# Investigation of molecular adaptations in *Pseudomonas aeruginosa* cystic fibrosis isolates

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# Declaration

I certify that the work described in this thesis describes original research work, the majority of which was undertaken under the supervision of Distinguished Professor Ian T. Paulsen in the Department of Molecular Sciences, a part of Faculty of Science and Engineering at Macquarie University. These results have not been previously submitted for any degree, and will not be submitted for any other degree or qualification. Unless otherwise specified in the text, all studies reported within this thesis were performed by the author. The assistance and contribution of others have been appropriately acknowledged. The research was financially supported by the Australian Research Council (FS110200026) and the Australian Cystic Fibrosis Research Trust with a Postgraduate Studentship Award.

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Sheemal Shanista Kumar

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# Conference proceedings

***Oral Presentations***

- **Kumar S**, Penesyan A, Venkatakrishnan V, Packer N, Paulsen I. Lifestyle choices of *Pseudomonas aeruginosa* in the cystic fibrosis lung. BacPath13, Melbourne, Sep 2015.

***Posters***

- **Kumar S**, Penesyan A, Venkatakrishnan V, Kamath K, Krisp C, Paulsen I. Lifestyle choices of *Pseudomonas aeruginosa* in the cystic fibrosis lung. MQ Biofocus Research Conference, Macquarie University, Sydney, Dec 2014.
- Penesyan A, **Kumar S**, Kamath K, Shathili A, Venkatakrishnan V, Krisp, Packer N, Molloy M, Paulsen I. Genetically and phenotypically distinct *Pseudomonas aeruginosa* cystic fibrosis isolates share a common proteomic signature. ASM Conference, Washington, DC, Sep 2015.
- Penesyan A, **Kumar S**, Kamath K, Shathili A, Venkatakrishnan V, Krisp, Packer N, Molloy M, Paulsen I. Genetically and phenotypically distinct *Pseudomonas aeruginosa* cystic fibrosis isolates share a common proteomic signature. JAMS 5$^{th}$ Annual Symposium and Dinner, Sydney, Mar 2016.

# Achievements

- Awarded the Postgraduate Research Fund ($5000 AUD) for conference travel from Macquarie University, Australia
- Awarded the Australian Cystic Fibrosis Research Trust Postgraduate Studentship Award ($15 000AUD)

# Contributions

**Chapter 2: Genetically and phenotypically distinct *Pseudomonas aeruginosa* cystic fibrosis isolates share a core proteomic signature.** (Published in PLoS One Journal on 2 October 2015)

This work was conceptualised by Paulsen, Penesyan, Molloy, Packer, Kumar and Kamath. Experimental work including design and troubleshooting was conducted by Penesyan, Kumar, Kamath, Shathili and Venkatakrishnan. The experimental work specifically conducted by me was the carbon utilisation assessment of *P. aeruginosa* CF isolates, protein extraction of *P. aeruginosa* CF isolates including 1D SDS-PAGE and in-gel digestion. The mass spectrometer was run by Krisp and DNA sequencing was conducted at the Ramaciotti Centre for Genomics. Data analyses were performed by Penesyan, Kumar, Kamath, Shathili, Krisp and Paulsen. The proteomic data analysis was specifically conducted by me, Kamath and Krisp. The manuscript was written by Penesyan with contributions from Kamath, Shathili, Krisp, Packer, Molloy, Paulsen, as well as, me on proteomics data and methodology writing of binding of *P. aeruginosa* to mucin and proteomics workflow with Shathili and Kamath, respectively.

**Chapter 3: A cystic fibrosis *Pseudomonas aeruginosa* isolate specialised to catabolise nucleic acids.**

This work was conceptualised by Paulsen, Penesyan and Kumar. Experimental design and troubleshooting was conducted by Penesyan and Kumar. The experimental work specifically conducted by me with *P. aeruginosa* CF isolate PASS4 was protein extraction and label-free shotgun proteomics, Whole Genome Sequencing, growth of PASS4 in adenosine, generation of PASS4 mutants and Phenotype microarray of PASS4 mutants. The mass spectrometer was run by Kumar with support from the technical staff at the Australian Proteome Analysis Facility and RNA and DNA sequencing was conducted at the

Ramaciotti Centre for Genomics. Data analyses were performed by Penesyan, Elbourne and Kumar. I performed analysis of all the Phenotype microarray data of *P. aeruginosa* CF isolates and PASS4 mutants, proteomics data analysis of PASS4 grown in two different conditions, functional analysis of proteomics and transcriptomics data of PASS4 grown in two different conditions, single nucleotide polymorphism analysis of PASS4 mutants with PASS4. The manuscript was written by Kumar with contributions from Paulsen, Penesyan and Elbourne.

**Chapter 4: *Pseudomonas aeruginosa* inhibits the growth of *Scedosporium aurantiacum*, an opportunistic fungal pathogen isolated from the lungs of cystic fibrosis patients.** (Published in Frontiers microbiology on 25 August 2015)

The work was conceptualised by Nevalainen, Paulsen, Penesyan, Kautoo, Sunna and Kaur. Experimental work including design and troubleshooting was conducted by Kaur, Kumar, Pethani, Kim. Specifically, the minimum inhibitory concentration testing of *P. aeruginosa* CF isolate PASS1 and yellow fluorescent protein tagging was undertaken by me. The data analyses were conducted by Kaur, Penesyan and Nevalainen. The manuscript was written by Kaur and Penesyan. The methodology of fluorescent protein tagging of *P. aeruginosa* CF isolate PASS1 was written by me.

**Chapter 5: Dual Transcriptomics of host-pathogen interaction of cystic fibrosis isolate *Pseudomonas aeruginosa* PASS1 with *Danio rerio***

This work was conceptualised by Kumar, Tandberg, Penesyan, Hanne Winther-Larsen and Paulsen. All of the experimental work including design and troubleshooting were undertaken by me, with technical advice on confocal laser scanning microscopy provided by Suarez-Bosche, and zebrafish infection by Skadberg and Cole. The RNA samples were sequenced at Australian Genome Research Facility. All data analyses were performed by

Kumar. The manuscript was written by Kumar with contributions from Paulsen and Penesyan.

**Chapter 6: Proteomics of hosts and pathogens in cystic fibrosis.** (Published in Proteomics Clinical Application on 9 February 2015)

The review was written by Kamath, Kumar, Kaur, Venkatakrishnan, Paulsen, Nevalainen and Molloy. I contributed in writing of the introduction and representation of Figure 6.1 and Supplementary table ST6.1.

# Abstract

*Pseudomonas aeruginosa* is the major pathogen contributing to morbidity and mortality among patients with cystic fibrosis (CF) lung infections. The opportunistic pathogen, *P. aeruginosa* is metabolically versatile and broadly found in the environment and on various hosts. The metabolic versatility can be attributed to its relatively large genome. In the CF lung *P. aeruginosa* is exposed to a variety of selective pressures including the host immune response, different microorganisms, environmental heterogeneity and antibiotics. This work explores the genetic and phenotypic differences among *P. aeruginosa* strains isolated from the sputum of CF patients with the model *P. aeruginosa* strain PAO1. Comparison of *P. aeruginosa* CF isolates PASS1-4 reveal differences in genetic and phenotypic adaption to the CF lung environment, including phenotypic variation in virulence related traits such as swimming motility, biofilm formation and mucin binding. Proteomic analyses revealed patterns in protein expression common to the CF isolates, but distinct from PAO1.

Investigation of the catabolic capabilities of the CF isolates revealed that PASS4 showed a dramatically decreased capacity for utilisation of carbon sources, growing well only on adenosine and inosine out of the substrates tested. This strain was also shown to grow well on DNA suggesting it may be adapted to growth on DNA in the CF lung. Transcriptomic and proteomic analyses of PASS4 and PAO1 grown on DNA revealed increased expression of many virulence genes. Mutants of PASS4 were obtained that had an expanded carbon catabolic phenotype, and sequencing of these mutants showed they all contained a mutation in the *purK* gene involved in purine biosynthesis, suggesting that the inability of PASS4 to grow on a range of carbon sources because it is a purine auxotroph.

The exploration of bacterial-fungal interaction revealed the highly virulent CF isolate PASS1 inhibited the growth of the fungal pathogen *Scedosporium aurantiacum.* PASS1 also has a significant effect on eukaryotic model organism zebrafish. Dual host-pathogen transcriptomics

revealed an arsenal of virulence factors contributing to PASS1 infection, while the zebrafish host expressed an array of innate immunity mechanisms involved in inflammation, infection and tissue injury. Overall, this thesis provides insights into the various adaptations and mechanisms of *P. aeruginosa* involved in surviving in the CF host environment.

# Abbreviations

| | |
|---|---|
| ABC | ammonium bicarbonate |
| ABC | ATP- binding cassette |
| ACN | acetonitrile |
| AHL | acylated homoserine lactone |
| AIDS | acquired immunodeficiency syndrome |
| APR | acute phase response |
| ASL | airway surface fluid |
| ATP | adenosine triphosphate |
| BALF | bronchoalveolar lavage fluid |
| BCC | *Burkholderia cepacia* complex |
| BLAST | basic local alignment search tool |
| cDNA | complementary deoxyribonucleic acid |
| CF | cystic fibrosis |
| CFTR | cystic fibrosis transmembrane conductance regulator |
| CFU | colony forming unit |
| CLSM | confocal laser scanning microscopy |
| COG | cluster of orthologous group |
| c-di-GMP | cyclic dimeric GMP |
| DAMPs | damage-associated molecular patterns |
| dATP | deoxyadenosine triphosphate |
| DNA | deoxyribonucleic acid |
| DNase | deoxyribonuclease |
| DPI | days post infection |
| eDNA | extracellular deoxyribonucleic acid |

| | |
|---|---|
| EPS | exopolysaccharide |
| EPS | extracellular polymeric substances |
| FA | formic acid |
| FDR | false discovery rate |
| g | grams |
| *g* | gravity |
| GB | gigabases |
| h | hour |
| $H_2O_2$ | hydrogen peroxide |
| HCD | higher-energy collisional dissociation |
| IDA | information-dependent acquisition |
| LB | Luria Bertani |
| LPS | lipopolysaccharide |
| LSCM | laser scanning confocal microscopy |
| MDR | multidrug resistance |
| ml | millilitre |
| MLST | multilocus sequence typing |
| mm | millimetre |
| mM | millimolar |
| MS | mass spectrometry |
| m/z | mass charge |
| µ/g | microgram |
| µmol | micromole |
| L | litre |
| MCS | multiple cloning site |
| NCBI | National centre for biotechnology information |

| | |
|---|---|
| NET | neutrophil extracellular traps |
| NTM | Nontuberculous mycobacterium |
| PAMPs | pathogen-associated molecular patterns |
| PBS | phosphate buffered saline |
| PCA | phenazine-1-carboxylic acid |
| PCN | phenazine-carboxamide |
| PDA | potato dextrose agar |
| PM | Phenotype MicroArrays |
| PMN | polymorphonuclear neutrophil |
| PRRs | pathogen recognition receptors |
| PYO | pyocyanin |
| QS | quorum sensing |
| RNA | ribonucleic acid |
| RND | resistance-nodulation division |
| ROS | reactive oxygen species |
| RPM | revolutions per minute |
| rRNA | ribosomal ribonucleic acid |
| RSV | respiratory syncytial virus |
| SCFM | synthetic cystic fibrosis medium |
| SCX | strong cation exchange |
| SDS | sodium dodecyl sulphate |
| SNP | single nucleotide polymorphism |
| SOD | superoxide dismutase |
| T3SS | type III secretion system |
| T4P | type IV pili |
| TCA | tricarboxylic acid |

| | |
|---|---|
| TLR | toll-like receptor |
| UHPLC | Ultra high-performance liquid chromatography |
| v/v | volume/volume |
| WGS | Whole genome shotgun |
| w/v | weight/volume |
| XIC | extracted ion chromatograms |
| YFP | yellow fluorescent protein |

# 1
# Introduction

## 1.1 Overview of cystic fibrosis

Cystic fibrosis (CF) is one of the most common autosomal genetic disorders especially among Caucasian populations, where one in 25 people are carriers of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene mutation (1). The disease was first described in 1936 by Fanconi and others (2), and in 1989, the *CFTR* gene responsible for CF was discovered (3). The cystic fibrosis transmembrane conductance regulator (CFTR) protein functions as an ion channel to assist in movement of chloride ions across the membrane, as well as the transport of glutathione, thiocyanate and bicarbonate ions (4-6).

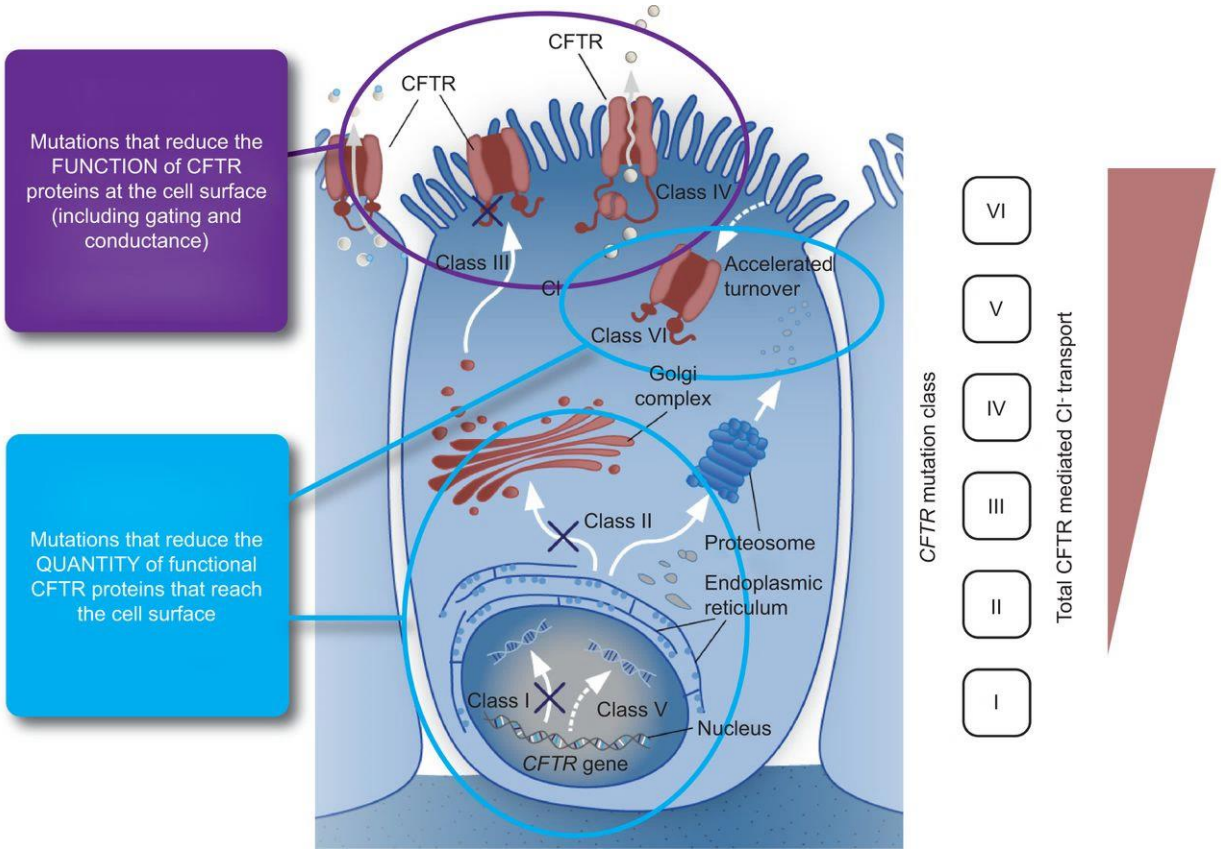


**Figure 1.1:** The cystic fibrosis transmembrane conductance regulator (*CFTR*) gene mutants can be grouped into six classes. The mutation classes III and IV (outlined with purple) result in reduced functioning of CFTR at the surface, whereas mutation classes I, II, V and VI (outlined in blue) result in absence or decreased amount of CFTR protein at the cell membrane. A normal-functioning CFTR channel can transport chloride ions to the outside of the cell while a mutant CFTR results in a large amount of chloride ions inside the cell. The lack of chloride ions outside the cell leads to build up of thick tenacious mucus. The mutation class I has disrupted chloride transport, however, classes II-VI display a gradual increase chloride ion transport capability. This figure is reprinted from Derichs et al., (2013), European respiratory review (copyright 2013, European respiratory society, UK).

The CFTR membrane protein is a unique member of the ATP-binding cassette (ABC) protein superfamily as its ATP-gated chloride channel has intrinsic enzymatic activity which regulates channel closure (7, 8). Mutations in the *CFTR* gene associated with CF results in sub-optimal or a non-functional CFTR at the apical membrane in epithelial cells (9). Typically, the *CFTR* gene mutations are categorised into six classes, with mutation classes I, II, V and VI having no or reduced levels of CFTR protein at the cell membrane, and mutation classes III and IV have a negative impact on the function or activity of CFTR at the cell membrane (10) (Figure 1.1).

The pathophysiology linked with a dysfunctional *CFTR* gene is an imbalance in electrolyte secretion through the plasma membrane of numerous epithelial cell types leading to an accumulation of sticky tenacious mucus consequently affecting multiple organ systems. The clinical problems that arise include sinusitis, complications of hepatobiliary system, high sodium concentration in the sweat, fat malabsorption due to pancreatic insufficiency, intestinal obstruction, male infertility and chronic pulmonary infection (2, 4). In the lungs, decreased chloride secretion and increased sodium absorption from the airways causes a decrease in airway surface liquid (ASL) and reduces mucociliary clearance leading to a build-up of hyperosmolar viscous mucus in the airway epithelium. The latter, can obstruct the airways and provide a favourable environment for the growth of microorganisms (11-14).

The thick mucus in the CF lung has regions of varying oxygen availability ranging from, low levels of oxygen (hypoxic conditions) to no oxygen (anoxic conditions) (14). There are also additional host-related factors which contribute to heterogeneity in the spatially heterogenous CF lungs. The varying compositions of the CF airway however does not hinder microbial adaptation and persistent colonisation which has a damaging effect on lung tissue leading to failure of lung function, inevitably becoming one of the main causes of limited life expectancy among the CF patients (2, 15).

The lung disease severity among CF patients increases with age and eventually results in respiratory failure (16). In recent years, advancements in treatment regime for CF patients has

improved life expectancy (17). Sheppard and Nicholson (2002) reported the mean life expectancy for CF patients in 2001 to be 35 years (4). In 2012 Chotirmall et al., reported the life expectancy for CF patients in the United States to be 39 years (18). Future advances in CF research may continue to improve treatments, raise the life expectancy and improve the quality of life among CF patients.

## 1.2 Cystic fibrosis lung infection

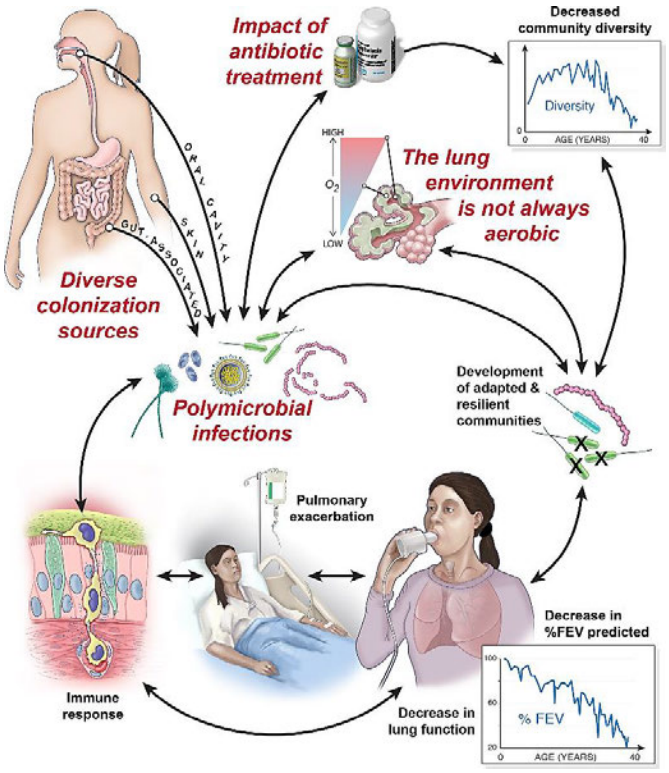


**Figure 1.2**: The cystic fibrosis lung consists of diverse polymicrobial communities which include bacterial, fungal and viral organisms. The microorganisms colonising the CF lungs can be microorganisms found to have an association with the gut and those found within the oral cavity and on the skin. The diverse CF lung microbiota has an impact on the choice of antibiotic treatment. Antibiotic treatment leads to a decrease in CF lung microbiota diversity, consequently leading to the development of resilient microbial communities which have an impact on lung function. This leads to a decrease in ability of a person to exhale normal volume of air, otherwise known as forced expiratory volume (FEV). The varying availability of oxygen in the lung and presence of host immune response has an influence on the resilience of the polymicrobial community, leading to negative effects on lung function and subsequently a decrease in FEV. This figure is reprinted from Filkins et al (2015), PLoS pathogens (copyright 2015, PLoS corporation, US).

Infection is the major cause of morbidity and mortality among CF patients (19). There are several processes involved in clearing microbes in the airways, these include aerodynamic

filtering and airway reflexes, such as coughing and sneezing, and mucociliary clearance (3). However, due to impaired mucociliary clearance, mucus builds up in the CF airway allowing for persistent microbial colonisation. Recent advances in molecular identification of microorganisms has instigated emphasis on the polymicrobial nature of infections in the CF lung environment (6) (Figure 1.2). Inhabitants of the CF lung include aerobic and anaerobic bacteria as well as yeasts, fungi and viruses (20, 21). These microbes have the ability to form highly resistant biofilm consortia (22). By forming biofilms microbes are able to establish chronic infections and avoid evasion in the CF airways, consequently leading to bronchitis, bronchiectasis, and finally, pulmonary fibrosis with respiratory failure (4).

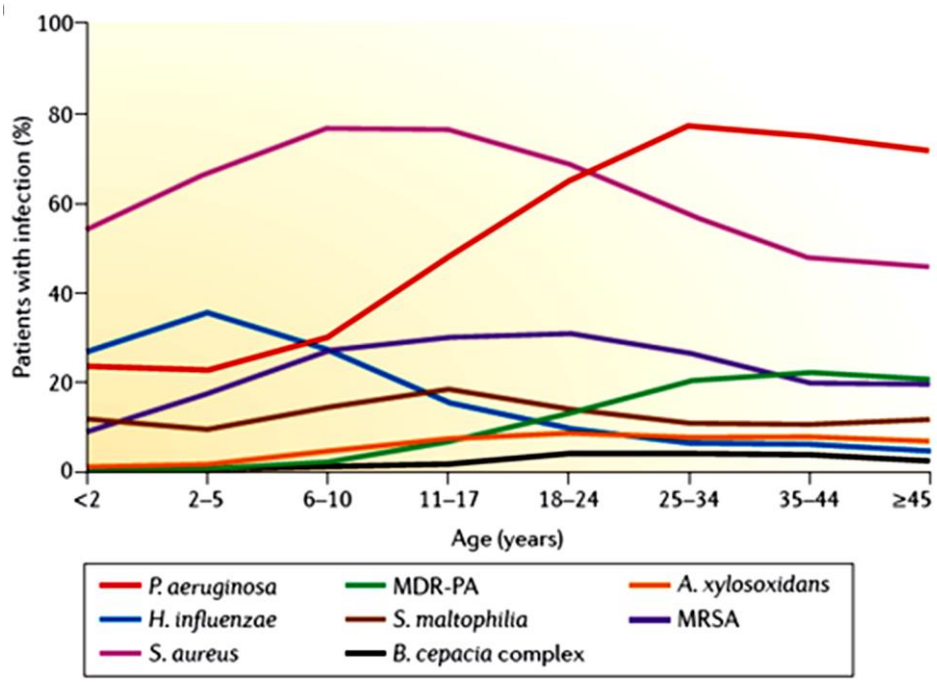


**Figure 1.3:** Occurrence of several common bacterial species present in CF patients from infancy to adulthood. The percentage of patients infected with *P. aeruginosa* increased in adulthood, however a higher percentage of patients in early infancy through to adolescence consisted of *S. aureus, H. influenza* and MRSA infections. Different colours represent different bacterial species commonly present in the CF lung from infancy to adulthood. This figure is reprinted from Folkesson et al (2012), Nature review microbiology (copyright 2012, Nature publishing group, UK).

The CF lung microbiome is continually evolving and longitudinal studies have shown that infants in the first one to two years of life mainly become infected with non- encapsulate and

non-type B capsulate *Haemophilus influenza* and *Staphylococcus aureus*, as well as enteric organisms *Klebsiella pneumoniae* and *Escherichia coli* (3, 5, 19, 20) (Figure 1.3). Chronic colonisation of CF lungs leads to lung injury (4). While *Pseudomonas aeruginosa* is present intermittently in the CF airway of children (19), almost half of these *P. aeruginosa* infections clear spontaneously (23). Eventually, species diversity decreases in the lungs of nearly all CF patients and other opportunistic pathogens such as *P. aeruginosa* and *Burkholderia cepacia* complex (BCC) associated with higher mortality become more prevalent (4, 5). *P. aeruginosa* is an opportunistic bacterial pathogen capable of causing a wide range of acute and chronic infections, and leads to premature death in patients with severe burns, organ transplants, AIDS and cancer (24). *P. aeruginosa* is often associated with chronic lung injury and reduced survival in CF patients as it is rarely eradicated by challenges of the immune defences and antibiotic therapy in the highly compartmentalised CF lung environment (12, 25, 26). *Burkholderia cepacia* infections are much less common in CF patients than *P. aeruginosa* infections (14) (Figure 1.3), however they have been linked with rapid decline in pulmonary function and a high mortality rate (27).

The above-mentioned bacteria are traditional pathogens in CF lung infection, however CF patients are susceptible to infection by other opportunistic bacterial species reviewed by Bittar and Rolain, 2010 (28). Microorganisms also present in the CF lung include: Nontuberculous mycobacteria (NTM) (*Mycobacterium abscessus*, *Mycobacterium avium*, and *Mycobacterium intracellulare*) and Gram-negative bacilli *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans* and *Pandoraea apista* (6, 19, 29-32). Several other Gram-negative bacteria were also isolated from CF sputum including species of the taxa: *Chryseobacterium* sp. (*C. meningosepticum*, *C. indologenes* and *C. gleum)* (33)*;* environmental species *Comamonas testosteroni* (34), *Moraxella catarrhalis* (35); *Acinetobacter* sp. found both in nature and in the hospital (34), *Bordetella* sp. (*B. bronchioseptica*, *B. parapertussis*, *B. hinzii*, *B. petrii* and *B. avium*) (36), *Inquilinus limosus* (34); members of the *Enterobacteriaceae* family such as

*Serratia marcescens* (34) and *Ralstonia* sp. (*R. pickettii, R. mannitolilytica, R. respiraculi, R. gilardii, R. paucula, R. taiwanesis* and *R. insidiosa*) (19, 34, 37-41). In addition, several anaerobic species, including the taxa *Prevotella oris* an oral-associated microbe (42), *Veillonella* (*V. atypica* and *V. dispar*) (43), *Propionibacterium* and *Actinomyces* such as *A. israelii, A. meyeri, A. naeslundii* and *A. odontolyticus* are found to naturally reside in the pharynx of humans (13, 43-45). There is information lacking regarding the pathogenicity or treatment of the above-mentioned bacteria in the CF context. Even though NTM was found to colonise the CF patient lung for 15 months, its clinical impact remained unclear with no observed decline in respiratory function (46). *S. maltophilia* is commonly found to be co-infected with *P. aeruginosa* in older patients (47). However, the pathogenic role of *S. maltophilia,* as well as *A. xylosoxidans,* and inherently colistin-resistant *P. apista* (19) when co-infected with *P. aeruginosa* remains elusive (46, 48). Also found to be abundant in adult sputum samples are the genera *Prevotella* and *Veillonella* (14).

The frequency of fungal isolation from adolescent and adult CF patients is greater than in children (20). CF patients are susceptible to pulmonary fungal infection due to extensive lung damage and long-term antibiotic therapy (4). Fungal colonisation occurs within the airways and occasionally as intra-cavity fungal balls (4). A significant rate of morbidity and mortality in immunocompromised patients is thought to be caused by the biofilm-forming fungal pathogens *Candida albicans* and filamentous fungus *Aspergillus* spp. (5, 49, 50). The cutaneous and mucosal surfaces of healthy individuals carry *C. albicans,* an opportunistic pathogen that can cause systemic and chronic infections and has the potential to thrive under anaerobic conditions (13, 49). The most common *Aspergillus* species, *Aspergillus fumigatus* is cultured from CF sputum. *A. fumigatus* spores are frequently found in the air, water and are usually derived from rotting vegetation (19). The increase in the use of immune-suppressive and aggressive antipseudomonal therapy in CF has been linked to an increase in Aspergillus lung disease, known as aspergillosis (4). Apart from these two prominent species, *Candida parapsilosis,*

*Exophiala dermatitidis* (51), *Penicillium emersonii* (52) and species of other genera can also be found in CF airway, such as *Alternaria*, *Trichosporon*, *Malassezia* and *Scedosporium* (6, 14). Of rising concern is the emerging pathogen and a highly virulent member of the *Scedosporium* sp. complex, *Scedosporium aurantiacum* (11). *Scedosporium* sp. complex is the second most common filamentous fungi associated with CF in Australia (5) and are resistant to many antifungal agents (53). *S. aurantiacum* is an opportunistic pathogen (54) that can be found in diverse ecological niches including soil, sewage and polluted waters (5). *S. aurantiacum* are capable of causing a range of localised and superficial infections, such as malignant otitis externa, osteomyelitis, invasive sinusitis, keratitis and pneumonia (54). However, it is unclear if the rates of *S. aurantiacum* carriage in the CF lung may have long been under-reported due to lack of sensitive fungal culture techniques available to examine the sputum specimens from CF patients (11).

Primary CF airway epithelial cells compared to non-CF airways are more susceptible to respiratory viruses (6). Infants through to adult CF patients with viral respiratory infections are at higher risk of pulmonary exacerbations as well as length and frequency of hospitalisations (55). Viral infections in CF patients cause a decline in pulmonary function, and have an influence on secondary bacterial infection (55) which consequently leads to morbidity (56). Viruses present in the lung include respiratory syncytial virus (RSV), adenovirus, influenza and rhinovirus (57). The respiratory syncytial virus is the most common cause of viral lower respiratory tract disease in children (58). Though, among the viruses present in the CF airways, rhinovirus is a major viral pathogen linked to pulmonary aggravations in CF (6).

Studies have revealed that the CF lung environment of each CF patient is individualised as the polymicrobial communities present among them are varied (14). There is a complex interplay between these polymicrobial communities and their host with other factors including the lung environment, antibiotic treatment and the immune response (Figure 1.2). Therefore, the use of molecular techniques such as rRNA sequencing to understand the lung microbiota of CF

patients has become increasingly important for trying to unravel the complexities of this polymicrobial disease.

## A. *Pseudomonas aeruginosa* - the major cystic fibrosis airway pathogen

Polymicrobial infections in the CF patients cause progressive lung damage and death (59). *P. aeruginosa* is the pathogen most frequently associated with chronic infection (60) resulting in a steady decline in CF lung function (59). However there remains substantial variation in the subsequent rate of decline in CF lung disease state, with some patients dying in adolescence and others surviving into their forties and beyond (59). A study comprising of 45 CF paediatric patients revealed the bacterial community composition in the respiratory tract to be also shaped by *P. aeruginosa* (59). Although, the specific mode of acquiring *P. aeruginosa* is unknown (60), the early-infecting strains have shown to resemble those found in the environment (61).

*P. aeruginosa* is an opportunistic Gram-negative bacterium with a genome size of ~ 6.3 million base pairs (62). The versatile bacterial saprophyte is capable of thriving in diverse environments ranging from soil, marshes and coastal marine habitats, sewage, mammalian gut, as well as on plant and animal tissues (24, 62, 63). Its survival can be attributed to its metabolic versatility, genome plasticity, adhesion factors, quorum sensing (QS) signals influencing a biofilm lifestyle, expression of virulence factors, and their capacity to out-compete other pathogens for trace elements (5, 64). The bacterium is intrinsically resistant to antibiotics and can generate spontaneous mutations following prolonged exposure to varying and challenging environments (65). Also, compartmentalisation of *P. aeruginosa* isolates and prolonged infection causes *P. aeruginosa* populations in the CF lung to display heritable phenotypic diversity within a single patient (64, 66, 67). Numerous factors influencing phenotypic changes are oxygen and carbon dioxide tension, drug concentration, osmotic shock, oxidative stress or the degree of inflammation and tissue damage (60, 68). Phenotypic changes in *P. aeruginosa* associated long term carriage in the CF lung include the ability to transition from nonmucoid to a mucoid alginate-overproducing phenotype, changes to B-band and O-antigen of its lipopolysaccharide

(LPS), loss of flagellar motility, loss of virulence factors, emergence of auxotrophic mutations and hypermutators (2, 61, 69, 70). The mucoid *P. aeruginosa* in chronic infections causes tissue damage and the deterioration of lung function (61).

**a. Genomic and phenotypic variability**

The chemically and spatially heterogeneous environment of the CF lung with non-uniform resource availability (66) plays a key role in driving *P. aeruginosa* diversification both genetically and phenotypically (12, 70, 71). The variations in nutrient availability, concentrations of penetrating antibiotics, other microorganisms and factors such as oxygen availability make the CF lung environment heterogeneous (70). The advent of genome sequencing has facilitated characterisation of the genetic adaptations occurring in *P. aeruginosa* populations in the CF lung (70, 72).

In 2000, the first *P. aeruginosa* genome (strain PAO1) was fully sequenced (66), revealing the complexity of *P. aeruginosa* genomes which allows it to succeed in a variety of environments. The *P. aeruginosa* strain PAO1 is a widely used reference organism currently (62) for study of *in vivo* and *in vitro* CF infections. However, advancement of DNA sequencing technology has facilitated sequencing and annotation of hundreds of *P. aeruginosa* genomes, and many of them from CF patients (5). At present, there are 1739 genome assemblies for the species *P. aeruginosa* listed on NCBI Genome database. Most recently, there has been an announcement of draft genome sequences of 63 *P. aeruginosa* isolates recovered from sputum of 15 CF patients over the course of 13 years (73). There are also reports of an astounding number of distinct *P. aeruginosa* strains, precisely 40, isolated from the sputum of a single CF patient over eight years (2007 to 2014) at Trentino Regional Support CF Centre (Rovereto, Italy) (74).

The availability of whole genome sequencing techniques has allowed for comparison of *P. aeruginosa* isolates, using comparative genomics methods including identification of the accessory genome (66), single nucleotide polymorphisms (SNPs), deletion events, reduction of

whole genome size (75), as well as identifying potential molecular mechanisms of resistance and prediction of antimicrobial susceptibility (74). The genome-wide analysis can be coupled with phenotypic characterisation for understanding of *P. aeruginosa* isolates adaptation in the CF lung environment (76).

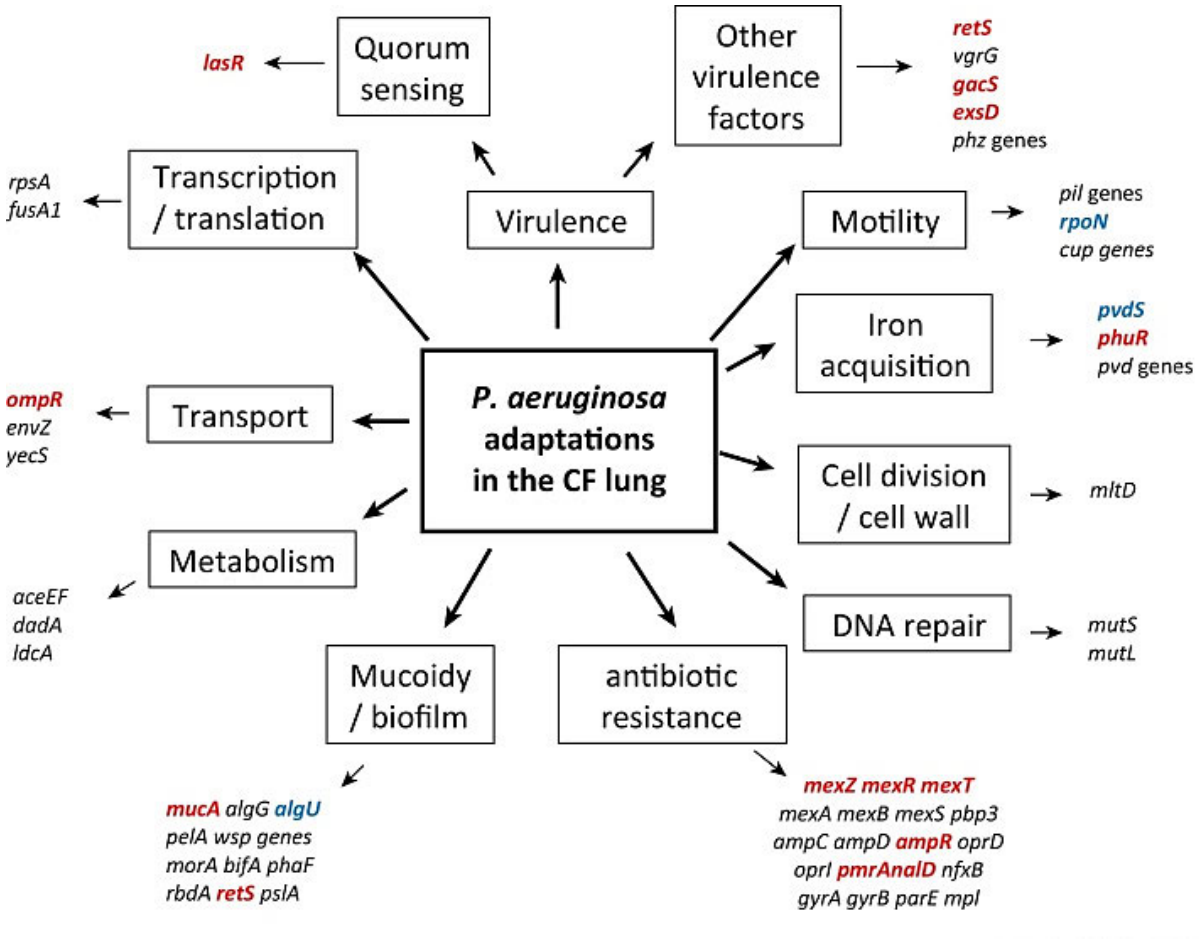


**Figure 1.4:** Mutations commonly occurring in *P. aeruginosa* as a result of its adaptation to the CF lung environment. The mutations can occur in genes involved in virulence, motility, iron acquisition, cell division, DNA repair, antibiotic resistance, mucoid phenotype, biofilm formation, metabolism, transport, transcription and translation. The genes encoding regulatory proteins are highlighted in red and the genes encoding sigma factors are highlighted in blue. This figure is reprinted from Winstanley et al (2016), Trends in microbiology (copyright 2016, Cell press, USA).

