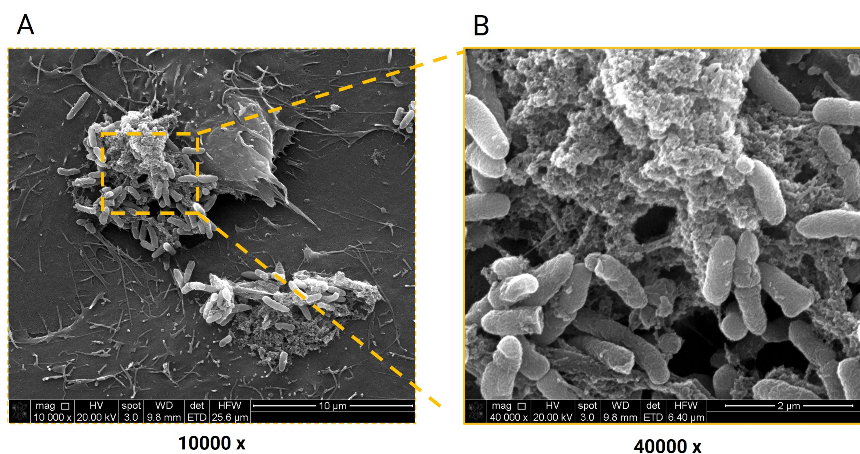
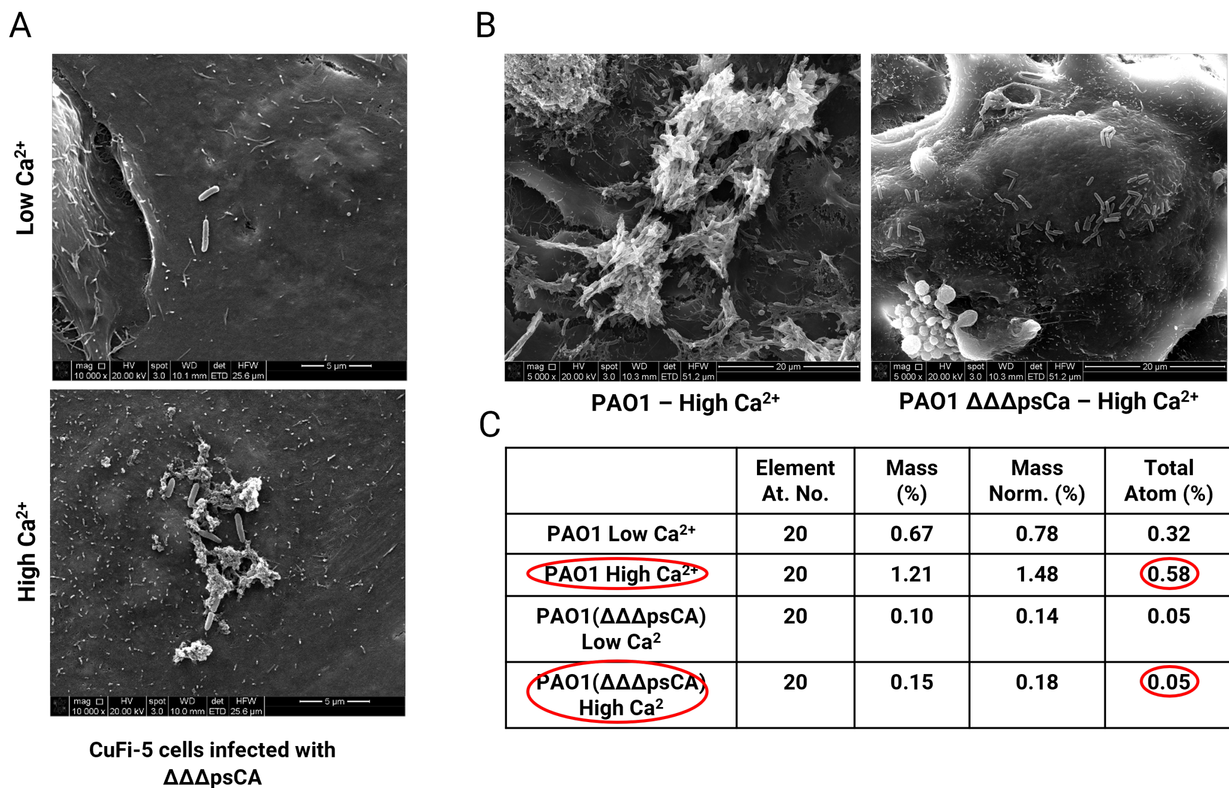


Figure 3.5: **SEM microscopy of PAO1 infecting CuFi-5 cells.** Mesh-like salt structures observed with CuFi-5 cells during infection at 5 mM Ca^{2+} at a magnification of 10,000x (A), further magnified to 40,000x (B), suggesting the possible development of salt crystals on the cell surface during infection.

As previously observed, Ca^{2+} deposition requires one of the three β-carbonic anhydrases of *P.*

aeruginosa, psCA1 (Lotlikar et al., 2019). To further investigate the role of these enzymes in Ca^{2+} deposition, CuFi-5 cells were set up for infection with PAO1 and the isogenic triple mutant lacking the three β-carbonic anhydrases, psCA1 (PAO102), psCA2 (PA2053), psCA3 (PA4676). The triple mutant *ps*ΔΔΔ*Ca* was used to eliminate possible contributions by the other two β-carbonic anhydrases. SEM analysis showed comparatively fewer *ps*ΔΔΔ*Ca*s mutant cells (Figure 3.6 A) adhering to the CuFi-5 cell monolayers when compared to PAO1 (Figure 3.6 B). Upon testing the elemental composition of the hypothesized salt formations,the deposition of Ca^{2+} was observed to reduce from 0.58% for PAO1 to 0.05% for *ps*ΔΔΔ*Ca* (Figure 3.6 C).

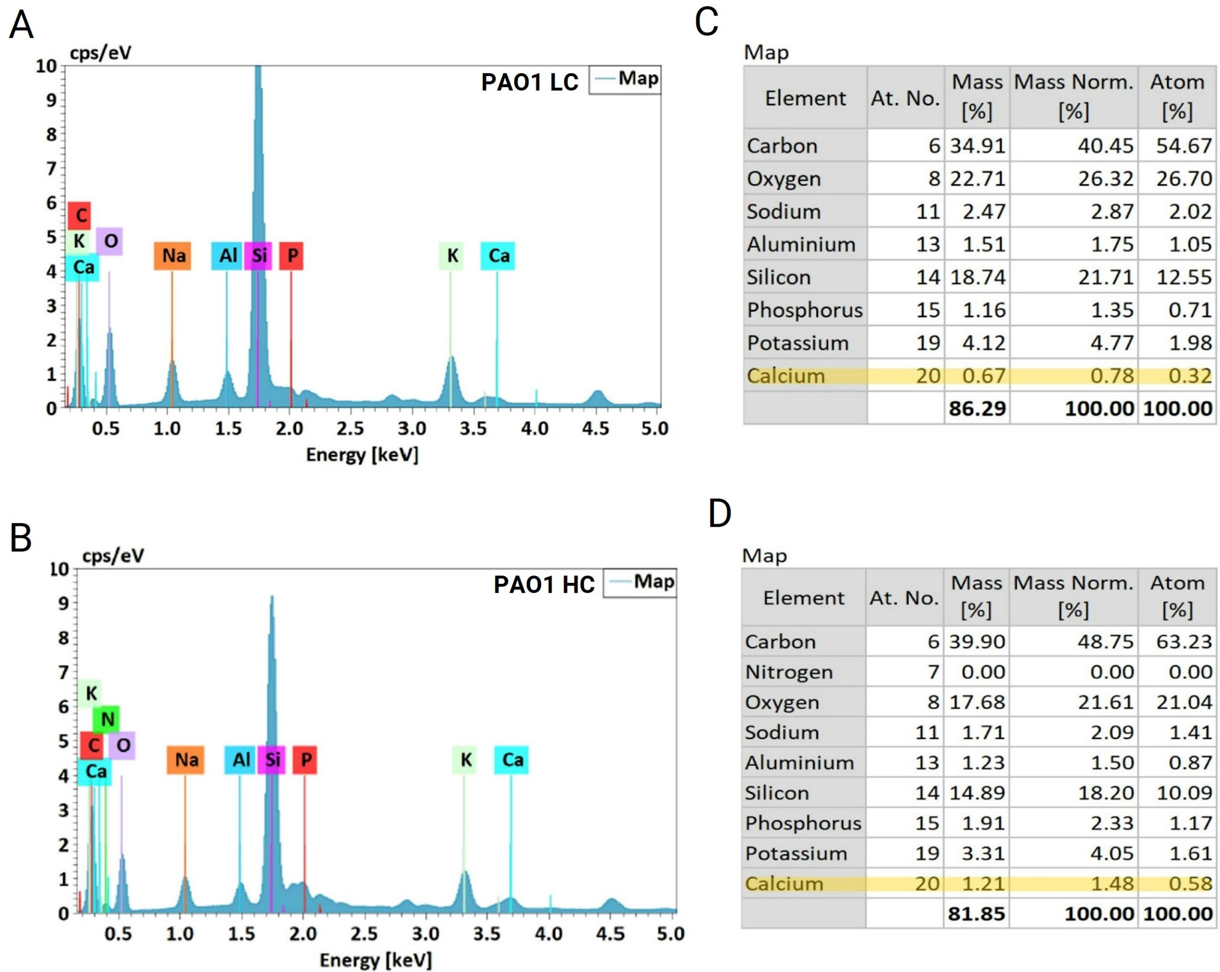


	Element At. No.	Mass (%)	Mass Norm. (%)	Total Atom (%)
PAO1 Low Ca^{2+}	20	0.67	0.78	0.32
PAO1 High Ca^{2+}	20	1.21	1.48	0.58
PAO1(ΔΔΔpsCA) Low Ca^{2}	20	0.10	0.14	0.05
PAO1(ΔΔΔpsCA) High Ca^{2}	20	0.15	0.18	0.05

Figure 3.6: **Deposition of Ca^{2+} by *P. aeruginosa* during infection.** The carbonic anhydrase triple mutant *ps*ΔΔΔ*psCa* (A) shows no mesh-like structures at a magnification of 10,000x in high Ca^{2+} (scale bar 5μm). In contrast, with PAO1 (B), the structures were visible at 5000x (scale bar 20μm) using SEM. (C) Energy dispersion X-ray spectroscopy of the samples showed negligible Ca^{2+} deposition by the *ps*ΔΔΔ*psCa*, (0.05 total atomic%), whereas a comparatively higher percentage of Ca^{2+} deposition by PAO1 (0.32% to 0.58%) was observed.