Advancements in genome sequencing have revealed the extensive genetic diversity among *P. aeruginosa* populations within individuals with CF, as well as identification of transmissible strains acquired by patient-to-patient transmission (70). In a study comparing clonally related

longitudinal isolates, mostly from early and late in the infection process, revealed common mutations among *P. aeruginosa* isolates, this included functional categories such as virulence (including QS and mucoidy), motility, transport, antibiotic resistance, iron acquisition, DNA replication or repair, transcription/translation, cell division or metabolism (70) (Figure 1.4). Also, there was often identification of mutations in genes encoding key global regulators (70) including *lasR*, *rpoN* (an alternative sigma factor that regulates various virulence factors) (77), *ampR* (regulator of β-lactamases, proteases, QS and other virulence factors) (78), *mucA* (the regulator of alginate biosynthesis) (79), *mexT* (MexEF multidrug efflux pump regulator) (80, 81), *mexZ* (MexXY multidrug efflux pump regulator) (82), *retS* (hybrid sensor kinase-response regulator protein that controls the switch between type III and type VI secretion via c-di-GMP signalling) (83) and *exsD* (a negative regulator of the type III regulon) (84) (Figure 1.4). Mutations of the metabolic and regulatory networks alter gene expression levels and detect metabolic fluxes (70). Similarly, a study of 474 longitudinal isolates from 34 CF children and young adults, representing 36 different lineages of *P. aeruginosa* revealed common evolution of numerous genes (70). There are several other studies which have examined genomic diversity in *P. aeruginosa* (85-90) and identified unique clonal lineages in individual CF patients (91).

The evolutionary phenotypic adaptations that are found in *P. aeruginosa* isolates from the CF lungs include changes in colony morphology, motility, protease activity, auxotrophy, siderophore levels, growth profiles, antibiotic resistance, type IV pili production, various modifications of the LPS toxin production, QS phenotype, mucoidity, as well as adoption of a biofilm lifestyle (6, 15, 70, 90, 92, 93) The well-known *P. aeruginosa* Liverpool Epidemic Strain (LES) from 10 adult CF patients was shown to be genetically and phenotypically diverse with variation in virulence factors, auxotrophy, hypermutability, antibiotic resistance, presence of prophage and colony morphotype (90).

One of the major hallmarks of *P. aeruginosa* adaption to the CF airways includes a switch to the mucoid phenotype (66, 90, 94). It has been reported that conversion of *P. aeruginosa* isolate

to a mucoid phenotype is a key step in the establishment of the chronic lung infection (12). The mucoid phenotype of *P. aeruginosa* provides protection from the relatively harsh CF environment where the bacterium is continually subjected to oxidative stress and attack by the immune system (94). The change to mucoid phenotype can be delayed by antibiotics, but it does not prevent mucoid conversion which can occur months or years after the initial colonisation (95). The mucoid phenotype occurs due to hyperproduction of alginate, an exopolysaccharide (EPS) consisting of a linear copolymer of mannuronic and guluronic acid joined by β 1-4 linkages (19, 94, 95). The molecular mechanisms involved in mucoid conversion is often associated with spontaneous mutations in *mucA*, which encodes an anti-sigma factor of AlgT (18, 94) which is required for expression of the alginate biosynthetic operon (94).

Understanding the consequence of various other phenotypes associated with *P. aeruginosa* adaptation to the CF lung is also essential for prevention of the deterioration of host lung function. Well-annotated and phenotypically characterised model strains are readily used to understand pathogenesis of *P. aeruginosa* infection in CF patients. Two well-annotated isolates used in CF lung infection studies include PAO1 and PA14, both are wound isolates (5). Like CF isolates, strain PAO1 has the ability to form biofilms on airway epithelial cells and strain PA14 are highly cytotoxic to epithelial cells (96). The pathogenic phenotypes of these individual strains are representative of their genomic content.

Although, *P. aeruginosa* isolates are frequently genetically and phenotypically different, their core genome is often highly conserved. For instance, comparative genomics of PAO1 and PA14 has revealed that approximately 90% of total genes are part of the ‘core genome’ (97). The collective name for genes that are not part of the core genome is the accessory genome. *P. aeruginosa* CF isolates typically contain a variety of DNA segments acquired through horizontal gene transfer including genomic islands and “pathogenicity islands”, which can carry genes encoding products important for virulence or survival in specific environmental niches

(91, 98). Genomic and pathogenicity islands typically have G+C content dissimilar to the host genome, and they frequently encode a phage-like integrase (91).

Although, it is essential to sample longitudinal isolates for characterisation of accessory genome and deeper assessment of genetic and phenotypic variation of populations of *P. aeruginosa* in the heterogenous CF lung environment, it is also important to specifically understand mechanisms (such as QS, biofilm formation, auxotrophy, etc.) that most commonly undergo genomic and phenotypic change. Understanding the mechanisms of *P. aeruginosa* adaption to CF environment may help us to design new therapeutic strategies.

Various studies have reported information on protein expression of *P. aeruginosa* in the CF context which has been discussed in detail in a co-authored review with Kamath (2015) (99) (Chapter 6). Various transcriptomic studies have been conducted with *P. aeruginosa* to address its persistence in the CF lungs. Previously, the transcriptome analysis of *P. aeruginosa* from fresh sputum samples of a CF patient were compared to those grown *in vitro* in 1× M9 medium containing citrate using Affymetrix *P. aeruginosa* GeneChips (100). This microarray technique showed increased expression in the sputum sample of genes related to virulence (including genes involved in alginate biosynthesis, iron acquisition, flagellar assembly and those involved in neutralising oxidative stress), drug resistance, and utilisation of multiple nutrient sources (lung surfactant lipids and amino acids) (100). There is also an *in vitro* CF mimicking media, in which the transcriptome of *P. aeruginosa* has been profiled using Affymetrix GeneChip microarrays, this investigation revealed the artificial CF sputum to activate the *Pseudomonas* quinolone signal cell-to-cell signalling cascade (101).

The gene expression of *P. aeruginosa* within the CF lungs can differ as they transition from planktonic to biofilm lifestyle. A study by Manos et al (2008) investigated *P. aeruginosa* CF isolates AES-1 and NC, as well as the model strain PAO1 grown planktonically and at 72 hours in biofilm state with the use of PAO1 microarrays. The CF isolates had a greater decrease in

differential expression of QS and virulence related genes compared with the model strain PAO1(102).

With the advent of RNA-sequencing technologies, there has been an improvement in resolution of information on the gene expression of *P. aeruginosa* in CF lung infection. In a study mimicking low fluid shear conditions and nutritional sources present in the CF lung with RNA sequencing revealed upregulation of genes involved in stress response, alginate biosynthesis, denitrification, glycine betaine biosynthesis, glycerol metabolism, and cell shape maintenance, and downregulation of genes involved in phenazine biosynthesis, type VI secretion, and multidrug efflux (103). Overall, transcriptomic and proteomic studies have provided insights into the physiology of *P. aeruginosa* which could not have been extrapolated from its genomic content.

**I. Quorum- sensing in *P. aeruginosa***

The complex bacterial cell signalling mechanism, otherwise known as QS controls the expression of numerous genes and phenotypes implicated in pathogenesis, this includes production of a variety of extracellular products known as public goods (104-111). With the advent of transcriptomic technologies such as DNA microarrays, the multicomponent communication and regulatory networks of *P. aeruginosa* QS has been shown to control expression of 3-10% of the *P. aeruginosa* genome (109).

The QS mechanism allows bacterial communities to coordinate population behaviour via small signalling molecules (autoinducers) which at sufficient concentrations in the external environment interact with the autoinducer-dependent transcriptional activator protein and alter gene expression (105, 106, 108, 112). This allows the majority of the bacterial population to simultaneously express a specific phenotype (109). In *P. aeruginosa*, two distinct yet interdependent LuxIR-type QS systems, the Las and Rhl systems, can respond to acylated homoserine lactone (AHL) signals (105, 106, 109, 111). The main QS system is Las system,

within this system the LasI protein catalyses the production of the AHL molecule *N*-3-oxododecanoyl- L -homoserine lactone (3O-C12-HSL) signal which diffuses into and out of cells and docks with the DNA-binding transcription factor LasR (a member of the LuxR family QS transcription factors). This allows LasR to bind to the promoters of QS-regulated genes to control expression of virulence factors such as pyocyanin (PCN), *lasB* (elastase), *lasA* (staphylolysin), *aprA* (alkaline protease), *toxA* (exotoxin A), *hcnABC* (hydrogen-cyanide synthase) and *lasI* itself (106, 109-113) (Figure 1.5). The Las circuit induces a positive feedback loop to produce more AHL, and also induces a secondary QS circuit, the Rhl system (109). The Rhl system consists of RhlI, which produces *N*-butyryl- L-homoserine lactone (C4-HSL) which accumulates in the external environment until it reaches sufficient concentration which allows for binding to its receptor, RhlR (109, 112, 113). As a result of this interaction, there is expression of *rhlAB* (rhamnolipid synthesis genes), *rhlI*, *lasB*, *rpoS* (the stationary-phase sigma factor), *lecA* (type-1 lectin), *lecB* (type-II lectin), *hcnABC* and genes involved in PCN production (109). These systems interact with a third analogous receptor, the QS regulator which operates with 3O-C12-HSL to modulate gene expression of a specific regulon which overlaps with the *las* and *rhl* regulons (111). The QS regulator can use 3O-C12-HSL to repress virulence factors when not required within the host (109, 111). The ability of the QS system to control expression of virulence factors has been shown in *in vivo* infection studies (106). The QS system has also been proven to be functional within the CF lung environment, since the QS signalling molecule, AHL has been detected in sputum samples and lung extracts of CF patients (106, 109).

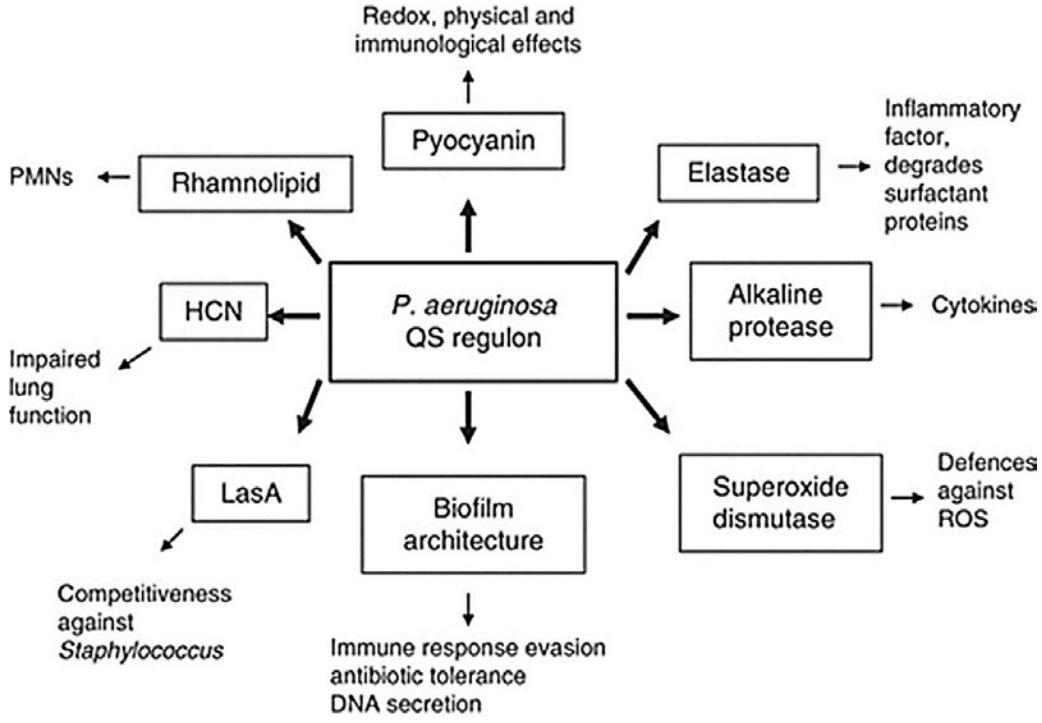


**Figure 1.5:** The *P. aeruginosa* quorum-sensing regulon stimulates expression of numerous virulence factors during CF infection. The products of *P. aeruginosa* such as elastase, alkaline protease, rhamnolipid and pyocyanin (PCN) affect the host immune response, whereas hydrogen cyanide (HCN) contributes to impairment of lung function. The virulence factors superoxide dismutase, LasA and biofilm architecture play a role in protection of *P. aeruginosa* from the host immune responses. ROS, reactive oxygen species; PMN,polymorphonuclear leukocyte (neutrophil). This figure is reprinted from Winstanley et al (2008), FEMS Microbiology Letters (copyright 2008, Federation of European microbiological societies, The Netherlands).

An additional *P. aeruginosa* QS system is the *Pseudomonas* quinolone signal (PQS) system which is mediated by 2-alkyl-quinolones, and, is also dependent on the balanced production of 3O-C12-HSL and C4-HSL (107, 109). This QS system is arranged hierarchically with the Las and Rhl system, where the Las system controls the Rhl system, and the PQS system, as an intermediary, is positioned between Las and Rhl system (111). In the PQS system, the PQS molecule (2-heptyl-3-hydroxy-4-quinolone) binds to the transcriptional regulator PqsR and plays a significant role in the transcription of Rhl-dependent *P. aeruginosa* virulence genes such as those encoding production of PCN and rhamnolipid (109, 113). The presence of high concentrations of PCN in the sputum of infected patients, contributes to the greenish colour of

CF sputa and inhibits the activity of cilia in the respiratory mucosa (109). The heat-stable haemolytic glycolipids with detergent-like activity known as rhamnolipids are also detected in the sputum of CF patients (106, 114). The quinolone signal, PQS, can also be detected in the lungs of CF patients, further supporting an active role of QS in chronic lung infections (109).

Chronic colonisation of *P. aeruginosa* leads to mutations in genes within the QS system. A typical mutation that arises is mutations in *lasR*, with approximately 1 in 3 chronically infected CF patients harbouring *P. aeruginosa* isolates with mutations in *lasR* (110). The disease state of patients harbouring *P. aeruginosa* isolates with *lasR* mutations display worse lung function (110). Whole-genome sequencing of early- and late-stage isolates of *P. aeruginosa* from a CF patient showed that *lasR* was inactivated in the late-stage infection isolate (109). In a follow-up study, comparing isolates from the same patient over an 8 year period, *lasR* mutants could be detected in isolates obtained after approximately 2 years of infection (109). However, wild-type strains could also be detected until approximately 5 years post-infection, although after this time period all of the isolates tested had a single *lasR* mutation (109). In a study consisting of 30 CF patients, there were 63% identified to consist of *P. aeruginosa* isolates with *lasR* mutation (110). Alarmingly, in a separate study, 31% CF patients infected with *P. aeruginosa* isolates consisting of mutations in *lasR* were associated with worse age-specific lung function (110). Mutations in *lasR* provides *P. aeruginosa* with growth advantages on amino acids found in the CF airways (115).

The complexity of the *P. aeruginosa* QS sensing system also enables it to mediate cross-communication with other bacterial species such as *B. cepacia*, as well as fungi (106). Thus, the intricacy of the QS system and its ability to adapt to the CF environment makes it an essential mechanism to investigate to combat challenges of CF infection.

## II. Biofilms

Biofilms are complex community of microorganisms embedded in an EPS matrix (116). The early and mature *P. aeruginosa* biofilm development is a coordinated behaviour dependent on the QS system (117, 118). *P. aeruginosa* adapts a biofilm lifestyle in the CF lung for survival from external stresses such as antimicrobial attack, killing effects of leukocytes and protection against other harmful conditions (19, 95, 119-122). Mature bacterial biofilms can be up to 1,000 times more resistant to antibiotics than early biofilms and their free-living counterparts (94, 118). The change in antimicrobial resistance phenotype can be attributed to its efflux pump systems and EPS matrix (123).

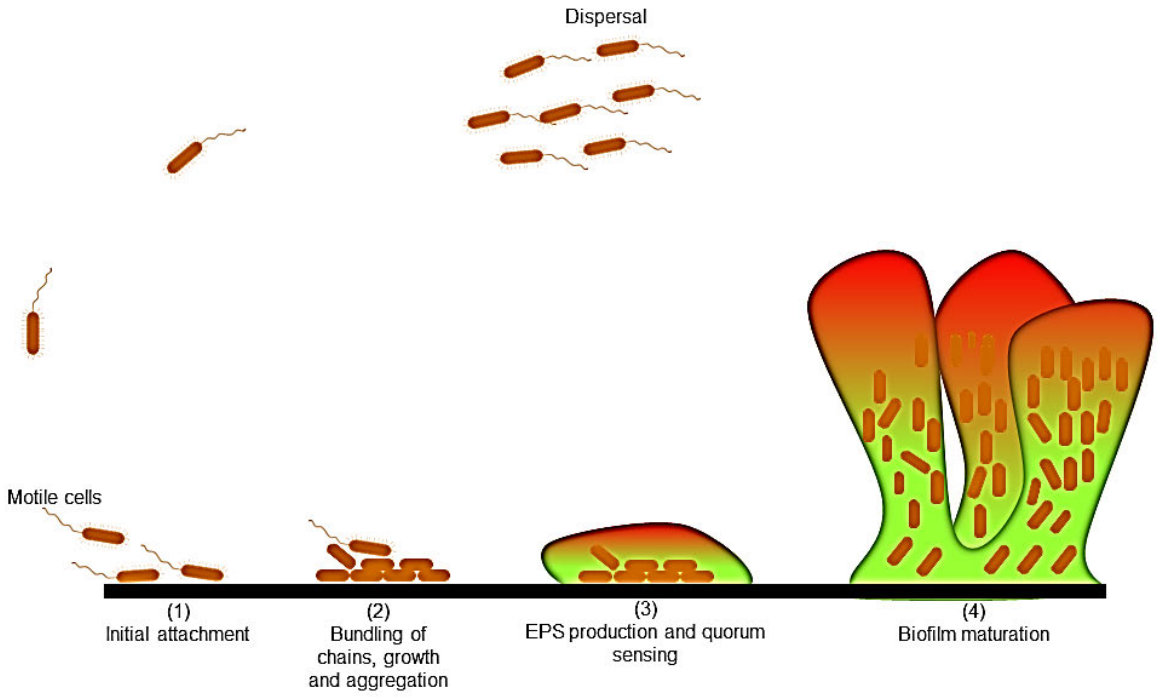


**Figure 1.6:** The biofilm development of *P. aeruginosa* occurs in four different stages. The process includes (1) initial bacterial attachment and (2) irreversible adhesion to a substratum, (3) microcolony development, (4) maturation of the biofilm and at this point bacterial cells are dispersed, to once again, commence the cycle of biofilm formation.

Typically, biofilm communities are either attached to a substratum or each other whilst encased in a matrix of extracellular polymeric substances (EPS) (95, 107). EPS is composed mainly of biomolecules, EPS, lipids, metabolites, extracellular DNA (eDNA), cellular debris and

polypeptides that form a highly hydrated polar mixture that contributes to the overall structural scaffold and architecture of biofilms (107, 117, 119). The presence of eDNA has been shown to be essential for early development of *P. aeruginosa* biofilms (124).

The physiology of biofilm formation in *P. aeruginosa* is associated with high levels of the secondary messenger cyclic dimeric GMP (c-di-GMP) (125). The biofilm formation process has been divided into various stages, including bacterial attachment and irreversible adhesion to a substratum, microcolony development, maturation of the biofilm demonstrated by the appearance of large structures resembling mushrooms and stalks with water channels, and dispersion of bacterial cells from the biofilm which occurs due to carbon starvation, oxygen limitation, sudden up-shifts in carbon-substrate concentration, nitric oxide as well as reduction in c-di-GMP (117, 125-127) (Figure 1.6). Biofilms are usually attached to the epithelial surface in CF lungs (18). The process of biofilm formation requires initial attachment where repulsion barrier between a negatively charged bacterial cell and a surface must be overcome (95). *P. aeruginosa* machineries required for initial attachment, and subsequently biofilm formation are flagella and pili (5, 117). Following attachment these complex communities embedded in extracellular matrix increase in strength and size through secretion of EPS.

The microenvironment within a mature *P. aeruginosa* biofilms has varying gradients of oxygen and nutrient availability (19). Thus, giving *P. aeruginosa* the ability to grow both in oxygen-limited microaerobic and anaerobic conditions (6). Low oxygen conditions lower *P. aeruginosa* strains metabolic growth and division, rate limiting its sensitivity to antibiotics targeting dividing cells (123).

The biofilm phenotype of *P. aeruginosa* strains can vary depending on their genomic content (107). *P. aeruginosa* biofilms can be formed by isolates with either mucoid and non-mucoid phenotype (118), however, the biofilm architecture of non-mucoid *P. aeruginosa* isolates is

different from mucoid ones (121). *P. aeruginosa* isolates with a mucoid phenotype form complex-structured biofilms with alginate as a matrix component (117).

Thus, to eradicate resistant biofilms of *P. aeruginosa* in the CF lung environment, it is essential to establish the molecular mechanisms behind various biofilm phenotypes which could allow early intervention in use of appropriate antimicrobial therapy.

**ii. Exopolysaccharides and their role in biofilm formation.**

EPS are a major component of biofilm matrix. The synthesis of EPS occurs either extracellularly or intracellularly, after which they are secreted into the external environment (123). *P. aeruginosa* biofilm matrix consists of EPS alginate, Psl and Pel. Collectively, they play an important role in maintenance of biofilm structure, mediate cell-cell and cell-surface interactions and provide protection from antibiotics and phagocytosis (13, 95, 107, 128). Internal and external stress imposed upon *P. aeruginosa*, such as, host-defence and antimicrobial agents succumb to varying properties of the EPS matrix that confer protection (95).

In mucoid *P. aeruginosa* strains, alginate is the major EPS playing a role in biofilm formation (120). The well characterised EPS, alginate, synthesised by the AlgC enzyme, is composed of mannuronic acid and guluronic acid (123, 129). In biofilms, alginate contributes to the structural stability and protection of biofilms from host immune response as well as to the retention of water and nutrients (107, 123). The Pel and Psl EPS, however, serve as key structural frameworks for biofilm development and are involved in early stages of biofilm formation (107).

Bacterial machineries influencing the production of EPS include QS systems and two-component regulatory systems (107). Psl expression can be controlled by the QS regulator LasR as it can bind to the promoter region of the *psl* operon (107). The *psl* gene cluster consists of 15 co-transcribed genes (*pslA* to *pslO*) (121). The gene products of the *psl* gene cluster are required

for biofilm matrix biosynthesis, mediating cell-surface and cell-cell interactions (95, 121). The molecular structure of exopolysaccharide Psl is a repeating pentasaccharide containing D-mannose, D-glucose and L-rhamnose (130). Biofilm formation by non-mucoid *P. aeruginosa* requires a *pslA* gene (121). When *P. aeruginosa* is migrating from one region on a surface to another it deposits Psl, permitting bacterial exploration, leading to the formation of microcolonies (130). In order to form these complex communities, Psl can also interact with DNA from *P. aeruginosa* and other organisms (130). Genotypic and phenotypic observations by Penesyan et al (2015) show that absence of Psl from *P. aeruginosa* isolate PASS2 genome results in a defect in biofilm formation (5). The Rhl system enhances Pel polysaccharide biosynthesis (107). Biosynthesis of Pel is encoded by a gene cluster comprising of seven genes (*pelA-G*) (131). It is a glucose-rich polysaccharide with most of its protein sequences predicted to encode enzymes involved in carbohydrate processing (95, 120).

**ii. Extracellular DNA and its role in biofilm formation**

Extracellular DNA is important for initial establishment of *P. aeruginosa* biofilms (95). Sources of eDNA in the CF airway include cell debris and lysed polymorphonucleocytes (PMNs), as well as DNA derived from *P. aeruginosa* cells (123, 132). This viscous 'slime' supports the structure of the biofilm matrix and has several other multifaceted roles including contribution to cation gradients, antibiotic resistance and source of organic nutrients during starvation (119, 128, 133). In *P. aeruginosa* antimicrobial peptide resistance can be obtained once eDNA chelates $Mg^{2+}$ which activates PhoPQ/PmrAB two-component systems (123).

Rhamnolipid are *P. aeruginosa* virulence factors that can be induced in the presence of PMN and is responsible for lysis of PMNs from which DNA is released (119). Bacterial machinery influencing the eDNA release during biofilm development includes flagella, type IV pili, the PQS system, as well as AHL QS systems, as has been shown for *P. aeruginosa* PAO1 (107, 123, 134, 135). The QS system allows *P. aeruginosa* to release DNA from a small subpopulation of cells through explosive cell lysis (135). During bacterial surface exploration

eDNA assists in twitching motility-mediated biofilm expansion allowing the maintenance of coherent cell alignments (107). The virulence factor PCN is able to bind to eDNA (136). The interaction between PCN and eDNA leads to changes in cell surface properties of *P. aeruginosa* such as the enhancement of cell-cell interaction which consequently promotes aggregation of cells, stability and biofilm development (136).

The eDNA in the biofilm matrix can be degraded by extracellular deoxyribonuclease (DNase) enzyme, however bacteria tightly control the production of this enzyme. The extracellular deoxyribonuclease (PA3909) is released when eDNA is required as a nutrient source by *P. aeruginosa* (128). In *P. aeruginosa* biofilms, starvation leads to loss of viable cells and an increase in dead cells and eDNA (126). However, there is lack of information on the physiology of *P. aeruginosa* when utilizing eDNA.

### III. Virulence factors

*P. aeruginosa* produces many recognised known virulence factors. These virulence factors can be bound to the cell surface, some are released, and others are injected via calcium- regulated *P. aeruginosa* type III secretion system (T3SS) (98). Surface structures playing a role in virulence in *P. aeruginosa* include a single polar flagellum, polar Type IV pili, chaperone/usher pili (cup) fimbriae and lipopolysaccharides (LPS) (98, 137). Secreted virulence factors which are governed by QS transcriptional regulator LasR include elastase and alkaline proteases, hydrogen cyanide (HCN) and exotoxins such as PCN (11, 137, 138). The expression of hydrogen cyanide encoded by *hcnABC* and PCN is also controlled by the PQS system (101).

In the initial stages of infection, surface appendages such as flagella and pili, are critical for colonisation (139). Flagella are ‘tails’ that allow cell motility and are major antigenic determinants toward the host immune response (5, 140). The attachment to eukaryotic cells is facilitated by expression of polar pili by *P. aeruginosa* (139). *P. aeruginosa* pathogenesis also requires the use of lectin, which is regulated by both QS and the alternative sigma factor RpoS

for host cell recognition and adhesion (141). *P. aeruginosa* harbours the lectins LecA and LecB. LecA is a D-galactose-binding lectin with high affinity for terminal glycans presenting an α-galactose residue at the nonreducing end (141). Studies have shown LecA to cause respiratory epithelial injury by imposing a cytotoxic effect on respiratory epithelial cells. The LecB lectin has an affinity for L-fucose and its derivatives (141), and is involved in pilus biogenesis and protease IV activity (141).

The structural components of the lung tissue and blood vessels succumb to damage as elastin is destroyed by secreted virulence factors such as the LasB elastase and LasA protease working in concert, and lipids are broken down by phospholipase C (encoded by gene *plcH*) (140). Other contributors to tissue damage include alkaline proteases, haemolysins (phospholipase and lecithinase), cytotoxin (leukocidin), siderophores with their uptake systems, and diffusible PCN pigment (139). The exotoxin, PCN, a blue-coloured pigment which is member of a class of redox-active compounds known as phenazines. These are produced in concentrations up to 100 μmol/L in the CF airways and can be responsible for eDNA release in *P. aeruginosa* strain PAO1 and PA14 (142-144). PCN encoded by *phzABCDE* genes generates reactive oxygen species (ROS), such as superoxide and hydrogen peroxide ($H_2O_2$) (101, 143, 145). The secretion and toxic effects of both PCN and its precursor, phenazine-1–carboxylic acid (PCA) are detrimental to the host and competing microorganisms (143). In the host, the proinflammatory effect of PCN disrupts the bronchial epithelium, and impairs ciliary function (139). Nevertheless, individual *P. aeruginosa* isolates can vary in phenazine production (143). Another important virulence factor is exotoxin A, encoded by *exoA*, which can inhibit host protein biosynthesis, leading to cell death (140).

The acquisition of iron is essential for *P. aeruginosa* to sustain its growth. *P. aeruginosa* can obtain iron from the host as well as other microbial pathogens present in the host (146). In the process of iron acquisition *P. aeruginosa* expresses an array of virulence factors. The process of iron acquisition involves *P. aeruginosa* synthesising and secreting two siderophores

(pyoverdine and pyochelin) which can scavenge and solubilize ferric iron (146). The binding of siderophore pyoverdine to the ferripyoverdine receptor FpvA activates gene expression via PvdS sigma factor which induces the production of iron-regulated protease, exotoxin A and pyoverdine biosynthesis proteins (146). The availability of phosphate is also essential for cell survival (147). The uptake and utilisation of phosphate is controlled by complex regulatory pathways which overlap complex regulatory routes (147) and consequently lead to expression of virulence factors. In one study, where *P. aeruginosa* was exposed to a low-phosphate medium, it overexpressed PQS and pyoverdine genes leading to enhanced killing of *Caenorhabditis elegans* (147). The absence of phosphate also causes *P. aeruginosa* to lyse the phospholipids in eukaryotic membranes using phospholipases, such as *plcB, plcH,* and *plcN* (147).

In the host, *P. aeruginosa* has to protect itself from oxidative stress produced by macrophages and other phagocytic cell attack (148). Host-pathogen interaction study of silkworm *Bombyx mori and P. aeruginosa* revealed that superoxide dismutase (SOD) is important for virulence (148). SODs are able to protect *P. aeruginosa* against toxic effects of superoxide by converting superoxide into hydrogen peroxide and oxygen (148). Rhamnolipids are also involved in the defence against the host immune system, particularly against PMNs which dominate the CF lungs (111).

Overall, either cell-surface attached virulence factors or secreted factors can cause a negative effect to the host and cohabitating microorganisms, and can trigger a host inflammatory response which leads to host tissue damage.

## IV. Antimicrobial resistance

The treatment of CF infections is a major problem due to innate resistance of *P. aeruginosa* to multiple classes of antibiotics and development of antibiotic resistance (149-151). The persistence of *P. aeruginosa* in the presence of antimicrobial agents can be largely attributed to

various mechanisms such as biofilm mode of growth, mucoid phenotype, lack of membrane porins, lower outer membrane permeability, cell wall, lipopolysaccharide, small colony variants, two-component systems, antibiotic efflux pumps, the natural occurrence of an inducible chromosomal β-lactamase, AmpC, pili-mediated microcolony formation, the microorganism's chromosome and mutational changes in regulatory genes (13, 15, 150, 151). The *P. aeruginosa* outer membrane has the ability to restrict penetration of small hydrophilic antibiotics which can only pass through the aqueous channels provided by porin proteins (150). The Mex antibiotic efflux systems are made up of three protein components, an energy-dependent pump located in the cytoplasmic membrane, an outer membrane porin and a linker protein which couples the two membrane components together (150). The toxic molecules present in the cytoplasm, cytoplasmic membrane and the periplasm are able to be extruded with the antibiotic efflux systems (150). *P. aeruginosa* consists of 12 genetically resistant resistance-nodulation-division (RND) family efflux pumps which are able to remove a wide range of antibiotics from inside the cell (151). *P. aeruginosa* RND efflux pumps include MexAB-OprM, MexXY-OprM, MexCD-OprJ and MexEF-OprN (150). Both MexAB–OprM and MexXY–OprM can collectively efflux β-lactams, chloramphenicol, fluoroquinolones, macrolides, novobiocin, sulfonamides, tetracycline, and trimethoprim and aminoglycosides (151).

To reduce antibiotic resistance CF patients are given two anti-pseudomonal antibiotics as different antibiotics display different modes of action and, therefore, different 'rates of resistance' (13, 19). However, antibiotic therapy complicates treatment for CF lung infection as it propagates the emergence of multidrug resistance (MDR) among inherently antibiotic resistant *P. aeruginosa* strains (24).

**B. Microbial interactions in the CF lungs**

The bacterial, fungal and viral pathogens colonising the CF lung can interact with each other. These microbial interactions during an infection can be either classified as neutral, synergistic or antagonistic. *P. aeruginosa* displays some synergistic interactions with other bacterial

species. Its interaction with BCC promotes BCC pathogenesis as it up-regulates the expression of virulence factor genes (57). The co-infections of mucoid *P. aeruginosa* with *S. maltophilia*, *A. xylosoxidans*, and *M. abscessus* show significant decline in the condition of the lung as opposed to infections with *P. aeruginosa* alone (57, 152). The bacteriophage present in the CF airways have an influence on *P. aeruginosa* pathogenesis as some phage are capable of inducing modifications to the *P. aeruginosa* serotype leading to a change in polyagglutinability – a trait linked with increased patient mortality (57). Besides their direct pneumopathological effect, viruses such as respiratory syncytial virus (RSV) augment the adherence of *P. aeruginosa* to CF airway epithelial cells and render some patients more susceptible to chronic *P. aeruginosa* infection (6, 57). Overall, the existing data suggests that co-infection of *P. aeruginosa* with other microorganisms can synergistically lead to a greater decline in CF lung condition.

*S. aureus* establishes infection in the CF lung in early childhood, however it is later outcompeted by antagonistic behaviour of *P. aeruginosa* (153). During co-infection of both organisms, *P. aeruginosa* produces 4-hydroxy-2-heptylquinoline-N-oxide which protects *S. aureus* against aminoglycoside antibiotics and helps select for small colony variants (6). However, it is not long until the functional quinolone biosynthesis system, possibly mediated by multiple factors like PCN and antimicrobial quinolones, allows *P. aeruginosa* to lyse *S. aureus* cells (153). Cell lysis of *S. aureus* provides *P. aeruginosa* with iron to support its growth (57). *P. aeruginosa* has been suggested to act as an opponent of *C. albicans* in the CF context. In this antagonistic relationship, *P. aeruginosa* produces a range of secondary metabolites such as the antifungals phenazine and PCN to inhibit or even kill *C. albicans* (154). Recently, interaction of *P. aeruginosa* with the fungal pathogen *S. aurantiacum* has revealed that bacteria elicits a specific inhibitory response to establish physical contact with fungal hyphae for colonisation and consequently inhibition of *S. aurantiacum* growth (11). The effects of *S. aurantiacum* and *P. aeruginosa* interaction are yet to be studied to elucidate their joint impact on the host.

## 1.3 Impact of microbial colonisation on the cystic fibrosis lung environment

The epithelial surfaces in the airways comprise a single layer of polymeric gel otherwise known as mucus (155). Mucus is a viscoelastic material that is few tens of micrometres thick and covers the epithelial glycocalyx, which includes a class of mucins bound to the apical plasma membrane (155, 156). Various other components of mucus are secreted by epithelial and glandular cells (155). Due to its characteristics and composition, mucus is considered as a barrier for protection of epithelial cells (155).

Microbial colonisation is detrimental to the CF lung function. The process of microbial invasion of the host includes first penetrating the secreted mucus barrier and then either attaching to the apical surface of epithelial cells or releasing toxins that disrupt epithelial integrity (157). *P. aeruginosa* adherence to the host can lead to altered morphology, fluid loss, induction of cytokine release, up-regulation of adhesion molecules, and apoptosis (157). The expression of an inflammatory response by the host to eliminate microbial communities can lead to damage of host tissue (4, 5). Inflammation due to infection can lead to hyperproduction of mucus which can also be deleterious to the host (155). In the CF patients, overproduction of mucus can lead to congestion in the airways. Thus, host-pathogen defences can have a negative effect on the CF lung environment.

## 1.4 Cystic fibrosis lung environment: a nutrient source

Mucus is not only a defence mechanism but also serves as a nutrient source for microbes. Often the stage of disease and clinical treatment methodologies can cause variation in nutrients available in sputum covering the epithelial cells (153). Typically, the CF sputum consists of mucin and non-mucin proteins, lipids, carbohydrates, pus, ions, cellular debris, and DNA and amino acids in concentrations above the mean of a healthy individual (15, 155, 156). Also, present in the sputum are biologically active components including lactoferrin, oligopeptides and other lipids besides lecithin which possibly are consumed as nutrients by microbes (15).

The high amount of iron found in the CF lung environment is mostly derived from transferrin, lactoferrin and ferritin (6).

The adaptation of *P. aeruginosa* to the host environment is influenced by the availability of nutrients (158). Carbon catabolism impacts *P. aeruginosa* biofilm development, production of extracellular virulence factors (101, 159), surface motility (160) and quorum sensing (101). Palmer et al. 2005, reported similarity in gene expression of carbon catabolism genes, chemotaxis/flagellar motility, iron regulated and QS genes in *P. aeruginosa* during growth in CF sputum and synthetic cystic fibrosis medium (SCFM). Aromatic acids present in the CF sputum have been reported to mediate QS signalling in *P. aeruginosa* (101). The gene expression study of *P. aeruginosa* grown in artificial sputum medium with the addition of mucin and DNA in comparison to CF sputum shows upregulation of nutrition related QS and T3SS genes in the exponential phase, and the upregulation of iron transport, fimbrial biogenesis and alginate genes with downregulation of virulence related genes and QS regulators in the stationary phase (161).

The adaption of *P. aeruginosa* is able to lead to phenotypic changes in its growth preference. Barth et al. (1995)(162) reported the ability of *P. aeruginosa* CF isolates to be auxotrophic in nature. Many studies have reported *P. aeruginosa* CF isolates to be auxotrophic to arginine and methionine (93, 163). An investigation into the diversity of metabolic profiles of *P. aeruginosa* CF isolates from early and late stages of infection revealed metabolism of amino acids, alanine, asparagine and glucose to be affected at late stages of infection in comparison to isolates from initial stages of infection (158). Nevertheless, the auxotrophic nature of the *P. aeruginosa* CF isolates remains to be elucidated. Thus, a complete characterisation of *P. aeruginosa* CF isolates can provide valuable insights to changes in metabolic diversity in from initial to the late stages of infection.

## 1.5 Host-defence mechanisms

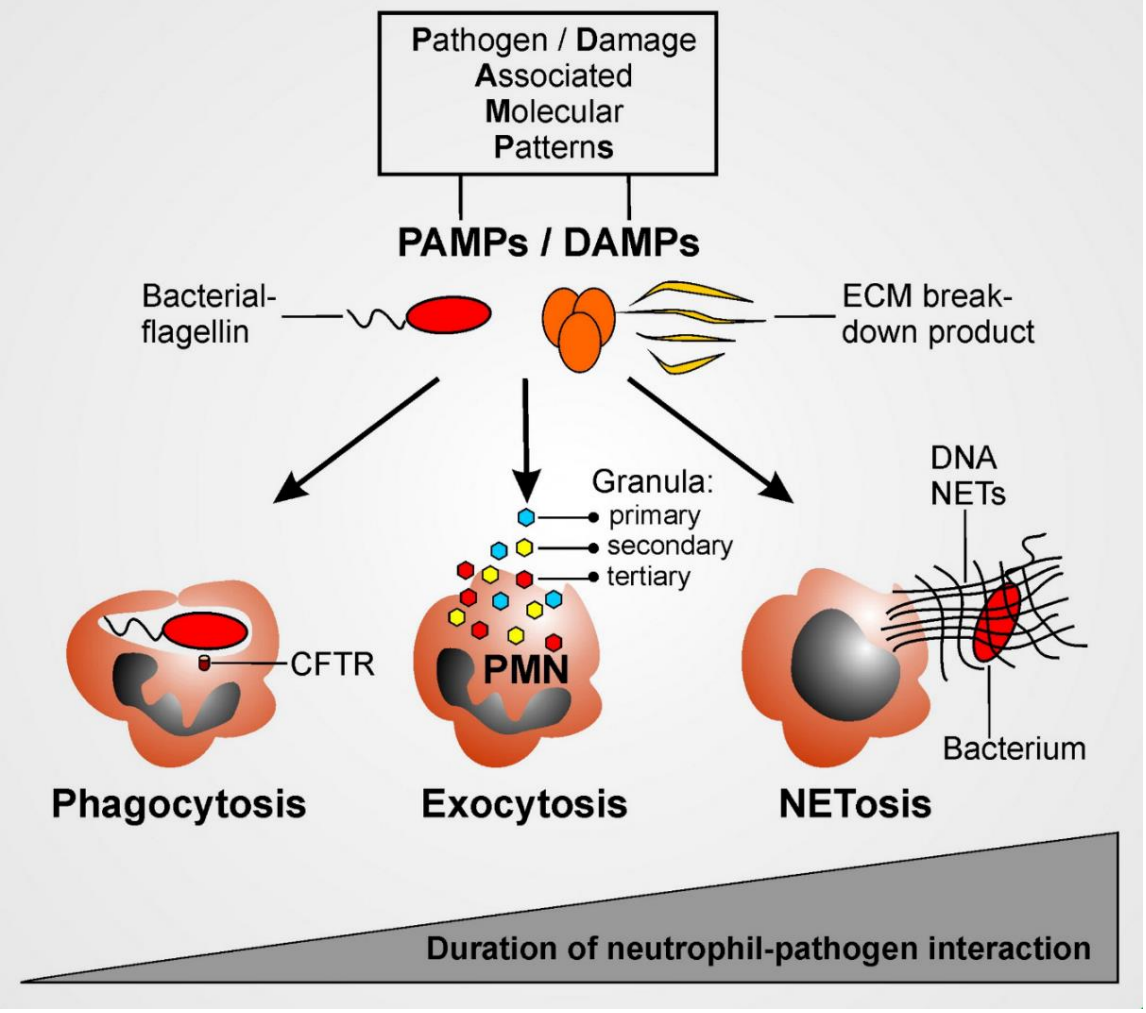


**Figure 1.7:** Neutrophils degrade invading bacteria through phagocytosis, exocytosis and NETosis. The presence of bacteria in the host causes pathogen associated molecular (PAMPs) and damage associated molecular patterns (DAMPs) to interact with neutrophils for bacterial degradation. The granule release from polymorphonuclear neutrophils (PMN) attack the bacteria and consequently the host tissue. Neutrophils also release DNA -based neutrophil extracellular traps (NETs) for bacterial killing. The three processes of neutrophil mediated bacterial killing, phagocytosis, granule release and NET formation, are employed depending on the duration and severity of host–pathogen interactions. This figure is reprinted from Hartl et al (2012), Journal of cystic fibrosis (copyright 2012, European cystic fibrosis society, Denmark).

The loss of CFTR functionality in the lungs results in a wide range of dysfunction in host defence mechanisms and immune functions, thus permitting pathogen colonisation and persistence (6). Changes within the CF lung, such as a decrease in ASL volume, increase in mucus viscosity, and impaired mucociliary clearance contribute to microbial colonisation (6).

In a normal lung, the process of mucociliary clearance involves moving foreign matter trapped in the mucus up the respiratory tract with the beating of cilia which also relies on ciliary function and sufficient amount of quality ASL (164). Host innate immune mechanisms such (i) secretion of antimicrobial peptides and pro-inflammatory cytokines due to interaction of pathogen-associated molecular patterns (PAMPs) with pathogen recognition receptors (PRRs) (such as toll like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors (NLRs), complement receptors, Fc receptors, RIG-like helicases, nucleic acid receptors and others), (ii) host-derived danger signals (DAMPs), (iii) the secretion of chemokines and cytokines that orchestrate the recruitment of leucocytes, (iv) phagocytosis by host cellular components such as neutrophils and macrophages, (v) neutrophil granule release and neutrophil extracellular traps (NET) formation (Figure 1.7), (vi) humoral immune response including secretion of antibacterial cationic peptides and surfactant proteins, and (vii) secretion of complement by epithelial cells are also involved in combating microbial colonisation (2, 3, 165-168). In addition to phagocytosis, macrophages contribute to the migration of neutrophils into the alveolar space, as well as the activation of dendritic cells and T cells for an adaptive immune response (167).

In the process of clearing microbes from the lungs alveolar macrophages, neutrophils, lymphocytes and circulating antibodies cause inflammation (2). Chronic bacterial infection can lead to hyperinflammation, where excessive recruitment of neutrophils to the airways with subsequent release of oxidants and enzymes causes tissue damage (138). CF infection with *P. aeruginosa* and *S. aureus* has been shown to initiate an inflammatory response and the subsequent release of tissue-damaging oxidants and enzymes from recruited inflammatory cells (164). When the host succumbs to infection, tissue injury and trauma, the acute phase response (APR) is a prominent systemic reaction which occurs to maintain homeostasis (169).

In the CF lung, host defence mechanisms play a vital role in combating *P. aeruginosa* infection. The rapid development in host-pathogen interaction studies has allowed for identification of molecular mechanisms key to *P. aeruginosa* pathogenesis.

### 1.6 Models available for studying host-pathogen interactions

The genetically tractable models that have been used as infection systems to study *P. aeruginosa* pathogenesis include *Mus musculus* (mouse), *Drosophila melanogaster* (fruit fly), *Bombyx mori* (silkworm), *Galleria mellonella* (greater wax moth), *Dictyostelium discoideum* (amoebae), plant model *Arabidopsis thaliana* (thale cress), *Danio rerio* (zebrafish) and the nematode *C. elegans* (roundworm) (98, 168, 170, 171). The invertebrates, *C. elegans* and *D. melanogaster* do not have an adaptive immune system and possess a very simple innate immune system which does not have macrophages or neutrophils, but instead uses one primitive cell type known as the haemocyte which carries out functions of the two phagocytes (138).

Previously, study of *P. aeruginosa* infection with mice as a model organism has revealed the essentiality of QS signals in causing chronic CF lung infection (170). The *P. aeruginosa* T3SS has also been shown to inject exotoxins into the cytoplasm of eukaryotic cells which has led to tissue damage and infection of the mouse lung (138). Both QS and T3SS have shown to be important in *P. aeruginosa* infection of *D. discoideum* (171). Further, demonstrating the importance of virulence factors in a host, a study with *C. elegans* revealed PCN to lead to host mortality (172). Phenazines are also important in *P. aeruginosa* pathogenesis in plants and mice (173). The similar expression of a subset of virulence factors in both plants and animals following infection studies validates the use of nonvertebrate hosts such as the plant *A. thaliana* for screening of virulence factors important in pathogenesis in mammals (173).

It has been widely reported that *P. aeruginosa* causing chronic CF lung infections gradually become less virulent over time (70). A similar adaptation with *P. aeruginosa* displaying slow

and gradual loss of virulence has been observed using a *D. discoideum* model system (171). In another study, *P. aeruginosa* longitudinal strains displayed a reduction in acute virulence in their late strains relative to the respective early isolates using *C. elegans, G. mellonella* and *D. melanogaster* as infection models (174).

Although, use of different hosts as infection models can provide insights to *P. aeruginosa* virulence, it should be recognised that they do not represent the entire mechanism of virulence involved in lung infection of a CF human host. Some of the factors leading to variation in expression of virulence by *P. aeruginosa* includes the temperature at which the host model organism is maintained and lack of specific receptors and pathways compared to mammals (174). Therefore, while use of model organism as infection hosts for *P. aeruginosa* can provide valuable insights, any findings should be validated in human hosts where possible.

### A. Zebrafish as a vertebrate model for *P. aeruginosa* infections

The vertebrate *Danio rerio* more commonly known as zebrafish has become recognised as a useful model for the study of infectious disease (138). Its 1.412 Gb genome is quite well-annotated, and 69% of zebrafish genes have at least one human orthologue with high degree of conservation between innate and adaptive related genes (175, 176). A difference between human and zebrafish immune system is the development of the immune system. In zebrafish, the innate immune system develops post-fertilisation with active macrophages and neutrophils, however after 30 days they gain a functional adaptive immune system (138, 175). This is advantageous in defining the role of innate and adaptive immune response during infection. The additional reasons for considering zebrafish embryos as an animal model includes characteristics such as high rate of fecundity, small size, easy maintenance, fast development, optical transparency, ability to be genetically manipulated, and since zebrafish embryos in early development stages do not experience pain, suffering, or distress, an ethics approval is not required (138, 175, 176) (Figure 1.8). Their optical transparency allows real-time visualisation of microbial infection using the microscope (138, 175). In order to see the interaction of

neutrophils and macrophages with the pathogen, both phagocytes and the pathogen have to be fluorescent protein tagged (175).

Although zebrafish lack lungs, an organ system where development of CF lung infections takes place, it has allowed for successful screening of virulence genes in bacterial lung pathogens such *Streptococcus pneumoniae, S. aureus, Mycobacterium tuberculosis, P. aeruginosa* and *S. maltophilia* (177-181). Most of the infections established in zebrafish for the study of CF lung infection are systematic infections. In zebrafish embryos, the establishment of a virulent infection by *P. aeruginosa* requires a high dose of pathogens as many will be killed by macrophages and neutrophils (182). The zebrafish embryo, like other model organisms above mentioned are susceptible to T3SS during *P. aeruginosa* infection (138). Previously, *P. aeruginosa* infection in zebrafish Cftr morphants and wild-type zebrafish embryos revealed higher bacterial burden in Cftr morphants than in control embryos at 8 h post infection (hpi) (138). Other model organisms for which Cftr morphants have shown more susceptibility to *P. aeruginosa* include mice and human bronchial epithelial cells. These studies together indicate that zebrafish embryos are useful model organisms for the study of CF infection.

Murine models, like zebrafish, have also been criticised also for differences among target organs and receptors to humans. Thus far, no single model organism is able to provide a complete portrayal of human infections, therefore, combing information of host-pathogen interaction studies can help extrapolate on human infections (175).

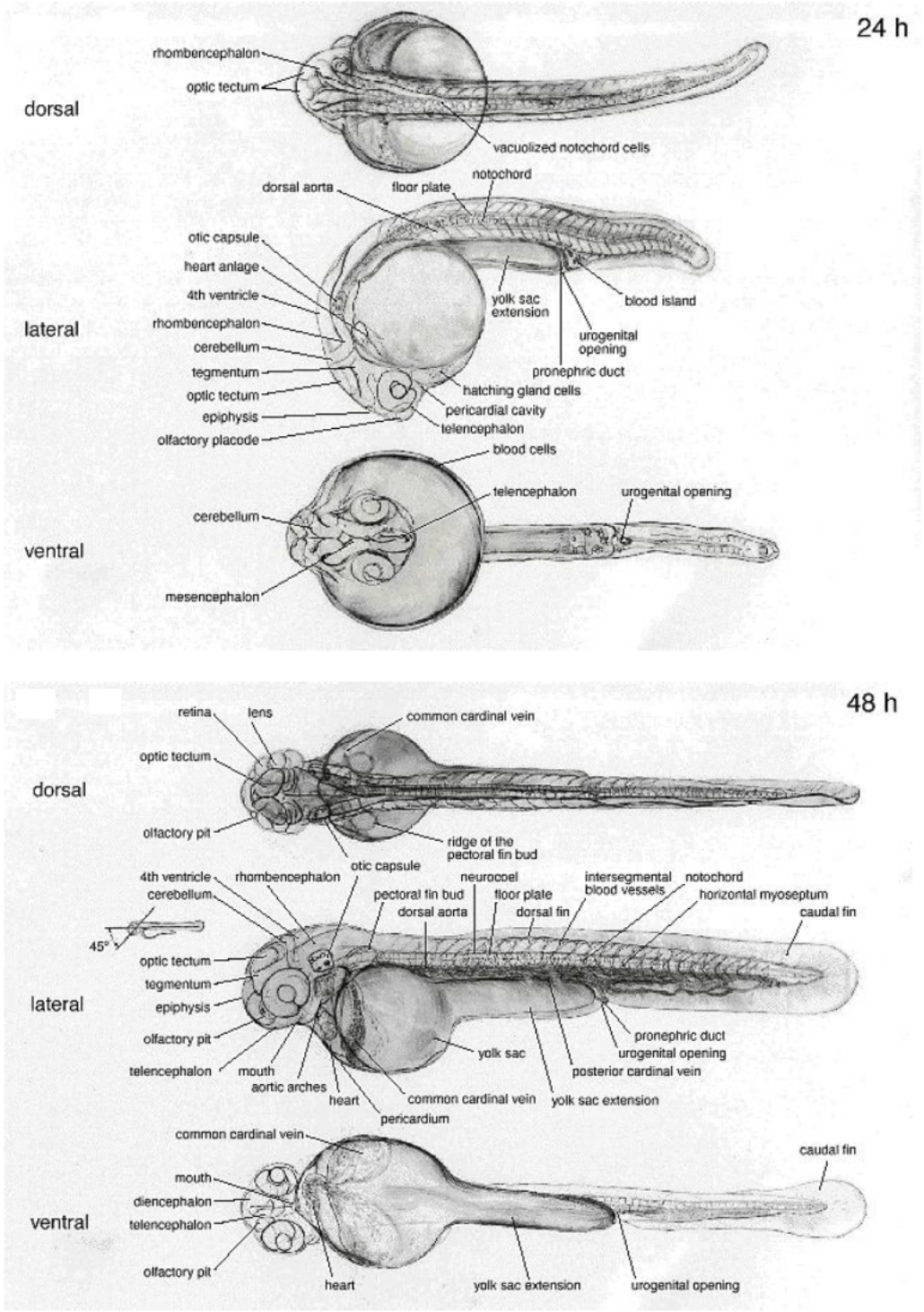


**Figure 1.8:** The development of zebrafish embryos in the first 24 and 48 hours post fertilisation. Highlighted in these images are anatomical features of a zebrafish embryo from the dorsal, lateral and ventral positions at 24 and 48 hours post fertilisation. This figure is reprinted from Dynamic development (http://people.ucalgary.ca/~browder/vir tualembryo/dev_biol.html).

## 1.7 Rationale and objectives

*P. aeruginosa* is a major cause of morbidity and mortality in patients with CF lung infection. Thus, it is important to understand the various mechanisms deployed by this organism in adaption and survival in the CF lung environment. To date, *P. aeruginosa* model organisms such as PAO1 and PA14 have been largely utilised to study pathogenesis in relation to the context of CF lung infection. Since the CF lung environment is vastly different from routine laboratory conditions, it is important to show differences in the genome, phenotype and physiology among freshly isolated *P. aeruginosa* CF isolates as well as laboratory maintained models strains. The various phenotypes of *P. aeruginosa* can include differences in cell morphology, colour, phenazine production, utilisation of carbon sources etc. Previous studies have shown that carbon catabolism phenotypes of *P. aeruginosa* CF isolates can be different. Thus, understanding the molecular mechanism involved in the catabolism of preferred carbon sources by *P. aeruginosa* CF isolates is essential. In addition to understanding the adaptations of *P. aeruginosa* to nutrients in the environment it is also important to reveal their interaction with other microorganisms in the CF lung. The interaction of *P. aeruginosa* with various bacterial and fungal pathogens have been previously explored, however, the molecular mechanisms involved in interaction of *P. aeruginosa* with *S. aurantiacum* remains largely unknown. It is important to gain insights into this interaction since *S. aurantiacum* is a recently recognised CF isolate which could lead to expression of various virulence mechanisms by *P. aeruginosa* and this could have implications on the CF host. The host has various cellular and humoral immune responses that help combat invading pathogens. Numerous model organisms have been utilised to study *P. aeruginosa* interaction with the host, however there is yet to be a global expression level study of simultaneous host-pathogen interaction in recently recognised model organism, zebrafish for study of disease.

Specifically, the objective of this thesis is to on understand the *P. aeruginosa* lifestyle in the CF lung:

To investigate the genetic, phenotypic or physiological adaptations of *P. aeruginosa* CF isolates. [Chapter 2]

To identify the molecular basis of reduced carbon catabolism phenotype displayed by *P. aeruginosa* CF isolate PASS4. [Chapter 3]

To investigate the effect of *P. aeruginosa* on fungal CF pathogen *S. aurantiacum* in host mimicking conditions. [Chapter 4]

To examine the molecular mechanisms involved in the interplay between a virulent *P. aeruginosa* CF isolate and a zebrafish model host during infection. [Chapter 5]

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# 2

# Genetically and phenotypically distinct *Pseudomonas aeruginosa* cystic fibrosis isolates share a core proteomic signature

PLOS | ONE

RESEARCH ARTICLE

# Genetically and Phenotypically Distinct *Pseudomonas aeruginosa* Cystic Fibrosis Isolates Share a Core Proteomic Signature

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## Abstract

The opportunistic pathogen *Pseudomonas aeruginosa* is among the main colonizers of the lungs of cystic fibrosis (CF) patients. We have isolated and sequenced several *P. aeruginosa* isolates from the sputum of CF patients and compared them with each other and with the model strain PAO1. Phenotypic analysis of CF isolates showed significant variability in colonization and virulence-related traits suggesting different strategies for adaptation to the CF lung. Genomic analysis indicated these strains shared a large set of core genes with the standard laboratory strain PAO1, and identified the genetic basis for some of the observed phenotypic differences. Proteomics revealed that in a conventional laboratory medium PAO1 expressed 827 proteins that were absent in the CF isolates while the CF isolates shared a distinctive signature set of 703 proteins not detected in PAO1. PAO1 expressed many transporters for the uptake of organic nutrients and relatively few biosynthetic pathways. Conversely, the CF isolates expressed a narrower range of transporters and a broader set of metabolic pathways for the biosynthesis of amino acids, carbohydrates, nucleotides and polyamines. The proteomic data suggests that in a common laboratory medium PAO1 may transport a diverse set of "ready-made" nutrients from the rich medium, whereas the CF isolates may only utilize a limited number of nutrients from the medium relying mainly on their own metabolism for synthesis of essential nutrients. These variations indicate significant differences between the metabolism and physiology of *P. aeruginosa* CF isolates and PAO1 that cannot be detected at the genome level alone. The widening gap between the increasing genomic data and the lack of phenotypic data means that researchers are increasingly reliant on extrapolating from genomic comparisons using experimentally characterized model organisms such as PAO1. While comparative genomics can provide valuable information, our data suggests that such extrapolations may be fraught with peril.

## Introduction

Cystic fibrosis (CF) is an autosomal recessive genetic disorder affecting most critically the lungs, and also the pancreas, liver, and intestine. It is characterized by abnormal transport of chloride and sodium ions across the epithelium leading to thick, viscous secretions. It is most common among the Caucasian population where, according to the World Health Organisation, approximately 1 in 2500 children are born with the genetic mutation leading to the development of CF. CF is caused by mutations in the gene encoding for the CF transmembrane conductance regulator protein (CFTR) involved in the regulation of the movement of chloride and sodium ions across epithelial membranes. The thick mucus that forms as a result of *CFTR* mutations represents a breeding ground for various microorganisms that cause chronic infection in lungs, thus leading to complications associated with the disease.

*Pseudomonas aeruginosa*, a Gram-negative opportunistic human pathogen, is one of the main colonizers of lungs in CF patients. It was found that by the age of 3 years, over 95% of children with CF show evidence of intermittent *P. aeruginosa* infection [1]. Moreover, early colonization with *P. aeruginosa* has been strongly correlated with poor prognosis in CF [2, 3]. By the age of 25, according to the 2010 Cystic Fibrosis Foundation Patient registry Annual Data Report, *P. aeruginosa* becomes the most dominant microorganism in the respiratory tract of CF patients.

Due to its metabolic versatility, innate resistance to the majority of drugs used in clinical practice, and extensive biofilm formation, infections caused by *P. aeruginosa* are especially hard to treat using conventional treatment regimes and are often destined to fail. As a result, CF is ranked among the most widespread life-shortening genetic diseases with the current life expectancy often not exceeding mid 40s.

Successful CF pathogens, including *P. aeruginosa*, have developed an effective arsenal to establish infection and evade the host response, together with an ability to adapt readily to the lung environment [4]. Thus, according to reports, isolates of *P. aeruginosa* that are involved in the acute infection and initial colonisation of CF lungs in early childhood differ from those found in adults with established chronic infections, the latter often showing adaptations specific to the CF lung environment [5, 6].

Considering its role in the disease progression, it is not surprising that *P. aeruginosa* has become a focal point for research in CF and other biofilm related complications. Improvements in DNA sequencing technology have led to the sequencing of hundreds of *P. aeruginosa* genomes in recent years, many of them from CF patients. However, for the vast majority of these strains there is little or no published experimental data on their phenotypic features (Fig 1). Instead most phenotypic experimental work on *P. aeruginosa* has focused on common well-characterised model strains, such as PAO1. Our knowledge of these sequenced CF isolates is thus largely based on extrapolations from model strains via *in silico* genome comparisons.

Strain PAO1 is a widely used model organism and the first *Pseudomonas* species to have its genome sequenced [7]. In 2014 alone 155 articles were published, as listed on NCBI PubMed, using PAO1 as a model *P. aeruginosa* strain, and 15% of those articles made direct correlations to CF. As strain PAO1 does not have a CF origin but was isolated from a wound infection in 1955 [8] and has been maintained in laboratories worldwide since then, PAO1 may have questionable relevance to CF.

In this study we have obtained fresh isolates of *P. aeruginosa* from the sputum of CF patients, subsequently named PASS1-4, and compared them with each other and with the model laboratory strain PAO1. The study aimed to identify specific adaptations developed by *P. aeruginosa* during chronic infection of CF lungs with an overarching aim of better understanding the mechanisms that render *P. aeruginosa* a successful CF lung colonizer. Significant

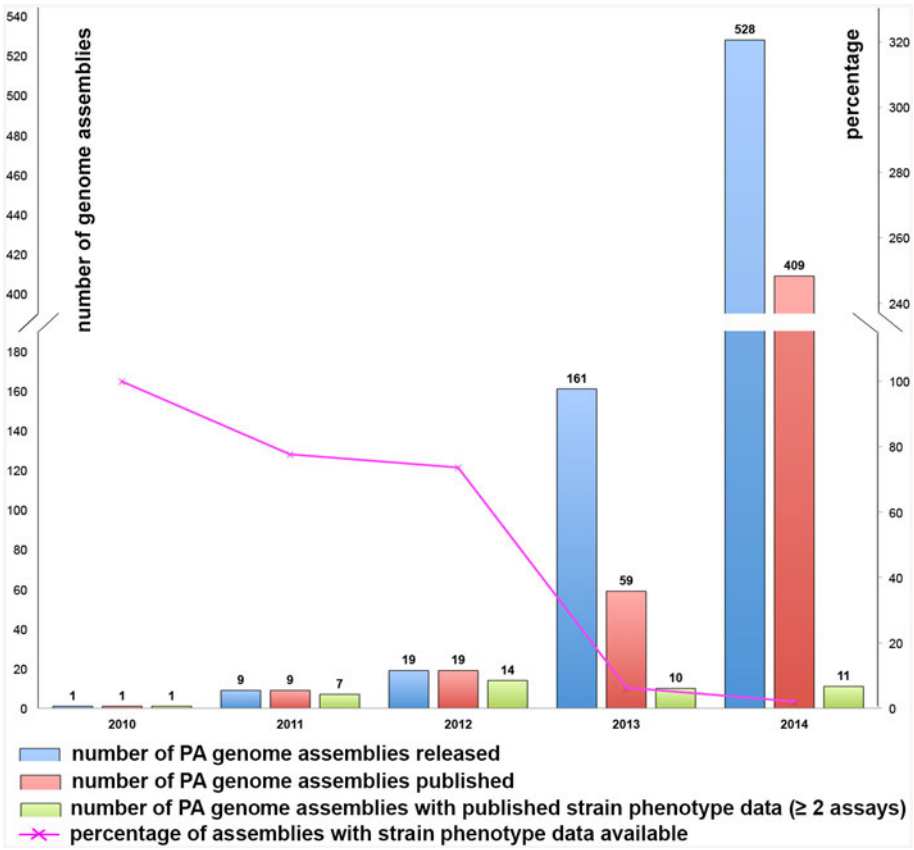


**Fig 1. Data on *P. aeruginosa* (PA) genome assemblies released in the last 5 years (2010–2014 inclusive).**

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differences were observed in genomes and phenomes amongst the CF isolates reflecting varying adaptation paths. At the same time, the CF isolates shared a common proteomic signature that was remarkably distinct from the proteome of PAO1.

Our study highlights the limitations of using model organisms when examining the role of bacteria in the context of their natural host/environment. Isolates from hosts/environments of interest may have developed specific adaptations that may not be present in model strains, or may have been lost during prolonged cultivation. Understanding these adaptations would be crucial for the successful treatment of infections caused by pathogens; therefore, the use of freshly obtained isolates is an important adjunct to work on model strains.

## Materials and Methods

### Strains used in this study

*P. aeruginosa* isolates PASS1-4 were previously obtained from the sputum of adult CF patients: PASS1 was obtained from a 40-year old female patient, PASS2 –from a 27-year old male, PASS3 –from a 23 year old male and PASS4 –from a 23-year old female [9]. Strains PASS1-4, as well as *P. aeruginosa* PAO1, were maintained in stock in -80°C freezer and grown on Luria Bertani (LB) solid or liquid media whenever needed unless otherwise stated, allowing minimal passaging before the assays.

## Genome sequencing, assembly and comparative genomics

DNA was isolated from *P. aeruginosa* strains using the Invitrogen PureLink Genomic DNA kit and sequenced at the Ramaciotti Centre for Gene Function Analysis (UNSW, Sydney) on an Illumina HiSeq 2000 platform. Sequence data was assessed for quality using FastQC (Babraham Bioinformatics) and assembled *de novo* using the VELVET algorithm [10]. Genome assembly quality and statistics were further evaluated using QUAST [11].

Genome sequencing data for strains PASS1-4 have been deposited to the NCBI Whole Genome Shotgun (WGS) database and are accessible under BioProject ID numbers PRJNA295120, PRJNA295121, PRJNA295122 and PRJNA295123 respectively (http://www.ncbi.nlm.nih.gov/bioproject).

The phylogenetic relationship of PASS1-4 strains to other *P. aeruginosa* strains in the MLST database (http://pubmlst.org/paeruginosa) was inferred via maximum likelihood analysis using the Arb software package [12]. The phylogenetic analysis was based on concatenated nucleotide sequences of seven genes utilized in the multilocus sequence typing (MLST) of *P. aeruginosa*. These included *acsA* (acetyl coenzyme A synthetase), *aroE* (shikimate dehydrogenase), *guaA* (GMP synthase), *mutL* (DNA mismatch repair protein), *nuoD* (NADH dehydrogenase I chain C, D), *ppsA* (phosphoenolpyruvate synthase), *trpE* (anthralite synthetase component I). Further analysis has been performed by assigning a number to each distinct allele within a locus according to the number available in the *P. aeruginosa* MLST database. As a result, each isolate was given up to seven numbers that represented its strain type. Any strain type that did not have a match in the existing database was designated as a "new" type.

Multiple genome alignments were performed using BRIG [13] and MAUVE [14] software.

Reciprocal similarity searches of all predicted and expressed proteins were performed using BLAST algorithm [15], as well as Proteinortho5 tool [16] followed by the visualization of results using FriPan (http://www.vicbioinformatics.com/software.fripan.shtml).

## Carbon source utilization

Carbon source utilization was assessed by growing bacteria in M9 minimal medium (Sigma) with the addition of the substrate of interest as a sole carbon source, in biological triplicates. Cultures were incubated on a horizontal shaker for 48 hours at 37°C after which the growth was assessed.

## Biofilm formation in flow cells

To assess formation of biofilms by *P. aeruginosa*, strains PASS1-4 and PAO1 were grown in continuous flow-cell system [17] in LB medium at 37°C for 48 hours and biofilms formed on the surface of coverslips were imaged using Olympus FV1000 Laser Scanning Confocal Microscopy (LSCM) System after staining with the BacLight Live/Dead stain (Molecular Probes). Three-dimensional rendering of LSCM images and the subsequent quantification were performed using the Imaris software (Bitplane). Quadruplicate images were used in the quantitative assessment of LSCM images of each sample; these included two independent flow cell chambers that were inoculated from separate culture stocks, and two distant fields of view in each chamber.

## Binding of *Pseudomonas aeruginosa* to mucin

*P. aeruginosa* isolates were cultured in 10 ml of LB broth at 37°C for 8 hours with shaking at 185 rpm. Bacterial cells were pelleted by centrifugation at 3000 x *g* for 5 min, washed twice with phosphate buffered saline (PBS), and then fluorescently labelled by resuspension in 1 ml

of PBS with SYBR® Green (0.1% w/v, Sigma) for 3 min. Labelled cells were collected by centrifugation at 3000 x g for 3 mins, and then washed thrice in PBS to remove residual dye. Meanwhile, PVDF membranes were placed into the wells of a 96-well microtiter plate and activated by soaking in methanol followed by washing three times in PBS. Fifty microliters of 1 mg/ml porcine gastric mucin (PGM, Sigma) were added into each well containing a PVDF membrane. Bacteria were resuspended in PBS to an $OD_{600}$ = 1.0, applied to the wells of 96-well microplate containing the mucin-coated PVDF membranes and incubated for 30 minutes while slowly shaking at 100 rpm at RT. Unbound bacteria were washed off the membrane three times with PBS and the attached bacteria were fluorescently measured (Ex 485nm, Em 520nm) using a Fluorostar Galaxy plate reader (BMG Labtech, Offenburg, Germany). Wells containing immobilised mucin (no bacteria applied) were used as a negative control. The binding to the mucin by the different bacterial strains was normalized against the maximum bacterial binding measured on each plate.

## Flagella mediated motility

Flagella mediated motility was assayed by point inoculation of LB plates containing 0.3% (w/v) agar as previously described [18]; zone sizes were observed after 48 hours of incubation at 37°C. The assay was performed in biological triplicates.

## Virulence against the nematode eukaryotic model *Caenorhabditis elegans*

To assess toxicity against the nematode *C. elegans*, a selective grazing assay was performed as previously described [19]. Briefly, all strains of *P. aeruginosa* were spotted from frozen stock cultures on a single LB agar plate, followed by incubation at 37°C for 4 days. At day 4, 5 μl of M9 medium with four to five L4-stage nematodes were added next to each colony. The plates were stored at room temperature and checked daily. A strain was considered positive if the colony was not grazed after 14 days of incubation with *C. elegans*, across all three biological replicates. The assay was performed in three biological replicates.

## Assessment of phenazine production

Phenazine compounds were extracted from *P. aeruginosa* cultures (in biological triplicates) after 40 hours of growth in LB broth, at 37°C with shaking. Two millilitres of chloroform were added to 5 ml of culture and incubated with shaking for 20 minutes. Chloroform fractions were collected, dried under reduced pressure and dissolved in 80% v/v acetonitrile in 25 mM ammonium acetate. Filtered samples were applied to the Zorbax Eclipse Plus C18 Rapid Resolution column (Agilent, 2.1 x 50 mm, 1.8 micron) and analysed using the Infinity 1290 Ultra High Performace Liquid Chromatography (UHPLC) instrument equipped with the Infinity 1290 photodiode array detector (Agilent). The separation program was adopted for UHPLC based on the previously published protocol [20]. The solvent flow rate was 0.5 ml/min and consisted of 0.25 minutes of 8% v/v acetonitrile–25 mM ammonium acetate, followed by a 3-min linear gradient to 80% v/v acetonitrile–25 mM ammonium acetate. UHPLC gradient profiles were monitored at spectral peak maxima of 257.0 and 313.0 nm.

## Pyoverdine production

Pyoverdine production was assessed by growing strains on iron-limited King's medium at 37°C for 40 hours, in biological triplicates, followed by visualization of pyoverdine under UV light, as previously described [21].

## Protein extraction for proteomics

Overnight cultures of *P. aeruginosa* strains (PAO1, PASS1, PASS2, PASS3 and PASS4) in LB broth, in biological triplicates, were inoculated into fresh LB broth and grown to mid-logarithmic phase with incubation at 37°C and shaking at 200 rpm. Cells were collected by centrifugation at 2500 x *g* for 10 min at 4°C and washed thrice with PBS, pH 7.4. The cell pellet (~ 0.5g) was weighed and resuspended in 0.5ml of PBS (pH 7.4) containing complete protease inhibitor cocktail (EDTA-free, Roche), benzonase (1:100 v/v, Sigma) and equal amounts of acid washed glass beads (Sigma Aldrich). Cells were lysed by bead-beating thrice at 4.5 Throw for 20 seconds using a FastPrep FP120 bead-beater apparatus (Savant) with 10 min breaks on ice. Cell debris and unbroken cells were removed by centrifugation at 2500 x *g* for 8 min at 4°C. Proteins in the supernatant were precipitated by the addition of ice-cold acetone in the ratio of 1:9 (v/v) and incubated overnight at -20°C followed by centrifugation at 2500 x *g* for 10 min at 4°C. The pellet was washed twice and resuspended in 1% (w/v) SDS in deionized water. Total protein content was measured using Pierce BCA Protein Assay Kit (Thermo Scientific).

## 1D SDS-PAGE and in-gel digestion

Thirty micrograms of protein from each sample was diluted using NuPAGE Laemmli loading buffer (Life Technologies) containing 50 mM DTT and denatured at 95°C for 5 min. Samples were spun down and loaded into a NuPAGE (4–12% T Bis-Tris) precast ready gel. Electrophoresis was performed using NuPAGE MOPS SDS running buffer to run the sample 10 mm into the gel. After electrophoresis the gels were stained with colloidal Coomassie blue (Sigma Aldrich) and the band was cut into 1 $mm^3$ cubes and transferred into 1.5 ml microcentrifuge tubes to perform in-gel tryptic digestion.

Trypsin in-gel digestion and peptide extraction were performed as previously described [22]. Briefly, gel bands were destained using acetonitrile (ACN) and 100 mM ammonium bicarbonate (ABC) in the 1:1 ratio, followed by reduction with 10 mM DTT prepared in 100 mM ABC at 56°C for 30 min and alkylation with 55 mM iodoacetamide prepared in 100 mM ABC for 20 min in the dark. The gel plugs were washed twice with ACN, with 5 minutes of incubation during each wash. The gel plugs were subsequently dried in a vacuum centrifuge, and digested overnight with sequencing-grade modified trypsin (Promega) in the ratio 1:30 at 37°C. Products of digestion were collected in 1.5 ml microcentrifuge tubes and combined with extracts from a consecutive extraction with ACN and 5% (v/v) formic acid at 37°C for 30 min. The total extract was then concentrated in a vacuum centrifuge and reconstituted with loading buffer (2% v/v ACN, 0.1% v/v FA) for mass spectrometry.

## Mass spectrometry

Peptides were analysed using a data-independent acquisition method known as SWATH-MS [23]. Five spectral ion libraries were generated, one for each strain by pooling biological replicates and separating the peptides into five salt fractions by online strong cation exchange chromatography (SCX) These libraries were later used for proteomics data analysis. Fractionated samples were analysed using a nanoLC ultra 2D cHiPLC system (Eksigent, part of SCIEX) in conjunction with a TripleTOF® 5600 (ABSciex) using positive nanoflow electrospray analysis and an information-dependent acquisition (IDA) mode. In data dependent MS/MS acquisition 20 most intense m/z values exceeding a threshold $>$ 150 counts per second (cps) with charge stages between 2+ and 4+ were selected for analysis following a full MS survey scan and excluded for 20 sec to minimize redundant precursor sampling.

For SWATH-MS, each biological triplicate was analyzed within a 60 min increasing ACN RP gradient (5% to 45% using 90% v/v ACN 0.1% v/v FA) using 60 variable window m/z

ranges (400–1250 m/z) selected based on intensity distribution of precursor m/z in the IDA data sets. Collision energies were calculated for 2+ precursors with m/z values of lowest m/z in window + 5 m/z and a collision energy spread of 5 eV was used.

### Protein identification

Spectral libraries for SWATH-MS quantitation were generated with ProteinPilot™ software 4.2 using the Paragon™ algorithm (ABSciex) thorough ID mode including biological modifications [23]. MS/MS data were searched against the *Pseudomonas aeruginosa* strain PAO1 protein sequence database retrieved from GenBank (January 2013) and Pseudomonas Genome Database (www.pseudomonas.com) [24] and *in-silico* translated genome databases of PASS1-4 strains. Carbamidomethylation of Cys residues was selected as a fixed modification. An Unused Score cut-off was set to 2.0 (99% confidence), equivalent to a protein false discovery rate (FDR) $< 1\%$.

### Proteomic data analysis

Generated protein libraries for each strain were imported into PeakView™ software 2.1 using the SWATH MicroApp 2.0 (release 27 November 2013) and matched against SWATH-MS data for each individual replicate. After retention time calibration with endogenous peptides, data were processed using the following settings; a maximum of 100 peptides per protein, maximal 6 transitions per peptide, peptide confidence threshold of 60%, transition FDR $< 1\%$, 10 min extraction window and fragment extraction tolerance of 75 ppm.

Expressed proteins were determined by comparing summed protein areas from extracted ion chromatograms between PAO1 and PASS1-4 strains to identify those with a +/- 5-fold change and ANOVA $p < 0.01$.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository [25] with the dataset identifier PXD002865.

In order to reveal similarities and differences in the proteomics profiles of *P. aeruginosa* strains tested, the reciprocal Blast searches and orthologue analyses were performed as was previously described for the predicted proteome.

Proteins identified as being expressed by *P. aeruginosa* strains during the growth in LB medium were mapped onto the *P. aeruginosa* PAO1 curated database in BioCyc [26] and metabolic pathways visualized using the Pathway Tools 17.5 cellular overview diagram and Omics Viewer [27].