The Ca^{2+} deposition by PAO1 in low (LC) and high (HC) Ca^{2+} conditions was further explored by analyzing the X-ray spectra generated by EDS. The spectra show a relative abundance

of all elements detected on the surface of the epithelial cell during infection, including carbon (C), nitrogen (N), oxygen (O), sodium (Na), aluminum (Al), silicon (Si), potassium (K), phosphorus (P), and calcium (Ca). During PAO1 infection under low and high Ca^{2+} conditions, we observed a lower and a higher Ca^{2+} peak, respectively (Figure 3.7 A and B). PAO1 deposited a total of 0.32 and 0.58 atomic % of Ca^{2+} per sample in low and high Ca^{2+}, respectively, whereas the *ps*ΔΔΔ*Ca* mutant deposited trace amounts of 0.05% in both conditions. (Figure 3.7 C and D).



Element	At. No.	Mass [%]	Mass Norm. [%]	Atom [%]
Carbon	6	34.91	40.45	54.67
Oxygen	8	22.71	26.32	26.70
Sodium	11	2.47	2.87	2.02
Aluminium	13	1.51	1.75	1.05
Silicon	14	18.74	21.71	12.55
Phosphorus	15	1.16	1.35	0.71
Potassium	19	4.12	4.77	1.98
Calcium	20	0.67	0.78	0.32
		86.29	**100.00**	**100.00**

Element	At. No.	Mass [%]	Mass Norm. [%]	Atom [%]
Carbon	6	39.90	48.75	63.23
Nitrogen	7	0.00	0.00	0.00
Oxygen	8	17.68	21.61	21.04
Sodium	11	1.71	2.09	1.41
Aluminium	13	1.23	1.50	0.87
Silicon	14	14.89	18.20	10.09
Phosphorus	15	1.91	2.33	1.17
Potassium	19	3.31	4.05	1.61
Calcium	20	1.21	1.48	0.58
		81.85	**100.00**	**100.00**

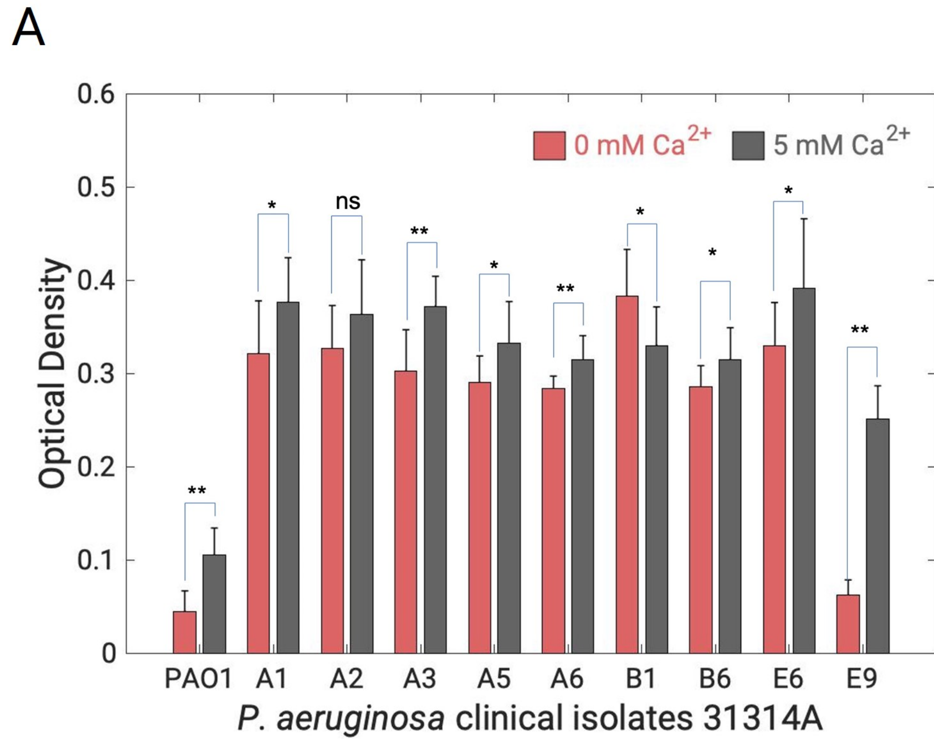
Figure 3.7: **Energy dispersion X-ray spectroscopy confirming the deposition of Ca^{2+} by PAO1 infecting CuFi-5 cells.** The difference in the Ca^{2+} peaks by spectra generated by PAO1 in low Ca^{2+} (A) and high Ca^{2+} (B) and the mass % deposition (C and D) varying from 0.78% to 1.48% in low and high Ca^{2+} affirming the possible deposition of Ca^{2+} during infection of CuFi-5 cells with PAO1.

Figure 3.8: **Alginate production by *P. aeruginosa* clinical isolates.** Image source: self-captured.

3.3.4 Increased biofilm production by *P. aeruginosa* clinical isolates in the presence of Ca^{2+}

Motile bacteria, such as *P. aeruginosa*, form biofilms on the biotic and abiotic surfaces during growth in microtiter plates. Biofilms defend bacterial communities by inhibiting their access to immune cells and against antimicrobial action. An earlier study showed that Ca^{2+} ions regulate the extracellular biofilm matrix of *P. aeruginosa*, using Ca^{2+}-induced virulence factors (Sarkisova et al., 2005). In this section, we present our findings on the effect of Ca^{2+} on biofilm formation by clinical isolates of CF. Sputum samples of patients with CF were obtained from Oklahoma Children's Hospital (Pulmonary and cystic fibrosis division) in 2014. It should be noted that samples 31314A and 22014B correspond to their collection dates of March 3rd 2014 and February 20th 2014, respectively. Samples were streaked on *Pseudomonas* isolation agar (PIA), where isolates produced alginate-producing mucoid colonies that were morphologically different from PAO1 lab strain Figure 3.8. The isolated colonies were checked for *P. aeruginosa* by 16S-rRNA sequencing. Sequences were analyzed using NCBI nucleotide BLAST, and compared with 16S-rRNA sequences from PAO1.

A



B

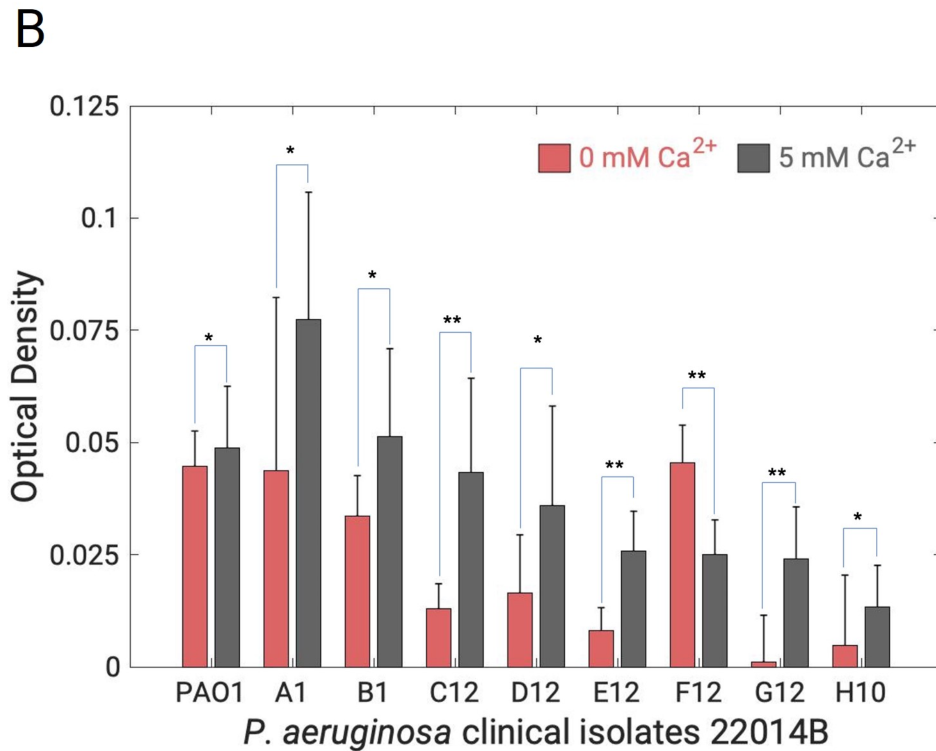


Figure 3.9: **Increased biofilm production by *P. aeruginosa* clinical isolates grown in high Ca^{2+}**. PAO1 was used as a control. The data were analyzed using MATLAB, and ANOVA was used to calculate *p* values with $p<0.05$ (*) and $p<0.01$ (**).

To quantify biofilm formation by clinical isolates of *P. aeruginosa* (grown in 0 and 5 mM Ca^{2+}), the crystal violet biofilm assay was performed with 24-hour-old biofilms. Subsequently, the absorbance of the solubilized solution was measured after the addition of acetic acid, which solubilized the stained biofilms. Figure 3.9 shows that biofilm production for clinical isolates *31314A* was significantly higher than that of the laboratory strain, PAO1. However, biofilm formation in the case of clinical isolates *22014B* was higher or lower than in the laboratory strain, depending on the strain type. Furthermore, the optical density of the biofilm increased significantly with the addition of Ca^{2+}, indicating an increase in the formation of biofilms regardless of the type of strain. This is an important insight that highlights the role of Ca^{2+} in biofilm formation by *P. aeruginosa*.

3.4 Discussion

P. aeruginosa adapts to the environment of the lung to form biofilms in the airways, causing chronic infections. Despite the significant pathological differences between bacteria isolated from acute versus chronic infections (Smith et al., 2006), its pathogenicity island (PAPI-1) encodes all virulence factors in all *P. aeruginosa* species. One of the determinants of the virulence of *P. aeruginosa* is the flagella, which are anchored to the cell wall through the basal body attached to the filament (Yonekura et al., 2002; Song and Yoon, 2014) to provide motility during infection. Flagella are composed of various flagellin proteins that bind to Muc1 mucin on the surface of the epithelial cell (Lillehoj et al., 2002). Together, these proteins form a single polar flagellum, responsible for swarming motility in *Pseudomonas*.

Ca^{2+} helps prokaryotic cells in motility, cell structure, differentiation, gene expression, and growth (Dominguez, 2004). The disruption of Ca^{2+} homeostasis in *P. aeruginosa* modulates swarming motility (Guragain et al., 2013). Moreover, the importance of Ca^{2+} in up-regulating *fliC* gene expression has been discussed earlier in chapter 2. Here, our findings on Ca^{2+}-mediated flagellation of *P. aeruginosa* further strengthen the importance of Ca^{2+} in enhancing virulence of *P. aeruginosa*. However, no flagella were observed in host cells infected by *P. aeruginosa* and visualized by SEM under high Ca^{2+} conditions (Figure 3.5). Therefore, we infer that flagella were

primarily involved in early colonization and the establishment of infection, where they act as a tether to the epithelial membrane. This is the first reported observation that suggests the effect of Ca^{2+} on the function of flagella to establish infection in the host, a characteristic of CF lung infections.

Furthermore, the generation of reactive oxygen species by prokaryotes is considered a survival mechanism for bacterial persistence. Hydrogen peroxide (an ROS) plays a significant role as a messenger to transduce signals from cell surface receptors that direct changes in the movement of cells (Hurd et al., 2012). ROS also interferes with cell signaling, leading to oxidative stress, altered gene expression, apoptosis, and activation of signaling cascades. In CF, a high concentration of intracellular Ca^{2+} leads to the activation of ROS-generating enzymes and the formation of free radicals, essential for many pathophysiological conditions (Chinopoulos and Adam-Vizi, 2006; Raturi et al., 2014).

A redox-active virulence factor of *P. aeruginosa*, pyocyanin, causes oxidative stress on the airway epithelium in CF, leading to tissue damage and cell death (Rada et al., 2008). Pyocyanin is also associated with mucin production by *P. aeruginosa* LPS, which modulates ROS generation (Yan et al., 2008), leading to blockage of the airways in CF. Sarkisova et al. (2005) showed that Ca^{2+} significantly up-regulates pyocyanin production in *P. aeruginosa*. It regulates the transcription of the β propeller protein of *P. aeruginosa*, carP, responsible for maintaining oxidative stress, and contributes to the tolerance of hydrogen peroxide (King et al., 2021). Our findings suggest that Ca^{2+} leads to the overproduction of ROS free radicals by *P. aeruginosa*, and its Ca^{2+} binding protein, EfhP modulates this ROS generation by PAO1 and FRD1 strains. Furthermore, the bacterial density increases the redox potential, adding to the virulence capacity of *P. aeruginosa*.

The biomineralization of soft tissues is often associated with severe health risks and diseases. More than 200 types of bacteria are involved in calcium carbonate mineralization (Li et al., 2015). This occurs in aggregates of microbial-forming biofilms, including *Pseudomonas* species. This microbial biomineralization is associated with metabolic pathways such as nitrification, urea hydrolysis, and photosynthesis (Bai et al., 2017). *Pseudomonas* biofilms often colonize medical

equipment in hospitals and tissues during infection (Stickler, 2008). Li et al. (2015) reported the deposition of Ca^{2+} by *P. aeruginosa* for the first time, which aids its ability to form biofilms. Further molecular investigations suggested that *P. aeruginosa* can deposit extracellular Ca^{2+} in the form of calcium carbonate, using β-carbonic anhydrase, psCA1, in stationary conditions (Lotlikar et al., 2019).

Our work showed the deposition of Ca^{2+} by *P. aeruginosa* cultured in high Ca^{2+} concentration. This is the first study to demonstrate the ability of the pathogen to deposit extracellular Ca^{2+} during interactions with human lung epithelial cells. This deposition was not observed with triple carbonic anhydrase mutant, *ps*ΔΔΔ*Ca*, which agrees with the previous knowledge of the involvement of carbonic anhydrases of *P. aeruginosa* in the deposition of Ca^{2+} (Lotlikar et al., 2019). This knowledge is of great clinical relevance, as bodily fluids in CF lungs contain elevated Ca^{2+}, and tissue calcification also leads to diseases such as atherosclerosis and endocarditis. As mentioned above, biomineralization is often associated with biofilm formation and *P. aeruginosa* produces more biofilms in the presence of Ca^{2+}. Our study of Ca^{2+}-induced biofilm formation in CF clinical isolates further supports our hypothesis and potentially enables the survival of *Pseudomonas* in a complex lung environment.

In summary, we showed that the concentration of Ca^{2+} plays a key role in regulating virulence factors of *P. aeruginosa*, including its ability to induce reactive oxygen species, overexpression of *fliC* - leading to more flagellated bacterial cells and calcite mineral deposition during infection and biofilm formation. Our work advances the current understanding of bacterial adaptation in CF and Ca^{2+} signaling during infection in the host. Understanding the clinical importance of Ca^{2+} in CF lung infections will also help in the development of new therapeutic strategies to prevent and treat devastating *P. aeruginosa* infections.

CHAPTER 4

CALCIUM MEDIATED INVASION, INTRACELLULAR REPLICATION AND ESCAPE OF *PSEUDOMONAS AERUGINOSA* DURING INFECTION WITH HOST CELLS

4.1 Introduction

P. aeruginosa is a critically important pathogen for research and development, as listed by the WHO and CDC (Jesaitis et al., 2003; Stewart and Costerton, 2001). Otherwise known as an extracellular pathogen, it possesses the capability to form an intracellular niche for its survival and spread of infection. Recent advances in research suggest *P. aeruginosa* possesses the ability to invade epithelial cells and evade host immunity (Sharma et al., 2020; Lyczak et al., 2000).

P. aeruginosa utilizes a multi-target approach to breach epithelial cell junctions for internalization. Before the invasion, bacterial cells reach the tissue lining by motility, provided by flagella (swarming motility) and Type IV pili (twitching motility). Studies suggest that *P. aeruginosa* requires chemotactic flagellar motility for survival in the wound and burn infections (Turner et al., 2014). In contrast, Pili have various adhesive properties that help *P. aeruginosa* adhere. Both flagella and pili help *P. aeruginosa* move toward and adhere to the mucosal lining. Upon adhesion, bacterial cells form a biofilm matrix and are able to transform the apical membrane into a basolateral membrane (Kierbel et al., 2007). *Pseudomonas* utilizes its Type VI secretion system effectors (H2 and H3), which target the microtubule network to subvert membrane polarization; specifically, VgrG2b interacts with the microtubule network via gamma-tubulin ring complex γ-TuRC (Kollman et al., 2011). This activates PI3 kinase, thereby interfering with Akt signaling pathways, leading to actin polymerization (Sana et al., 2016).

Flagellar motility of *Pseduomonas* is shown to activate the phagocyte Phosphatidylinositol 3-kinase (PI3K)/Akt pathway for phagocytic engulfment (Lovewell, Hayes, O'Toole and Berwin,

2014). PI3K is involved in various cellular functions, including cell differentiation, proliferation, growth, motility, and intracellular trafficking. It is understood that activation of PI3K and protein kinase B/ Akt is important for *Pseudomonas* invasion (Kierbel et al., 2005). Furthermore, Ca^{2+} activates PI3K by converting calmodulin into phosphorylated pCaM (Zhang et al., 2017), which leads to the hypothesis that Ca^{2+} stimulates invasion and intracellular replication. Furthermore, PIPK leads to PIP3 synthesis for recruitment, phosphorylation, and activation of the master regulatory kinase Akt (protein kinase B). Akt activation in the apical plasma membrane controls various downstream targets that play a vital role in actin remodeling and cytoskeletal arrangements in the cell, leading to junction breach and allowing bacterial cells to internalize.

Although it is a primarily extracellular bacterium, *Pseudomonas* uses a multifactor process to actively invade epithelial cells. It manipulates epithelial cell signaling by activating novel Src kinases (specifically tyrosine kinase p60Src and p59Fyn) for internalization (Esen et al., 2001). The CFTR peptides as modulators subvert the activation and internalization of src tyrosine kinase. These CFTR peptides compete with *P. aeruginosa* lipopolysaccharide for specific binding to CFTR. The addition of src kinase inhibitors blocks internalization but not adhesion (Esen et al., 2001).

Our study sheds light on Ca^{2+} regulated virulence of *P. aeruginosa*. Ca^{2+} is a crucial player in cell survival and function. Various transporters, exchangers, and Ca^{2+} channels, together modulate intracellular Ca^{2+} levels in the lung of patients with CF. Any fluctuations in cytoplasmic Ca^{2+} make the epithelial cell membrane prone to microbial attack, cell death, and abnormal gene expression in pathogens. Some Ca^{2+} transporters, CalC, PA4614, AtPase PA2435, and PA3950 in *P. aeruginosa* have been shown to contribute to Ca^{2+} efflux (Guragain et al., 2013). Furthermore, *P. aeruginosa*'s Ca^{2+} binding protein and Ca^{2+} sensor, EfhP, significantly binds to intracellular Ca^{2+} and undergoes conformational changes as a result of binding. The Δ*efhP* mutant of *P. aeruginosa* showed an approx. 1.5-fold decrease in virulence in the worm model (Kayastha et al., 2022), and an increase in intracellular *efhP* gene expression after infection (Dr. Aya Kubo, data not published), indicating the role of EfhP in invasion and intracellular spread.

These convergent mechanisms are utilized by *P. aeruginosa* to cross the epithelial cell barrier for effective colonization of the mucosal, epithelial, and endothelial membranes of the host. However, the molecular mechanisms involved in *P. aeruginosa* internalization is greatly understudied. Thus, for a better understanding of how this opportunistic extracellular pathogen can act as an intracellular pathogen and spread infection, more work needs to be done. Here, this study highlights the importance of the *P. aeruginosa* Ca^{2+} binding protein EfhP in the invasion, intracellular survival, and escape (Figure 4.1). Additionally, the alteration in host signaling is regulated by *P. aeruginosa* invasion and the effect of Ca^{2+} explained, thus affirming the overall significance of this study.

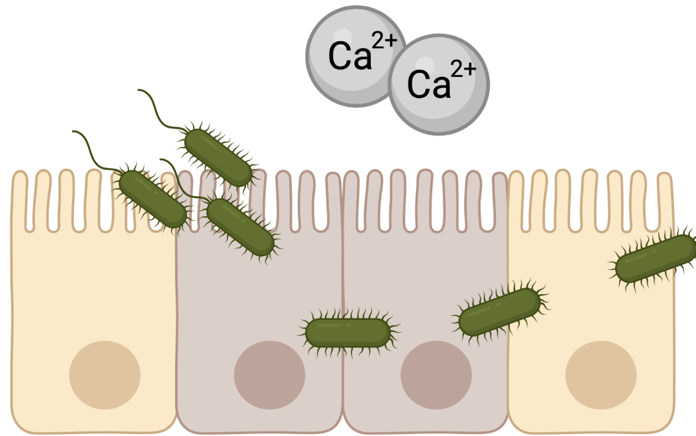


Figure 4.1: **Determine the role of Ca^{2+} in the invasion, intracellular survival and escape of epithelial cells during infection and the role of Ca^{2+} and Ca^{2+} binding gene, *efhP* in this interaction.** Image source: generated by author using BioRender.

4.2 Materials and Methods

4.2.1 *P. aeruginosa* strains and cell culture

The strains of *P. aeruginosa* used in this study were: PAO1 (laboratory strain isolated from wound infection) (Stover et al., 2000), FRD1 (mucA22) (mucoid pulmonary isolate of a CF patient) (Ohman and Chakrabarty, 1981), Δ*efhp* mutant (PAO1043, FRD1043) and complement (PAO1043.pMF 470.1, FRD143.pMF 470.1). It should be noted that Δ*efhp* and complement strains

were generated on PAO1 and FRD1 backgrounds. (Kayastha et al., 2022; Sarkisova et al., 2014). *P. aeruginosa* was cultured as described in §§2.2.1. A549 cells were grown in Roswell Park Memorial Institute 1640 (RPMI) with 5% FBS incubated at 37°C, and CuFi-5 cells were grown in Airway epithelial growth media (AEGM) with 5% CO_2 (+95% humidified air).