## Results and Discussion

### Colony morphology of isolated CF strains

Four *P. aeruginosa* isolates were obtained from the sputum of CF patients at the Westmead Hospital (Sydney, Australia) and were minimally passaged. Isolates displayed significant diversity in their colony morphology: PASS1 formed pale-green non-mucoid colonies when grown on LB agar, PASS2 –light brown non-mucoid colonies, PASS3 –mainly white mucoid colonies and PASS4 –dark green-blue non-mucoid colonies.

### Whole genome sequencing and phylogeny

*De novo* assembly of the sequencing reads obtained from Illumina HiSeq platform yielded draft genomes as per Table 1. Strain PASS3 was found to have the largest genome, and PASS2 genome was the smallest. Based on the MLST phylogeny, strains PASS1 and PASS3 were found to have an identical MLST sequence, while PASS2 and PASS4 were in different MLST clusters

**Table 1. Genome assembly statistics for PASS1-4 strains.**

| Strain | Number of contigs (> 1 kb) | N50 | Genome size (Mbp) | Number of ORFs |
|---|---|---|---|---|
| PASS1 | 81 | 205938 | 6.3 | 5792 |
| PASS2 | 96 | 186035 | 6.1 | 5795 |
| PASS3 | 76 | 255936 | 6.4 | 5847 |
| PASS4 | 93 | 171309 | 6.3 | 5936 |

Further MLST analysis revealed that strains PASS1-3 do not have an exact match in the MLST database (http://pubmlst.org/paeruginosa), and therefore represent new strain types, while PASS4 belongs to the strain type 649 (S1 Table). The latter currently comprises of 23 isolates one of which originates from the blood sample in Czech Republic and the rest are epidemic isolates obtained from lungs of CF patients in Australia.

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distinct from PASS1, PASS3 and PAO1 (Fig 2). Consistent with their isolation source, all CF isolates (PASS1-4) clustered with other Australian sputum isolates, while PAO1 was closely related to a more diverse set of isolates from various sources, including tissue infection strains.

## Comparative genomics and the analysis of predicted proteins

Multiple genome alignments using BRIG [13] and MAUVE [14] tools show extensive gene conservation and synteny between PAO1 and the PASS1-4 genomes (Fig 3). The analysis of strain PASS2 draft genome also revealed an absence of a ~ 160 kB genomic region corresponding to the nucleotide positions between 2439150 and 2602350 in the PAO1 genome and containing genes with locus tags PA2218-PA2354 (Fig 3). This region, absent in PASS2, contains a number of potential virulence and colonization determinants, including the *psl* cluster of genes responsible for the production of biofilm matrix component Psl, as well as genes encoding chitinase and those involved in biosynthesis of L-2-amino-4-methoxy-trans-3-butenoic acid (AMB) toxin (Fig 3). This may represent an adaptation to the CF lung via genome reduction and the loss of several virulence factors. Decreased virulence has been previously described among the characteristics of *P. aeruginosa* isolates from chronic lung infections as a way of minimizing the host immune response and limiting bacterial resource expenditure [28]. A similar large deletion of ~ 180 kB spanning from PA2272 to PA2410 has been previously reported in a different CF isolate [29].

BLAST searches of predicted proteins of PASS1-4 strains and the standard laboratory strain PAO1 revealed 4676 shared protein sequences between all 5 strains (Fig 4C). Ortholog analysis of all predicted proteins performed using Proteinortho5, followed by visualization of results via FriPan demonstrated distinct clustering of PAO1 at a significant distance from CF isolates PASS1-4. Interestingly, significant differences were also observed among PASS1-4 strains, *i.e.* PASS1 and PASS3 showed high degree of similarity, while PASS2 and PASS4 clustered together with a lower level of similarity (Fig 4A and 4B), in agreement with the MLST phylogeny (Fig 2).

## Carbon source utilization

Genomic analysis revealed that PASS2 lacks genes essential for the utilization of various carbon sources. Many of these genes were located in the ~160 kB region of DNA absent in strain PASS2. These include genes involved in transport and catabolism of mannitol (PA2337-PA2344); the *bkd* operon for utilization of valine, leucine and isoleucine; and genes involved in the utilization of gluconate and glycerol (PA2321-2322 and PA2352 respectively). Minimal media growth experiments confirmed that PASS2 was unable to grow in the minimal medium in the presence of the above-mentioned compounds as sole carbon sources (data not shown).

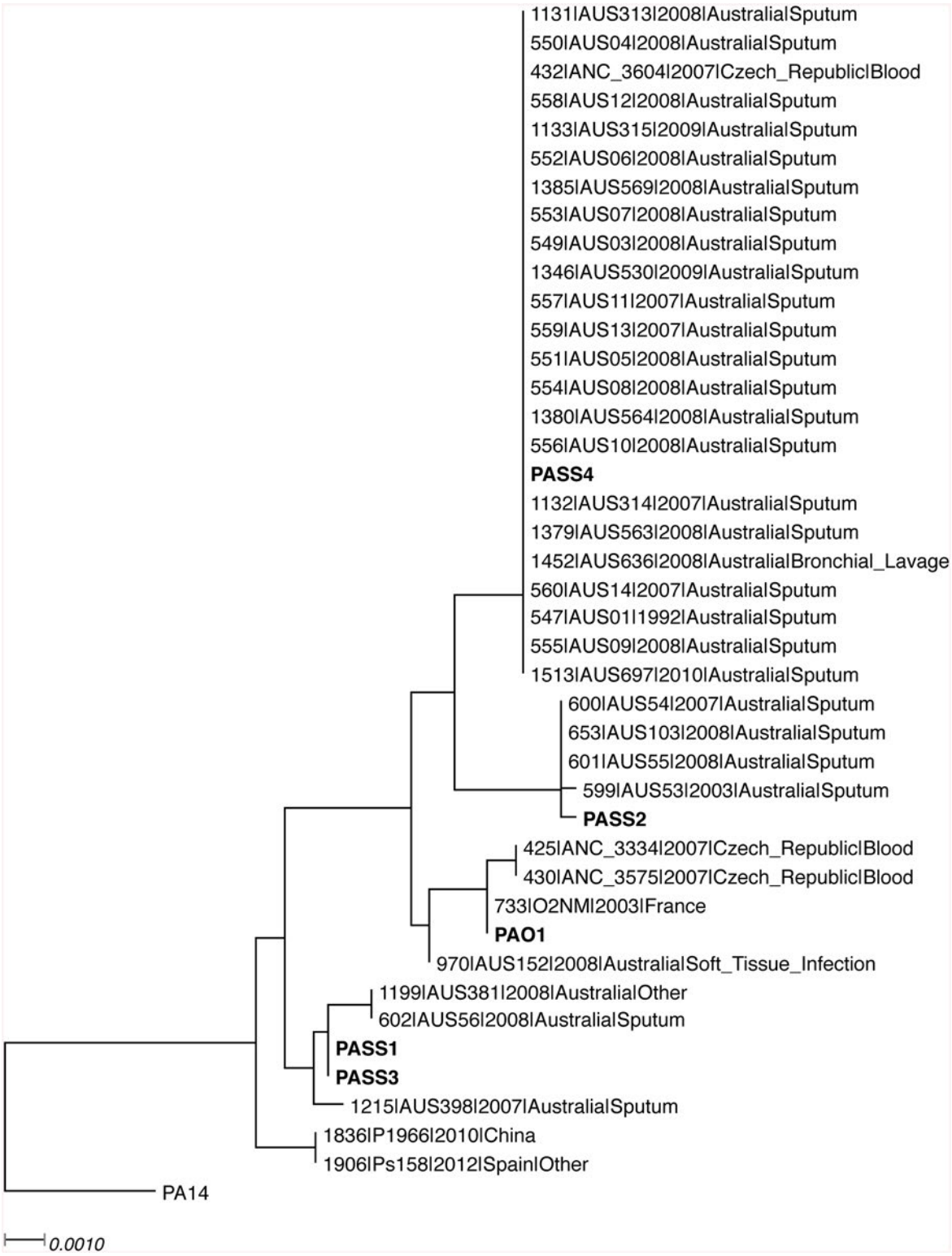


**Fig 2. The maximum likelihood phylogenetic tree generated using concatenated sequences of MLST genes in *P. aeruginosa*.** Isolates mentioned in this study are shown in bold; all the other closely related MLST sequences are retrieved from *P. aeruginosa* PubMLST database (http://pubmlst.org/paeruginosa). *P. aeruginosa* PA14 is used as an outgroup. The scale bar indicates the number of substitutions per nucleotide position.

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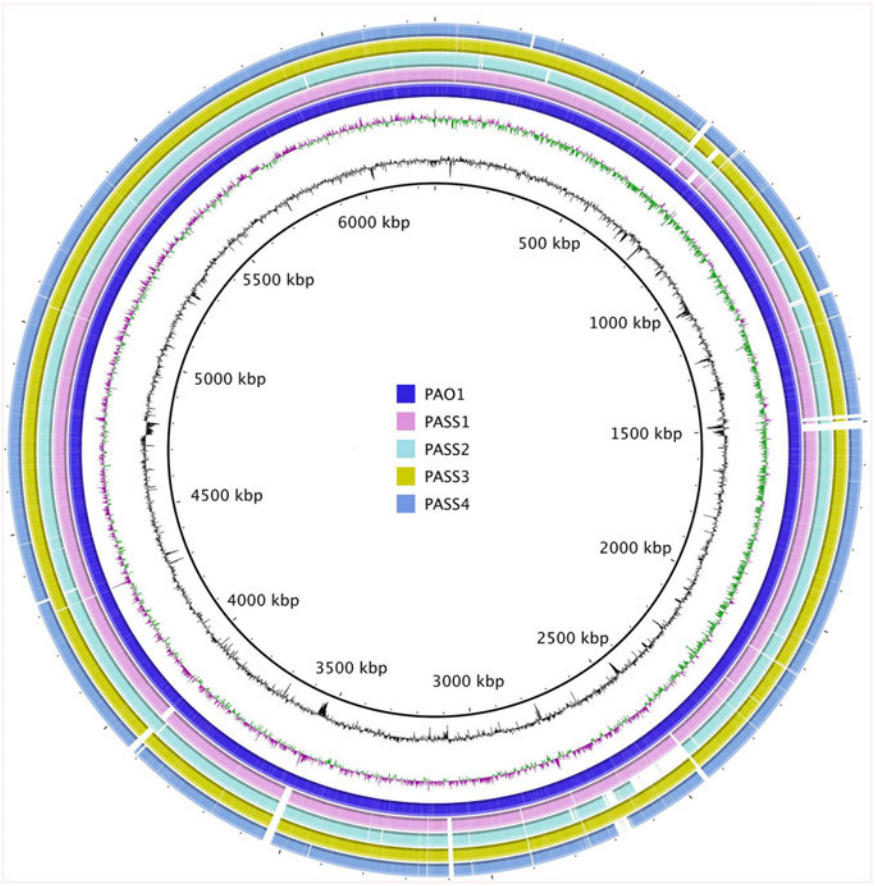


**Fig 3. Circular representation of *P. aeruginosa* PASS1-4 draft genomes aligned against the reference genome of PAO1, generated using BRIG software.** The two innermost circles represent the GC Content and the GC Skew respectively.

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## Biofilm formation

Biofilm formation in flow chambers highlighted differences in the biofilm forming ability and biofilm architecture of the *P. aeruginosa* strains. PASS1, PASS3 and PASS4 were shown to form thicker biofilms compared to PAO1 (Fig 5A and 5B). Conversely, strain PASS2 was not able to form a biofilm on the surface of glass coverslips in the flow chambers. This is likely due to the loss of the *psl* cluster of genes responsible for the biosynthesis of exopolysacharide Psl located within the PA2218-PA2354 region absent in this strain (Fig 3). Psl is one of the major exopolysaccharides involved in the formation of biofilm matrix in *P. aeruginosa*, along with alginate and Pel. Non-mucoid strains, such as PASS2, primarily utilize either the Psl or Pel polysaccharides for biofilm formation [30, 31]. Previous mutational analyses demonstrated that Psl plays an important role in surface attachment for most isolates and the Psl deficiency often leads to the lack of biofilm formation [30].

Extracellular substances like exopolysaccharide Psl, are often regarded as "public goods" as they can benefit the overall population and not only the single producer strain [32]. Production of such metabolites is often metabolically expensive. This inevitably leads to the emergence of "social cheaters"–phenotypes that do not produce certain public goods, and, therefore, do not bear the cost of their production, but still benefit from metabolites produced by other members of the community [33, 34]. Hence, it is possible that strain PASS2 may have evolved as a

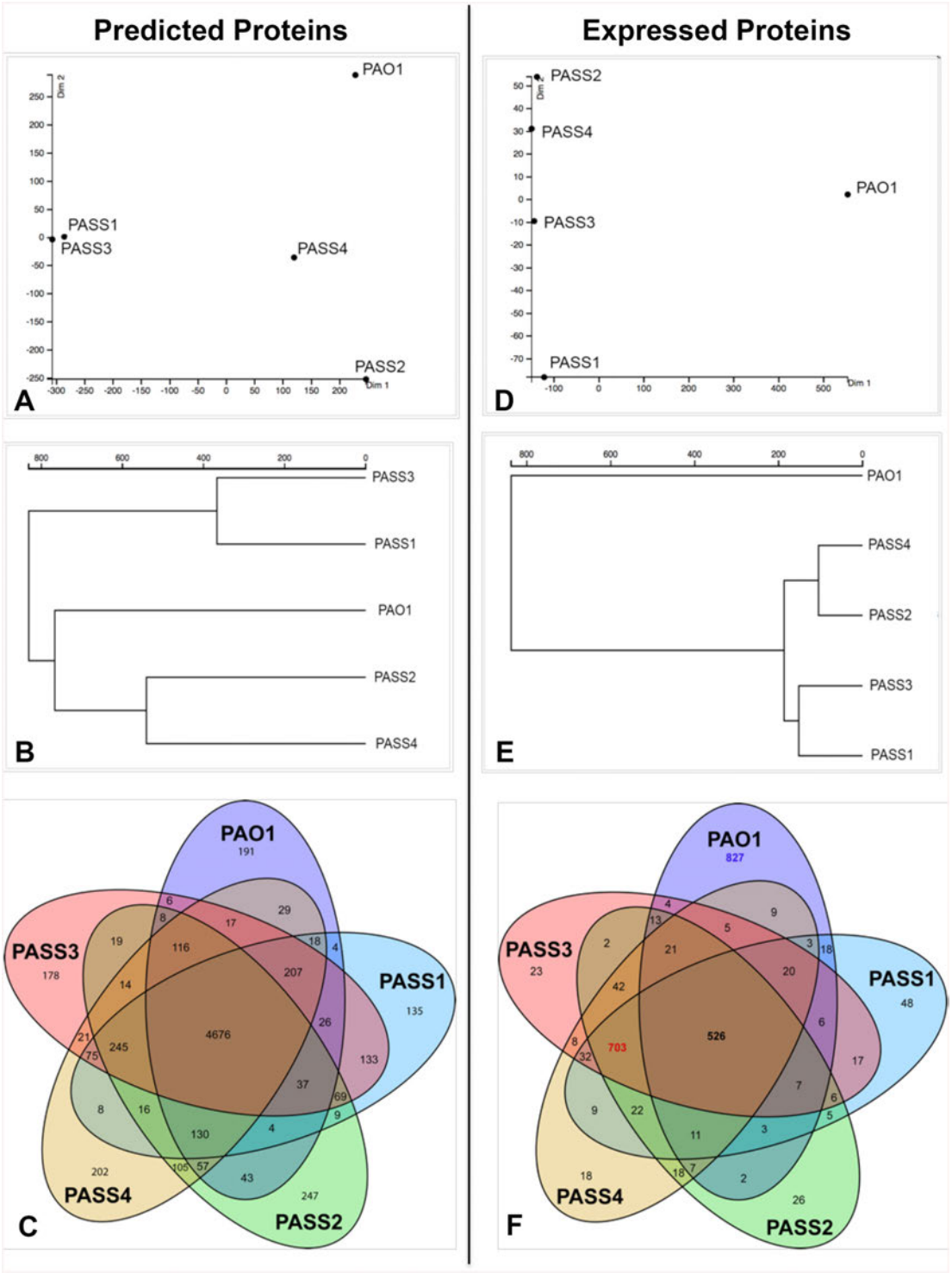


**Fig 4. Comparison of predicted and expressed proteins in CF isolates PASS1-4 and the reference strain PAO1.** The 2-dimensional plot (A) and clustering representation (B) of all predicted proteins of corresponding strains analysed via Proteinortho and FriPan software; (C)–Venn diagram representing the results of reciprocal BLAST searches of all predicted proteins in corresponding strains. The 2-dimensional plot (D) and clustering representation (E) of all expressed proteins of corresponding strains as assessed by proteomics and analysed via Proteinortho and FriPan software; (F)–Venn diagram representing the results of reciprocal BLAST searches of expressed proteins detected via proteomics; the number of proteins uniquely expressed by PAO1 is shown in blue, the number of proteins shared by PASS1-4 and not expressed by PAO1 is in red.

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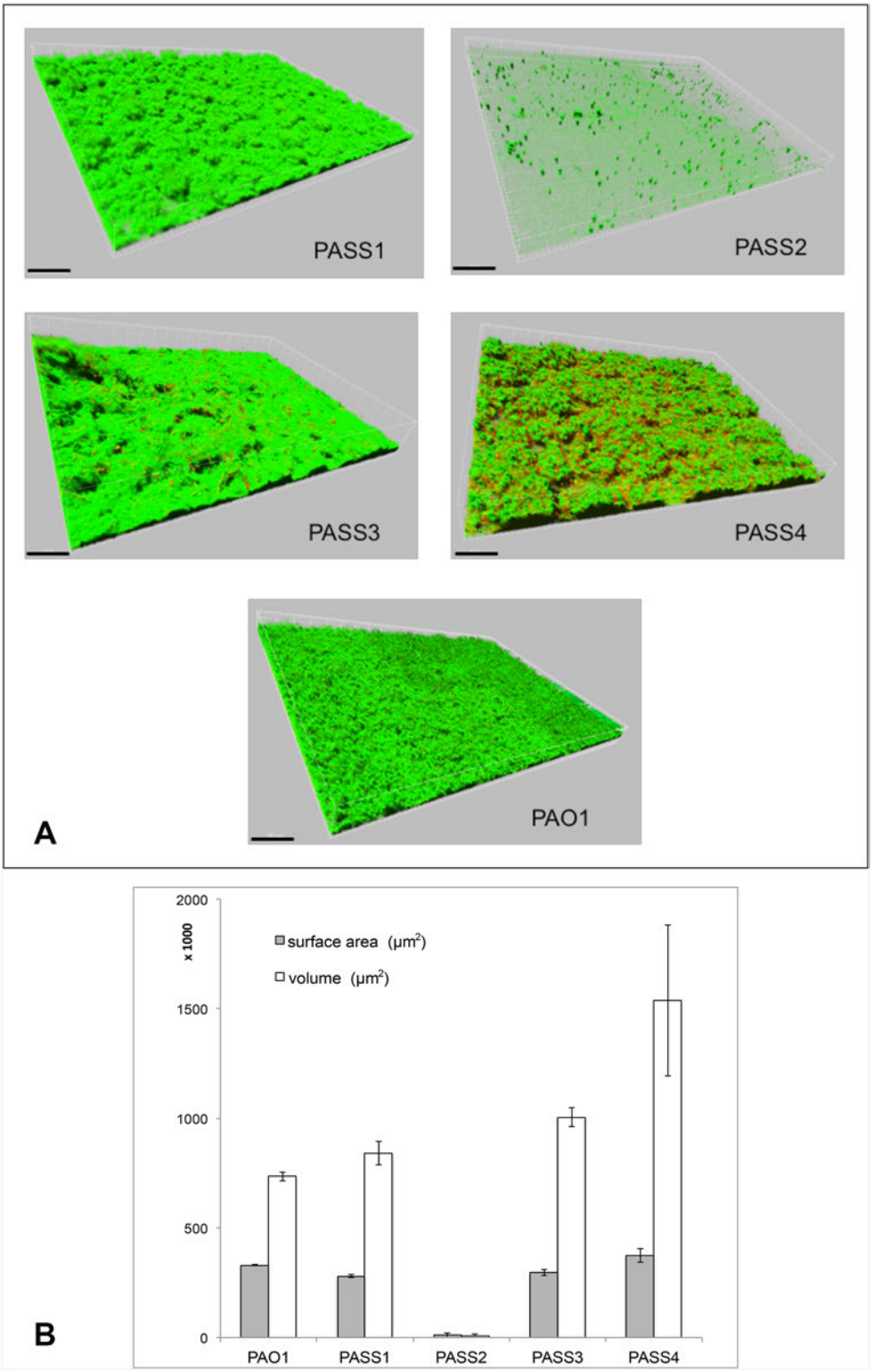


**Fig 5. (A) Representative images of biofilms formed in flow cells by strains PASS1-4 and PAO1 after 48 hours of growth in LB medium at 37°C. (B) Quantitative analysis of quadruplicate LSCM images (derived from two independent flow cell chambers that were inoculated from separate overnight cultures, and two distant fields of view in each chamber) was performed using Imaris software.** Images were taken using Olympus FV1000 confocal laser-scanning microscopy (LSCM) system, 3D pictures

were built using Imaris software package (Bitplane). Cells are stained using BacLight Live/Dead stain (Molecular Probes); live cells are presented in green, dead cells—in red. Scale bars on LSCM images represent 50 μm.

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"cheater" phenotype and may still be able to form biofilms in the mixed communities with other strains present in the CF lung, benefiting from Psl produced by other strains. PASS2 was the only *P. aeruginosa* strain isolated from that particular CF patient, so it is not possible to directly investigate this "cheater" hypothesis.

## Flagella and pili

Flagella and pili are important factors in biofilm formation, especially in the initial stages of attachment. Thus, flagella, besides being motility organelles, also play a direct role in virulence as major antigenic determinants for the immune response to *P. aeruginosa* infection [35]. PASS1, PASS3 and PAO1 shared a similar organization of flagellar biogenesis genes, while PASS2 and PASS4 showed distinct differences (S1A Fig). Based on the molecular weight and serological properties of flagellin, *P. aeruginosa* have been previously classified into two groups carrying A- and B- type flagellins [36, 37]. PASS1 and PASS3 share four genes orthologous to PA1088-PA1091 of PAO1, typical of B-type flagellins [38]. In contrast, the same region in PASS2 and PASS4 had a more complex polymorphic organisation characteristic of strains with a highly glycosylated and more heterogenous type A flagellin The duplication of the *fliS* gene observed in PASS2 and PASS4 was also characteristic for type A flagella [38].

It is largely accepted that glycosylation of flagellins is important for virulence and host specificity, however the precise physiological activity remains unclear [39, 40]. Strains PASS2 and PASS4 exhibited little or no flagellum-dependent swimming motility as opposed to PAO1, PASS1 and PASS3 (S1B Fig); whether this is a general feature of type A flagellin has yet to be elucidated.

Flagellar components have been shown to be important for adhesion to mucins [41]. Mucins are major macromolecular glycoprotein components of the mucus that line the surface of lung epithelium and that vary in their composition and bacterial adhesion properties in CF [42, 43]. Mucins are overproduced in the lungs of CF patients and binding of *P. aeruginosa* to lung epithelial mucins in CF has been previously reported [44]. The mucin-binding assay showed variations amongst the isolates, with PASS4 showing the highest level of binding to mucin. Notably, PASS2 binding to mucin was comparable to that of PASS3 (Fig 6) despite the decreased biofilm formation in flow cells (Fig 5A and 5B). This data suggests that adhesion to biological surfaces, such as to the mucins on the epithelium, is multifactorial and bacteria may employ various tools for attachment to surfaces that may allow them to at least partially compensate for a loss of another mechanism.

Bacterial type IV pili (T4P) have been extensively studied for their contribution to motility, biofilm formation and virulence [45]. T4P may, therefore, play a major role in the colonization, survival and virulence in the CF lung. Based on genomic context of T4P genes, pili are classified into groups I-V [46]. PAO1 has been shown to encode a group II pilin [46], this is also true for strains PASS1 and PASS3 based on our analyses, whereas strains PASS2 and PASS4 were found to encode group I pili. Interestingly, Kus et al. [46] have described the high prevalence of group I pili in isolates from CF patients, possibly representing a specific adaptation to the CF lung environment. Nevertheless, the significance and specific role of group I T4P in CF is currently unclear.

## Phenazine production

Phenazines are secondary metabolites produced by a variety of bacteria, most notably pseudomonads, and have been studied intensively because of their broad-spectrum antibiotic

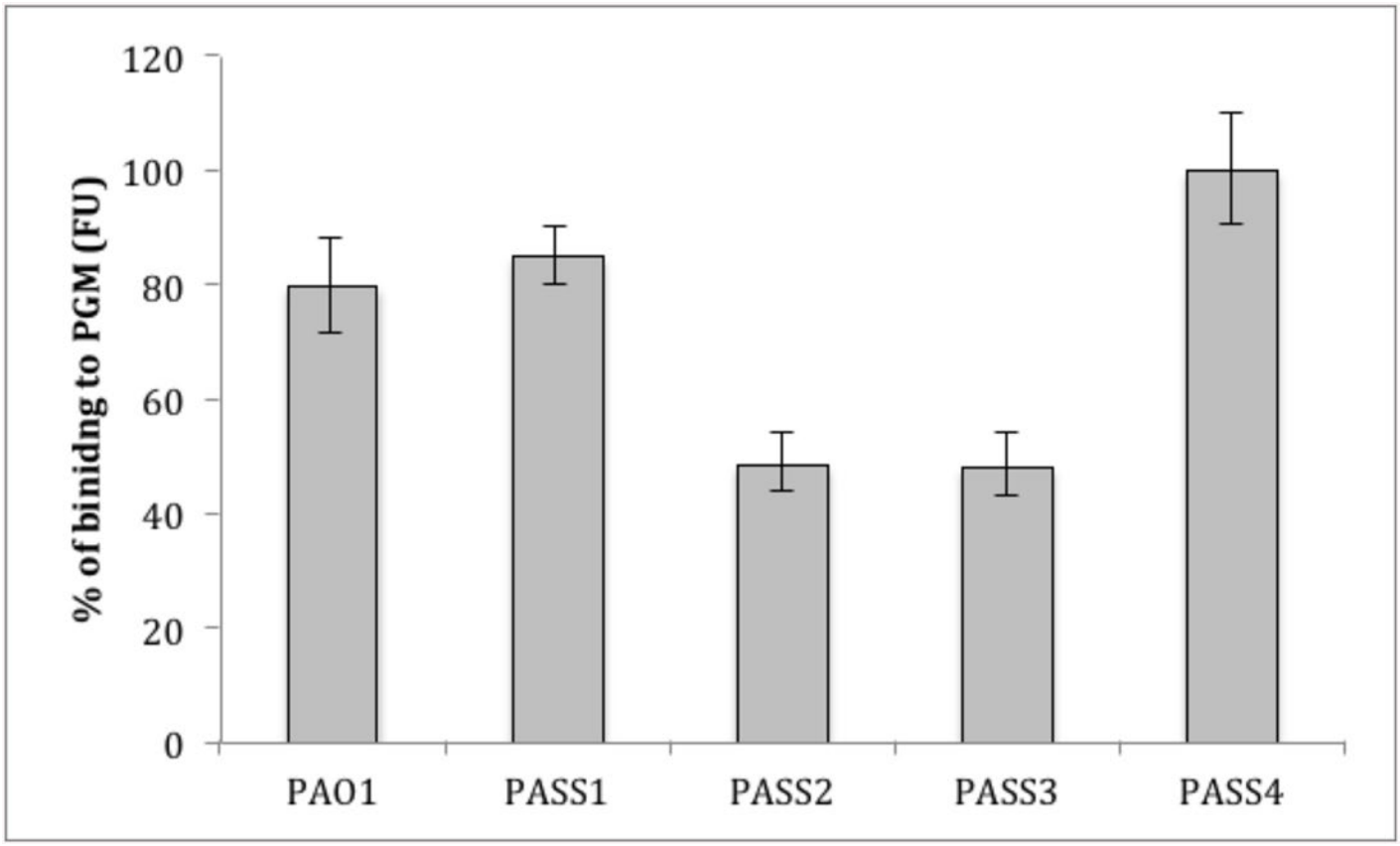


**Fig 6. Binding of isolates PASS1-4 and PAO1 to porcine gastric mucine (PGM).** The Y-axis represents the binding to mucin by the different bacterial strains as arbitrary fluorescent units (FU) normalised against the maximum bacterial binding of PASS4.

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properties and proven role in virulence. Many phenazine-producing bacteria are commonly found associated with host organisms [47]. The well-known phenazines produced by *P. aeruginosa* include phenazine-carboxylic acid (PCA), phenazine-carboxamide (PCN) and hydroxyl-phenazine (1-OH-PHZ), and especially pyocyanin (PYO). The latter is produced in concentrations close to 100 μmol/L during infection in CF [48, 49]; and its presence is associated with high morbidity and mortality in CF patients [50, 51].

Lau et al, using PYO-deficient mutants, have provided direct evidence that PYO is among the most potent virulence factors in the arsenal of *P. aeruginosa*. Mutants lacking PYO production were attenuated in their ability to infect mouse lungs in an acute pneumonia model of infection when compared with isogenic wild-type bacteria [52]. Ultra High Performance Liquid Chromatography (UHPLC) analyses revealed that PASS2 and PASS3 did not produce detectable phenazines (Fig 7). This was confirmed in a *Caenorhabditis elegans* selective grazing assay, where PASS2 and PASS3 were completely grazed by nematodes, indicative of a less-toxic/less-virulent phenotype, whereas all other *P. aeruginosa* colonies remained intact (Fig 8). The activity of phenazines as anti-nematode compounds against *C. elegans* has been recently demonstrated [53]. Production of the phenazine carboxamide was not observed in the strain PASS4. There is no evidence for absence or a significant disruption of genes involved in the production and modification of phenazines in any of the PASS1-4 genomes.

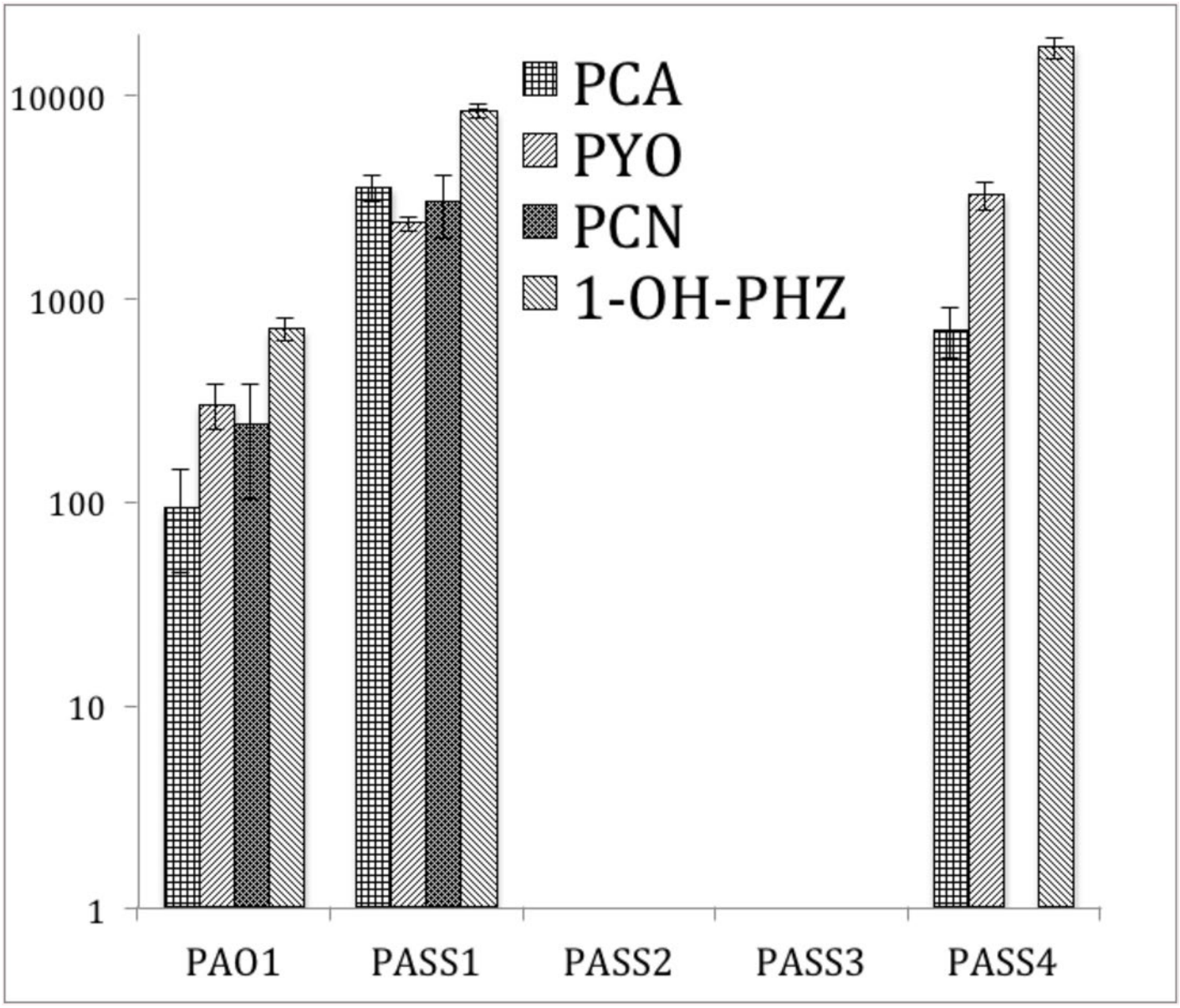


**Fig 7. Production of the phenazines phenazine-carboxylic acid (PCA), pyocyanin (PYO), phenazine-carboxamide (PCN) and hydroxyl-phenazine (1-OH-PHZ) as assessed by UHPLC.** The Y-axis represents arbitrary values based on chromatographic peak areas representative for each compound as observed at 257 nm, in logarithmic scale. Error bars represent standard deviations between three biological replicates.

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## Production of pyoverdine

One of the main characteristics of fluorescent pseudomonads is the production a fluorescent yellow-green siderophore, pyoverdine, important for iron acquisition in low iron environments [54]. PASS2 and PASS4 did not produce pyoverdine on iron-limited King's medium, while PASS3 showed only trace amounts of pyoverdine as compared to PAO1 and PASS1 based on a pyoverdine production assay (Fig 9).

Genomic analysis revealed the loss of several genes in the pyoverdine biosynthesis pathway in both strains PASS2 and PASS4 including either complete or partial loss of genes orthologous to PA2398-PA2402 encoding the ferripyoverdine receptor FpvA, pyoverdine synthetase PvdD,

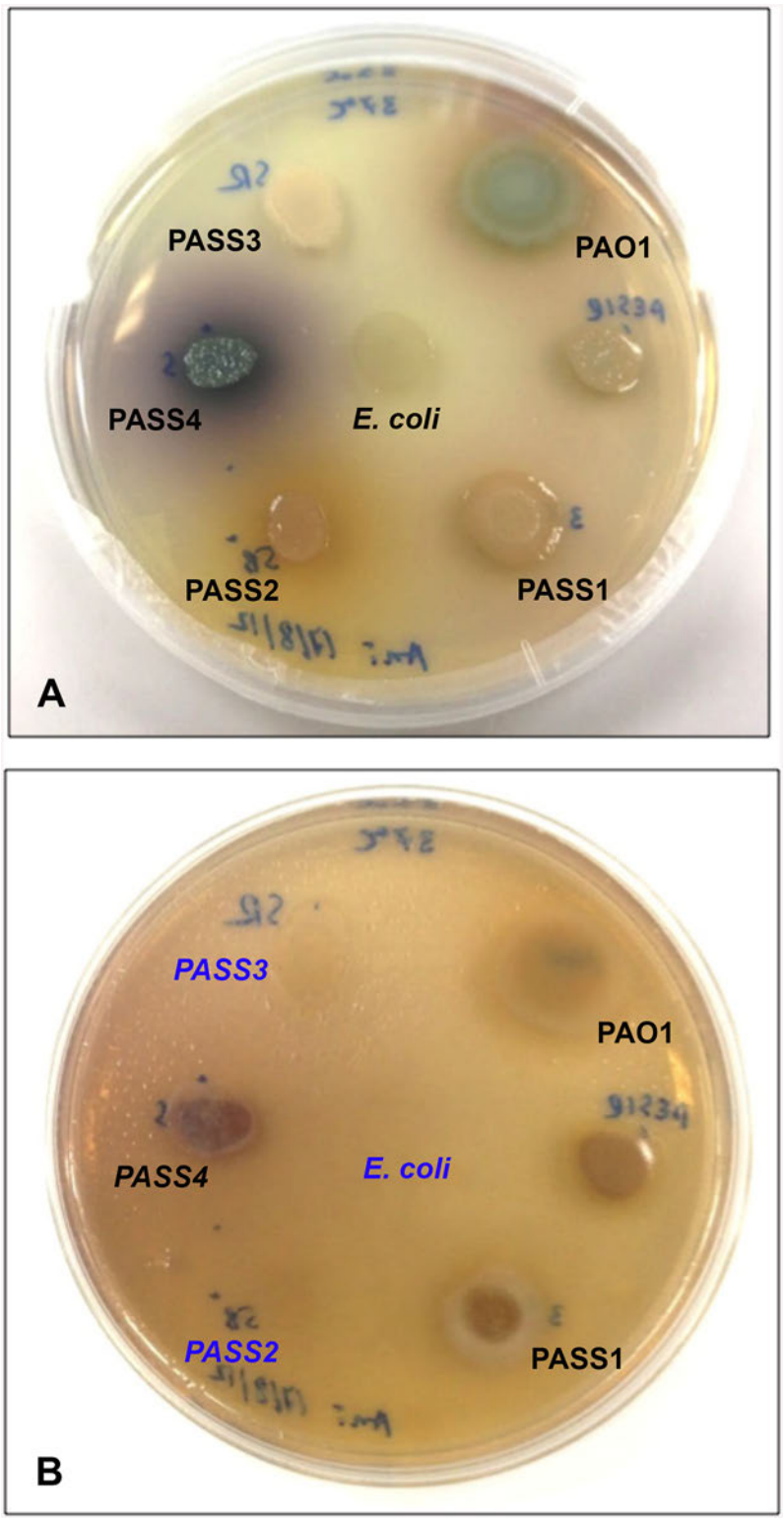


**Fig 8. Nematode selective grazing assay using *Caenorhabditis elegans*.** A representative Petri dish with colonies of various *P*. *aeruginosa* isolates before *C*. *elegans* inoculation (A) and after the 2-week incubation with *C*. *elegans* (B). Colonies of isolates that have been completely grazed by nematodes, and, thus represent the less-toxic/less-virulent phenotypes, are shown in blue. *Escherichia coli* OP50 was used as a positive control for *C*. *elegans* grazing.