4.2.2 Cytotoxicity Assay

The cytotoxic effects of *P. aeruginosa* on host cells were quantified using the Pierce lactose dehydrogenase (LDH) Cytotoxicity Kit (ThermoFisher Scientific, Waltham, MA) with slight modifications. A549 cells were seeded in round bottom 96-well plates at 95% confluency. The cell monolayers were then infected with *P. aeruginosa* PAO1 in triplicates under low (0.54-0.6 mM) and high (5 mM) Ca^{2+} conditions and centrifuged (500 g for 15 min) to synchronize infection. 2 hours after infection, 10 μl of lysis buffer was added to each set of triplicate wells and incubated for 30 minutes. The plates were then centrifuged at 250 g for 55 minutes. Furthermore, 50 μl media was gently transferred to another 96-well flat bottom plate, which was incubated for 15 min at room temperature. The reaction was stopped by adding 50 μl of the stop solution. DMSO was used as a loading control. The plates were read using a Biotek plate reader at an absorbance of 490 and 680 nm to quantify LDH release under both infection and control conditions at low and high Ca^{2+}. The background control values were subtracted from the experimental values to obtain the cytotoxicity as shown in equation 4.1.

$$\text{Cytotoxicity (\%)} = \frac{\text{Experimental value} - \text{Cell Control}}{\text{Cell Control}} \times 100 \tag{4.1}$$

4.2.3 Gentamycin protection assay

Gentamycin protection assay was used to quantify the number of internalized *P. aeruginosa* during infection with A549 cells. The method to culture *P. aeruginosa* strains is described in §§2.2.1. Bacterial cells were normalized to a multiplicity of infection (MOI) of approximately 100 bacteria

per cell in RPMI. To test the concentration of gentamycin that can effectively kill *P. aeruginosa*, bacterial cells were seeded in 24 well plates, without epithelial cells, and incubated at 37°C. Cells were washed five times with HBSS, followed by the addition of RPMI containing gentamycin at different concentrations, and lyzed by adding sterile water to release *P. aeruginosa*. 200 μl samples were collected in triplicate at times corresponding to 0, 10, 20, 30, 35, 40, 45, 50, 55, and 60 minutes after treatment and plated on Luria Bertanni (LB) agar after their serial dilution in PBS. Colony formation units (CFU) were counted for each dilution to estimate the total count, which was further used to determine the total count of *P. aeruginosa* recovered from each condition.

4.2.4 Invasion Assay

A549 human lung epithelial cells were seeded in 24 well plates at a confluence of 5×10^5 cells. Infection was carried out with *P. aeruginosa* strains under low (~0.54-0.6) and high Ca^{2+} (5 mM) conditions, as mentioned in §§2.3.2, which varies from batch to batch. Therefore, RPMI was supplemented with 1 M $CaCO_3$ to obtain a 5 mM high Ca^{2+} condition for each experiment. Well plates were centrifuged (10 min at 500 g) to synchronize infection and then incubated at 37 °C with 5% CO_2. After 30 min of infection, the RPMI in the well plates was replaced with gentamycin (200μg) containing media and incubated again at 37°C (see figure 4.2). Each condition had a few sets of wells in triplicate, (A) total *P. aeruginosa* used for infection, (B) non-adherent free swimming, (C) adherent *P. aeruginosa*, and (D) Invaded/ internalized *P. aeruginosa* in 1, 2, and 3 hours. Uninfected A549 and *P. aeruginosa* without epithelial cells were seeded in control wells. At different time points during the invasion, cell monolayers were gently washed five times with Hank's balanced salt solution (HBSS, pH 7.5, Cytiva, Marlborough, MI) with 5 min of incubation between washes to remove bacterial cells. Epithelial cells were lysed with 1 ml of sterile water and scraped with a cell scraper (Biologix Research Co, Lenexa, KS). Cell lysates were serially diluted and plated on LB agar using spin-wheel spotting. After 24 hours of incubation, recovered bacterial CFUs were counted for each dilution and the total count was extrapolated to calculate the total, planktonic, adhered and invaded *P. aeruginosa*. This assay was also used to determine intracellular

survival and replication of *P. aeruginosa*.

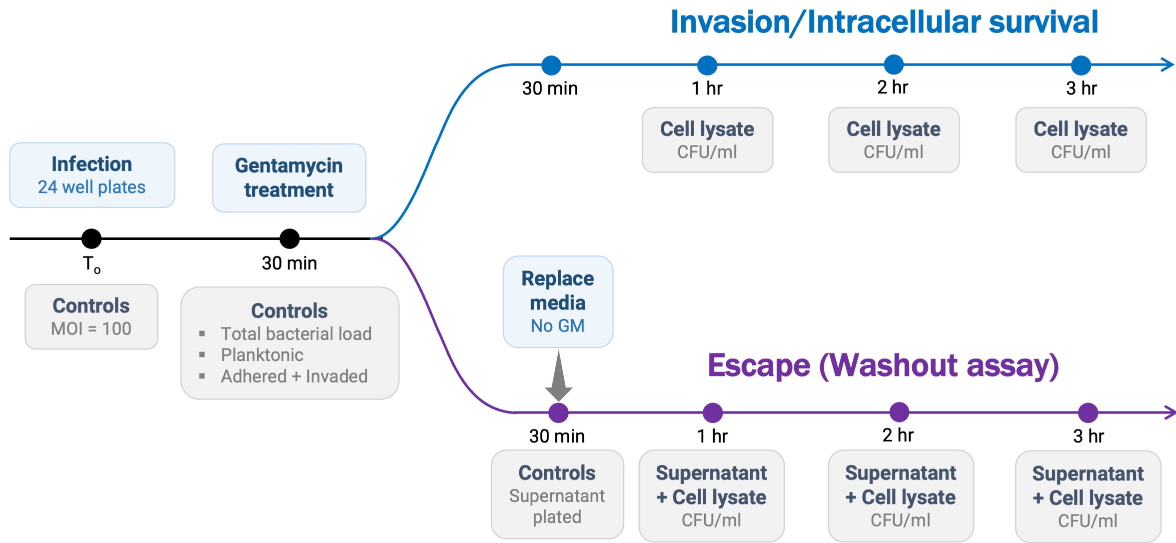


Figure 4.2: **Gentamycin protection assay.** A549 lung epithelial cells were infected with *P. aeruginosa* to perform invasion and escape (washout assay).

4.2.5 Escape Assay

A549 cells were seeded in 24 well plates and infected with *P. aeruginosa* strains in low and high Ca^{2+} conditions for 30 minutes, as described in §§4.2.4. After infection, cells are treated with gentamycin for 30 minutes and then incubated at 37 °C. To quantify the number of *P. aeruginosa* that escape from epithelial cells, the gentamycin washout assay was performed (Figure 4.2). The gentamycin-containing medium was replaced with fresh medium and incubated again to allow bacterial cells to escape. After 1, 2, and 3 hours, the medium (supernatant) was collected in microcentrifuge tubes and the epithelial cells were lysed with 1 ml of sterile water. The lysates and supernatant were serially diluted in triplicate, enumerated on LB agar plates, and incubated at 37°C. CFUs were counted after 24 hours for each dilution, and the total CFU was extrapolated for the entire sample to determine the number of escaped *P. aeruginosa* at each time point.

4.2.6 Western blotting

CuFi-5 cells, in 24-well plates, were infected with *P. aeruginosa* PAO1 at a MOI of 100. The infected cells were grown in RPMI containing 5% FBS under low and high Ca^{2+} conditions and washed with 1× Phosphate Buffer Saline (PBS) and lysis at different times during infection, such as 1, 2, 3 hpi. Mock infected A549 cells and *P. aeruginosa* grown without epithelial cells were used as a control. For each time point, cells were lyzed with cold 100 μL of 8 M Urea, including a 1× protease cocktail (ThermoFisher Scientific, Waltham, MA) per well in a 24-well plate. Cell surfaces were scraped with a cell scraper, and 100 μLof 2× Laemmli buffer containing β-mercaptoethanol was added, followed by incubation on ice for 15 minutes. Protein samples were denatured by heating at -95°C. Using SDS-PAGE, protein samples were resolved and transferred to a 0.2 μM nitrocellulose membrane (Bio-rad laboratories, Hercules, CA). The membranes were blocked with 5% skim milk in a 1× Tris buffered solution containing 0.1% Tween-20 (TBST) for 30 minutes at room temperature, followed by washing with 1× PBS and overnight incubation at 4°C with antibodies. RNA polymerase (RNAP) beta monoclonal antibody 8RB13 (Invitrogen Life Technologies, Carlsbad, CA), Phospho-Akt Substrate (RXXS*/T*) rabbit monoclonal antibody (Cell Signaling Technology, Danvers, MA) and Phospho-PI3K p85/p55 (Tyr 467, Tyr 199) rabbit polyclonal antibody (ThermoFisher Scientific, Waltham, MA) were diluted in 5% BSA in 1× TBST. Membrane blots were washed 3 times with 1× PBS for 15 minutes each and then blocked with horseradish peroxidase conjugated anti-rabbit antibodies. The blots were developed by chemiluminescence using SuperSignal™ West Pico PLUS (ThermoFisher Scientific) ECL reagents. Immunoblot images were obtained using BioRad ChemiDoc MP (Hercules, CA).

4.2.7 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). A one-way ANOVA with the post-hoc Newmann-Keuls test was performed to study the effect of calcium on invasion and escape of *P. aeruginosa*. P-values of $p = 0.05$ ($*$), $p = 0.01$ ($**$), and $p = 0.001$ ($***$) were used for statistical significance.

4.3 Results

4.3.1 Cytotoxicity of Ca^{2+} on epithelial cells

Pseudomonas aeruginosa clinical isolates are shown to differentiate into cytotoxic or invasive types (Fleiszig et al., 1997). Some studies have reported the cytotoxicity of PAO1 during infection and its dependence on the density of bacterial cells (Wang et al., 2013). To investigate the cytotoxic effects of PAO1 and its infection with A549 cells, an LDH cytotoxicity assay was performed. Figure 4.3A shows that the average cytotoxicity imparted by PAO1 to epithelial cells were ~6.8%, ~10.26%, ~8.39%, and ~5.63% to A549 cells for a multiplicity of infection (MOI) of 10, 50, 100 and 1000 bacteria per cell, respectively. The effect of MOI on cytotoxicity was statistically insignificant ($p > 0.05$).

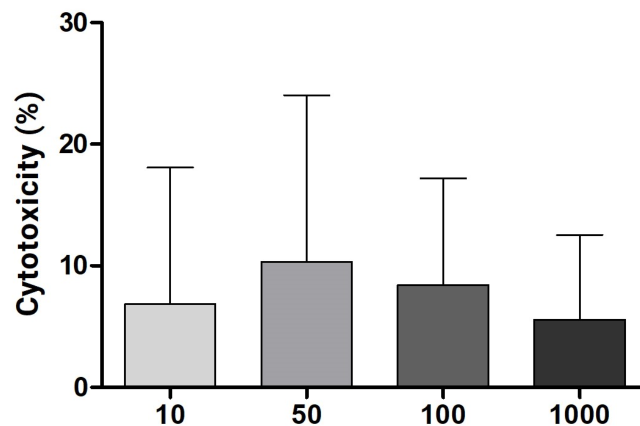


Figure 4.3: ***P. aeruginosa* induces cytotoxicity during infection of A549 cells.** Attenuation of *P. aeruginosa* cytotoxicity was observed with increasing MOI (x-axis).