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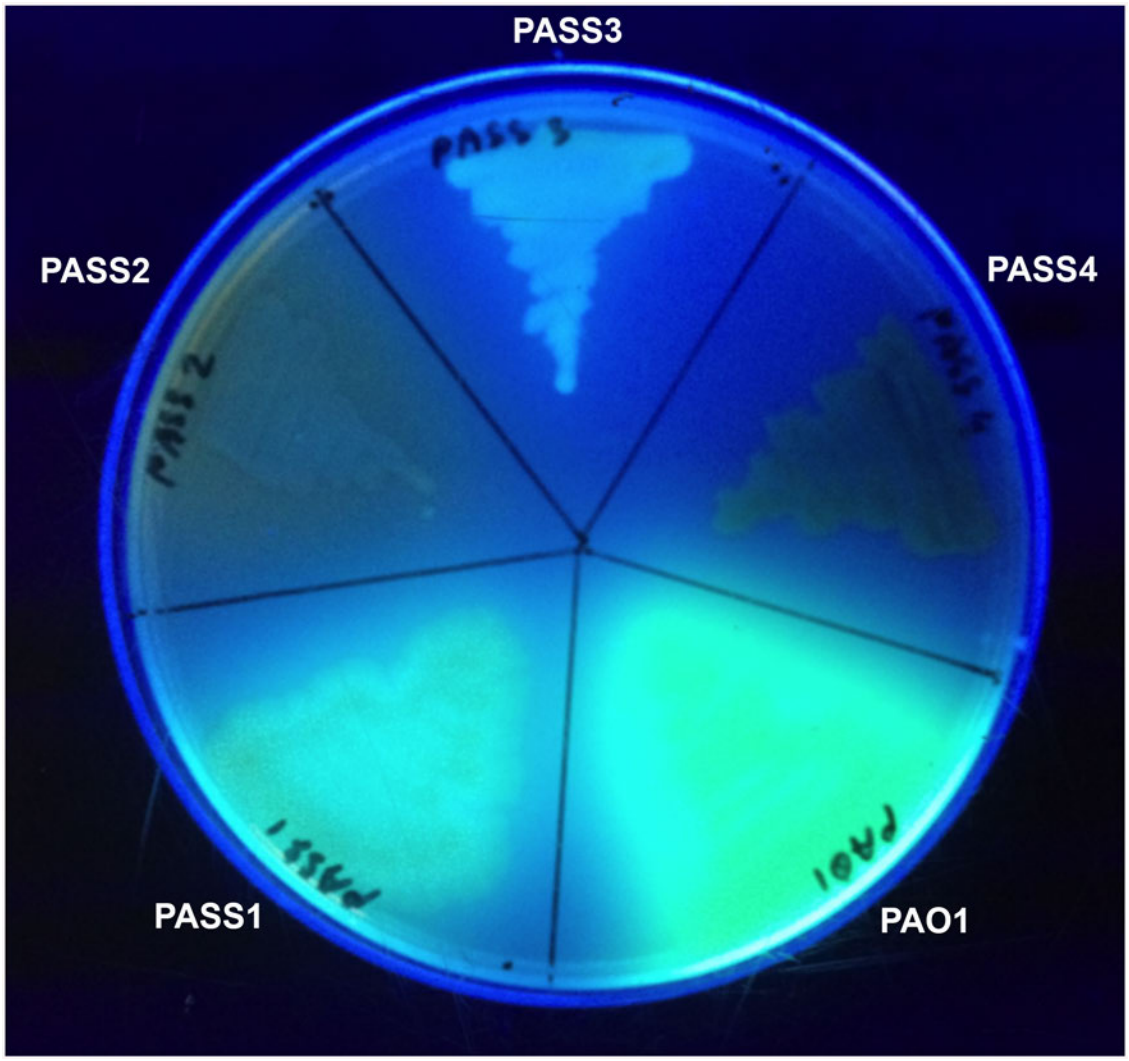


**Fig 9. Representative image showing the production of fluorescent siderophore pyoverdine as assessed under the UV light.**

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protein PvdJ, and the peptide synthase, respectively; which would explain the lack of pyoverdine production in strains PASS 2 and PASS4. Longitudinal studies of CF patients have reported an increased occurrence of pyoverdine-negative isolates with chronic *P. aeruginosa* colonization [55, 56]. The increased availability of heme in CF lungs may select for the use of hemophores rather than siderophores by *P. aeruginosa* in the CF lung environment [56, 57].

**Table 2. Summary of mutations in the *lasR* regulator gene correlated with the observed production of phenazines (PCA, PYO, PCN, 1-OH-PHZ) and pyoverdine (PVD), and the integrity of the pyoverdine biosynthesis gene cluster.**

| Strain | *lasR* variant | Predicted LasR effect | Production of phenazines | Production of PVD | PVD biosynthesis genomic deficiency (PA2398-PA2402) |
|---|---|---|---|---|---|
| PASS1 | c.61G>A | p.(A21T) | positive | positive | non-deficient |
| PASS2 | c.693 694insATGGCC | p.M231 A232insMA | negative | negative | deficient |
| PASS3 | c.[61G>A; 124del] | p.[(A21T; D43fs*114)] | negative | decreased | non-deficient |
| PASS4 | No changes | No changes | positive (no PCN) | negative | deficient |

doi:10.1371/journal.pone.0138527.t002

The LasR quorum sensing (QS) regulator has a role in regulation of the production of virulence factors such as phenazines and pyoverdine, and *lasR* mutants have been frequently described among *P. aeruginosa* CF isolates [58, 59]. Sequence analysis of *lasR* in PASS1-4 strains revealed a single nucleotide polymorphism (SNP) in PASS1 and a 6-nucleotide insertion in PASS2 (Table 2). The same SNP observed in PASS1 was also present in the *lasR* gene in PASS3, as well as an additional deletion leading to a frameshift error. The *lasR* gene in PASS4 was identical to that of PAO1. From this analysis, PASS1-2 strains may be attenuated in the LasR regulator, while PASS3 would most certainly be LasR deficient. This may explain the decreased pyoverdine production by PASS3, despite it carrying an intact pyoverdine biosynthetic gene cluster. The inability of PASS2 and PASS4 to produce pyoverdine is almost certainly due to the loss of several pyoverdine biosynthetic genes. The lack of phenazine production by PASS2 and PASS3 might be due to these two strains having the most severe mutations in the *lasR* regulator.

The deficiency in *lasR*, in conjunction with the deficiency in the *psl* cluster of genes responsible for the production of a major exopolysaccharide, may aid to the lifestyle of PASS2 as a potential "social cheater". LasR deficiency may further render PASS2 "deaf" to QS signals and, therefore, limit its response in production of various metabolically costly QS-controlled virulence factors and toxins benefiting the bacterial population [60, 61].

## Other virulence factors

Several other virulence factor genes were missing in PASS2 and PASS4, compared to the genome of PAO1. The colicin-like pyocin S5 and its cognate immunity protein (encoded by PA0985 and PA0984 in PAO1 genome respectively), pyocin S4 (PA3866), a probable non-ribosomal peptide synthetase (PA2402), and phospholipase D (PA3487) were absent in both PASS2 and PASS4. In addition, PASS2 also lacks genes for the production of paerucumarin (PA2254-PA2257), chitinase (PA2300), and AMB toxin (PA2302-PA2306).

## Distinct *P. aeruginosa* lifestyle strategies in the CF lung

Based on genomic and phenotypic comparisons the four CF isolates obtained directly from the sputum of CF patients revealed significant differences in lifestyle strategy. PASS2 and PASS4 shared some common features with the loss of various virulence determinants, and similar organization of their pili and flagellar loci. PASS2 showed significant genome reduction, with the loss of additional virulence factors, narrowing of potential carbon substrates that can be utilized and even significant reduction in biofilm forming capacity due to the lack of polysaccharide Psl production. The defect in the LasR QS regulator in PASS2 probably also impacts its virulence potential. These adaptations in PASS2 may limit the host immune response as well as reducing expenditure of cellular resources, possibly representing a passive, "cheater" strategy [28].

In contrast, based on the increased production of phenazines such as pyocyanin and increased biofilm forming capacity, isolates PASS1 and PASS4 (the latter even despite the loss of several other virulence genes) may have taken a different adaptation path by enhancing their virulence and colonization potential, possibly representing more aggressive phenotypes. The virulence of PASS1 and PASS4 was also demonstrated against the eukaryotic model *C. elegans* (Fig 8). The virulent phenotype of PASS4 in the *C. elegans* model, despite the clear loss of known virulence factors, highlights an issue in making virulence predictions based on genomic analyses alone.

PASS3 is closely related to PASS1 based on MLST and genomic comparisons, but differs significantly in phenotype. PASS3 displays low toxicity in the *C. elegans* model and is deficient in phenazine production, possibly as a result of mutations in *lasR*. However, PASS3 shows

enhanced biofilm formation and conversion to a mucoid phenotype, which likely enhances its survival and persistence, and therefore may have adopted a more "defensive" strategy.

Table 3 summarizes the overall genetic and phenotypic differences observed between PASS1-4 strains and PAO1. The diversity seen amongst the CF isolates in this and previous studies [62, 63] suggests that there is no single preferred adaptation pathway for success in a CF lung.

In order to enable researchers to broaden *P. aeruginosa* research beyond the limitations of model laboratory strains, recently a *P. aeruginosa* reference panel of 43 strains has been suggested, also based on the origin, in order to reflect its diversity [64]. Nevertheless, the diversity seen among our 4 isolates with each of these strains equipped with a unique set of defensive and offensive tools, suggests that differences that exist between strains that originate even from the same environment like CF can make it impossible to pinpoint a single phenotype/strain with a set of defined characteristics that would be representative for that environment.

## CF isolates share a core proteomic signature distinct from PAO1

A shotgun proteomic analysis was undertaken to investigate protein expression profiles of the four clinical isolates and the model *P. aeruginosa* strain. There were striking differences in protein expression between the common laboratory model organism PAO1 and the CF isolates PASS1-4. With ~ 1300–1400 proteins identified for each of these organisms, only 526 were shared among all five, while PAO1 showed 827 unique proteins not detected in any of the CF isolates when cultured in a common laboratory medium LB. Conversely, PASS1-4 strains shared 703 commonly expressed proteins, none of which were detected in PAO1 (Fig 4F, S2 Table). This trend can also be seen on FriPan 2D plot where PAO1 appears at a considerable distance from PASS1-4 strains, while CF strains PASS1-4 among themselves retained the overall similarity profiles with PASS1 and PASS3, and PASS2 and PASS4 clustering together (Fig 4D and 4E), consistent with the overall pattern observed in bioinformatics analysis for all predicted proteins (Fig 4A and 4B).

The proteins uniquely expressed by PAO1 (827 in total) and those uniquely shared by PASS1-4 strains (703 in total), were mapped onto the metabolic pathways of PAO1 using Pathway Tools 17.5 (Fig 10, S2 and S3 Figs). The analysis revealed distinct differences in cell physiology between the CF strains and the model strain PAO1. PAO1 expressed an array of membrane transport proteins involved in the uptake of a broad range of amino acids, polyamines, carbohydrates and other organic nutrients, whose expression was not detected in any of the PASS1-4 strains. There was essentially no expression of biosynthetic genes involved in amino acid, polyamine, carbohydrates, nucleoside or nucleotide biosynthesis (Fig 10A, S2 Fig).

In contrast, PASS1-4 expressed only a small select group of transporters for compounds such as sugars, dipeptides, and heme. Instead the PASS1-4 strains expressed many proteins involved in biosynthetic pathways for compounds such as nucleosides and nucleotides, amino acids, carbohydrates and polyamines (Fig 10B, S3 Fig).

The proteomic data indicates that in a conventional laboratory medium PAO1 may transport a diverse set of "ready-made" nutrients from the rich LB medium, whereas CF isolates PASS1-4 require only a limited number of nutrients from the LB medium, relying mainly on their own metabolism for synthesis of other essential nutrients. Interestingly, despite dramatic differences in the proteins involved in biosynthesis, in general, the expression of proteins involved in the catabolism of various compound groups were similar. This might indicate that PAO1 and PASS1-4 strains all have access to a variety of compound classes, but, as mentioned above, may vary significantly in the means of obtaining these compounds: PAO1 –via transport, and PASS1-4 –via intracellular biosynthesis. This probably reflects specialization in the

**Table 3. Summary of genomic and phenotypic characteristics of *P. aeruginosa* CF isolates PASS1-4 and the model strain PAO1.**

| | PASS1 | PASS2 | PASS3 | PASS4 | PAO1 |
|---|---|---|---|---|---|
| **Colony morphology on LB solid medium** | pale-green non-mucoid | light brown non-mucoid | white mucoid | green-blue non-mucoid | green non-mucoid |
| **Biofilm formation in flow cells** | positive | negative | positive | positive | positive |
| **Flagella** | type B | type A | type B | type A | type B |
| **Type IV pili** | group II | group I | group II | group I | group II |
| **Swimming motility** | positive | deficient | positive | deficient | positive |
| **Binding to mucin** | high (80–100%) | low (≤50%) | low (≤50%) | high (80–100%) | medium (50–80%) |
| **Phenazines production** | positive | negative | negative | positive (no PCN) | positive |
| **Pyoverdine production** | positive | negative | decreased | negative | positive |
| **LasR deficiency** | uncertain | possibly deficient | possibly deficient | non-deficient | non-deficient |
| **Loss of additional virulence factors** | | pyocin S5, pyocin S4, NRPS, phospholipase D, paerucumarin, chitinase, AMD toxin | | pyocin S5, pyocin S4, NRPS, phospholipase D, | |
| **Toxicity/ virulence*** | positive | negative | negative | positive | positive |

* as assessed in *C. elegans* selective grazing assay

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CF strains geared towards utilization of select types of nutrients that might be abundant in CF lungs, such as highly glycosylated mucins, and further highlights the preference for heme as a possible source of iron in CF isolates. Conversely, PAO1, having been grown and maintained in laboratories worldwide for decades, may have adapted to using the broad variety of nutrients present in common growth media, such as LB. The proteomics data reveals significant differences in the metabolic strategies of CF isolates compared with PAO1 and emphasises the limitations presented by the use of laboratory model organisms for studying processes in specific hosts or environments.

## Concluding remarks

Significant phenotypic differences were observed amongst the *P. aeruginosa* CF isolates PASS1-4 including differences in traits important for successful CF lung colonization and survival. Phenotypic diversity has been previously shown among the *P. aeruginosa* CF isolates [62, 63], particularly with respect to the decreased virulence in chronic infections [65–67]. While the classical reduction of virulence seen in many CF isolates was observed in PASS2, and the conversion to mucoid phenotype by PASS3, a more aggressive phenotype was shown by PASS1 and PASS4 with increased production of known virulence factors such as phenazines, and increased biofilm formation. This suggests that there is no single pathway of adaptation to an environment such as the CF lung; instead, strains acquire adaptations that allow them to pursue different lifestyles ranging from passive, defensive to aggressive. This makes it extremely difficult to predict the development of certain traits in a particular environment or host, and, hence, make assumptions for the suitable treatment strategies.

Relying on genomic data to make predictions about phenotypes can be problematic. *In silico* analysis of genomes of all 5 isolates in this study revealed a high degree of similarity between the strains with 80% of shared predicted proteome, which is in the range commonly observed for strains in the same species implying possible phenotypic similarities. However, actual protein expression by these strains grown in a common laboratory medium revealed dramatically

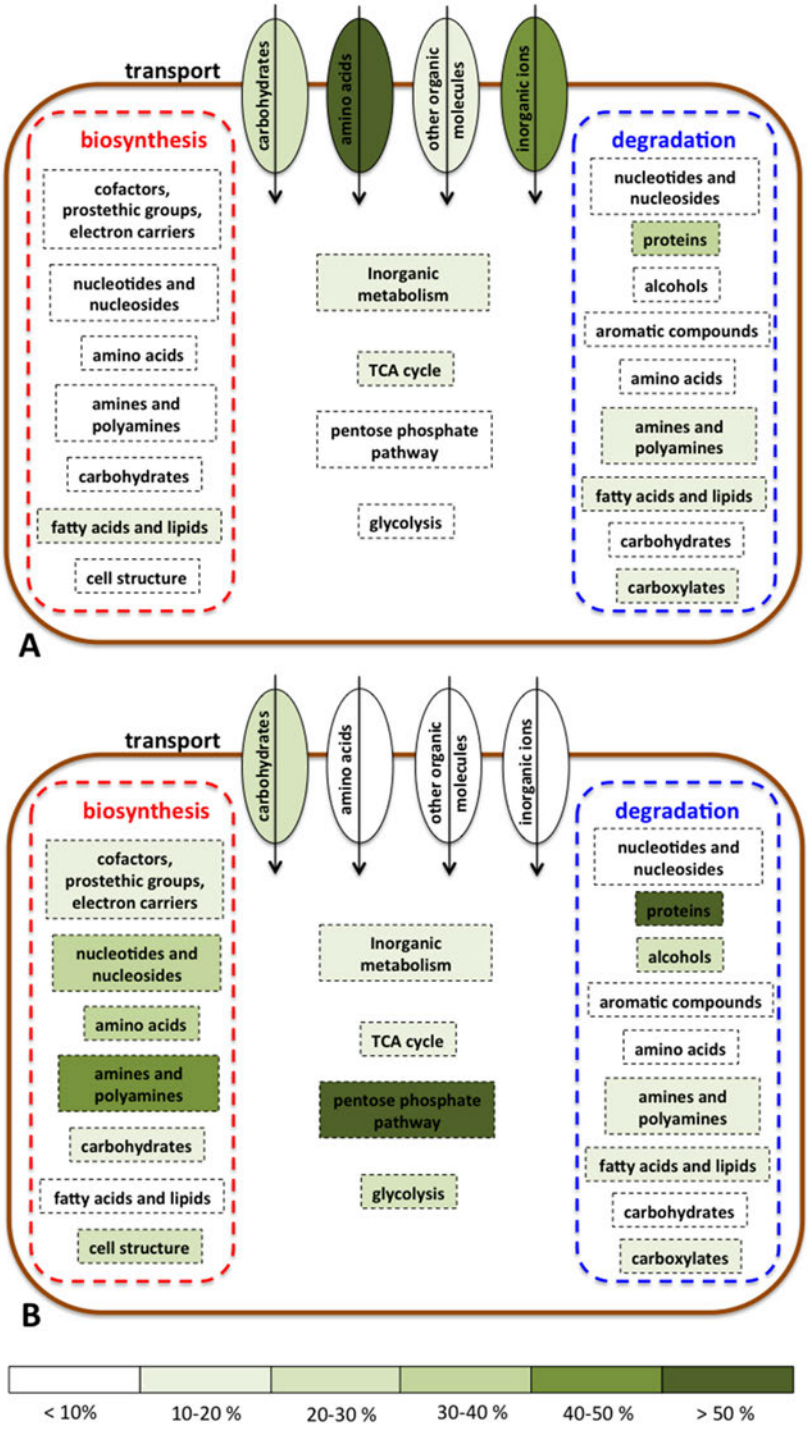


**Fig 10. Schematic metabolic overview showing differences in protein abundance between PAO1 (A) and the CF isolates PASS1-4 (B) as assessed via proteomics.** Shading represents the percentage of expressed proteins within each metabolic category. Assignment to metabolic categories is based on analysis using Pathway Tools [27]. Full details of the Pathway Tools analysis are shown in S2 and S3 Figs, and the full list of express proteins for each strain is provided in S2 Table.

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different expression profiles suggesting distinct physiological and metabolic states that were not predictable at the genomic level via *in silico* analysis. This further highlights the limitations of model laboratory strains and the need to complement analyses in model organisms with direct experimental work on isolates from the relevant host or environment.

## Supporting Information

**S1 Fig. Genomic alignment of flagella biogenesis genes in strains PASS1-4 as compared to PAO1 using MAUVE.** Sequences conserved among all 5 isolates are presented in mauve, sequences shared between isolates PAO1, PASS1 and PASS3 are in green, sequences shared between isolates PASS2 and PASS4 are presented in blue **(A)**. **Flagella-mediated swimming motility assay for strains PASS1-4 and PAO1 (B).**
(TIF)

**S2 Fig. Pathway Tools 17.5 cellular overview diagram showing proteins/pathways expressed uniquely by PAO1, in red.** The shapes on the diagram represent specific types of compounds as follows: square—carbohydrate, triangle—amino acid, upside down triangle—cofactor, ellipse (horizontal)—purine, ellipse (vertical)—pyrimidine, diamond—proteins, T-shape—tRNA, circle—all other compounds. The shading of the icon indicates the phosphorylation state of the compound: shaded compounds are phosphorylated; unshaded compounds are unphosphorylated. Shapes/paths located on outer rectangles represent transporters/membrane proteins. The arrows indicate the direction of the transport: pointing in—uptake, pointing out—export.
(TIF)

**S3 Fig. Pathway Tools 17.5 cellular overview diagram showing proteins/pathways expressed uniquely by PASS1-4 strains, in red.** The shapes on the diagram represent specific types of compounds as follows: square—carbohydrate, triangle—amino acid, upside down triangle—cofactor, ellipse (horizontal)—purine, ellipse (vertical)—pyrimidine, diamond—proteins, T-shape—tRNA, circle—all other compounds. The shading of the icon indicates the phosphorylation state of the compound: shaded compounds are phosphorylated; unshaded compounds are unphosphorylated. Shapes/paths located on outer rectangles represent transporters/membrane proteins. The arrows indicate the direction of the transport: pointing in—uptake, pointing out—export.
(TIF)

**S1 Table. MLST allelic profiles and strain types of *P. aeruginosa* isolates obtained from the CF sputum in this study.**
(DOCX)

**S2 Table. Proteins identified in strains PASS1-4 and PAO1 grown in LB medium via proteomics ($p < 0.01$).** + protein identified in the strain culture (shaded),—protein not identified in the strain culture (unshaded), NA—protein does not have an ortholog in the PAO1 genome or did not map to an ortholog in PAO1 due to differences in the sequence.
(DOCX)

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## Author Contributions

Conceived and designed the experiments: AP SSK KK NHP MPM ITP. Performed the experiments: AP SSK KK AMS VV. Analyzed the data: AP SSK KK AMS CK ITP. Wrote the paper: AP SSK KK AMS CK NHP MPM ITP.

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# 3

# A cystic fibrosis *Pseudomonas aeruginosa* isolate specialised to catabolise nucleic acids

# A Cystic Fibrosis *Pseudomonas aeruginosa* Isolate Specialised to Catabolise Nucleic Acids

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Running Head: Adaptation of *P. aeruginosa* for growth on DNA

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## 3.1ABSTRACT

*Pseudomonas aeruginosa* is a major cause of morbidity and mortality in patients with cystic fibrosis (CF). We undertook Biolog Phenotype Microarray testing of *P. aeruginosa* CF isolates to investigate their catabolic capabilities compared to *P. aeruginosa* laboratory strains PAO1 and PA14. One strain, PASS4, displayed an unusual phenotype, only showing strong respiration on adenosine and inosine. Further testing indicated that PASS4 could grow on DNA as a sole carbon source, with a higher biomass production than PAO1. This suggested that PASS4 was specifically adapted to metabolise extracellular DNA, a substrate present at high concentrations in the CF lung. Transcriptomic and proteomic profiling of PASS4 and PAO1 when grown with DNA as a sole carbon source identified a set of upregulated genes, including virulence and host-adaptation genes. PASS4 was unable to utilise N-Acetyl-D-glucosamine, and when we selected PASS4 mutants able to grow on this carbon source, they also displayed a gain in ability to catabolise a broad range of other carbon sources. Genome sequencing of the mutants revealed they all contained mutations within the *purK* gene, encoding a key protein in the *de novo* purine biosynthesis pathway. This suggested that PASS4 was a purine auxotroph. Growth assays in the presence of 2 mM adenosine confirmed this conclusion. Purine auxotrophy may represent a viable microbial strategy for adaptation to DNA rich environments such as the CF lung.

## 3.2 IMPORTANCE

*Pseudomonas aeruginosa* is an opportunistic pathogen associated with complications in cystic fibrosis (CF) and is a major cause of death among CF patients. We found that *P. aeruginosa* CF isolate PASS4 has very limited carbon catabolism capabilities, and may have become specialised at utilising nucleic acids as a source of carbon. The CF lung contains viscous sputum which includes an abundance of DNA from epithelial and bacterial cells, and therefore provides an excellent niche for such an adaptation. We found that many *P. aeruginosa* virulence genes have increased expression in response to external DNA. Characterisation of *P. aeruginosa* PASS4 revealed that the molecular basis of its metabolic specialisation is a defect in biosynthesis of

purines, precursors for DNA synthesis. Better understanding of *P. aeruginosa* adaptations in the CF lung will help in the development of specialised treatment regimes aimed at eradication of *P. aeruginosa* infections.

## 3.3 INTRODUCTION

Cystic fibrosis (CF) is a genetic disorder most common among Caucasian populations (1). It is caused by mutations in the cystic fibrosis transmembrane regulator (*CFTR*) gene which encodes a cAMP-dependent chloride channel (2). Dysfunction in the chloride channel leads to dehydrated and thickened airway surface liquid (ASL) hampering mucociliary clearance from the conducting airways (3). The thickened ASL enhances microbial colonisation, leading to continuous stimulation of the immune system, and resulting in chronic lung inflammation (4). This hyperactive inflammatory response leads to a decline in lung function and eventual lung failure (5).

*P. aeruginosa* is one of the primary causes of acute and chronic lung infections in CF patients, resulting in significant morbidity and mortality (1, 6). *P. aeruginosa* is a metabolically versatile Gram-negative opportunistic pathogen that is common in various environments, such as soil and water (5-8). It can metabolise a broad range of carbon sources and grows both aerobically and anaerobically (6). Its metabolic versatility is conferred by a diverse set of transport systems and catabolic pathways encoded within a relatively large genome, typically more than 6 Mb (9).

Most CF patients who develop a lung infection by adolescence can live with the infection for 20 or more years (5). During this period, *P. aeruginosa* continues to adapt to the CF lung environment. This results in the emergence of diverse phenotypes including increased mucoidy, auxotrophy, loss of motility, emergence of hypermutators, resistance to antimicrobials, and defects in key virulence factors such as quorum sensing regulation and type III secretion (5-7, 9).

Adaptation and chronic or recurrent infection of *P. aeruginosa* in the CF lung is facilitated by its ability to grow as biofilms (6). Biofilms are highly organised, structured bacterial communities

attached to one another and to a solid surface (1, 5, 6). Cells in biofilms are held together by a matrix of extracellular polymeric substances (EPS) consisting mainly of polysaccharides, extracellular DNA (eDNA), lipids, proteins, cellular debris and membrane vesicles (5, 6, 10, 11). EPS protect bacterial cells from damage or death caused by surfactants, biocides, grazing predators and host defenses (6, 12).

Extracellular DNA plays an adhesive role in the initial stages and development of *P. aeruginosa* biofilms (6, 8, 10). The source of eDNA has been proposed to be due to either prophage-induced cell lysis or the release of membrane vesicles which contain DNA (10, 13). Investigations into *P. aeruginosa* eDNA release by Turnball et al (2016) suggests explosive cell lysis-mediated MV production in biofilms and planktonic cultures are independent of the Pseudomonas Quinolone Signal (10).

Extracellular DNA in CF lungs is derived from both microorganisms, and also lysed host cells (11, 14). The concentration of eDNA in the CF lung can be as high as 14 mg/ml (14, 15), and could serve as a nutrient source for bacterial growth (11, 14). *P. aeruginosa, Escherichia coli* and *Shewanella spp*. have all been shown to be able to utilise DNA as a nutrient source (8, 14, 16, 17).

We recently described *P. aeruginosa* CF isolates (PASS1-4) that showed significant variability in colonisation and virulence-related traits (18). To investigate the metabolic capabilities of these strains, their ability to respire on 190 carbon sources was tested (Fig. 3.1). One of the strains examined, PASS4, had lost the ability to utilise a broad range of carbon sources, and strong respiration was only observed on the purines inosine and adenosine. We found that PASS4 grew better on eDNA compared with other *P. aeruginosa* strains such as PAO1, suggesting that this strain may have become specialised for growth on DNA in the CF lung. Transcriptomic and proteomic analysis of PASS4, and genome sequencing of PASS4 mutants were undertaken to investigate the molecular basis of this metabolic specialisation.

## 3.4 RESULTS AND DISCUSSION

### Phenotypic comparison of the PASS1-4 *P. aeruginosa* CF isolates.

We used Biolog phenotype microarrays to compare the carbon utilisation profiles of four *P. aeruginosa* CF isolates (PASS1-4) with two *P. aeruginosa* model strains (PAO1 and PA14). Strains were screened for their ability to utilise 190 sole carbon sources, including a range of carbohydrates, amino acids, carboxylic acids and miscellaneous compounds (19). *P. aeruginosa* strains PAO1, PA14 and PASS1 had similar metabolic fingerprints. The other three CF isolates (PASS2-4) displayed reduced capabilities for carbon utilisation (Fig. 3.1). In particular, PASS4 showed a striking reduction in catabolic capability, only showing strong respiration on two compounds, the nucleosides adenosine and inosine. To confirm the capacity of PASS4 to grow on nucleosides, we cultured PASS4 on solid M9 minimal medium supplemented with 13 mM adenosine as a sole carbon source.

The carbon utilisation profiles of PASS2-4 suggest that reduction in metabolic capabilities might be a common adaptation for *P. aeruginosa* in the CF lung. Biolog phenotype testing of 10 *P. aeruginosa* CF isolates in a previous study showed metabolic reduction was common in CF isolates, with extensive heterogeneity amongst substrate utilisation profiles (20).

We hypothesized that PASS4 might be specialised to grow on DNA, which can be readily found in the CF lung environment. To investigate this possibility, PASS4 and PAO1 were grown in M9 minimal medium supplemented with 1.5 mg/ml of salmon sperm DNA as a sole carbon source. Based on optical density measurement at $OD_{600}$, the total biomass of PASS4 cells was 18% greater than PAO1 following growth in DNA over 7 days with constant aeration at 37°C. Both strains tested negative for production of extracellular DNase, suggesting that they uptake DNA where it is subsequently degraded intracellularly**.**

## The transcriptome and proteome of PAO1 and PASS4 following growth in DNA.

The global transcriptional response and protein abundance of *P. aeruginosa* strains PASS4 and PAO1 grown to an $OD_{600}$ = 0.6 with DNA as a sole carbon source was assessed using RNA sequencing (Table 3.1) and whole-cell proteomics. Growth on asparagine was used as a control, since this was one of the few carbon sources that PASS4 was able to utilise, according to the Biolog Phenotype MicroArray analysis (Fig. 3.1).

The transcriptomic analysis identified a total of 576 genes that were differentially transcribed by PASS4 when grown in the presence of DNA (*P* value < 0.01, $\log_2$ fold-change 1< to < -1), with 322 genes upregulated and 254 genes downregulated (Fig. 3.2, Table ST3.1). There were a total of 423 genes differentially expressed by PAO1 when grown in DNA (*P* value < 0.01, $\log_2$ fold-change 1< to <-1), with 359 genes upregulated and 64 genes downregulated (Fig. 3.3, Table ST3.1). The transcriptional response of PASS4 and PAO1 grown in DNA in comparison to growth in asparagine had a correlation value of $R^2$= 0.5983 (Fig 3.4a). A total of 129 transcripts displayed similar expression patterns in both organisms, with 112 being upregulated and 17 downregulated (Table ST3.1). The two strains displayed differential expression of a hypothetical protein (PA3783), which was downregulated 3-fold in PAO1 and upregulated 4-fold in PASS4 during growth in DNA.

The shotgun proteomic analysis detected a total of 1962 proteins in PASS4, of which 307 displayed significant differential abundance between the two conditions (P< 0.05, $\log_2$ fold-change 1< to <-1) (Fig. 3.5, Table ST3.1). A total of 239 proteins showed greater abundance when the cells were grown on DNA as a sole carbon source in comparison to 68 proteins which displayed a decrease in abundance. A total of 2112 proteins were detected from PAO1, of 293 proteins were significantly differentially abundant (*P*< 0.05, $\log_2$ fold-change 1< to <-1), with an increase in abundance of 232 and decrease in abundance of 61 proteins (Fig. 3.6, Table ST3.1). A correlation analysis of the protein expression of PAO1 and PASS4 grown in DNA presented an $R^2$ value of

0.4072 (Figure 3.4b). Only six proteins showed significant differences in protein abundance between PAO1 and PASS4 (Figure 3.4b), for example a C5 dicarboxylate transporter showed an 6.5-fold increase in abundance in PAO1 but showed a 10.9-fold decrease in abundance in PASS4 when these strains were grown in DNA.

**Increased expression of iron and sulphate acquisition genes in response to DNA.**

The pyoverdine (*pvd*) and pyochelin (*pch*) biosynthetic gene clusters encode the two major *P. aeruginosa* siderophores (21, 22). Expression of the *pch* gene cluster and pyochelin receptor (*fptA*) was significantly induced by exposure to DNA in both PAO1 and PASS4 (Fig. 3.7). The *pvd* gene cluster was significantly upregulated by DNA in PAO1, but not in PASS4 (Fig. 3.7). PASS4 grown in DNA displayed a significant increase in the abundance of the PvdE and PvdF proteins. The iron-responsive small RNAs PrrF1 and PrrF2 (23) were upregulated in both PASS4 (11-fold and 19-fold, respectively) and PAO1 (both by 6-fold) (Fig. 3.7). During iron limitation, these small RNAs enable inhibition of genes that encode "nonessential" iron-containing proteins (24). Additionally, PASS4 grown in DNA displayed an increased abundance of iron acquisition proteins including the putative TonB-dependent receptor family protein (PA0781), TonB-dependent siderophore receptor (PA4837), FeoB $Fe^{2+}$ transporter (PA4358), FecA ferric citrate receptor (PA3901) and FiuR iron transport protein.

DNA as a sole carbon source led to the upregulation of an array of genes involved in sulphate metabolism in both PASS4 and PAO1. For example, the *tauABC* genes, encoding an ABC-transporter for the sulphate-containing amino acid taurine were upregulated more than 2- fold in both PASS4 and PAO1. The *tauD* gene, encoding taurine dioxygenase was upregulated more than 2-fold and had an 8-fold increase in protein abundance in PASS4. The expression of (*tauABCD*) is known to be regulated by sulphate starvation (25). Expression of other genes involved in the utilisation of alternate sulphate sources, such as alkane sulphonates, were also increased in the presence of DNA. The *lsfA* gene encoding a thio-specific antioxidant was upregulated 4-fold in PASS4 and 30-fold in PAO1 following growth in DNA.

Sulphate regulatory systems also showed significant changes in gene expression in DNA grown cells. The ECF sigma factor PA2093 was 8-fold upregulated by DNA in PASS4. This sigma factor has previously been shown to be upregulated by sulphate limitation (26). Typically, the TonB-dependent transducers PA2089 and PA2590 (both have increased protein abundance and significant upregulation of transcripts in PASS4 grown in DNA) sense an extracellular signal which is transmitted via the anti-sigma factor leading to the activation of ECF sigma factor (PA2093) and to the subsequent transcription of target genes such as PA2090 (upregulated significantly in PAO1 and PASS4 grown in DNA). The putative sulphonatase (PA2090) is part of a predicted extracellular sulphate (ECS) locus (PA2083-PA2094) (27). All members of the ECS locus were upregulated at the transcriptional level in PASS4, with some upregulated in PAO1. The protein abundance of members of the ECS locus was also increased in PASS4.

During growth, most heterotrophic bacteria are known to maintain specific elemental ratios of carbon, nitrogen, sulphate, phosphorus and iron (28). Compared to most other carbon sources, DNA is a rich source for nitrogen and phosphorus. Thus, the increased expression of genes for iron and sulphate acquisition may reflect an attempt of the *Pseudomonas* cells to balance their elemental ratios in a phosphorus/nitrogen rich setting.

**Increased expression of virulence genes in response to eDNA.**

The set of genes that were induced at least 4-fold by DNA in both PAO1 and PASS4 included two clusters of phenazine biosynthesis genes *phzA1B1C1D1E1F1G1* and *phzA2B2C2D2E2F2G2* (Fig.3.7). Several genes from the *phz1* and *phz2* gene clusters had significant increases in protein abundance in PAO1. Phenazine redox pigments such as pyocyanin and 1-hydroxyphenazine are important virulence factors: 1-hydroxyphenazine constrains mammalian cell respiration; and pyocyanin hinders epidermal cell growth and plays a role in acquisition of iron from transferrin (29). Pyocyanin has also been shown to bind directly to the phosphate backbone of DNA, altering DNA viscosity. During biofilm growth, a change in viscosity of DNA facilitates electron transfer

through DNA for maintenance of redox homeostasis between anoxic and oxygenated regions of the biofilms (15, 30).

The RhlR quorum-sensing system regulates the phenazine biosynthesis and receptor genes (31, 32), elastase *lasB*, protease *lasA*, and rhamnolipid biosynthesis genes *rhlAB* (33, 34). The *rhlR* transcripts were upregulated in both PAO1 and PASS4 grown in DNA, *rhlAB* transcripts and proteins showed increased abundance in both PASS4 and PAO1 when grown on DNA (Fig. 3.7). The *lasA* and *lasB* transcripts were upregulated in PASS4 and PAO1 grown in DNA, the protein abundance of LasA was significantly increased in PASS4 and LasB was significantly increased in PAO1.

The *mxtR* (PA3271) gene encoding a sensor kinase was upregulated 5-fold in PASS4. The MxtR protein showed 8-fold and 16-fold increase in abundance in PAO1 and PASS4, respectively. MxtR has been shown to modulate the production of interbacterial 2-alkyl-4(1*H*)-quinolone (AQ) signal molecule via the LysR-type transcriptional regulator MexT (35) (no significant differential expression observed in our study). MxtR-induced AQ has an influence on the *P. aeruginosa* regulatory network, including the transcription of virulence genes coding for pyocyanin and rhamnolipids (35). PAO1 grown in DNA showed increased abundance of the PqsA, PqsE and PqsL proteins (13-fold, 15-fold and 3-fold, respectively). The *pqsABCDE* operon is positively regulated by the expression of 2-heptyl-4-hydroxyquinoline (HHQ) and is required for AQ biosynthesis (36). Although *pqsE* does not play a role in AQ biosynthesis, it has been shown to influence the production of virulence factors such as pyocyanin, phenazines and rhamnolipids (37).

A QS dependent lysine-specific endoprotease (38), *piv* was upregulated 4.5-fold when PAO1 grown in DNA. This protease has been shown to cause killing of *Tenebrio molitor* larvae within 4 days post infection (39). PIV also play a role in scavenging for nutrients (40).

The *lecB* gene, encoding the fucose-binding lectin, showed 15-fold higher expression in PASS4 grown on DNA, but only two-fold increased expression in PAO1 (Fig 3.7). The LecB lectin has

been shown to have cytotoxic effects on host respiratory epithelial cells, and plays an important role in facilitating adhesion to the airway mucosa. The *lecA* gene encoding a galactose-binding lectin was not significantly differentially expressed in either PAO1 or PASS4 when grown in DNA. This was surprising, as a previous study (47) has shown the induction of the *lecA* lectin by adenosine.

The type 4 pilus (T4P) transcripts *pilA, pilG* and *pilZ* were upregulated in PAO1 grown in DNA. Following growth of PASS4 in DNA there was has an increase in expression of the *pilM* transcript and an increase in protein abundance of PilH and PilM. The T4P plays a role in cell adhesion, host cell invasion (41), biofilm formation, as well as DNA uptake (42, 43). The *aceA* gene encoding isocitrate lyase (48), which is specific to the glyoxylate shunt pathway, is 11-fold upregulated in PASS4 and 3-fold upregulated in PAO1 when grown in DNA. The glycoxylate shunt is known to be upregulated under conditions of oxidative stress, antibiotic stress, and host infection (49). The *aceA* gene has been shown to be critical for *P. aeruginosa* infection in an alfalfa seedling model (50).

An arginine-specific autotransporter, AaaA showed an 11-fold higher protein abundance, and 2-fold downregulation of the transcript in PAO1. Previously, a gene knockout of *aaaA* led to attenuation of *P. aeruginosa* in a mouse chronic wound infection suggesting it plays a role in virulence (44).

**Other changes transcriptional changes in response to DNA.**

As expected, the *ansB* gene, encoding L-asparaginase, showed 16-fold and 7-fold decrease in gene expression in DNA-grown cells compared with asparagine-grown cells for PAO1 and PASS4, respectively. Other genes in the same regulon as *ansB*, such as the *aatM* gene, encoding for acidic L-amino acid uptake, were also upregulated by asparagine. The branched chain amino acid catabolism genes *liuA, liuB, liuC, bkdA1, bkdA2, bkdB, lpdV, mmsB* and *mmsA* were upregulated in asparagine-grown cells, suggesting a regulatory link between asparagine and branched chain

amino acid degradation. The transport and utilisation of branch chain amino acids, arginine and ornithine has been reported to be governed by the CbrAB/Crc regulatory system (45).

The PA0622-PA0624 genes involved in the production of the R-type pyocin were downregulated by a more than 4-fold in both PAO1 and PASS4 during growth in DNA compared to asparagine. These genes, along with other genes from the R- and F-pyocin gene cluster, encode a prophage endolysin which is essential for explosive cell lysis that leads to increased availability of public goods such as cytosolic proteins, eDNA, and membrane vesicles (10). The transcriptomic data suggests that the concentration of eDNA is an important factor regulating the expression of genes controlling explosive cell lysis.

**Comparison of *P. aeruginosa* growth in DNA to *in vivo* transcriptomic data**

Previously, Turner *et al.* (46) have generated *P. aeruginosa* transcriptional profiles in acute and chronic murine infections. Our transcriptomic data of PASS4 and PAO1 grown in DNA was compared to the *P. aeruginosa* acute and chronic murine infection data to identify the overlap between up- and downregulated transcripts. This revealed 10 and 1 commonly up- or downregulated transcripts across all of the datasets (Table ST3.3). The upregulated genes common to these four datasets included PA4142 encoding a probable secretion protein, *ampOP* operon involved in regulation of beta-lactamase activity, and the virulence genes *phzA1, aceA, pchA, pchH, pchF, fptA, cbpD*.

**Carbon utilisation by *P. aeruginosa* mutants.**

The transcriptomic and proteomic analyses of PASS4 grown on DNA identified a broad range of genes whose expression might be regulated by DNA including many virulence factors. However, it did not provide any clear answers as to the molecular basis of metabolic specialisation in PASS4. To further investigate the specialisation of PASS4 for growth on DNA, we screened mutants of PASS4 to isolate strains that had broader substrate utilisation capabilities. To obtain such mutants, we inoculated *P. aeruginosa* PASS4 on minimal M9 medium containing 20 mM N-Acetyl-D-

glucosamine (GlcNAc), a carbon source that PASS4 cells were unable to utilise (Fig. 3.1). Within 7 days, spontaneous mutant colonies were observed and subsequently cultured into liquid M9 minimal salts medium containing 20 mM GlcNAc. This culture was consecutively subcultured for 14 days until cells reached an $OD_{600}$=0.7 within 35 hours. At the end of this process, we obtained eight *P. aeruginosa* PASS4 mutants which had gained the ability to grow on GlcNAc.

The eight mutants displayed much broader carbon catabolic profiles than the parental PASS4 strain when tested on Biolog Phenotype Microarray plates. All of these mutants had gained the ability to utilise a wide range of carbon sources including amino acids, carbohydrates and carboxylic acids (Fig. 3.8). The substrate utilisation profiles of the mutants resembled those of most *P. aeruginosa* strains, including PAO1, PA14 and PASS1. This dramatic change in carbon utilisation capability suggests that the mutation(s) in these PASS4 mutants had apparently circumvented the genetic specialisation in the parental PASS4 isolate that allowed it to grow well on DNA, but not on most other carbon sources.

**PASS4 mutants have mutations in the *purK* gene.**

Genome sequencing was undertaken on the Miseq v2 platform of eight PASS4 mutants that had gained the ability to utilise GlcNAc and an array of other carbon sources. In parallel, we also resequenced the PASS4 genome as a reference. Bioinformatic analysis identified single nucleotide polymorphisms (SNPs) between the mutants and the PASS4 genome. All of the PASS4 mutants contained a mutation in the *purK* gene, located at codon 214 (for PASS4 mutants 2 and 3) or codon 354 (for PASS4 mutants 1 and 4 to 8) (Table 2, Fig. 3.9). Additional SNPs were also detected in mutants 1 and 7, in a chemotaxis transducer (*pctB*) and a hypothetical protein, respectively. The *purK* gene encodes a subunit of the phosphoribosylaminoimadazole carboxylase enzyme, a critical step in the *de novo* synthesis of purines (47). This suggests that PASS4 is defective in purine biosynthesis, which explains why it grows well only on DNA and purines, while growing poorly

or not at all on essentially all other tested carbon sources, as would be expected for a purine auxotroph.

To test whether the inability of PASS4 to grow on various carbon sources was due to a defect in purine biosynthesis, we repeated the Biolog phenotype testing but including supplementation with 2 mM adenosine. This concentration of adenosine was not sufficient as a sole carbon source to support the growth of PASS4. The supplementation of 2 mM adenosine enabled PASS4 to grow on a wide range of carbohydrates, amino acids, and carboxylic acids that it previously was unable to utilise (Fig. 3.8). This provides further evidence that the underlying growth defect in PASS4 is due to a defect in *de novo* purine biosynthesis. Since all of the PASS4 mutants had changes within the *purK* gene, it is highly likely that PurK is defective in PASS4. Future studies involving the complementation of a non-defective *purK* gene into the parental PASS4 strain with subjection to Biolog phenotype testing may provide complete evidence of its role in purine auxotrophy of PASS4.

The CF lung environment contains high concentrations of eDNA. The defect in purine biosynthesis in PASS4, coupled with an ability to grow faster on DNA as a sole carbon source compared with other *P. aeruginosa* strains, probably reflects a niche adaptation to this DNA-rich environment. Species from the genera *Chlamydia* and *Rickettsia*, the parasitic flagellate protozoa *Trypanosoma*, *Treponema pallidum*, *Mycoplasma*, *Ureaplasma*, *Mesoplama*, *Borrelia*, and even *Lactobacillus*, all appear to exist without the presence of the classical *de novo* purine nucleotide biosynthesis pathway (47). These organisms are either obligate parasites or are associated with mucosal epithelial layers of the host (47). Scavenging purines or nucleic acids from their host may be a common adaptation in many parasites, pathogens and commensals.

The PASS4 strain showed modest growth on asparagine as a sole carbon source. Indeed, this compound was used as a growth nutrient in the transcriptomic and proteomic experiments in this study. Asparagine is required for the conversion of inosine monophosphate to adenylo-succinate, in the first enzymatic step in dATP synthesis after *de novo* purine biosynthesis (Fig. 3.10). The

ability of PASS4 to grow on asparagine as a sole carbon source could be due to high levels of asparagine enabling sufficient flux through the purine biosynthesis pathway to allow growth. In turn, this suggests a decrease in the efficiency of the PurK enzyme in PASS4, rather than a complete loss of function.

## 3.5 CONCLUSIONS

Phenotypic analysis revealed that the *P. aeruginosa* CF isolate PASS4 was only able to grow on a limited range of carbon sources, including purines and DNA. On these substrates it showed higher growth rates than other *P. aeruginosa* isolates. This suggested that this strain is specialised to live on eDNA available in the CF lung. We investigated the genetic basis of this apparent metabolic specialisation. Transcriptomic and proteomic studies of *P. aeruginosa* PASS4 and PAO1 grown in DNA revealed that eDNA affected the expression of many genes, particularly virulence and host-adaptation genes. This suggests that eDNA may be an important signal in the CF lung for expression of virulence factors by *P. aeruginosa*.

The transcriptomic and proteomic expression analyses did not provide any direct insights into the metabolic specialisation of PASS4. Genome sequencing of PASS4 mutants that had gained the ability to catabolise GlcNAc, as well as a wide range of unrelated carbon sources, indicated that they all contained mutations in the *purK* gene, which encodes a key enzyme in the *de novo* purine biosynthesis pathway. This suggested that PASS4 was a purine auxotroph, and this was confirmed by phenotypic testing showing addition of 2 mM adenosine could rescue growth of PASS4 on a broad range of carbon sources. Purine auxotrophy may represent a viable microbial strategy for adaptation to DNA-rich environments such as the CF lung.

## 3.6 MATERIALS AND METHODS

### *Pseudomonas aeruginosa* strains and media.

*P. aeruginosa* strains used for this study were PAO1 (48), PA14 (49) and PASS1-4 (18). The PASS1-4 isolates were obtained from the sputum of CF patients at the Westmead Hospital

(Sydney, Australia). The *P. aeruginosa* isolate PASS1 was obtained from a 40-year old female patient, PASS2 from a 27-year old male, PASS3 from a 23-year-old male and PASS4 from a 23-year old female (18, 50). These isolates were maintained in glycerol stocks at -80°C. *P. aeruginosa* isolates were routinely cultured on Luria Bertani (LB) media, solid or liquid, from the frozen stock, allowing minimum passages during cultivation.

**Production of exoenzyme DNase.**

The test for the production of exoenzyme DNase by *P. aeruginosa* PAO1 and PASS4 was conducted with a use of DNase test agar (Oxoid). Briefly, as per manufacturer's instructions, the strains were streaked on a DNase test agar plate and incubated at 37°C for 24 hours. The DNase test agar contained tryptose, DNA and NaCl. The production of DNase leads to the hydrolysis of DNA in the media. Therefore, following incubation, the plates were flooded with 1N HCl to observe for a clearing/hydrolysis of DNA by the bacterium.

**RNA extraction and RNA-Seq transcriptomics.**

*P. aeruginosa* strains PAO1 and PASS4 were simultaneously inoculated into 5 ml of LB liquid medium from the frozen stock and grown overnight. Overnight cultures were used to inoculate 15 ml of M9 minimal medium supplemented with either L-Asparagine (20 mM) or DNA (1.5 mg/ml) (Salmon sperm DNA, Sigma), both in biological triplicates. Cultures were grown until mid-exponential phase ($OD_{600}$ = 0.6). RNA was extracted from these cultures using the miRNEasy RNA extraction kit (Qiagen) according to the manufacturer's protocol. To remove any residual genomic DNA, the samples were treated with DNAse using the TurboDNAse kit (Invitrogen). The quality and quantity of extracted RNA was assessed on a Nanodrop spectrophotometer. To remove highly abundant ribosomal RNA from the RNA extracts before sequencing, the samples were treated using RiboZero GN Magnetic rRNA depletion kit (Epicentre). The rRNA depleted samples were purified using RNeasy MinELute Cleanup kit (Qiagen) and re-assessed on Nanodrop and submitted to the Ramaciotti Centre for Genomics for paired-end RNA Sequencing on the HiSeq

2000 platform. Paired-end RNA Sequence files obtained from Ramaciotti were assessed for quality using FastQC software (Babraham Bioinformatics) and processed by trimming the first 10 nucleotides using Fastx Toolkit. Trimmed sequences were tiled against the complete genome of PAO1 via EdgePro software (51) and differential expression calculated using DESeq software (52). *De novo* transcript assembly and differential gene expression analysis was performed for PASS4 strain using the Rockhopper 2.03 tool (53, 54) to supplement the data on PASS4 genes absent in PAO1 genome. The RNA-seq raw data reported here are accessible under the Gene Expression Omnibus submission accession number GSE100287.

**Protein extraction and label-free shotgun proteomics.**

*P. aeruginosa* strains PAO1 and PASS4 were inoculated into M9 Minimal salts media with 100 µM calcium chloride ($CaCl_2$) and 2 mM magnesium sulphate ($MgSO_4$) containing either L-Asparagine (20 mM) as a control condition or DNA (1.5 mg/ml) as an experimental condition. Cultures were grown aerobically ($n$=3) at 37°C with constant shaking at 200 rpm. Overnight cultures were subcultured into the same respective medium, prior to harvesting cells in logarithmic phase ($OD_{600}$ = 0.6). For proteomic analysis, cells were harvested from 60 ml of culture by centrifugation at 3220 *g*, 10 min at 4°C (Beckman centrifuge, USA). Cells were washed with phosphate buffered saline (pH 7.4) and stored at -80°C until further processing. Proteins were extracted by lysing cell pellets with sodium dodecyl sulphate (SDS) lysis buffer (2.3% w/v SDS, 0.12 M Tris, 0.4 mM EDTA, 4% w/v glycerol and 0.05% v/v β-mercaptoethanol (pH 6.8). Followed by four 30 s rounds of bead beating at 5.5 m/sec (FastPrep FP120, USA), with intermittent cooling. Cellular debris was removed by centrifugation at for 10,000 *g* for 10 min (Eppendorf, model 5804R) and the supernatants were collected and stored at -20°C for further processing. The extracted proteins were precipitated using a methanol/chloroform/water protocol (55). Resultant proteins pellets were resuspended in 1% w/v SDS, 13% w/v glycerol and 33 mM Tris (pH 6.8) and quantitated using a BCA Protein assay kit as per manufacturer's instructions (Thermo Fisher Scientific, USA). 30 µg of each sample was diluted with sample loading buffer,

(1% w/v SDS, 13% w/v glycerol and 33 mM Tris, 20 mM β-mercaptoethanol, 0.004% bromophenol blue (pH 6.8)), denatured by boiling (95°C, 4 min) and separated using SDS-PAGE (Bio-Rad, Australia). After electrophoresis, proteins were visualised using colloidal Coomassie Blue and processed further for tryptic digestion as detailed in Mirzaei et al (2012). Briefly, each gel lane corresponding to individual sample was cut into 16 pieces, chopped and placed into a well of a 96-well plate. The gel pieces were briefly washed with 100 mM $NH_4HCO_3$, followed by washing twice with acetonitrile (ACN) (50%)/100 mM $NH_4HCO_3$ (50%) for 10 min. Finally, gel pieces were dehydrated with 100% ACN and air-dried. Proteins were reduced with 10 mM dithiothreitol (DTT) in $NH_4HCO_3$ (50 mM) at 37°C for 1h, followed by alkylation with 50 mM iodoacetamide in $NH_4HCO_3$ (50 mM), in the dark at RT for 1h. Samples were then washed with 100 mM $NH_4HCO_3$, followed by ACN (50%)/100 mM $NH_4HCO_3$ (50%) for 10 min, dehydrated with 100% ACN and then air-dried. Finally, samples were digested with 20 μL of trypsin (12.5 ng/mL 50 mM $NH_4HCO_3$), overnight at 37°C. Proteolytic peptides were extracted twice with ACN (50%)/formic acid (2%), dried using a vacuum centrifuge and reconstituted to 10 μL with 2% formic acid for LC-MS/MS analysis.

Peptides were analysed using a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer coupled to a high pressure liquid chromatography unit (Thermo Scientific, USA). Peptides were separated on a 60-min reverse phase gradient of 1-50% solvent B (acetonitrile in 0.1% formic acid) gradient. In each data collection cycle, one full MS scan (350-2000 m/z) was recorded in the Orbitrap. Subsequently, MS2 analysis was conducted for top 10 most intense ions and were fragmented by higher-energy collisional dissociation (HCD) with following settings; normalized collision energy of 30%, isolation window 3.0 m/z, maximum ion accumulation time 60 ms with a dynamic exclusion for 10 seconds.

Protein identification and quantification was performed on Proteome Discoverer 1.3 (Thermo Fisher Scientific) using Mascot search engine (56). *P. aeruginosa* strain PAO1 protein sequence database retrieved from GenBank (January 2013) and *Pseudomonas* Genome Database

(www.pseudomonas.com) and *in-silico* translated genome databases of PASS4 (18) were used as the search databases. Database searching against the decoy database was also performed to evaluate the false discovery rate (FDR) of peptide identification. All searches were performed using a static modification for cysteine alkylation and methionine oxidation, acetylation (protein N-term) as dynamic modifications, precursor ion tolerance of 10 ppm and a fragment ion tolerance of 0.02 Da were used. Peptide matches were filtered with peptide and protein FDR<1%. Then for each identified peptide, its abundance (peak area) was estimated by calculating the area under the extracted ion chromatograms (XIC) curve.

The mass spectrometry proteomics data has been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (57) with the dataset identifier PXD006742.

For statistical analyses, log-transformed quantitative values were used to conduct two-sample *t*-tests comparing protein expression of PASS4 or PAO1 in DNA relative to L-Asparagine using the in-house developed program based on R modules (58). Proteins with log fold change ±1 and *t*-test *p*-value < 0.05 were considered to be significantly differentially expressed.

**Functional analysis.**

The transcripts and proteins were mapped to virulence factor annotations and to cluster of orthologous groups (COG) categories obtained from the Pseudomonas Database (59).

**Generation of *P. aeruginosa* PASS4 mutants.**

Single colonies of *P. aeruginosa* strain PASS4 were inoculated into 10 ml of LB liquid medium, in triplicate, and grown overnight with constant shaking of 200 rpm at 37°C. Overnight cultures were centrifuged at 4000 *g* for 7 minutes at 4°C in an Eppendorf centrifuge Model 5430. Once the supernatant was discarded the cells were washed with phosphate buffered saline and spun at 4000 *g* for 7 minutes at 4°C. The cells were then resuspended in 5 ml phosphate buffered saline and 2.04 × $10^8$ washed cells were spread on 20 mM GlcNAc. Once spontaneous mutant colonies were obtained, colonies were cultured into M9 minimal salts medium with 100 uM calcium chloride

($CaCl_2$) and 2 mM magnesium sulphate ($MgSO_4$) containing 20 mM GlcNAc. This culture was consecutively subcultured till cells were able to reach an $OD_{600}$=0.7 within ~35 hours.

**Whole genome sequencing and SNP analysis of PASS4 mutants.**

Single colonies of 8 *P. aeruginosa* strain PASS4 mutants and the parental PASS4 strain were inoculated into 10 ml of LB liquid medium, in triplicates, from LB agar and grown overnight with constant shaking of 200 rpm at 37°C. DNA was extracted from the samples according to DNA Isolation DNeasy Blood and Tissue Kit protocol for Gram-negative bacteria (QIAGEN). Total DNA was quantified using the Nanodrop and submitted to the Ramaciotti Centre for Genomics for paired-end DNA Sequencing on MiSeq v2 platform. Paired-end DNA sequence files obtained from Ramaciotti were assessed for quality using FastQC software (Babraham Bioinformatics). The pair-end reads for each sample were merged by FLASH-1.2.11 (60). The merged reads were filtered with the FASTQ quality filter with minimum quality score being 20, and 90 being the minimum percent of bases with a quality score of 20. The reads were then trimmed with fastq quality trimmer ensuring minimum length of sequence of 200 and quality thresholds of nucleotide of 28. The fastq files were converted to fasta and then subjected to SNP analysis according to the user guide of kSNP3 software (61).

**Growth of *P. aeruginosa* PASS4 in adenosine.**

To identify the lowest concentration of adenosine as a sole carbon source that supported growth of PASS4, growth assays were undertaken in liquid media in M9 minimal salt medium with 100 uM calcium chloride ($CaCl_2$) and 2 mM magnesium sulphate ($MgSO_4$) supplemented with 0.02 mM – 20 mM adenosine using a broth dilution method essentially as previously described (62). Cell growth was determined spectrophotometrically.

**Phenotype microarray.**

Biolog Phenotype analysis was carried out for *P. aeruginosa* parental strain PASS4 and 8 PASS4 mutants using PM1 and PM2A MicroPlate™ Carbon Sources (Biolog, USA) containing a total of

190 substrates (carbohydrate, carboxylic acid, amino acid, fatty acid, amine, alcohol, polymer, amide and ester) and a negative control for each plate (19). Bacterial cell suspensions (absorbance of 0.07 at 600 nm) were prepared in the inoculating fluid (IF-0a, Biolog, USA) and 100 ul of the inoculum was dispensed into each well of the plate using a multichannel pipette. After inoculation, the plates were incubated in the OmniLog incubator/reader (Biolog) for 48 hours at 37˚C. Cell respiration was recorded every 15 minutes by a charge-coupled device camera. The changes in the colour of inoculated wells due to the conversion of the tetrazolium dye present in the wells into the purple derivative during cell respiration, were plotted over the whole period of incubation yielding kinetic curves representative of the metabolic activity of the strain in the presence of a particular carbon source. Raw values were imported from the OmniLog reader for heatmap generation. For the adenosine-supplemented experiments, *P. aeruginosa* PASS4 was suspended in inoculating fluid supplemented with 2 mM adenosine before dispensing 100 µl to each well of the PM1 and PM2A plates (Biolog, USA), which were subjected to Biolog Phenotype MicroArray analysis as described above.

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We confirm that we have no conflicts of interest.

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## 3.10 FIGURES

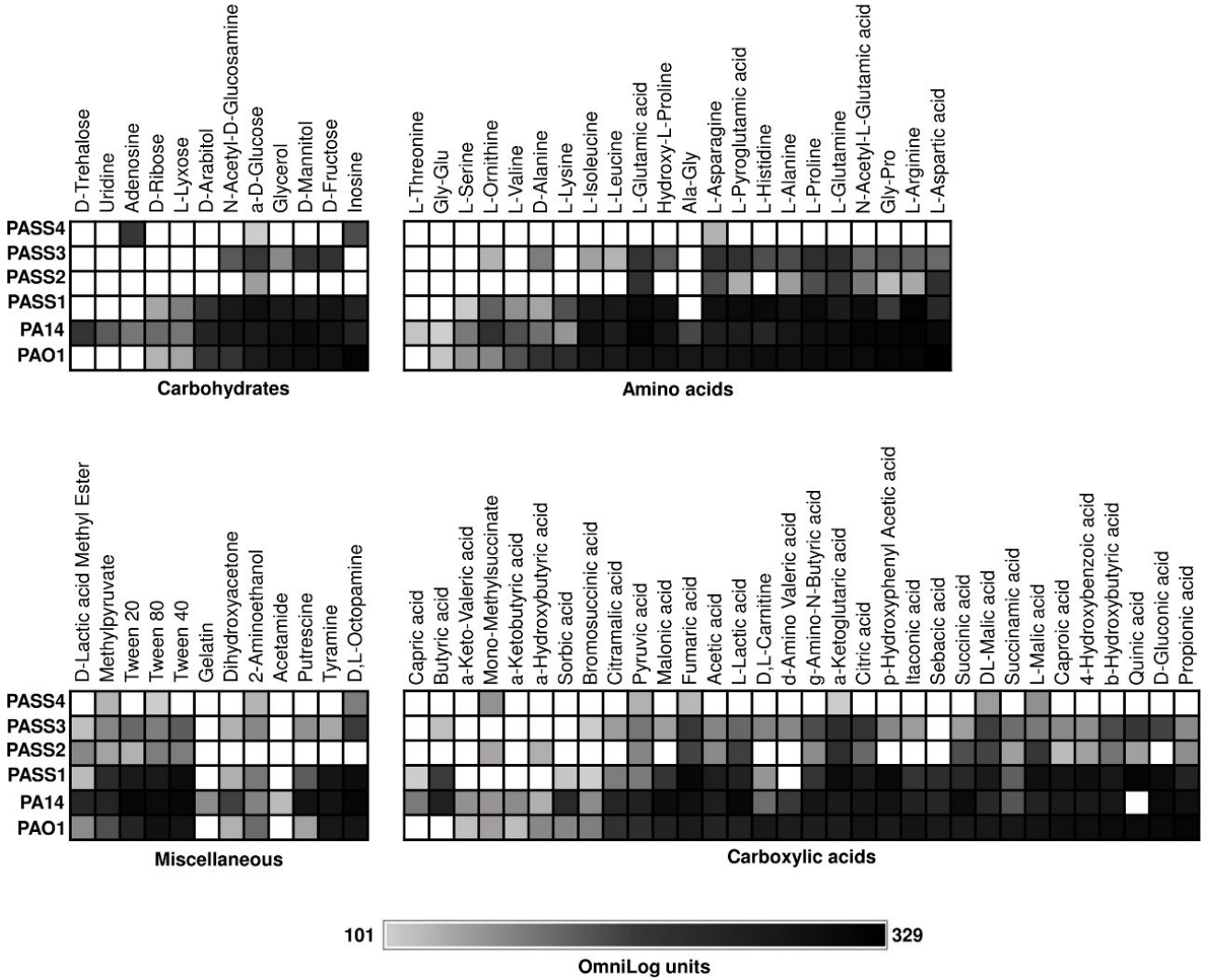


**FIG 3.1** The catabolic phenome of *P. aeruginosa* wild-type strains. The maximal kinetic curve height of *P. aeruginosa* wild-type strains is expressed as a greyscale ranging from 101 (grey) to 329 (black) OmniLog units following growth at 37°C for 48 hours. Phenotypes < 101 OmniLog units (white) showed no detectable respiration.

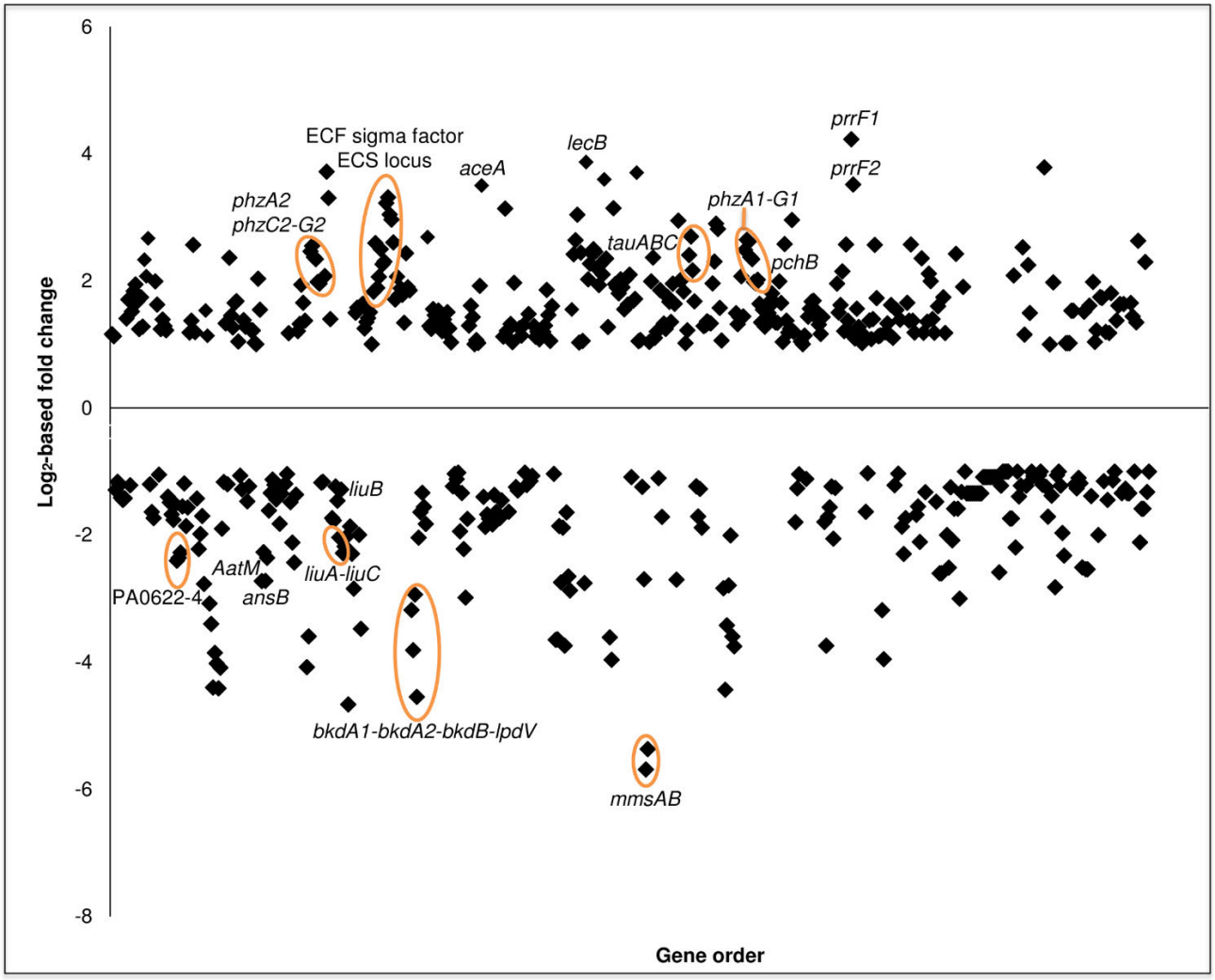


**FIG 3.2** Differential gene expression in *P. aeruginosa* PASS4 cells grown in DNA compared to cells grown in asparagine. Each dot represents a gene within the *P. aeruginosa* PASS4 genome (x-axis) and its fold-change ($log_2$) expression in DNA. Only significantly differentially expressed genes are shown (*P* value < 0.01, $log_2$ fold-change 1< to < -1).

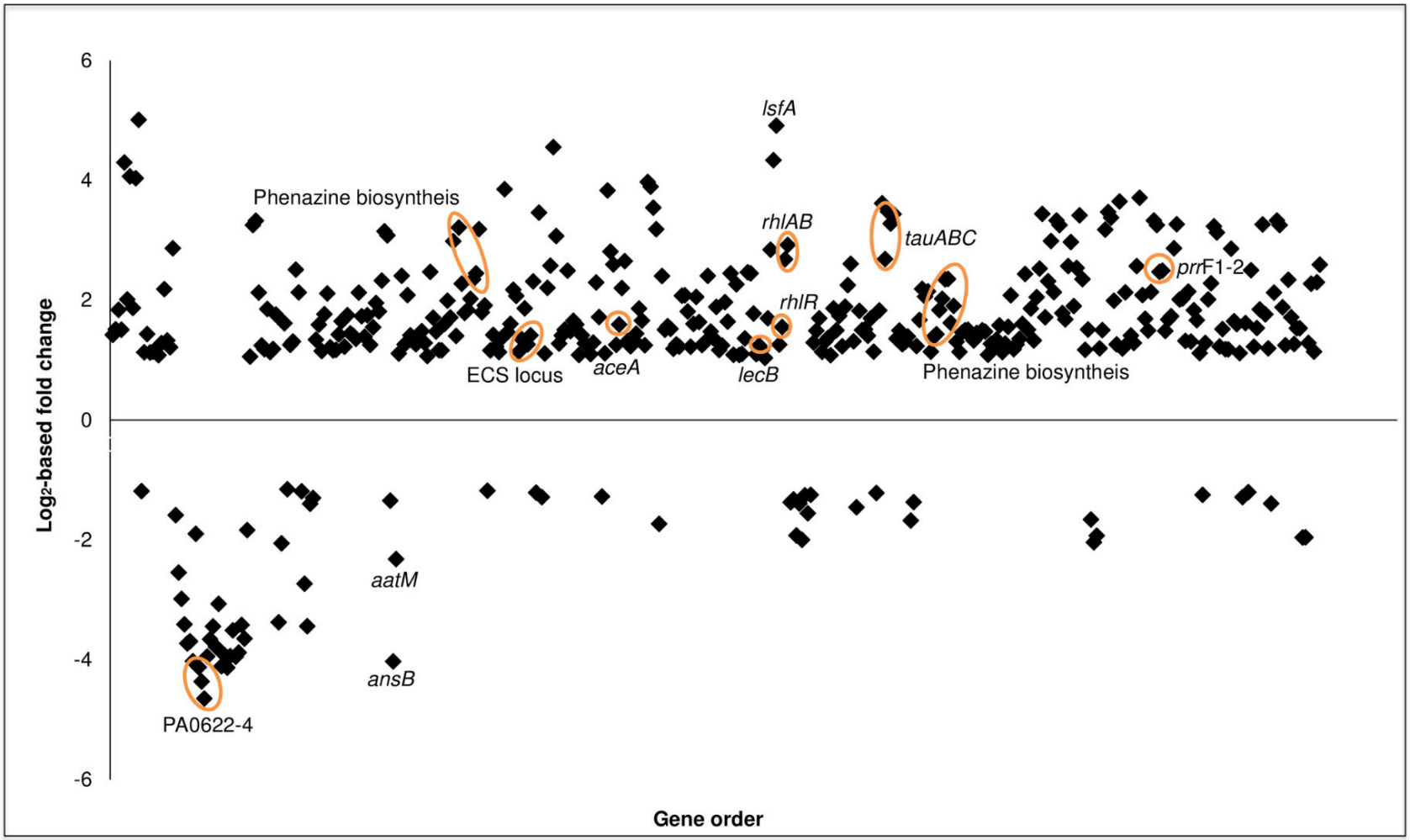


**FIG 3.3** Differential gene expression in *P. aeruginosa* PAO1 cells grown in DNA compared to cells grown in asparagine. Each dot represents a gene within the *P. aeruginosa* PAO1 genome (x-axis) and its fold-change ($log_2$ scale on the y-axis) expression in DNA. Only significantly differentially expressed genes are shown *(P* value < 0.01, $log_2$ fold-change 1< to < -1).

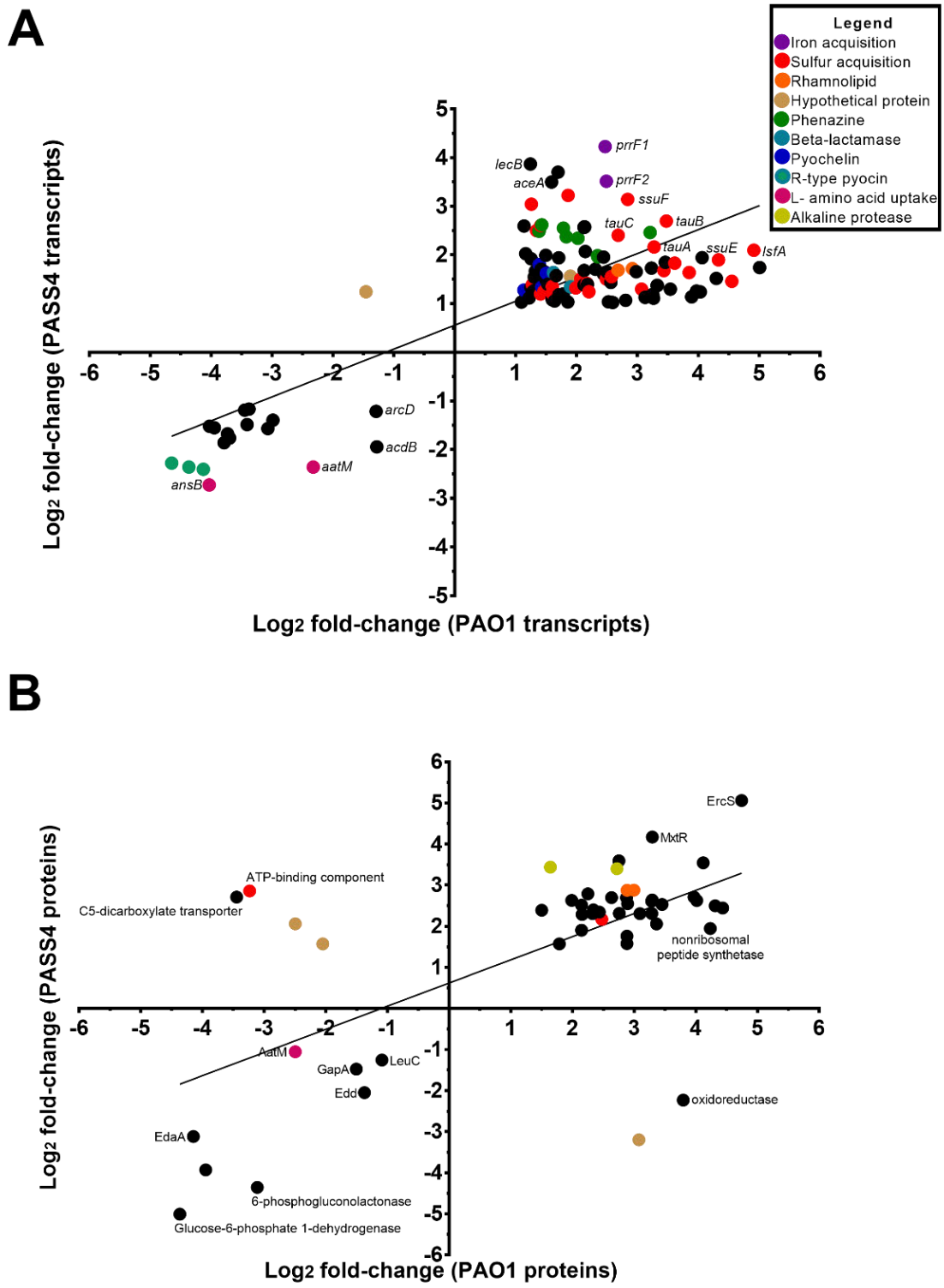


**FIG 3.4** Comparison of the changes in expression at a transcript and protein level in *P. aeruginosa* PASS4 and PAO1 following growth in DNA; growth in asparagine was used as a reference. (A) The chart shows $\log_2$ fold changes of transcript data from RNA-sequencing ($P < 0.01$, $\log_2$ fold-change 1< to <-1). Correlation of PASS4 and PAO1 transcript data was moderately similar ($R^2$=0.5983). (B) The chart shows log2 fold changes of shotgun protein data ($P < 0.05$, $\log_2$ fold-change 1< to <-1). Correlation of PASS4 and PAO1 protein data was moderately similar ($R^2$=0.4072).