During infection with A549 cells at low and high Ca^{2+} for 4 hours or more, *P. aeruginosa* does not show a significant dose-dependent response. The cytotoxicity of MOI=100 at high Ca^{2+} was observed to be -70%. This unexpected observation instilled our curiosity to further investigate the effect of Ca^{2+} on A549 cells (Figure 4.3). To study the effect of Ca^{2+} on the health of A549 cells, uninfected A549 was treated with different concentrations of Ca^{2+} for 4 hours. An increase in Ca2 + concentration was found to lead to an increase in the percentage of cytotoxicity. At 5 mM Ca^{2+}, ~70% of A549 cells were cytotoxic, while at 0.55 mM, which is the Ca^{2+} concentration

in a normal lung, the cells did not show cytotoxicity (Figure 4.4). This was also confirmed by differential interface microscopy (data not shown).

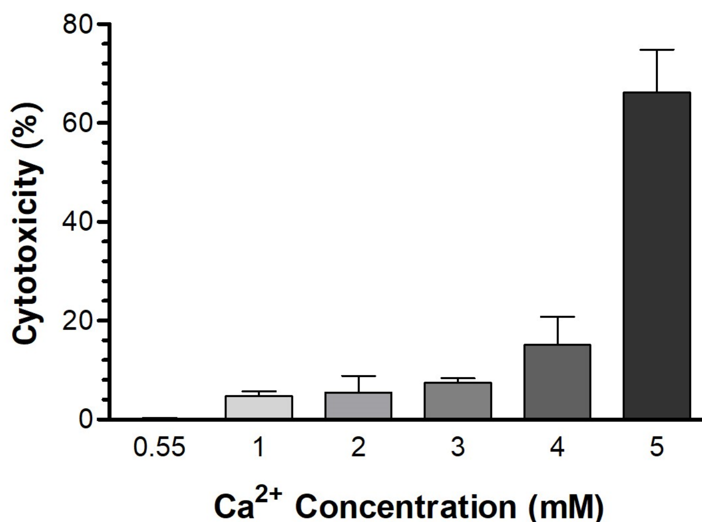


Figure 4.4: **Calcium is cytotoxic to A549 cells at high concentration.** Increasing levels of Ca^{2+}, induces cytotoxicity of epithelial cells in vitro. Additionally, 5 mM Ca^{2+} is substantially cytotoxic to epithelial cells at 4+ hr of incubation (data not shown)

4.3.2 Effect of Ca^{2+} on the invasion rate of *P. aeruginosa*

To study the effect of Ca^{2+} on the invasion of *Pseudomonas*, gentamycin protection assay (also known as a gentamycin killing assay) was performed to eliminate planktonic adhered *P. aeruginosa*. As high levels of Ca^{2+} are cytotoxic to epithelial cells, the concentration of gentamycin needed to kill *P. aeruginosa* in a short period of time was initially determined to reduce the time of Ca^{2+}-mediated invasion studies. Using different concentrations of gentamycin (10, 50, 100, and 200 μg) in RPMI, the growth of *P. aeruginosa* was tested at various time intervals to generate a time course of *P. aeruginosa* survival. Figure 4.5 shows that 200 μg/ml of gentamycin in RPMI effectively killed *P. aeruginosa* 30 minutes after treatment, making it the preferred concentration for invasion studies.

Cell monolayers of A549 ($\sim$ 95% confluence) were infected with PAO1 for an hour (MOI = 100) to examine the effect of Ca^{2+} on the invasion of this *P. aeruginosa*. Cell culture medium,

RPMI, contained 0.54-0.6 mM Ca^{2+}, as mentioned in §§2.3.2. Under high Ca^{2+} conditions, the recovered PAO1 CFU count was higher (although statistically insignificant) at 1, 2, and 3 hours of gentamycin treatment (Figure 4.6.B). Furthermore, the growth of the PAO1 strain in biofilm minimal medium (BMM) and RPMI was different under different Ca^{2+} conditions (§§2.3.2). We hypothesize that this could possibly be the reason for the insignificant difference ($p > 0.05$) between total, planktonic, adhered, and invaded bacterial cells under different Ca^{2+} conditions (Figure 4.6. A).

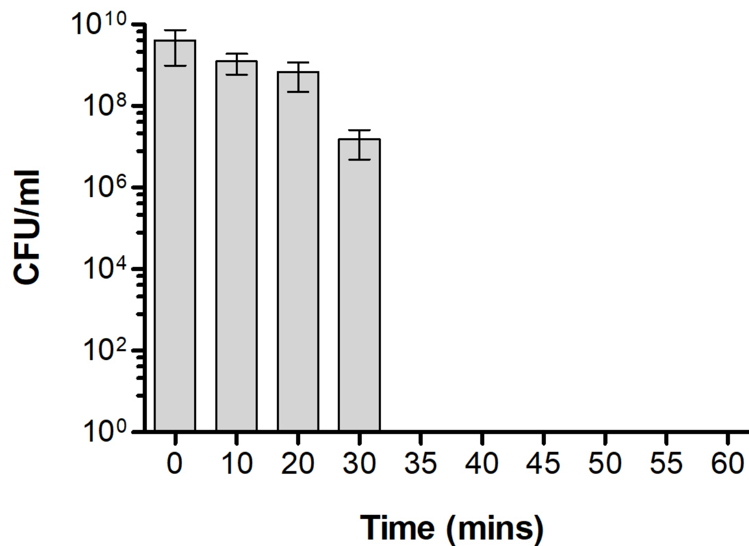


Figure 4.5: **Gentamycin at a concentration of 200 μg/ml can effectively kill *P. aeruginosa* in 30 minutes post-gentamycin treatment.**

4.3.3 Intracellular survival and replication of *P. aeruginosa*

After confirmation of invasion, we tested intracellular persistence and replication of PAO1 in A549 cells. Intracellular bacteria were counted at 1-, 2-, and 3- hours post-gentamycin treatment and compared with 1 hour to estimate intracellular PAO1 growth. CFU counts of invasive PAO1 in high Ca^{2+} increased from $\sim 2 \times 10^5$ (1 h) to $\sim 5.8 \times 10^5$ (3 h) (Figure 4.6).

Interestingly, the increasing abundance of RNA polymerase (RNAP) in the total protein encoding *P. aeruginosa* housekeeping gene, rpoB, suggests intracellular multiplication of *P. aeruginosa*.

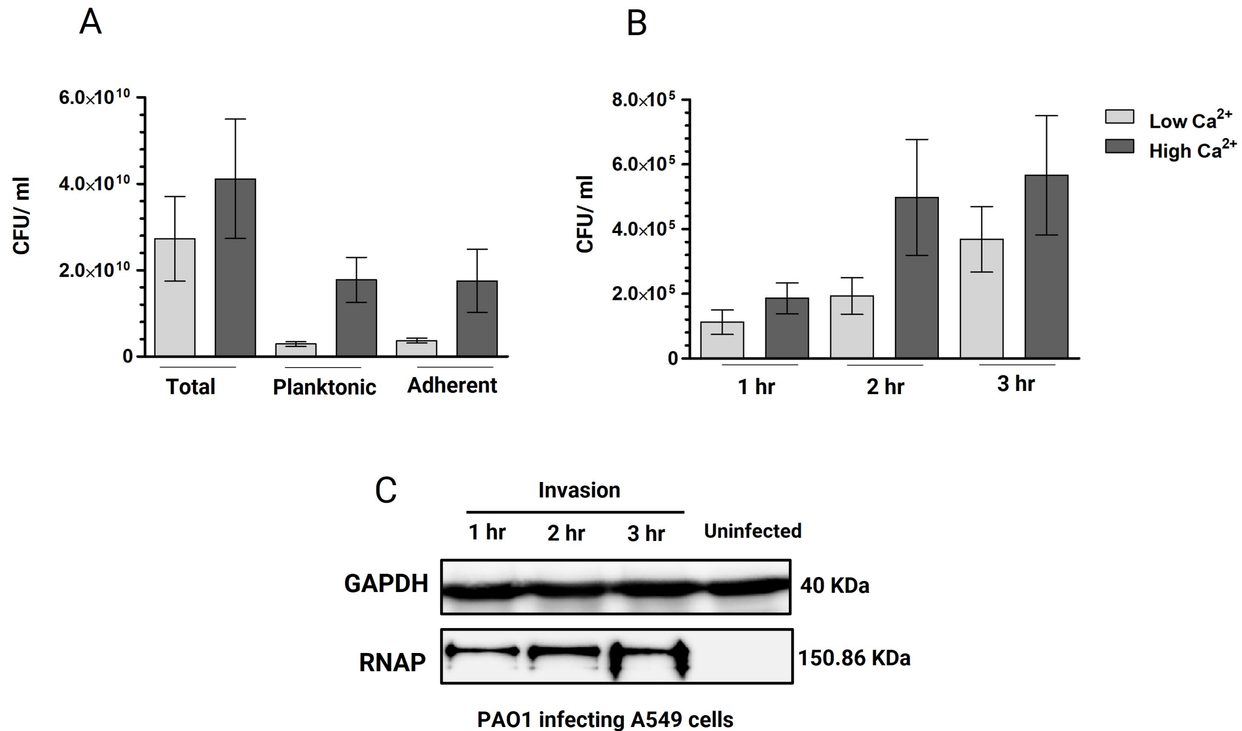


Figure 4.6: **Calcium upregulates *P. aeruginosa* invasion and survival with higher recoverable CFU counts at every hour during infection in high Ca^{2+}** (A) Total CFU/ml of *P. aeruginosa* recovered post-infection, along with planktonic and adhered *P. aeruginosa* as a control. (B) Invasion and intracellular multiplication of *P. aeruginosa*. (C) The abundance of RNAP in total protein obtained after lysis of epithelial cells infected with *P. aeruginosa*. GAPDH was used as an epithelial cell control.

The consistency in the abundance of GAPDH (glyceraldehyde 3-phosphate dehydrogenase, epithelial cell loading control) confirmed that epithelial cells were healthy during infection in high Ca^{2+} conditions (Figure 4.6 C).

4.3.4 Evasion of *P. aeruginosa* during infection

To assess the fate of intracellular *P. aeruginosa* PAO1, a gentamycin washout assay was performed. After 30-, 60-, and 90-min post-gentamycin washout, the supernatant was plated to assess the escape of *P. aeruginosa* from epithelial cells. Figure 4.7.A shows that *P. aeruginosa* escaped epithelial cells within 30 minutes and the CFU count increased with time under different Ca^{2+} conditions. *P. aeruginosa* escaped epithelial cells at a rate of $\sim$ 1000 bacteria every 30 minutes in high Ca^{2+} (Figure 4.7.A).