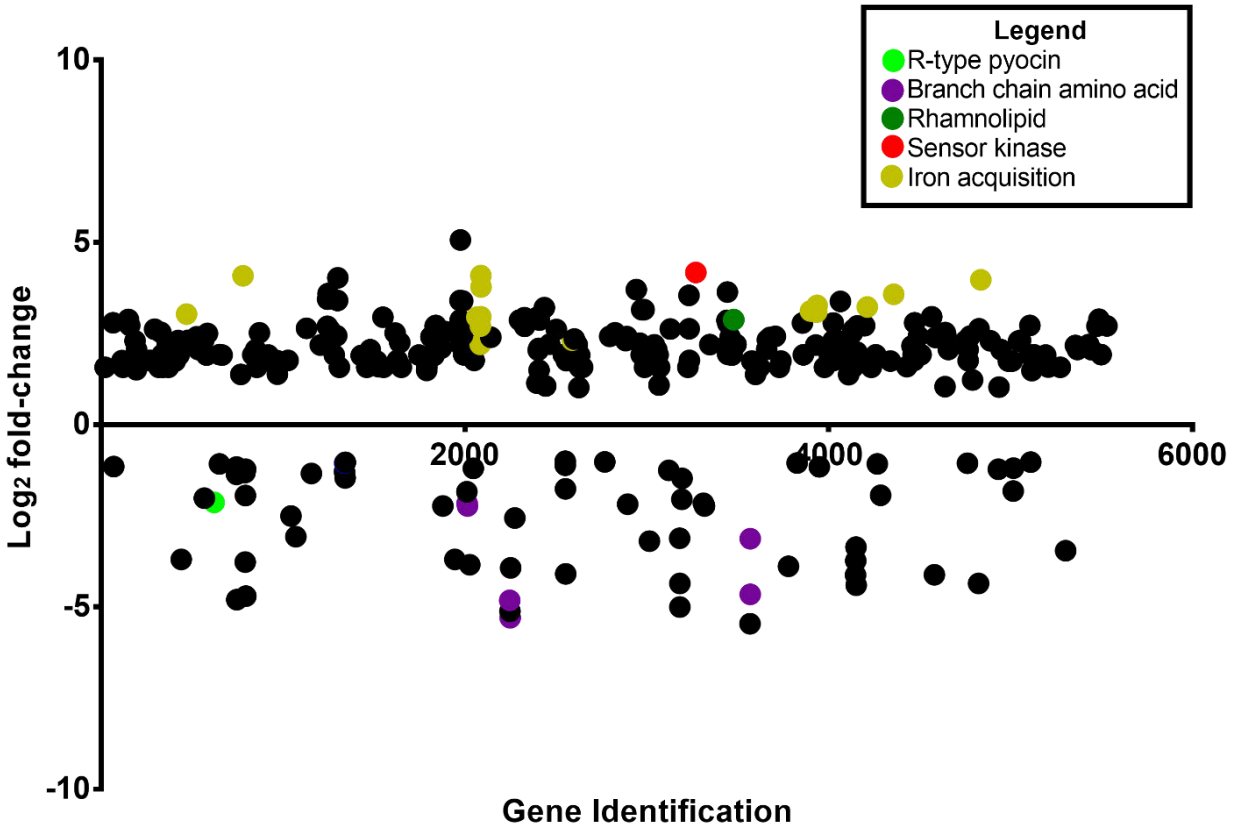


**FIG 3.5** Protein expression in *P. aeruginosa* PASS4 cells grown in DNA compared to cells grown in asparagine. Each dot represents a protein within the *P. aeruginosa* PASS4 genome (x-axis) and its fold-change ($log_2$ scale on the y-axis) expression in DNA. Only significantly differentially expressed genes are shown (*P* value < 0.05, $log_2$ fold-change 1< to < -1).

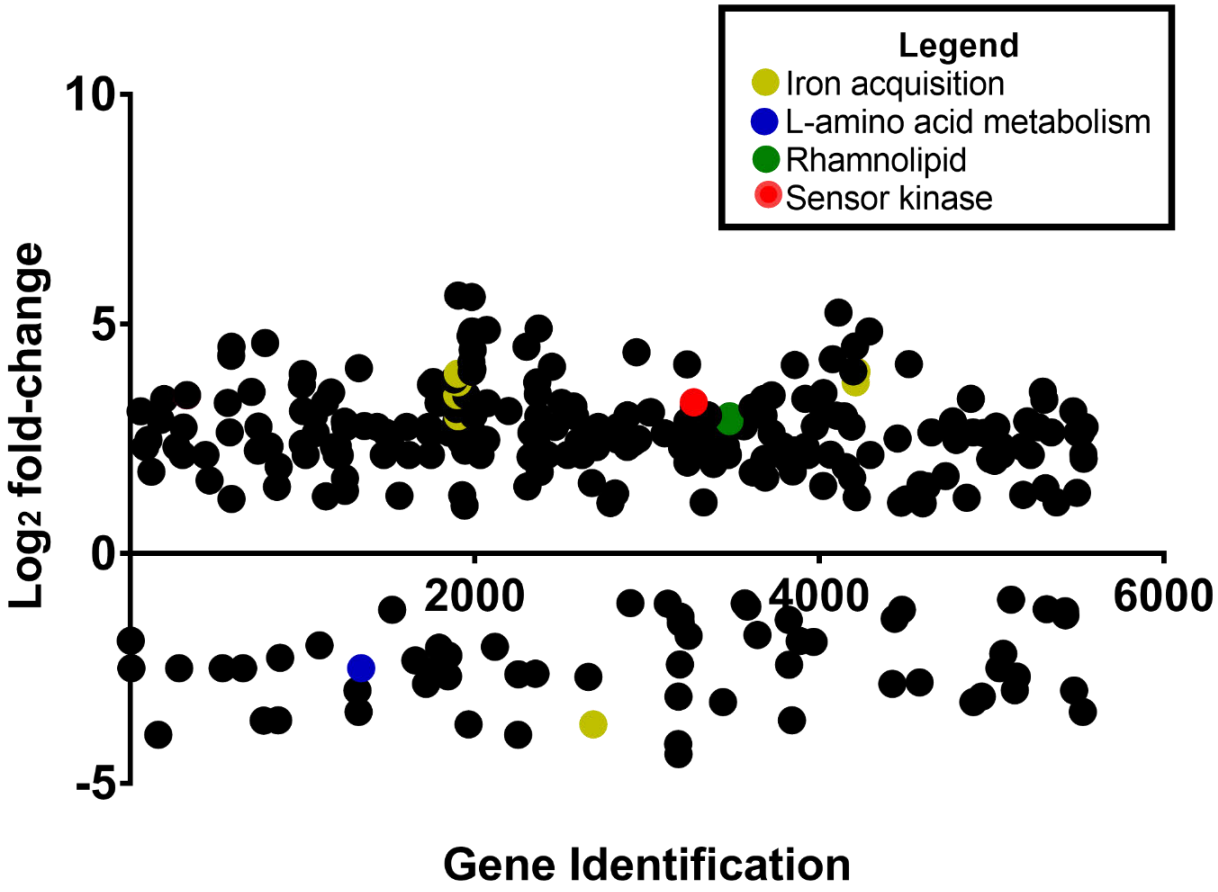


**FIG 3.6** Protein expression in *P. aeruginosa* PAO1 cells grown in DNA compared to cells grown in asparagine. Each dot represents a protein within the *P. aeruginosa* PAO1 genome (x-axis) and its fold-change ($log_2$ scale on the y-axis) expression in DNA. Only significantly differentially expressed genes are shown (*P* value < 0.05, $log_2$ fold-change 1< to < -1).

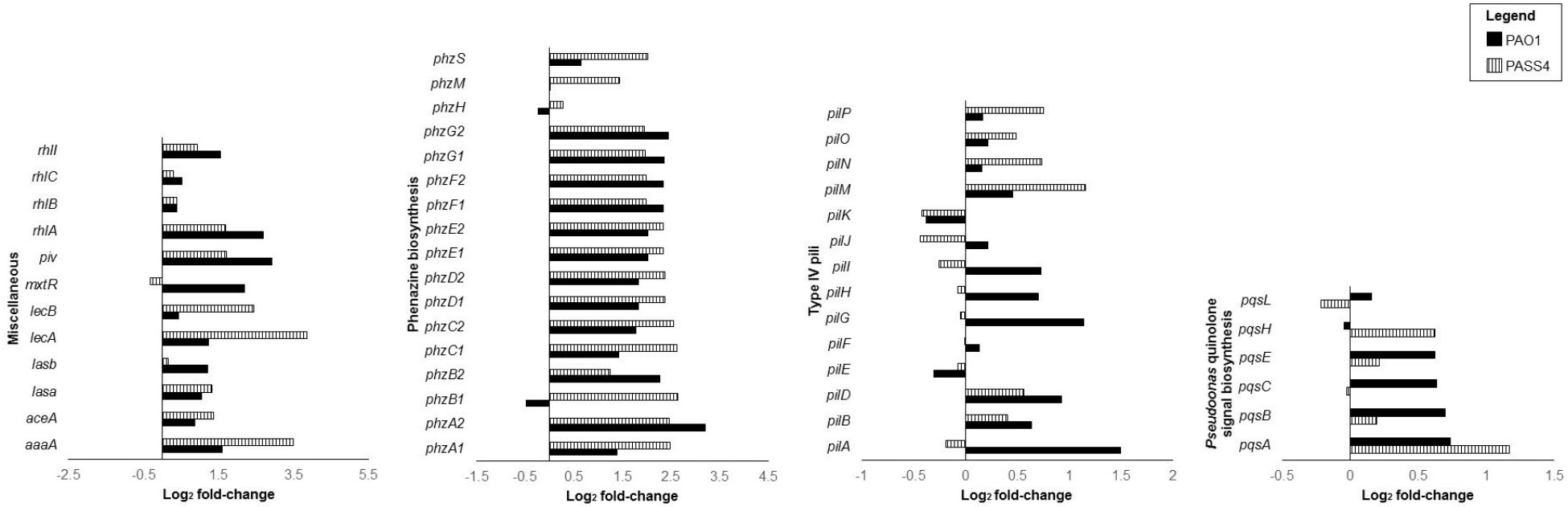


**FIG 3.7** Differential gene expression ($log_2$ fold-change) of virulence genes when *P. aeruginosa* strains PASS4 and PAO1 were grown in DNA compared to asparagine.

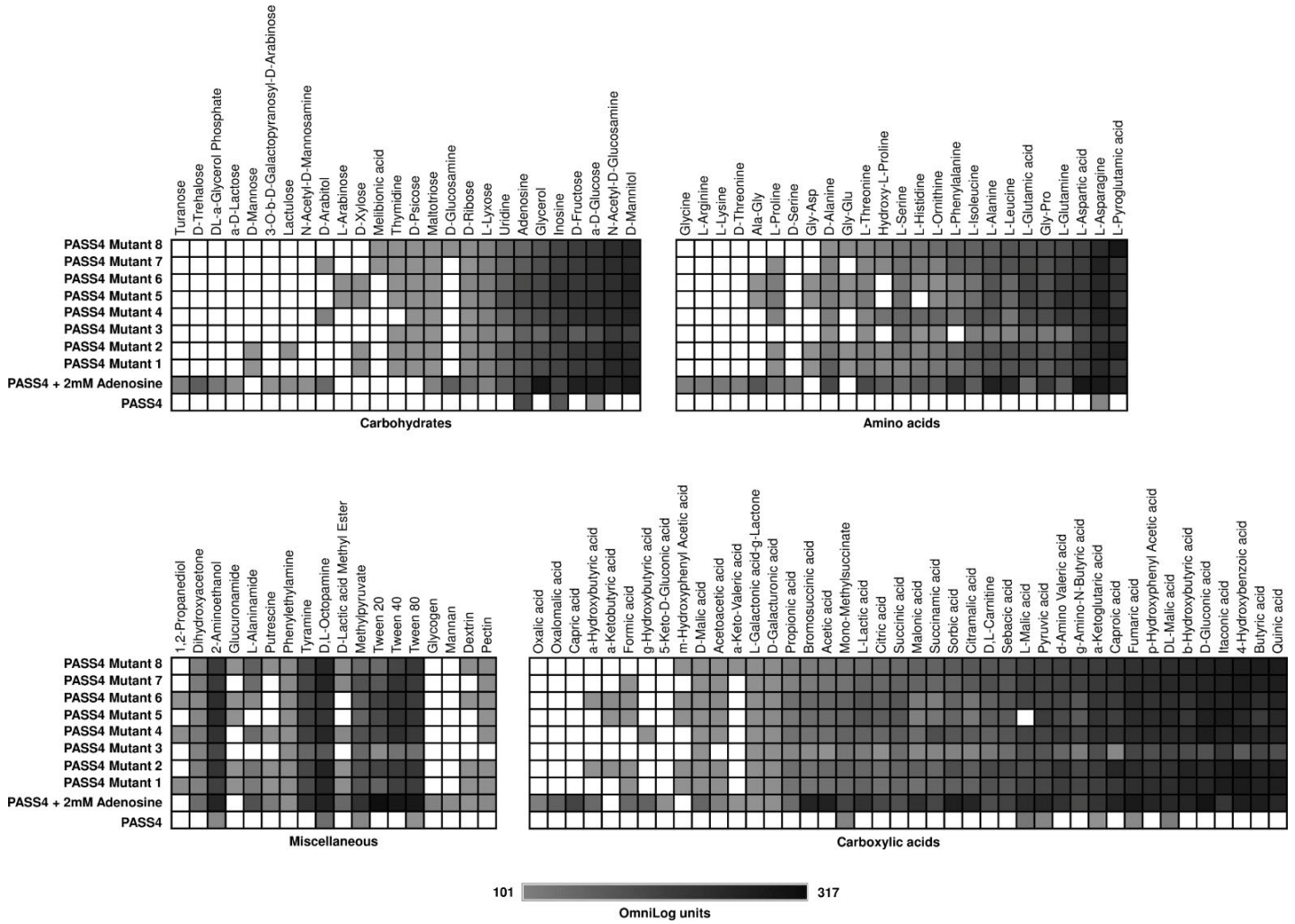


**FIG 3.8** The catabolic phenome profile of *P. aeruginosa* PASS4 mutants, PASS4 supplemented with 2mM adenosine and *P. aeruginosa* PASS4. The maximal kinetic curve height difference to *P. aeruginosa* wild-type strain PASS4 is expressed as greyscale ranging from 101 (grey) to 317 OmniLog units (black). White represents no detectable respiration.

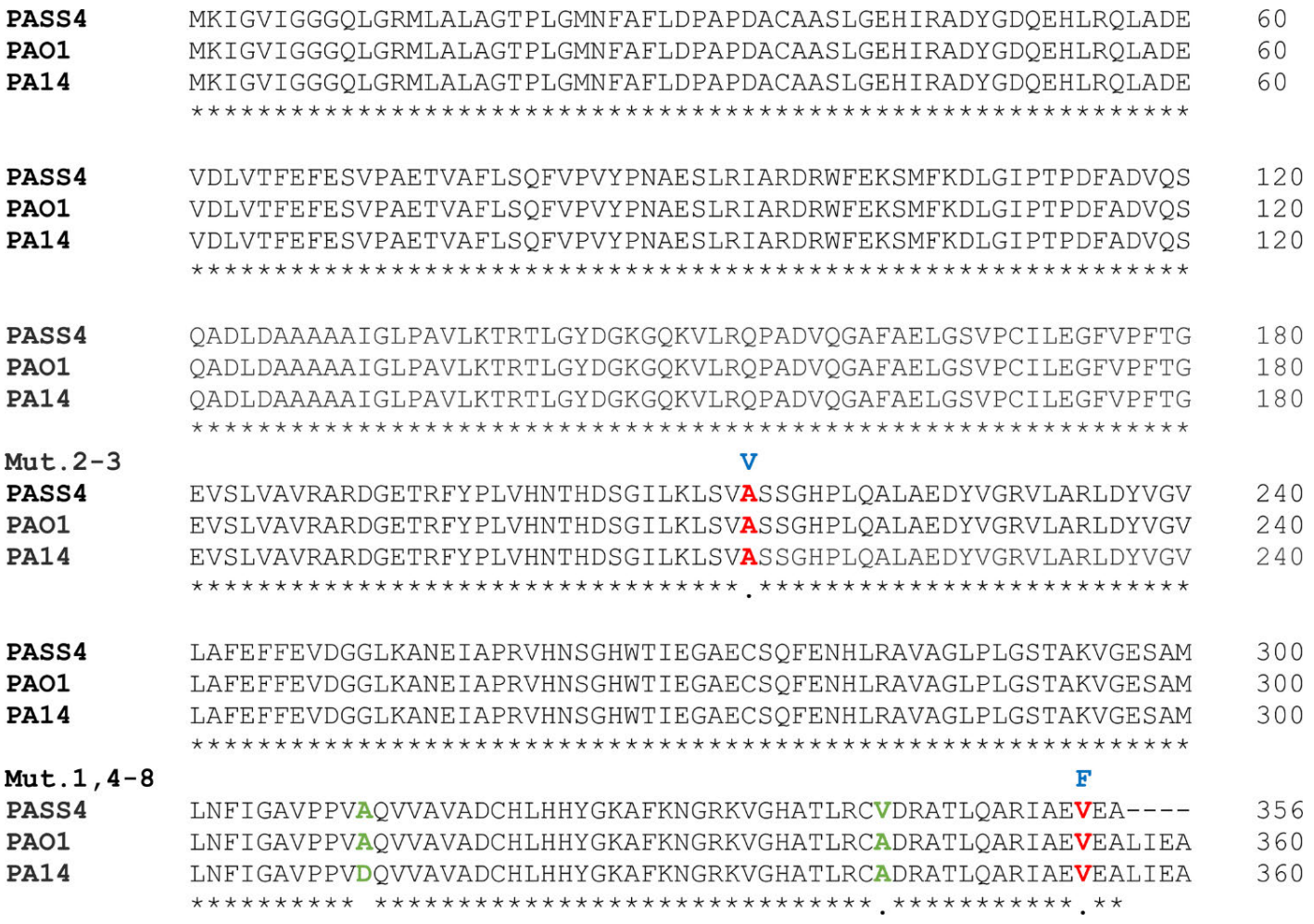


**FIG 3.9** Amino acid sequence alignment of the predicted amino acid sequences from the *purK* gene in *P. aeruginosa* PASS4, PAO1 and PA14. Non-identical residues are coloured green. The location of altered amino acid residues for the PASS4 mutants selected for growth on N-Acetyl-D-Glucosamine are shown above the alignment in blue, with the corresponding residue in the alignment highlighted in red.


**FIG 3.10** Graphic overview of the super-pathway of the *de novo* purine biosynthetic pathway in *P. aeruginosa*. The PASS4 mutants selected for growth on N-Acetyl-D-Glucosamine all possessed mutations in the *purK* gene, which together with its protein product are highlighted in red in this figure

## 3.11 TABLES

**Table 3.1.** Summary of *P. aeruginosa* PAO1 and PASS4 mapped reads following growth in DNA or asparagine

| Condition | Replicate | Paired reads | Percentage aligned |
|---|---|---|---|
| **PAO1 grown in Asparagine** | 1 | 14532003 | 98.80 |
| | 2 | 11886289 | 98.77 |
| | 3 | 12821286 | 98.95 |
| **PAO1 grown in DNA** | 1 | 13805330 | 80.69 |
| | 2 | 12869272 | 89.53 |
| | 3 | 12660666 | 89.75 |
| **PASS4 grown in Asparagine** | 1 | 11436333 | 97.95 |
| | 2 | 13152398 | 97.08 |
| | 3 | 11153482 | 97.96 |
| **PASS4 grown in DNA** | 1 | 11846213 | 92.70 |
| | 2 | 11599891 | 91.39 |
| | 3 | 12826627 | 86.20 |

**TABLE 3.2** SNP mutations in *P. aeruginosa* PASS4 mutants

| Mutant | PASS4 Wild-type | PASS4 Mutant | Position (codon) | Gene Identification | Gene | Product |
|---|---|---|---|---|---|---|
| **1** | G | T | 354 | AOA76_RS03255 | *purK* | Phosphoribosylaminoimidazole carboxylase |
| **1** | G | A | 584 | AOA76_RS29575 | *pctA/pctC* | Chemotaxis transducer |
| **2** | G | A | 214 | AOA76_RS03255 | *purK* | Phosphoribosylaminoimidazole carboxylase |
| **3** | G | A | 214 | AOA76_RS03255 | *purK* | Phosphoribosylaminoimidazole carboxylase |
| **4** | G | T | 354 | AOA76_RS03255 | *purK* | Phosphoribosylaminoimidazole carboxylase |
| **5** | G | T | 354 | AOA76_RS03255 | *purK* | Phosphoribosylaminoimidazole carboxylase |
| **6** | G | T | 354 | AOA76_RS03255 | *purK* | Phosphoribosylaminoimidazole carboxylase |
| **7** | G | A | 1279/ 1853 | PA1874 | | Hypothetical protein |
| **7** | G | T | 354 | AOA76_RS03255 | *purK* | Phosphoribosylaminoimidazole carboxylase |
| **8** | G | T | 354 | AOA76_RS03255 | *purK* | Phosphoribosylaminoimidazole carboxylase |

# 4

# *Pseudomonas aeruginosa* inhibits the growth of *Scedosporium aurantiacum*, an opportunistic fungal pathogen isolated from the lungs of cystic fibrosis patient

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ORIGINAL RESEARCH
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# *Pseudomonas aeruginosa* inhibits the growth of *Scedosporium aurantiacum*, an opportunistic fungal pathogen isolated from the lungs of cystic fibrosis patients

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The filamentous fungus *Scedosporium aurantiacum* and the bacterium *Pseudomonas aeruginosa* are opportunistic pathogens isolated from lungs of the cystic fibrosis (CF) patients. *P. aeruginosa* has been known to suppress the growth of a number of CF related fungi such as *Aspergillus fumigatus, Candida albicans,* and *Cryptococcus neoformans*. However, the interactions between *P. aeruginosa* and *S. aurantiacum* have not been investigated in depth. Hence we assessed the effect of *P. aeruginosa* reference strain PAO1 and two clinical isolates PASS1 and PASS2 on the growth of two clinical *S. aurantiacum* isolates WM 06.482 and WM 08.202 using solid plate assays and liquid cultures, in a synthetic medium mimicking the nutrient condition in the CF sputum. Solid plate assays showed a clear inhibition of growth of both *S. aurantiacum* strains when cultured with *P. aeruginosa* strains PASS1 and PAO1. The inhibitory effect was confirmed by confocal microscopy. In addition to using chemical fluorescent stains, strains tagged with yfp (*P. aeruginosa* PASS1) and mCherry (*S. aurantiacum* WM 06.482) were created to facilitate detailed microscopic observations on strain interaction. To our knowledge, this is the first study describing successful genetic transformation of *S. aurantiacum*. Inhibition of growth was observed only in co-cultures of *P. aeruginosa* and *S. aurantiacum*; the cell fractions obtained from independent bacterial monocultures failed to initiate a response against the fungus. In the liquid co-cultures, biofilm forming *P. aeruginosa* strains PASS1 and PAO1 displayed higher inhibition of fungal growth when compared to PASS2. No change was observed in the inhibition pattern when direct cell contact between the bacterial and fungal strains was prevented using a separation membrane suggesting the involvement of extracellular metabolites in the fungal inhibition. However, one of the most commonly described bacterial virulence factors, pyocyanin, had no effect against either of the *S. aurantiacum* strains. This study shows that *P. aeruginosa* has a substantial inhibitory effect on the growth of the recently described CF fungal pathogen *S. aurantiacum*. The findings also highlighted that *P. aeruginosa* biofilm formation is important but not crucial for inhibiting the growth of *S. aurantiacum* in a lung- mimicking environment.

**Keywords: co-culture, *S. aurantiacum*, *P. aeruginosa*, interactions, growth inhibition, phenazines, SCFM, biofilms**

## Introduction

Cystic fibrosis (CF) is one of the most common, potentially lethal, genetically inherited disorders affecting mainly the European Caucasian population (O'Sullivan and Freedman, 2009). Although the disease affects a number of organs and systems in the human body, lungs remain the main site of infection in CF patients (Quinton, 1999). The inherited condition stems from the mutation of the CF transmembrane conductance regulator (CFTR) gene, which regulates the transport of chloride ions across the plasma membrane of the epithelial cells (Boucher, 2007). Impaired ion exchange reduces the mucociliary clearance, which leads to accumulation of hyper-viscous mucus in the airway surfaces, thus providing ideal conditions for the growth of microorganisms (Delhaes et al., 2012). Various molecular and microbiology based approaches have revealed the polymicrobial nature of the infections in CF with the identification of complex microbiota including bacteria, fungi, and viruses (Lynch and Bruce, 2013). Most of these microorganisms are either acquired from the environment or through contact with other infected patients (Lipuma, 2010).

Bacteria constitute the major portion of the microorganisms associated with CF. The most common bacterial inhabitants of the CF airways include *Haemophilus influenzae*, *Staphylococcus aureus*, *Pseudomonas Aeruginosa,* and *Burkholderia cepacia* complex (BCC) (Harrison, 2007; Lipuma, 2010). Among them, *P. aeruginosa* is the most dominant bacterial species known to cause chronic respiratory infections in more than 50% of adult CF patients (Coutinho et al., 2008). *P. aeruginosa* is a ubiquitous Gram-negative bacterium possessing a wide variety of pathogenicity factors to evade the host defense system (Davies, 2002). During the early stages of infection, the bacterium attaches itself to lung epithelial cell surface receptors through specific adhesins and secretes extracellular products to prolong its survival in the CF airways (Tang et al., 1995). The extracellular products secreted by *P. aeruginosa* include enzymes such as elastase and alkaline protease, exotoxins, siderophores, and phenazines such as pyocyanin with a known role in virulence (Haas et al., 1991). Moreover, *P. aeruginosa* cells form biofilms in order to proliferate inside the lungs and protect themselves from antibiotic agents (Singh et al., 2000).

In addition to bacteria, some fungal species are also known to colonize the respiratory tracts of CF patients (Cimon et al., 2000; Pihet et al., 2009). Mycological examination of the specimens obtained from CF patients have shown that *Aspergillus fumigatus* is the most predominant fungal colonizer of the CF lungs as it has been recovered from 6 to 71% of CF patients (Bakare et al., 2003; Horre et al., 2010). However, the presence of non-*Aspergillus* fungal species often remains unnoticed owing to the lack of sensitive culture techniques to examine the sputum specimens from CF patients (Delhaes et al., 2012). Recently, a more targeted approach has been developed by combining molecular techniques with laboratory culture methods, which can now identify a wide range of fungal pathogens in the expectorated sputa (Middleton et al., 2013). Studies conducted on CF patients in Australia and certain parts of Europe have confirmed the emergence of a new fungal genus *Scedosporium* (originally called *Pseudallescheria*) that causes infections in the lungs of immunocompromised hosts (Blyth et al., 2010a; Paugam et al., 2010; Lackner et al., 2014). *Scedosporium* sp. have been isolated from the sputum specimens of 14.7–17.4% of Australian CF patients which makes it the second most common fungal respiratory pathogen associated with CF (Blyth et al., 2010a,b). *Scedosporium aurantiacum* is a recently identified, highly virulent member of the *Scedosporium* sp. complex recovered from one in six CF patients in Sydney (Heath et al., 2009; Blyth et al., 2010b; Harun et al., 2010). The clinical consequences of the *S. aurantiacum* colonization or infections in the CF patients remain to be explored (Harun et al., 2010).

According to the clinical reports, the prevalence of fungi in the respiratory tracts of CF patients is mainly affected by the bacteria present, and the interactions between the bacteria and fungi potentially impact the disease outcome (Sibley et al., 2006; Chotirmall et al., 2010; Leclair and Hogan, 2010). Several *in vitro* studies have reported an inhibitory effect of *P. aeruginosa* against the common lung co-inhabitants such as *A. fumigatus* or the yeasts *Candida albicans,* and *Cryptococcus neoformans* (Hogan and Kolter, 2002; Bandara et al., 2010; Cugini et al., 2010). Similar data for *S. aurantiacum* are lacking. Reflecting the increasing importance of *S. aurantiacum* in CF, we examined the effect of clinical *P. aeruginosa* CF isolates PASS1 and PASS2 and laboratory reference strain PAO1 on the growth of two clinical *S. aurantiacum* isolates WM 06.482 and WM 08.202 using solid plate assays and liquid co-cultures containing medium that mimics the nutritional content of human CF sputum (Palmer et al., 2007).

## Materials and Methods

### Growth and Maintenance of Strains

Strains used in the study are listed in **Table 1**. *P. aeruginosa* PASS1 and PASS2 were isolated from the sputum samples of CF patients (Penesyan et al., under review). A common laboratory 'reference' strain PAO1 (Lewenza et al., 2014) was also included in the study. *S. aurantiacum* strains WM 06.482 and WM 08.202 were obtained from the culture collection of the Medical Mycology Research Laboratory, Centre for Infectious Diseases and Microbiology, Westmead Hospital, Sydney, NSW, Australia (Kaur et al., 2015). Virulence levels of all *P. aeruginosa* strains used in this study have been tested previously using *Caenorhabditis elegans* based infection model (Lewenza et al., 2014; Penesyan et al., under review). Virulence studies of *S. aurantiacum* have been performed using *Galleria mellonella* larvae model (Kaur et al., 2015).

*Pseudomonas aeruginosa* strains were revived from frozen stocks stored at −80°C by streaking on LB (Luria Bertani, Sigma) plates and incubation overnight at 37°C. Bacterial colonies were inoculated into LB broth and incubated at 37°C on an orbital shaker (200 rpm) overnight. Following fractions were prepared from overnight cultures of the *P. aeruginosa* strains: (1) Heat killed cells were obtained by incubating 1 ml of an overnight cell culture at 80°C for 60 min. Absence of any viable cells was confirmed by plating on LB agar medium; (2)

**TABLE 1 | *Pseudomonas aeruginosa* and *Scedosporium aurantiacum* strains used in the study.**

| Strain | Strain name | Source | Virulence level | Reference |
|---|---|---|---|---|
| PASS1 | *P. aeruginosa* | Sputum sample of a cystic fibrosis (CF) patient Sydney, NSW, Australia | High | Penesyan et al. (under review) |
| PASS2 | *P. aeruginosa* | Sputum sample of a CF patient Sydney, NSW, Australia | Low | Penesyan et al. (under review) |
| PAO1 (ATCC 15692) | *P. aeruginosa* | Wound exudate Melbourne, VIC, Australia | High | Holloway (1955) |
| WM 06.482 | *S. aurantiacum* | Invasive clinical isolate from CF patient Sydney, NSW, Australia | High | Kaur et al. (2015) |
| WM 08.202 | *S. aurantiacum* | Type strain from a wound exudate Santiago de Compostela (Spain) | Low | Kaur et al. (2015) |

Cell lysates were obtained after sonicating the cells (50 ml) on ice for 10 min in an ultrasonic processor followed by collection of the supernatant after centrifugation at 10,000 × *g* for 30 min; (3) Cell culture supernatants were collected by centrifuging 50 ml of overnight cultures of *P. aeruginosa* strains at 10,000 × *g* for 30 min. Supernatants were then freeze dried and resuspended in 100 μl of 1x PBS and stored at 4°C until use.

Fungal strains were maintained on PDA (potato dextrose agar, BD, Difco™) plates at 37°C. After 5 days of growth, the conidia were scraped into sterile saline solution (0.9% w/v NaCl and 0.01% v/v Tween 80) and the suspension was filtered through a sterile cotton wool to separate the conidia from the hyphal debris. Conidia were washed with 1x PBS to remove traces of saline and the inoculum was adjusted to a McFarland standard concentration of $2.5 \times 10^5$ conidia/ml. Concentration of conidia was confirmed using Neubauer counting chamber and additional plate counting.

## Construction of Strains Tagged with Fluorescent Proteins

### *Pseudomonas aeruginosa* Strain Expressing Yellow Fluorescent Protein (YFP)

Plasmid pUCP*yfp* (Gloag et al., 2013) encoding yellow fluorescent protein (YFP) was used to transform the *P. aeruginosa* PASS1 strain. In order to make electrocompetent cells, PASS1 was cultured in 5 ml of LB broth overnight at 42°C and 200 rpm. Cells were harvested by centrifugation (14,000 *g* for 15 min at 4°C) and the ionic strength of the suspension was reduced by rigorous washing with 1x M9 minimal salts medium (Sigma) followed by two washes with ice-cold sterile milliQ water. Bacterial cells were transformed by electroporation as described by Dower et al. (1988) by adding 1 μg of the plasmid DNA to 20 μl of the washed cell aliquots. At the end of the procedure, cells were streaked on LB plates containing 8 mg/ml ampicillin and incubated for up to 48 h at 37°C to select for the transformants.

### Construction of the *S. aurantiacum* Strain Expressing mCherry

The *mCherry* gene was PCR amplified from the pmcherry-c1 vector (Clontech Laboratories, USA) using *mCherry.fwd* and *mCherry.rev* primers (**Table 2**) and was expressed under the *Trichoderma reesei* pyruvate kinase (*pki*) promoter, which was amplified from the pCBH1corlin vector (Te'o et al., 2000) using *pki.fwd* and *pki.rev* primers. In addition, a DNA fragment featuring the *pki* promoter together with the hygromycin B resistance gene (*pki-hph*) was PCR amplified using primer *pkihph.fwd* and *pki-hph.rev* to allow selection of transformants. The fragments were engineered to contain restriction sites as needed (**Table 2**).

**TABLE 2 | Sequence of primers used for the construction of transformation cassettes.**

| Primer name | Sequence (5′–3′) |
|---|---|
| *mCherry. fwd* | **GAA GAACCT CTT AAC CTC TAG (*pki* sequence)** ATG GTG AGC AAG GGC GAG G |
| *mCherry. rev* | CAT GCG GGT ACC (*Kpn***I**) CTA TTA CTT GTA CAG CTC GTC CAT GC |
| *pki. fwd* | TGC TGC GAT ATC (*Eco*R**V**) CTT AAG TTA G TA ACT AGT GGA TC |
| *pki.rev* | **CTC GCC CTT GCT CAC CAT (*mCherry* sequence)** CTA GAG GTT AAG AGG TTC TTC |
| *pki-hph. fwd* | TAC GCG GCG CGC C CT TAA G **(*Afl*II)** TT AG T AAC TAG TGG ATC |
| *pki-hph.rev* | CAT GCT AAG CTT **(*Hind*III)** CTA TTC CTT TGC CCG CGG AC |

*The primers contain engineered restriction sites shown in shading and the overlapping sequences are shown in bold.*

The primers *pki.fwd* and *mCherry.rev* were used to fuse the separately amplified *pki* and *mCherry* fragments in an overlap extension PCR as described by Thornton (2015). The fragment *pki-hph* was digested with restriction enzymes *Hind*III and *Afl*II (Fermentas, Thermo Scientific, USA) and fragment *pki-mcherry* was digested with *Eco*RV and *Kpn*I. The digested products *pki-hph* and *pkimcherry* were gel purified using QIAquick gel extraction kit (Qiagen, USA) and inserted into MCS-1 (multiple cloning site) and MCS-2 of the pETDuet-1 plasmid, respectively (Supplementary Figure S1). Finally, the purified vectors and inserts were ligated using T4 ligase (Fermentas, USA) at a 1:3 molar ratio for 2 h at room temperature. The final ligated vector (pETDuet-phpm) was introduced into *Escherichia coli* DH5α competent cells as described by Inoue et al. (1990). Selection of transformants was performed on LB agar plates containing ampicillin (100 μg/ml) and incubating at 37°C. Selected transformants were grown in 3 ml of LB and plasmid DNA was isolated using QIAprep Spin Miniprep kit (Qiagen, USA). The plasmid pETDuet-phpm was sequenced by AGRF, Sydney, NSW, USA to check sequence alignment of the inserted gene cassettes.

The pETDuet-phpm DNA was introduced into highly virulent *S. aurantiacum* WM 06.482 using protoplast-mediated transformation based on the method adopted from Penttilä et al. (1987) with modifications. The young hyphae obtained from an overnight culture of WM 06.482 on PDA plates with cellophane at 28°C were digested with 10 mg/ml of lysing enzyme from *T. harzianum* (Sigma–Aldrich, Australia) to obtain protoplasts which were then filtered through a sterile sintered glass filter (porosity 1). Osmotically stabilized protoplasts were transformed with 5 μg of plasmid DNA as described by Penttilä et al. (1987). Transformed protoplasts were mixed with 10 ml of molten agar (1.5% w/v $KH_2PO_4$, 0.5% w/v $NH_4SO_4$, 2% w/v glucose, 1 M sorbitol, pH 5.5) containing hygromycin B (410 U/ml) and overlayed onto PDA plates which were incubated at 28°C for 3–5 days. Hygromycin resistant colonies were restreaked onto fresh PDA plates containing hygromycin B (410 U/ml) for a second round of selection. Transformation efficiency was calculated as number of transformants per μg of plasmid DNA. Expression of the mCherry protein in selected transformants was confirmed using Fluoview FV1000 inverted confocal microscope (Olympus) with an excitation and emission wavelength 488/633 nm (HeNe).

## Growth Inhibition Assays

The effect of *P. aeruginosa* on the growth *of S. aurantiacum* was tested in different combinations on both solid and liquid growth media. Combinations of bacterial and fungal strains for the testing are presented in **Table 3**.

### Cross Streak Assay using Live Cells

The effect of bacteria on fungal growth was assessed using an agar plate method described by Kerr (1999), with slight modifications adopted from Chen et al. (2013). *P. aeruginosa strains* PASS1, PASS2, and PAO1; and *S. aurantiacum* strains WM 06.482 and WM 08.202, were cultured together on a synthetic cystic fibrosis medium (SCFM) that mimics the nutritional content of human CF sputum. SCFM contains average concentrations of ions, free amino acids, glucose, and lactate present in the CF sputum samples (Palmer et al., 2007). Solid SCFM agar plates were made with an addition of 2% w/v agar to liquid SCFM medium. A sterile cotton swab was used to draw a straight vertical line of *P. aeruginosa* cells ($1 \times 10^8$ CFU/ml=0.5 McFarland standard concentration) across the plate. At the same time, *S. aurantiacum* conidia ($2.5 \times 10^5$ conidia/ml = 0.5 McFarland standard concentration) were inoculated with a cotton swab horizontally across the upper part of the plate preventing any direct contact between fungi and bacteria. The plates were dried at room temperature for 15 min and incubated at 37°C. Digital photography was performed after 24 h to visualize the growth of both bacterial and the fungal strains tested on the plate.

### Disk Inhibition Method using Live Cells and Cell Fractions

Sterile filter paper disks (Whatman no. 1; Sigma–Aldrich), 7 mm in diameter, were impregnated with 20 μl of the *P. aeruginosa* PASS1, PASS2, and PAO1 cell fractions, i.e., cell lysates, cell culture supernatant and heat inactivated cells (see preparation in section 1.1) and placed on an SCFM plate that was freshly surface seeded with 100 μl ($2.5 \times 10^5$ conidia/ml) of *S. aurantiacum* conidia (WM 06.482 or WM 08.202). A suspension of live *P. aeruginosa* cells was included for comparison. The plates were incubated at 37°C for up to 3 days and observed at regular intervals for the appearance of any clear inhibition zones around the disks. Assays were repeated in three biological replicates. A relative inhibition index was calculated for each *P. aeruginosa* isolate by dividing the area of activity (difference between the area of the inhibition zone and area of the colony) by the area of the colony.