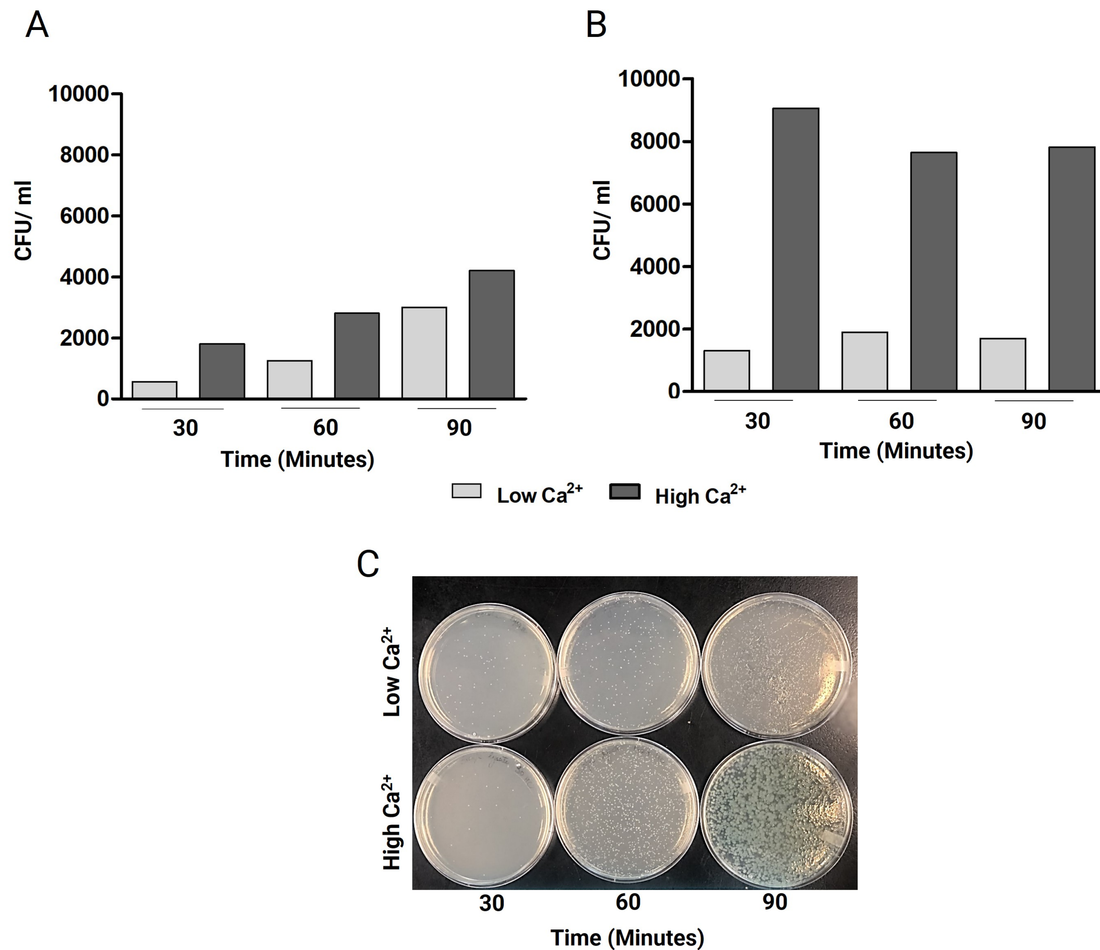


Figure 4.7: ***P. aeruginosa* escapes epithelial cells in 30 minutes post gentamycin washout.** CFU quantified from plating supernatant (A) and cell lysates (B) reported higher counts at every hour in high Ca^{2+}. (C) Supernatant obtained from 30-, 60-, and 90- minutes post washout was plated on LB agar.

Subsequently, epithelial cells were lysed with sterile water to release intracellular PAO1. Lysates when plated on LB agar also resulted in a higher CFU count under high Ca^{2+} conditions. Figure 4.7(B,C) shows that the count of CFU recovered after 30 to 90 minutes of escape was significantly higher ($p < 0.05$) than that observed for low Ca^{2+} conditions. The reason behind such higher counts in the lysates could be attributed to the intracellular multiplication of PAO1, as described earlier in §§4.3.3.

4.3.5 Invasion and Intracellular replication of (PAO1, Δ*efhP* and complement)

Kayastha et al. (2022) identified a Ca^{2+} binding protein, EfhP acts as a Ca^{2+} sensor and undergoes structural and conformational changes upon binding to Ca^{2+}. Furthermore, we showed that the role of EfhP in the adherence of PAO1 and FRD1 strains was not significant (see Chapter 2). In addition, recent preliminary studies by Dr. Aya Kubo (data not shown) showed an increase in the expression of Δ*efhP* transcription during internalization. This prompted the need to investigate the possible role of EfhP in the invasion.

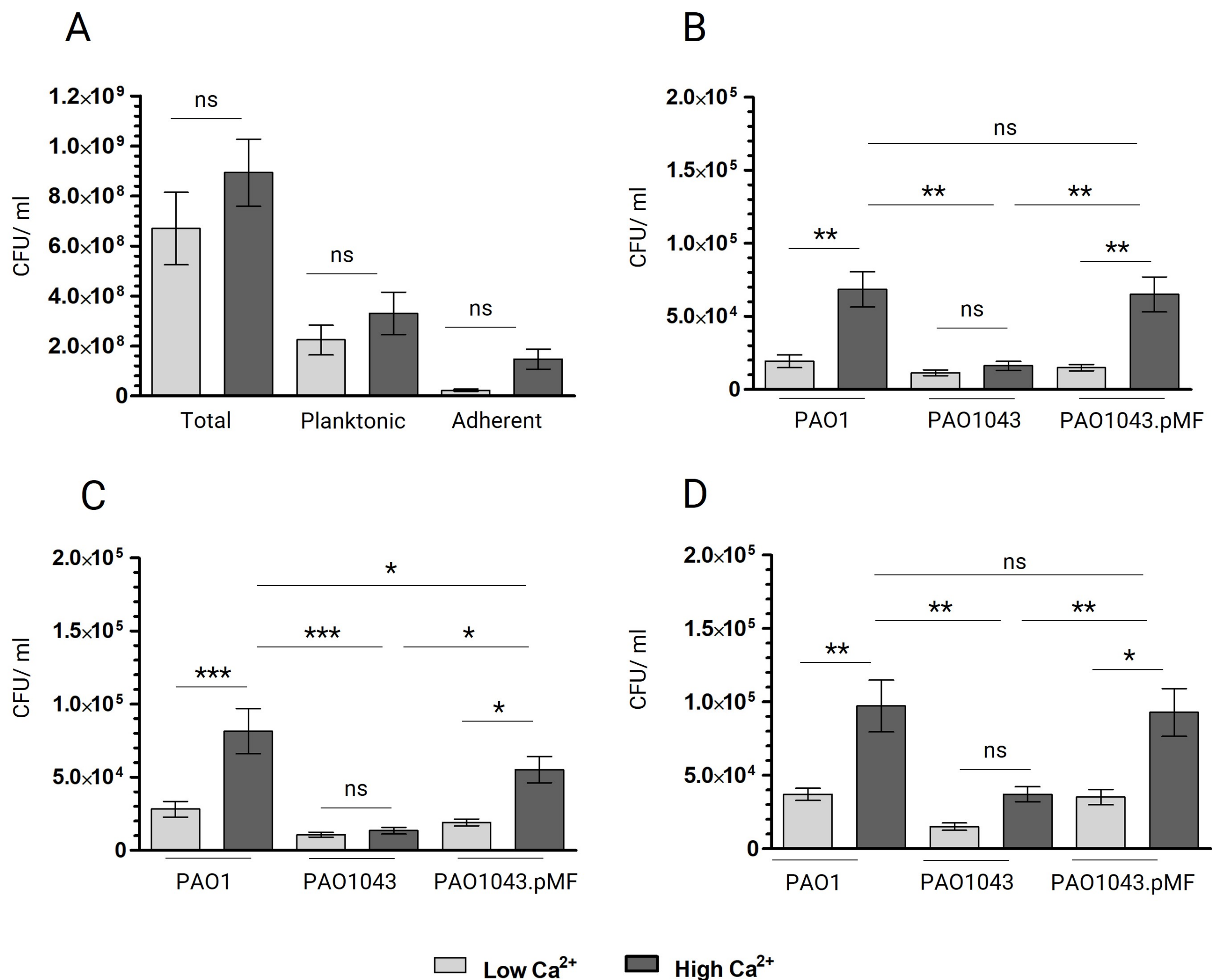


Figure 4.8: **Calcium binding protein, EfhP aids in invasion and multiplication of *P. aeruginosa* during infection with A549 lung epithelial cells.** (A) Total CFU/ml of *P. aeruginosa* recovered post-infection, along with planktonic and adhered *P. aeruginosa* as a control. Invasion of P. aeruginosa strains at 1 hr (B), 2hr (C), and 3 hr (D) post gentamycin treatment.

To study the role of EfhP in invasion, A549 cells were infected with the *efhP* mutant (PAO1034) and complement (PAO1043. pMF 470.1) generated in the PAO1 background as described earlier. The CFU count of recovered wild-type PAO1 from lysing epithelial cells at 1, 2, and 3 hr post-invasion not only showed a significant increase for high Ca^{2+} but also showed a significant difference ($p < 0.05$) in the CFU count obtained from PAO1 and PAO1043 (Figure 4.8 B, C, D). However, the difference between the CFU counts for the Δ*efhP* complement PAO1043.pMF and PAO1 was insignificant at 1 and 3 hr post-invasion in high Ca^{2+}, highlighting the importance of EfhP in invasion and intracellular survival of *P. aeruginosa* during infection.

4.3.6 Role of EfhP in Ca^{2+}-mediated escape of *P. aeruginosa*

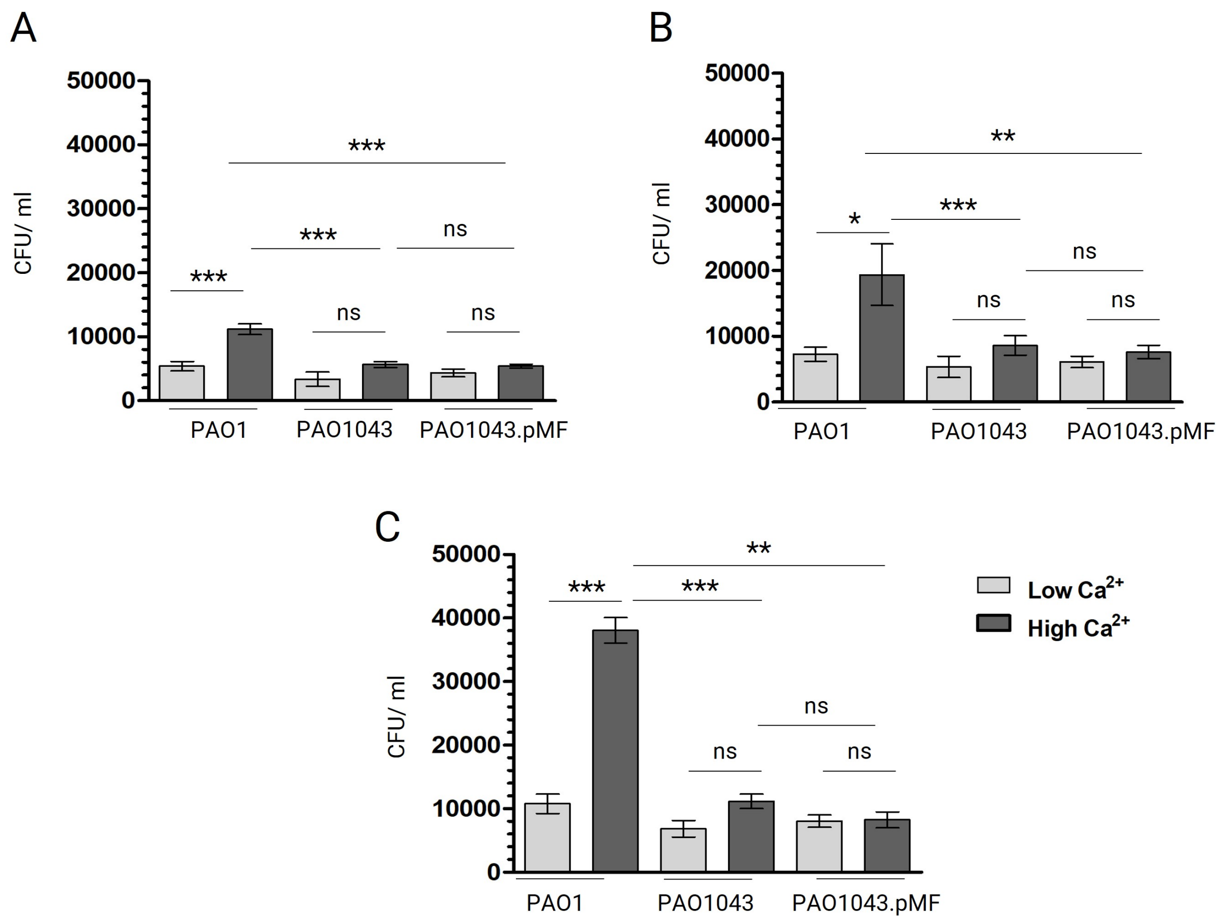


Figure 4.9: ***P. aeruginosa* escapes A549 lung epithelial cells.** Recoverable CFU/ml of *P. aeruginosa* in supernatant post gentamycin removal, suggesting escape of *P. aeruginosa* strains at 1 hr (A), 2 hr (B), and 3 hr (C) post gentamycin treatment.

In addition to invasion, the role of EfhP in Ca^{2+}-mediated escape of *P. aeruginosa* was investigated. Using PAO1 strains (wild type, Δ*efhP* mutant, and complement), gentamycin washout assays were performed to assess the ability of *Pseudomonas* to escape epithelial cells. Figure 4.9.A shows that the count of escaped wild-type PAO1 was $\sim 10,000$ one-hour post-washout which increased to ~20,000 and ~40,000 at 2 and 3 hours, respectively (Figure 4.9.B,C). For higher Ca^{2+}, the count of recovered CFUs increased significantly ($p < 0.05$) for the three strains at different times post-gentamycin washout. In addition to the Ca^{2+} mediated escape of PAO1, Δ*efhP* mutant shows a reduction in the count of *P. aeruginosa* escaped after different time points, implying the possible role of EfhP in escape.

4.3.7 Role of Ca^{2+} in host signaling during infection

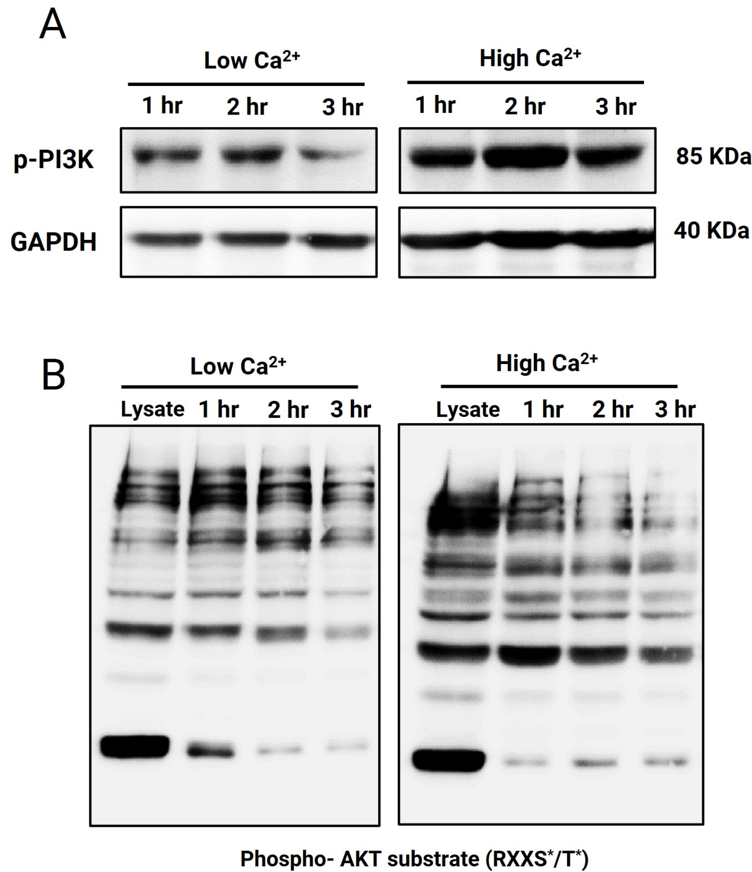


Figure 4.10: **Phosphorylation of PI3K and p-AKT substrates increases during infection in high Ca^{2+} when compared to phosphorylation in low Ca^{2+}** . Total protein was collected from *P. aeruginosa* PAO1 invasion of CuFi-5 cells for 1, 2, and 3 hr. (A) Activation of phospho PI3 Kinase (B) and abundance of total Phospho- AKT substrates increases in the presence of Ca^{2+}

PI3K activation for PIP3 generation and Akt recruitment crucial for *P. aeruginosa* invasion (Kierbel et al., 2005; Kannan et al., 2008). *Pseudomonas* interferes with intracellular signaling cascades that aid in its survival and replication. To determine the effect of Ca^{2+} on the activation of AKT/PIP3 signaling during the invasion, whole cell lysates were collected from CuFi-5 cells infected with PAO1 in low and high Ca^{2+} and prepared for immunoblotting. The phosphorylation of the PI3 kinase (motif p85/p55- Tyr 467.Tyr 199) for the production of phosphopeptides p85 and p55 was observed during the invasion at the three-time points, along with a higher abundance of PI3K in high Ca^{2+} (Figure 4.10 A).

GAPDH was used as an epithelial cell loading control that remained uniform at all time points under both conditions. Concomitantly, the phosphorylation of AKT substrates (RXXS*/T*) suggested the recruitment of AKT during PAO1 invasion in high Ca^{2+}. (Figure 4.10 B).

4.4 Discussion

Bacterial pathogens utilize an arsenal of virulence factors to transmigrate across tissues and body fluids. Opportunistic pathogens such as *Pseudomonas aeruginosa*, particularly known as an extracellular pathogen, have been reported to migrate across host barriers using their Type 3 (T3SS) and Type 6 (T6SS) secretion system effectors by interfering with signaling cascades that lead to junction break. *P. aeruginosa* incorporates several molecular mechanisms to invade epithelial cells, which are categorized into three main theories, utilization of lipid rafts (Kannan et al., 2008), activation of Src tyrosine kinases (Esen et al., 2001), and activation of PI3k that recruits AKT leading to remodeling of the cytoskeleton (Sana et al., 2016). The mechanisms utilized to transverse the host cell barrier are unknown. Upon adhesion, flagella activate the PI3K/Akt pathway for phagocyte engulfment (Lovewell, Hayes, O'Toole and Berwin, 2014). Additionally, internalization of *P. aeruginosa*, requires activation of signaling cascades that involve phosphoinositol-3-kinase (PI3K) and protein kinase B/Akt (Lovewell, Hayes, O'Toole and Berwin, 2014). PI3 kinase is involved in various cellular functions, including intracellular trafficking. Blocking PIP3 activity prevents *Pseudomonas* invasion (Kierbel et al., 2005) and PIP3 activators, Lyn, and cholesterol-rich membrane rafts regulate *P. aeruginosa* phagocytosis (Kannan et al., 2008).

Furthermore, Ca^{2+} fluctuations in the cytoplasmic fluid of eukaryotes are controlled by the *P. aeruginosa* transporter (CalC), the two-component system (CarSR) and the Ca^{2+} binding proteins. EfhP, a Ca^{2+} sensor of *P. aeruginosa*, has been shown to effectively bind to and regulate intracellular Ca^{2+} (Kayastha et al., 2022). Abnormalities in Ca^{2+} signaling can be detrimental to eukaryotic cells and contribute to cell death and diseases. Ca^{2+} activates PI3K by converting calmodulin to phosphorylated pCaM (Zhang et al., 2017). Therefore, it is important to investigate the role of EfhP in the invasion, intracellular survival, replication, and escape of *P. aeruginosa*. Immunoblot

analysis suggested the activation of PI3 kinase and phosphorylation of Akt substrates during the invasion and increased abundance of proteins in invasion and intracellular survival of *P. aeruginosa*. Furthermore, epithelial cell lysates showed a higher CFU count for high Ca^{2+}, which was not observed in low Ca^{2+} environment, strengthening our hypothesis of the importance of Ca^{2+} in this interaction.

Contrastingly, some *P. aeruginosa* strains are cytotoxic and lead to host cell apoptosis. Cytotoxic strains are often associated with the expression of ExoU, a transcriptionally regulated cytotoxin of the type III secretion system cytotoxin transcriptionally regulated by ExsA, while invasive strains lack ExoU (Finck-Barbançon et al., 1997). Attenuation of cytotoxicity of *P. aeruginosa* has been reported to cause an increase in bacterial cell density, which is often associated with the accumulation of phenylacetic acid (PAA) in *P. aeruginosa*. Accumulated PAA acts as an inhibitor of the Type 3 secretion system (T3SS) genes. Interestingly, T3SS is one of the main virulence determinants of *P. aeruginosa* responsible for the cytotoxicity and establishment of infection (Wang et al., 2013). Furthermore, LecA, the galactopoietic lectin from *P. aeruginosa*, induces cytotoxic effects on respiratory epithelial cells (Diggle et al., 2006). It is also known to induce epithelium permeability defects, causing absorption of endotoxin A and aiding in pathogenesis and immune evasion of *P. aeruginosa* (Laughlin et al., 2000). In contrast, not all *P. aeruginosa* strains are cytotoxic. Our study reported low PAO1-induced cytotoxicity during infection with A549 cells, while the invasive phenotype is highly expressed.

Overall, this work shows that the Ca^{2+} and Ca^{2+}-binding protein EfhP enhances the ability of *Pseudomonas* to invade and escape epithelial cells. To the best of our knowledge, our work is the first study to highlight the potential exit mechanism used by *Pseudomonas* to escape epithelial cells, possibly by regulating EfhP. However, the mechanisms involved in avoidance of host immune responses by *P. aeruginosa* to alter intracellular junctions are yet unknown and require further investigation.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Conclusions

In this dissertation, the effect of calcium on host-pathogen interactions of *P. aeruginosa* was investigated, which drives cystic fibrosis pulmonary infections. The work builds on the previous knowledge of the role of Ca^{2+} as an intracellular sensor, upregulating the expression of *P. aeruginosa* virulence (Kayastha et al., 2022; Guragain et al., 2013, 2016; Sarkisova et al., 2005, 2014; King et al., 2021; Patrauchan et al., 2007). It was anticipated that Ca^{2+} regulates *P. aeruginosa* virulence at the cellular level. To study such interactions, we shed light on the Ca^{2+} mediated mechanisms used by this pathogen to establish intracellular and extracellular niches, in addition to the virulence of *P.aeruginosa*. We used a variety of microbiology, molecular biology, microscopy, and genetic tools and techniques to investigate the role of Ca^{2+} in host-pathogen interactions. The main outcomes of this study chapter-by-chapter are as follows.

In this dissertation, the research aimed at investigating the importance of Ca^{2+} on the pathogenic interactions of *P. aeruginosa* with the host epithelial cells. Using various microbiology, molecular biology, microscopy, and genetic tools, we were able to establish the role of Ca^{2+} in host-pathogen interactions. The major findings of this study were divided into 3 main sections:

- In Chapter 1, we conducted adherence assays with wild-type PAO1 and FRD1 strain along with their Δ*efhP* mutant and complemented strains using two types of human lung epithelial cells under low and high Ca^{2+} conditions. We showed that under high Ca^{2+} conditions, adherence of *P. aeruginosa* to epithelial cells was enhanced. These results were supported by immunofluorescence and scanning electron microscopy. Furthermore, to genetically establish these findings, we tested the transcription of virulence genes associated with adhesion

(*fliC, pilA, lecA* using RNA-seq and RT-qPCR. Genetic data showed a strain- and cell-line-dependent role for Ca^{2+} in gene expression by *P. aeruginosa*.

- In Chapter 2, we report Ca^{2+} mediated upregulation of *P. aeruginosa* virulence factors, including its ability to induce reactive oxygen species, overexpression of *fliC* leading to more flagellated bacterial cells, calcite mineral deposition, and biofilm formation during infection. This study advances our understanding of bacterial adaptation in high concentrations of Ca^{2+}, to establish an anatomical niche in CF lung.

- In Chapter 3, we studied the intracellular lifestyle of this pathogen. Our findings suggest that Ca^{2+} upregulates the ability of *P. aeruginosa* to invade and increases its chances of replicating intracellularly and these interactions are mediated by Ca^{2+} binding protein, EfhP. We also reflect on Ca^{2+} mediated signaling of *P. aeruginosa* during infection, suggesting the activation of PI3K and Akt signaling pathways in the host in response to Ca^{2+}. To our knowledge, our work is one of the first to demonstrate the involvement of Ca^{2+} binding proteins of *P. aeruginosa* in its escape from epithelial cells.

Overall, our work advanced the knowledge of the role of Ca^{2+}, Ca^{2+} signaling, and Ca^{2+} binding protein (EfhP) in enhancing the pathogenicity of *P. aeruginosa* (Figure 5.1).

5.2 Limitations

Some of the limitations of this study include the differences in growth of the Δ*efhP* complemented strains, PAO1043.pMF 470.1 and FRD1043.pMF 470.1 in BMM and RMPI. The strains grew slightly differently in high Ca^{2+}, reflecting a poor restoration of the phenotype in the complemented strain. Having a chromosomal complement might eliminate this and result in a better understanding of the role of EfhP in various host-pathogen interactions.

Secondly, some of the adherence studies conducted using transposon mutants of the *fliC, pilA* and *rhlA* genes resulted in an inconsistent data set. An analysis of the entire sequence of these mutants indicated multiple mutations in the genome. To better understand the role of *fliC* and

pilA in adhesion to *P. aeruginosa*, clean deletion mutants can be generated in PAO1, and the gene expression of these mutants can be compared with wild-type strains.

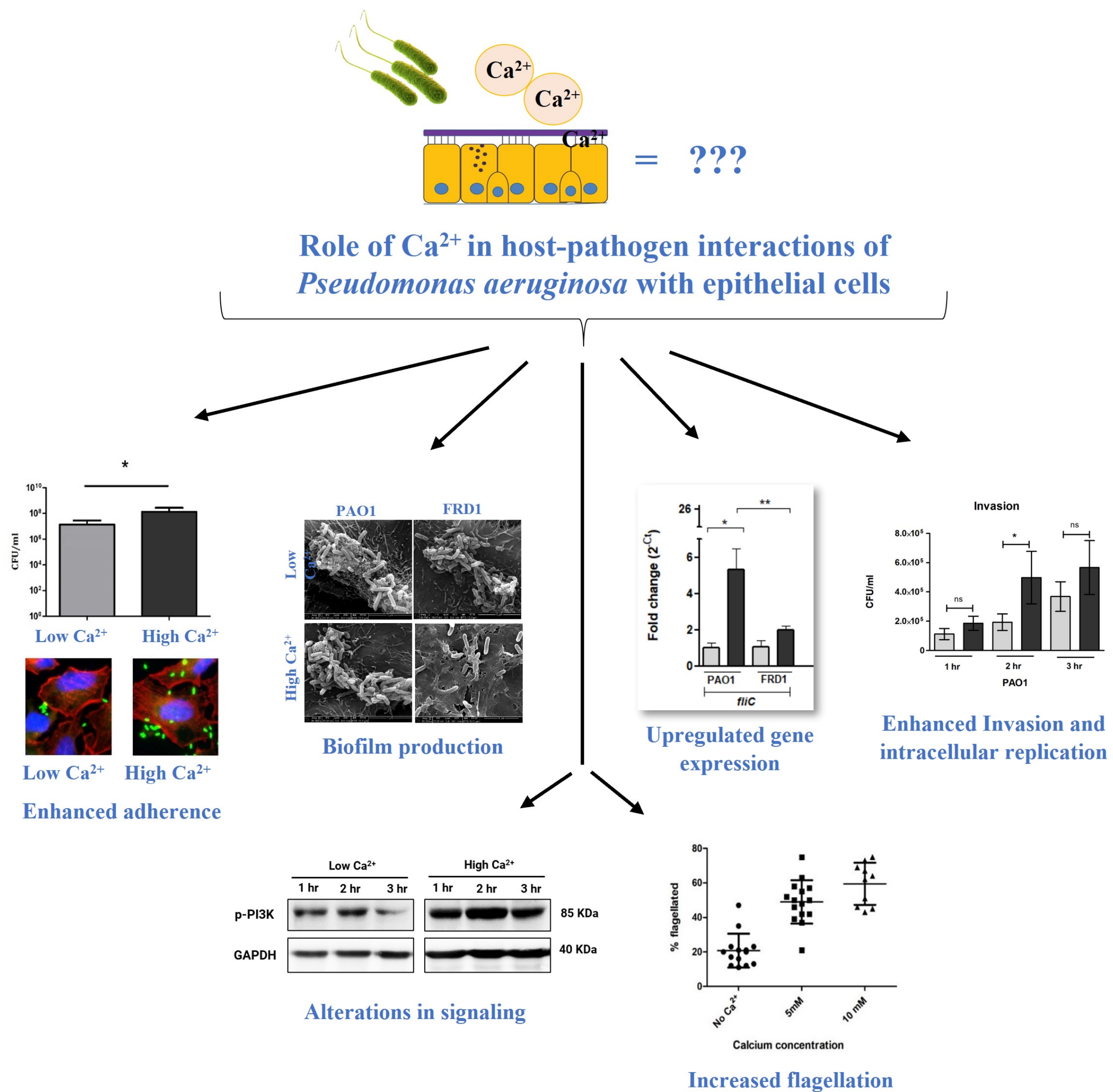


Figure 5.1: **Summary of research findings.** This research study reports the role of calcium in influencing host-pathogen interactions of *Pseudomonas aeruginosa*, by altering the expression of virulence factors, host signaling and its ability to adhere and invade epithelial cells.

5.3 Future Directions

The research findings presented in this dissertation advance the understanding of *P. aeruginosa* pathogenesis with the host in response to Ca^{2+}. Furthermore, we suggest that future work in this direction might benefit from the following studies.

- **Comparison of invasion, intracellular survival and escape of *P. aeruginosa* during infection with CuFi-5 cells**- Our data confirms that PAO1 invades, replicates intracellularly, and has the ability to escape A549 cells. We hypothesize that, unlike adherence studies, if this interaction is studied with CuFi-5 cells, which have the same Δ508 mutation in CFTR, there may be a significant increase in invasion and escape rates.

- **Microscopic visualization of intracellular *P. aeruginosa***- Preliminary data of in/out staining and fluorescence microscopy staining of PAO1 infected glass coverslips under high Ca^{2+} conditions further established the role of Ca^{2+} in influencing the intracellular lifestyle of *P. aeruginosa*.However, the methodology could have been improved for better visualization of intracellular *P. aeruginosa* for which we suggest a lower concentration ($<5\%$) of digitonin or using Triton X to prevent cross-staining.

- **Intracellular localization of *P. aeruginosa***- Previous studies have suggested that *Pseudomonas* induces membrane blebs to establish an intracellular niche in epithelial cells (Angus et al., 2008). Further investigations should employ live confocal microscopy to study Ca^{2+}-mediated invasion and intracellular replication of *Pseudomonas* and its localization within cell organelles. Fluorescence microscopy can also be used with mitochondria and lysosomal cell trackers.

- **Manipulation of host signaling by Ca^{2+}**- During this work, some preliminary findings suggested that Ca^{2+} not only activates PI3K/AKT but also manipulates PKA signaling during infection of PAO1 with A549 cells as tested using PKA substrate antibody. Further investigations of alterations in Ca^{2+} mediated host signaling in CuFi-5 cells will help in better

understanding of the mechanisms utilized by *P. aeruginosa* in causing the epithelial cell junction breach and intracellular transmigration.

- **Evasion of host immunity by *P. aeruginosa***- Multiple studies have reported how *P. aeruginosa* utilizes virulence factors to evade phagocytosis by neutrophils and macrophages (Rada, 2017), yet the contribution of alterations in Ca^{2+} signaling to evade host immunity has not yet been tested. Using macrophage cell lines, these host-pathogen interactions can be studied, followed by the use of flow cytometry to quantify changes in immune markers.

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