### Effect of Bacteria on the Fungal Growth in Liquid Co-cultures

Interactions between *P. aeruginosa* and *S. aurantiacum* were observed in liquid medium using both chemical fluorescent stains and genetically labeled strains of bacteria and fungi in a direct contact with each other. In case of fluorescently labeled co-cultures, $1 \times 10^8$ CFU/ml of *P. aeruginosa* PASS1, PASS2, and PAO1 and $2.5 \times 10^5$ conidia/ml of *S. aurantiacum* WM 06.482 and WM 08.202 were inoculated in 20 ml SCFM medium in 100 ml shake flasks and incubated for 24 h at 37°C on an orbital shaker at 150 rpm. Aliquots were taken on a sterile glass slide from the co-cultures after every 4 h, washed with 1x PBS and fixed using 2% v/v paraformaldehyde (Sigma–Aldrich). The co-cultures were stained with DNA specific Syto9 (0.6 μM) and mitochondria specific Mito-Tracker$^R$ Red FM (25 nM) for 15 min in the dark as per the manufacturer's protocol (Molecular Probes, Life Technologies). Bacterial cells were expected to stain with Syto9 whereas fungal cells would stain with Mito-Tracker$^R$ Red FM. Fixed specimens were imaged using Fluoview FV1000 inverted confocal microscope (Olympus) with an excitation and emission wavelength of 488 nm (Ar) and 633 nm (HeNe).

The genetically tagged *P. aeruginosa* PASS1yfp strain and *S. aurantiacum* WM 06.482mCherry strain were also cultured together in 20 ml of SCFM for 24 h at 37°C, shaking at 150 rpm. At the end of the incubation period, cells were washed and fixed on sterile glass slides as above. Imaging was performed with a confocal microscope using an excitation and emission

**TABLE 3 | Types of cultures used to investigate the effect of different *P. aeruginosa* strains on *S. aurantiacum*.**

| Type of co-culture | *P. aeruginosa* strains | *S. aurantiacum* strains |
|---|---|---|
| Solid plate (cross streak, disk inhibition assay) | PASS1, PASS2, PAO1 | WM 06.482, WM 08.202 |
| Liquid cultures (chemical fluorescent dyes) | PASS1, PASS2, PAO1(stained with Syto9) | WM 06.482, WM 08.202 (stained with Mito tracker FR) |
| Liquid culture (genetically tagged strains) | PASS1 (*yfp*-labeled) | WM 06.482 *(mCherry*-labeled) |
| Liquid culture (addition of an antibiotic) | PASS1 (*yfp*-labeled) | WM 06.482 *(mCherry*-labeled) |

wavelength of 488 nm (blue laser diode for yfp) and 561 nm (yellow–green laser for mCherry) respectively. Liquid co-cultures with genetically labeled PASS1 and WM 06.482 strains were also repeated by adding different concentrations of gentamicin (2.5–10 mg/ml), which is a commonly used antibiotic against bacteria (Doring et al., 2000; Lin et al., 2011). Image analysis for both types of co-cultures was performed using IMARIS imaging software.

### Transwell Assay with Polycarbonate Membranes

In order to explore the role of secreted bacterial metabolites on the fungi, *P. aeruginosa* strains PASS1, PASS2, and PAO1 and *S. aurantiacum* strains WM 06.482 and WM 08.202 were co-cultured in SCFM in sterile six-well Transwell plates (Corning) with polycarbonate cell culture inserts (0.4 μm, Sigma–Aldrich) in order to prevent direct contact between the fungal and bacterial strains. *P. aeruginosa* ($1 \times 10^8$ CFU/ml) and *S. aurantiacum* ($2.5 \times 10^5$ conidia/ml) were inoculated in the bottom and top of the membrane insert, respectively. The plates were incubated at 37°C for 24 h and any inhibition of the growth of *S. aurantiacum* was measured as a difference in the dry weight of the *S. aurantiacum* cultured with or without *P. aeruginosa*. The method of calculating dry weight was adopted from Kaur et al. (2015).

### Effect of Phenazines

Phenazines were extracted from all *P. aeruginosa* strains (PASS1, PASS2, and PAO1) cultured in 5 ml of LB (in three biological replicates) for 2 days at 37°C using chloroform according to a method described by Mavrodi et al. (2001). Crude phenazine extracts were dried under reduced pressure to remove the solvent, resuspended in 80% acetonitrile (ACN) and applied to the filter paper disks (Whatman paper no. 1). The presence of pyocyanin in the crude phenazine extracts was confirmed using Ultra High Performance Liquid Chromatography (UHPLC) as described by Penesyan et al. (under review). While the absolute concentration of pyocyanin in the crude phenazine extracts from the three *P. aeruginosa* strains was not known, major experimental discrepancy was minimized by using same amount (20 μl) of crude extracts for the testing. The effect of these crude extracts on the fungal growth was determined using a disk inhibition assay (as described in section Disk Inhibition Method using Live Cells and Cell Fractions) where disks containing 20 μl of phenazine extracts from different *P. aeruginosa* isolates were air-dried and placed on SCFM agar plates freshly spread with $2.5 \times 10^5$ conidia/ml of *S. aurantiacum* strains (WM 06.482 and WM 08.202). The activity of blank 80% ACN, blank LB medium extract and the solution of commercial pyocyanin (10 mM, Sigma–Aldrich) were also tested against *S. aurantiacum* for comparison. Plates were incubated for 48 h at 37°C and observed for the presence of clearing zones around the filter paper disks as an indication of inhibitory activity of the extracts on fungal growth.

### Statistical Analysis

Statistical significance between the means of different experimental datasets was analyzed using two-tailed Student's *t*-test. SD with *p*-value less than 0.05 was considered significant. All experiments were performed in biological triplicates.

## Results

### Inhibition of *S. aurantiacum* Growth by Live *P. aeruginosa* Cells

When *S. aurantiacum* strains WM 06.482 (high virulence) and WM 08.202 (low virulence) were cross streaked against three different *P. aeruginosa* isolates PAO1, PASS1, and PASS2 on the SCFM agar medium, an area of inhibition was observed in the growth of both *S. aurantiacum* strains after 24 h (**Figures 1A–F**). It was evident from the size of the inhibition area that the bacterial strains had lesser impact on the highly virulent *S. aurantiacum* strain WM 06.482 (**Figures 1A,B**) compared to the less virulent WM 08.202 strain (**Figures 1D,E**). Out of the three bacterial strains studied, *P. aeruginosa* PASS2 had the weakest inhibitory effect on the *S. aurantiacum* strains in the plate test as seen in **Figures 1C,F**.

### Effect of *P. aeruginosa* Cell Extracts on *S. aurantiacum*

The effect of different cell fractions, i.e., the culture supernatant and cell lysate, and heat inactivated cells of *P. aeruginosa* strains PASS1, PASS2, and PAO1 was further tested on the growth of *S. aurantiacum* WM 06.482 and WM 08.202 using the disk inhibition method. Following 48 h incubation, clear inhibition zones were observed on plates inoculated with living cells of *P. aeruginosa* PASS1 and the reference strain PAO1 and their respective cell lysates. The inhibitory effect of *P. aeruginosa* was expressed as a relative inhibition index (**Figure 2**).

Living cells of both PAO1 and PASS1 and their corresponding cell lysates displayed a higher inhibitory activity against the less virulent *S. aurantiacum* strain WM 08.202 compared to the high virulence strain WM 06.482. Cell supernatants and heat killed *P. aeruginosa* cells failed to elicit a response against either of the fungal strains. In a separate experiment, the effect of *S. aurantiacum* was also tested against *P. aeruginosa* by incubating filter disks impregnated with *S. aurantiacum* conidia and cell fractions on the plates freshly seeded with *P. aeruginosa* cells. As *S. aurantiacum* failed to display any inhibition against *P. aeruginosa*, these interactions were not studied further (data not shown).

### Effect of *P. aeruginosa* on Fungal Physiology

*Pseudomonas–Scedosporium* interactions were also studied using confocal microscopy by imaging cellular aggregates from liquid co-cultures labeled with fluorescent stains. Confocal images demonstrated an inhibitory effect of the *P. aeruginosa* PASS1 (isolated from sputum of a CF patient) and the reference strain PAO1 on the growth and development of both *S. aurantiacum* strains tested (**Figures 3A–F**). In the course of 24 h, the bacteria had attached to the surface of fungal hyphae and formed biofilm-like structures containing a high density of bacterial cells but very few fungal hyphae. The tested bacterial strains had a weaker impact on the more virulent WM 06.482 compared

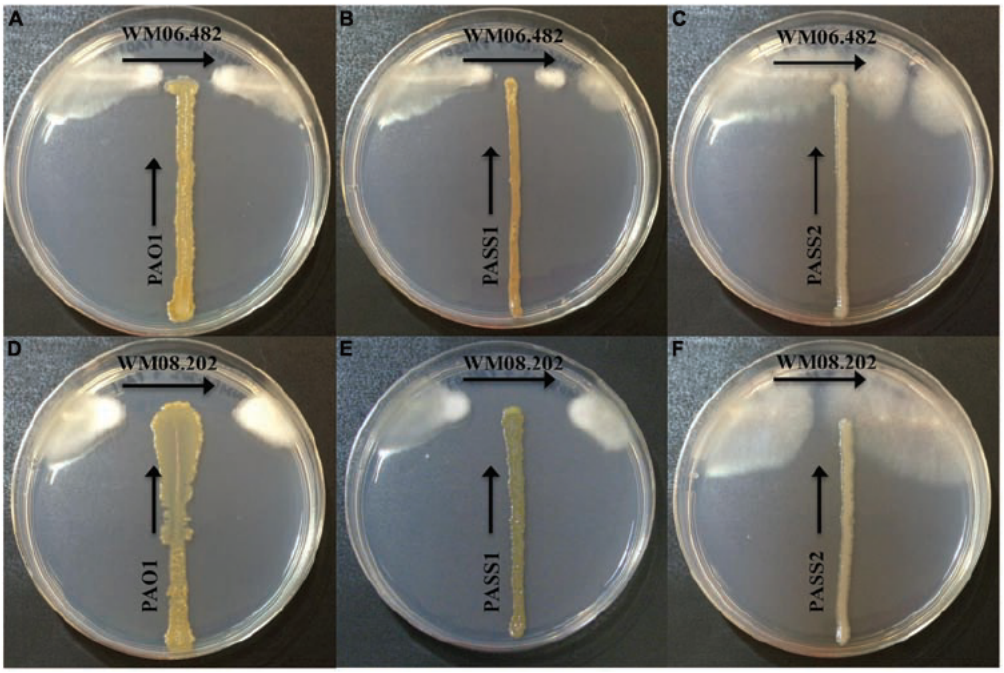


**FIGURE 1 | Cross-streak plate assay between different strains of *Pseudomonas aeruginosa* and *Scedosporium aurantiacum* on synthetic cystic fibrosis medium (SCFM) agar plates.** All bacterial strains were inoculated vertically whereas the fungal strains were streaked horizontally across the upper part of the SCFM agar plate. The plates were incubated at 37°C for 24–48 h. **(A–C)** Inhibition of *S. aurantiacum* strain WM 06.482 by PAO1, PASS1, and PASS2 strains of *P. aeruginosa*. **(D–F)** Inhibition of *S. aurantiacum* strain WM 08.202 by PAO1, PASS1, and PASS2 strains of *P. aeruginosa*.

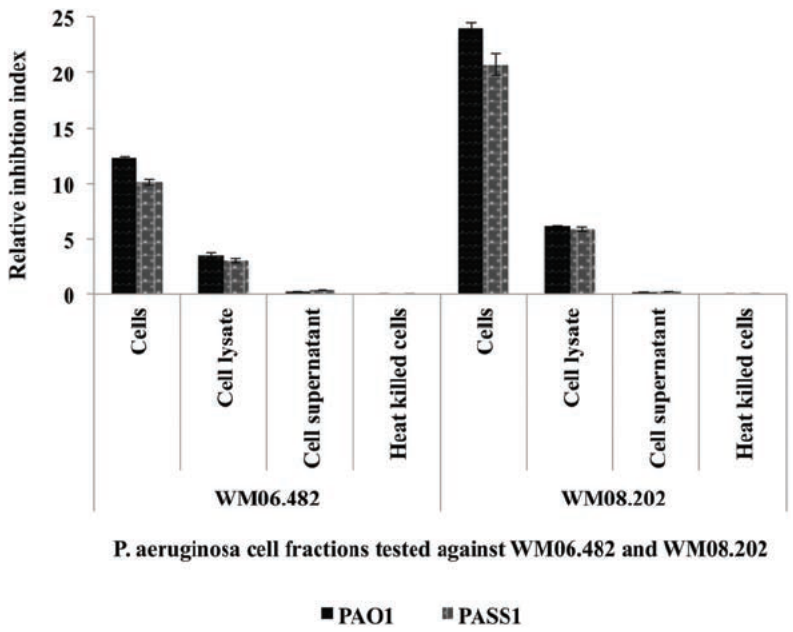


**FIGURE 2 | Susceptibility of *S. aurantiacum* (WM 06.482 and WM 08.202) to *P. aeruginosa* (PAO1, PASS1, and PASS2) and their cell lysate fractions.** Relative inhibition index was calculated as the average value of three replicates ($n = 3$) with a $p$-value $<0.05$ considered as significant.

to the less virulent WM 08.202, showing the resistant nature of the more virulent strain also highlighted in the plate tests. Although different fluorescent stains were used to distinguish between *P. aeruginosa* and *S. aurantiacum* in liquid cultures, it was difficult to visualize the detailed effect of bacteria on the fungal hyphae due to permeabilisation of the Syto9 dye by both

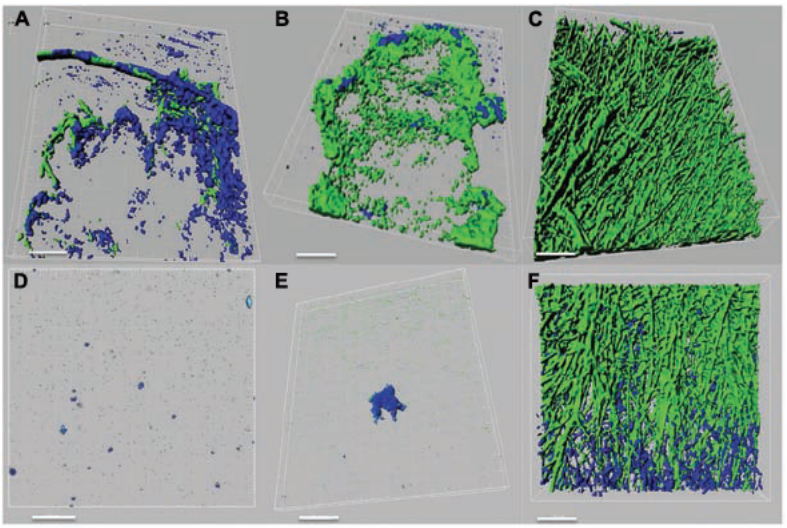


**FIGURE 3 | Confocal laser scanning microscope (CLSM) images of interactions between *P. aeruginosa* (PAO1, PASS1, and PASS2) and *S. aurantiacum* (WM 06.482 and WM 08.202) as observed after co-incubating both the organisms in SCFM liquid medium at 37°C for 24 h.** *P. aeruginosa* cells are stained with Syto9 (shown in green) and *S. aurantiacum* with Mito-tracker deep red FM (shown in blue). 3D re-construction of CLSM datasets was performed using IMARIS software package (Bitplane). Scale bar = 50 μm. **(A–C)** CLSM images of co-culture of WM 06.482 with PAO1, PASS1, and PASS2, respectively. **(D–F)** CLSM images of co-culture of WM 08.202 with PAO1, PASS1, and PASS2, respectively.

*P. aeruginosa* and *S. aurantiacum*. No growth inhibiting effect was observed when the fungal strains were co-cultured with PASS2 as indicated by dense growth of fungi in **Figures 3C,F**. These observations were also consistent with the results seen in the assays carried on plates.

## Interactions between Genetically Tagged *P. aeruginosa* and *S. aurantiacum* Strains

To circumvent the difficulty in differentiating between bacteria and fungi in liquid co-cultures, genetically tagged *P. aeruginosa* strain PASS1 expressing yfp and *S. aurantiacum* strain WM 06.482 expressing mCherry were developed. With this arrangement, it was observed that the bacteria started colonizing the fungal conidia soon after incubating them together in the SCFM (**Figure 4A**). Thus, within 8 h, bacteria began aligning themselves along the length of fungal hyphae as seen in **Figures 4B,C**. After 24 h, large clumps of *P. aeruginosa* cells were observed on *S. aurantiacum* hyphal filaments (**Figure 4D**), and the amount of hyphae was also reduced in number compared to the *S. aurantiacum* control without the bacteria (**Figure 4E**).

## Effect of Antibiotics used in Clinical Practice on Co-cultures

Analysis of the plate cultures and confocal images confirmed that *P. aeruginosa* had an inhibitory effect on the growth of *S. aurantiacum*. Therefore, in order to further validate this finding and to reveal the possible effect of antibiotic therapy on *S. aurantiacum* and *P. aeruginosa* mixed populations present in CF lungs, co-culturing was repeated with an addition of varying amounts of gentamicin (2.5–10 mg/ml) to selectively inhibit the growth of *P. aeruginosa*. *S. aurantiacum*-*P. aeruginosa* co-cultures were also maintained without the addition of gentamicin for comparison (**Figure 5A**). All bacteria were killed at a concentration of 8 mg/ml of gentamicin. As seen from **Figure 5B**, *S. aurantiacum* strain WM 06.482 was growing actively in the absence of *P. aeruginosa* strain PASS1 indicating the reversal of the inhibitory effect caused by live bacteria against the fungus.

## Indirect (non-physical) Interactions between *P. aeruginosa* and *S. aurantiacum*

To investigate whether physical contact between *P. aeruginosa* and *S. aurantiacum* was important to trigger growth inhibition, co-cultures were performed in six-well plates fitted with polycarbonate membranes to prevent direct contact between *P. aeruginosa* and *S. aurantiacum* cells while allowing free exchange of nutrients and extracellular molecules between the organisms. Growth of the less virulent *S. aurantiacum* strain WM 08.202 was inhibited when co-cultured with PAO1 and PASS1, evident from the substantial decrease in the fungal biomass (**Figure 6**) when compared to the culture of WM 08.202 maintained for the same amount of time.

*Pseudomonas aeruginosa* isolate PASS1 and the reference strain PAO1 showed a milder inhibitory effect against the high virulence *S. aurantiacum* strain WM 06.482. The PASS2 strain had little or almost no effect on growth of either of the *S. aurantiacum* strains. The results suggested that cell–cell contact was in fact not necessary to bring about inhibition of the growth of *S. aurantiacum* by *P. aeruginosa* and that the inhibition might involve bacterial metabolites and/or extracellular signaling molecules. In addition, *S. aurantiacum* strains WM 06.482 and WM 08.202 produced a red colored pigment when co-cultured with clinical *P. aeruginosa* strain PASS1 and reference strain PAO1. No such pigment was observed in the co-cultures

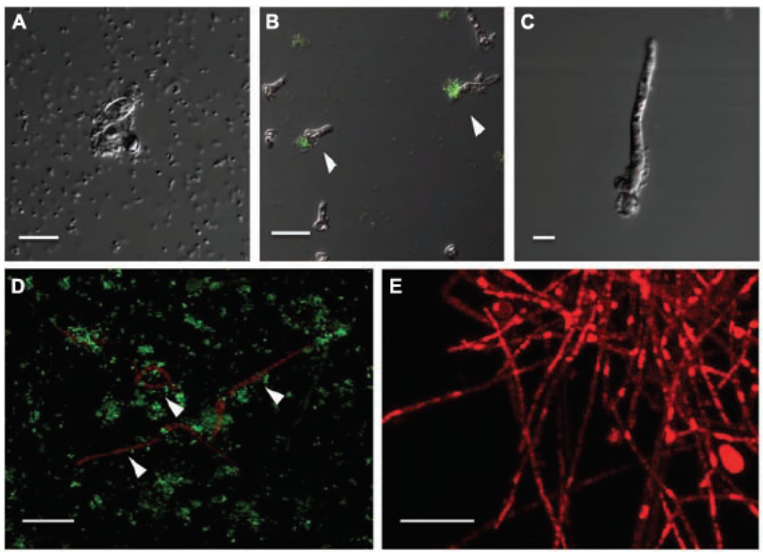


**FIGURE 4 | Adhesion and colonization of mCherry-tagged *S. aurantiacum* strain WM 06.482 (shown in red) by *P. aeruginosa* strain PASS1 tagged with yfp (shown in green) during coculturing in SCFM for 24 h at 37°C.** Scale bar = 20 μm. **(A)** *P. aeruginosa* cells adhered to germinating *S. aurantiacum* conidia after 2 h of incubation as viewed by DIC. **(B,C)** after 8 h, some young hyphae were surrounded by bacterial cells. **(D)** Bacteria can be seen attached to the hyphal filaments after incubation for 24 h. **(E)** Healthy growing culture of WM 06.482 expressing *mCherry* in the absence of bacteria. *White arrows indicate fungal filaments that are being colonized by the bacteria.

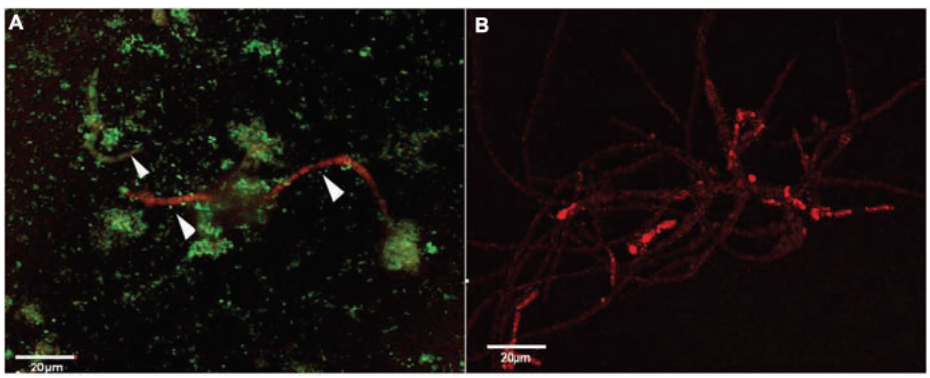


**FIGURE 5 | The effect of gentamicin on *P. aeruginosa* (PASS01) and *S. aurantiacum* (WM 06.482) co-cultures growing in SCFM at 37°C for 24 h.** Scale = 20 μm. **(A)** Co-culture of WM 06.482 and PASS01 without the antibiotic. **(B)** Active growth of *S. aurantiacum* in a co-culture treated with 8 mg/ml of gentamicin to eradicate the bacterial growth.

involving *S. aurantiacum* and PASS2 strain (Supplementary Figure S2).

### Effect of Phenazines on the Growth of *S. aurantiacum*

To test whether known virulence factors such as phenazines secreted by *P. aeruginosa* were involved in the inhibition of *S. aurantiacum* growth, the effect of crude phenazine extracts from *P. aeruginosa* strains PAO1, PASS1, and PASS2 were tested on the two *S. aurantiacum* strains using a disk inhibition assay. No inhibition was observed with disks saturated with the crude extracts as seen in **Figure 7**. All *S. aurantiacum* strains also showed resistance to a high concentration (10 mM) of commercial phenazine pyocyanin. Phenazines are known to have an inhibitory effect against a wide range of fungal species (Kerr et al., 1999).

## Discussion

Most of the studies targeting bacterial-fungal interactions *in vitro* have been performed with bacterial laboratory reference strains using either fungus-specific culture media (PDA, SABD) and/or minimal salts medium (Kerr et al., 1999; Hogan and Kolter, 2002; McAlester et al., 2008; Bandara et al., 2010; Manavathu et al., 2014). Differently to previous studies and to provide a better focus, we used a CF sputum-mimicking medium, i.e., SCFM to

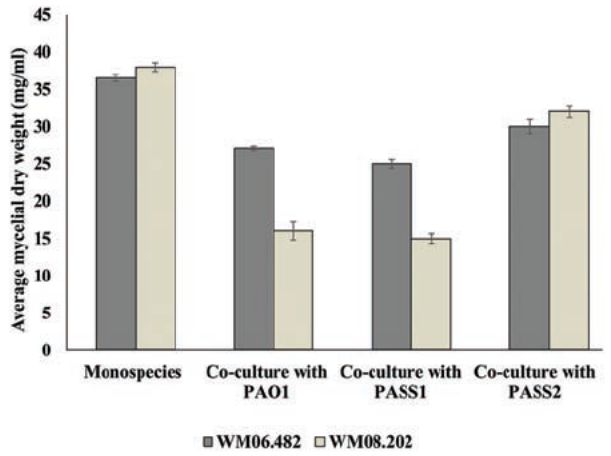


**FIGURE 6 | The inhibitory effect of *P. aeruginosa* on *S. aurantiacum* during co-culture in SCFM, determined by the change in dry weight of *S. aurantiacum* hyphae.** A sterile polycarbonate membrane was used to separate *P. aeruginosa* from *S. aurantiacum* in six well culture plates. Error bars on each data point represent SEM of three independent experiments. *P*-values were calculated by student's *t*-test, where $p < 0.05$ was considered significant.

explore the possible effect of *P. aeruginosa* on *S. aurantiacum* in the CF lung environment. We also used two recently isolated clinical CF strains of *P. aeruginosa*, PASS1 and PASS2, together with PAO1, a commonly used reference strain, and a clinical *S. aurantiacum* isolate (WM 06.482) with a high established virulence and a less-virulent type strain WM 08.202 to add to the clinical relevance of the findings.

Our results demonstrated that *P. aeruginosa* strains exhibit an inhibitory effect against *S. aurantiacum*. Consistent with the co-culture studies involving *P. aeruginosa* and other fungi, initial screening using plate assays suggested that presence of metabolically active (live) bacteria was necessary to inhibit the growth of the fungus as heat killed cells had no effect on *S. aurantiacum* growth (Mowat et al., 2010). Further on, extracts obtained from the bacterial monocultures failed to show any inhibitory effect. Thus it is possible that inhibition pathways might involve genes that are expressed only in bacterial-fungal co-cultures. In this respect our findings are similar to those of Rella et al. (2012) who showed that the growth of *C. neoformans* was not affected by the cell extracts obtained from *P. aeruginosa* strains PAO1 and PA14 cultured separately. The inhibition of *S. aurantiacum* by cell lysates of *P. aeruginosa* may be explained by the presence of bacterial exotoxins that are released during the cell lysis.

Confocal microscopy has been used to study interactions between chemically stained *P. aeruginosa* and major fungal lung pathogens such as *C. albicans* and *A. fumigatus* in liquid co-cultures (Bandara et al., 2010; Manavathu et al., 2014). However, the use of chemical stains was limited by the cross staining of bacteria and fungi thereby making it impossible to differentiate between them under a confocal microscope (Bandara et al., 2010). One of the key features of the current study is the use of *P. aeruginosa* and *S. aurantiacum* strains that were genetically tagged with fluorescent proteins in order to characterize the interactions in detail. To the best of our knowledge, this is the first report on successful genetic transformation of the newly described *S. aurantiacum* species. As no homologous promoters are available for this fungal species as yet, the fluorescent marker mCherry and the *E. coli hph* gene encoding hygromycin B phosphotransferase were expressed under a heterologous *pki* (pyruvate kinase) promoter derived from another ascomycetous fungus, *T. reesei* (Te'o et al., 2002; Boon et al., 2008; Klix et al., 2010). In previous studies, heterologous promoters such as *pki* and *gpdA* have been successfully used for gene expression across various phylogenetically close species (Punt et al., 1990; Jieh-Juen Yu, 1998; Ruiz-Diez and Martinez-Suarez, 1999; Almeida et al., 2007). The amount of hygromycin B required to inhibit the growth of *S. aurantiacum* was relatively high (410 U/ml) compared to some other fungi, which shows the highly resistant nature of *S. aurantiacum* also observed in antifungal susceptibility tests described in other studies (Lackner

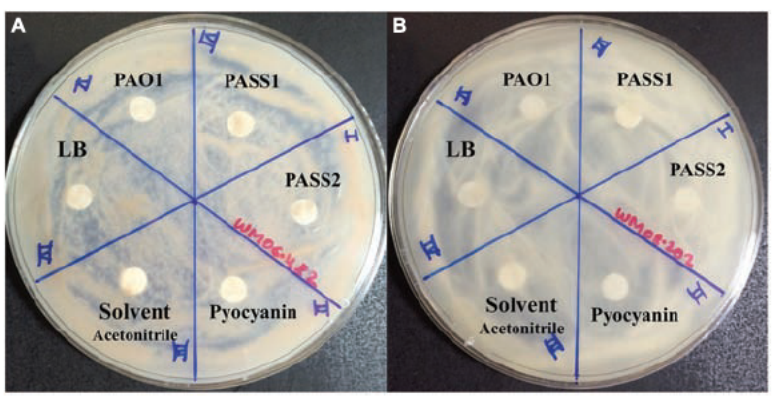


**FIGURE 7 | Effect of *P. aeruginosa* phenazines on two *S. aurantiacum* isolates (A) WM 06.482 and (B) WM 08.202.** Phenazines were extracted from different *P. aeruginosa* strains (PAO1, PASS1, and PASS2) and redissolved in acetonitrile (ACN). LB medium extract, ACN solvent and commercial phenazine (pyocyanin) were also tested against *S. aurantiacum*.

et al., 2012). Although the transformation efficiency was low (2.2 μg of plasmid DNA), transformant strains expressing the mCherry protein were obtained.

Confocal microscopy of the bacterial-fungal co-cultures revealed that bacteria elicit a specific inhibitory response by establishing a physical contact with the fungal hyphae. Similar types of interactions have also been observed in yeasts such as *C. albicans* and ascomycetous fungi such as *A. nidulans* and *Alternaria alternata* (Hogan and Kolter, 2002; Jarosz et al., 2011). This association might be directed toward utilization of the fungus by bacteria as an additional source of nutrients, or as an additional matrix support to form biofilms (Hibbing et al., 2010), or it may be a strategy to promote their own survival by inhibiting the fungal growth owing to nutrient limiting conditions in the medium (Brand et al., 2008).

Under nutrient limiting conditions, biofilm formation has been described as an important characteristic for *P. aeruginosa* mediated killing of other fungi such as *C. albicans* and *A. fumigatus* (Hogan and Kolter, 2002; Manavathu et al., 2014). Similarly, an inhibitory effect was also displayed by the biofilm forming strains of *P. aeruginosa* (PASS1 and PAO1) against the two *S. aurantiacum* strains in this study. PASS1 and PAO1 are high virulence strains, which share many similarities in their respective genomes. In contrast, the least virulent bacterial strain PASS2 (Penesyan et al., under review) that failed to show an effect against the fungi lacks several virulence related genes such as those encoding phenazines and the *psl* (polysaccharide synthesis locus) gene cluster which is required for biofilm formation (Ma et al., 2009; Penesyan et al., under review). The effect of bacteria on the growth of the less virulent *S. aurantiacum* strain WM 08.202 was much higher compared to the more virulent WM 06.482 both in the plate assays and in liquid co-cultures. This difference probably results from their different physiology as shown by Kaur et al. (2015) and possibly higher resistance to antifungals of the more virulent *S. aurantiacum* strain WM 06.482. These factors will be studied further when annotated *S. aurantiacum* genomes are available.

While biofilm formation and colonization of fungal hyphae in the nutrient limited SCFM liquid medium clearly contributed to the inhibition of *S. aurantiacum* by *P. aeruginosa*, it was not absolutely essential for the inhibitory effect as the cross streak assay with cultures not touching each other and disk inhibition experiments using cell lysates also resulted in inhibition of fungal growth. These indicated the possible involvement of secreted diffusible bacterial exoproducts/metabolites in fungal growth inhibition. One of these metabolites pyocyanin, a phenazine, is an extracellular redox-active virulence factor which is widely known to affect the growth of a large number of fungal species such as *A. fumigatus*, *C. albicans,* and *C. neoformans* (Kerr et al., 1999; Laursen and Nielsen, 2004; Gibson et al., 2009). Corroborating the highly resistant nature of *S. aurantiacum,* the amount of commercial pyocyanin (i.e., 10 mM) included in the test for comparison, was much higher than the MIC (minimum inhibitory concentration) of pyocyanin used for *C. albicans* and *A. fumigatus* (>0.3 mM; Kerr et al., 1999). These amounts are significantly higher than the amount of pyocyanin normally detected in the lungs of CF patients (100 μM; Wilson et al., 1988). However, neither crude phenazines nor pyocyanin showed an inhibitory effect against *S. aurantiacum* in our assays. A similar phenomenon has been observed in some ascomycetous fungi such as *A. sclerotiorum* (Hill and Johnson, 1969). Although it is not yet known if the phenazines are modified or sequestered by *S. aurantiacum*, the production of a red colored pigment in co-cultures could be due to a detoxification mechanism used by the fungus against bacterial phenazines. However, further studies into the chemical structure and UV and visible absorption spectra are required in order to ascertain if the red pigment indicates a modified phenazine.

In addition to phenazines, *P. aeruginosa* has also been reported to produce a wide variety of other exoproducts/metabolites such as proteases, elastases, haemolysin, and rhamnolipids that contribute to bacterial virulence (McAlester et al., 2008; Ben Haj Khalifa et al., 2011; Heeb et al., 2011; Rella et al., 2012; Mear et al., 2013). Their possible activity against *S. aurantiacum* will be worthy of a further study.

Most of the CF associated filamentous fungal species have been isolated from the lungs of patients with prolonged antibiotic therapies (Bakare et al., 2003). Previous clinical reports by Blyth et al. (2010b) have also showed an increased prevalence of *S. aurantiacum* in CF patients administered with antibacterial drugs indicating that the presence of bacteria has an effect on the susceptibility of the lungs to fungal infection. In support of this view, an increase in the growth of the fungus was observed upon a decline in the bacterial growth through addition of gentamicin to the co-culture medium in the present study. Therefore, it seems that the *P. aeruginosa* strains prevalent in CF patients during early stages of CF hinder fungal infection of lungs by inhibiting their growth.

## Conclusion

We have assessed the effect of clinically relevant strains of *P. aeruginosa* on a newly discovered fungal lung pathogen *S. aurantiacum* in a synthetic lung-mimicking medium (SCFM) that closely resembles the chemistry of CF sputum. An inhibitory effect of *P. aeruginosa* was observed on the growth of *S. aurantiacum*, which can be mediated by the production of biologically active metabolites. Biofilm formation and colonization of fungal hyphae by bacteria were also important for *S. aurantiacum* growth inhibition. Surprisingly, the toxic *P. aeruginosa* phenazine pigments, such as pyocyanin, known to have an inhibitory effect against other fungal species including *A. fumigatus* and *C. albicans,* proved to be ineffective against *S. aurantiacum*. This suggests involvement of other virulence determinants and emphasizes the resilient nature of *S. aurantiacum* compared to other fungi present in lung infections. Further research may include transcriptomic studies of *P. aeruginosa* – *S. aurantiacum* co-cultures in order to reveal detailed molecular mechanisms underlying these interactions; these studies will be facilitated by the upcoming annotated *S. aurantiacum* genome.

## Author Contributions

Conceived and designed the experiments: JK, LK, AP, AS, IP, HN. Performed the experiments: JK, SK, BP, MK. Analysed the data: JK, AP, HN. Wrote the paper: JK, HN.

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## Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00866

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

# 5

# Dual transcriptomics of host-pathogen interaction of cystic fibrosis isolate *Pseudomonas aeruginosa* PASS1 with *Danio rerio*

# Dual transcriptomics of host-pathogen interaction of cystic fibrosis isolate *Pseudomonas aeruginosa* PASS1 with *Danio rerio*

**Short title: Transcriptome of *Pseudomonas aeruginosa*-infected zebrafish**

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# Dual transcriptomics of host-pathogen interaction of cystic fibrosis isolate *Pseudomonas aeruginosa* PASS1 with *Danio rerio*

## 5.1 ABSTRACT

*Pseudomonas aeruginosa* is a significant cause of mortality in patients with cystic fibrosis (CF). To explore the interaction of the CF isolate *P. aeruginosa* PASS1 with the innate immune response, we have used *Danio rerio* (zebrafish) as an infection model. Confocal laser scanning microscopy (CLSM) enabled visualisation of direct interactions between zebrafish macrophages and *P. aeruginosa* PASS1. Dual RNA-sequencing of host-pathogen was undertaken to profile RNA expression simultaneously in the pathogen and the host during *P. aeruginosa* infection. Following establishment of infection in zebrafish embryos with PASS1, 3 days post infection (dpi), there were 6739 genes found to be significantly differentially expressed in zebrafish and 176 genes in PASS1. A range of virulence genes were upregulated in PASS1, including genes encoding pyoverdine biosynthesis, flagellin, non-haemolytic phospholipase C, proteases, superoxide dismutase and fimbrial subunits. Additionally, iron and phosphate acquisition genes were upregulated in PASS1 cells in the zebrafish. Transcriptional changes in the host immune response genes highlighted phagocytosis as a key response mechanism to PASS1 infection. Transcriptional regulators of neutrophil and macrophage phagocytosis were upregulated alongside transcriptional regulators governing response to tissue injury, infection and inflammation. The zebrafish host showed significant downregulation of the ribosomal RNAs and other genes involved in translation, suggesting that protein translation in the host is affected by PASS1 infection.

## 5.2 DATA SUMMARY

We provide two supplementary tables with the gene expression profile of *Pseudomonas aeruginosa* PASS1 and *Danio rerio* following host-pathogen interaction. The *P. aeruginosa*

PASS1 and *Danio rerio* transcriptomic data can be found in Dryad under the accession number doi:10.5061/dryad.3vk38 at http://datadryad.org/review?doi=doi:10.5061/dryad.3vk38.

**I/We confirm all supporting data, code and protocols have been provided within the article or through supplementary data files. ☒**

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## 5.3 IMPACT STATEMENT

This study represents the first global expression view of the molecular interactions between *Pseudomonas aeruginosa* and zebrafish. *P. aeruginosa* is an opportunistic pathogen that is the major cause of mortality among cystic fibrosis patients. Our transcriptomic data suggests that key virulence mechanisms for *P. aeruginosa* PASS1 in zebrafish include adherence to host cells via Cup fimbriae, iron and phosphate scavenging, protease cleavage of host proteins such as collagen, and superoxide dismutase as a defence mechanism against oxidative killing. The host gene expression during *P. aeruginosa* PASS1 infection shows upregulation of an array of genes including transcriptional regulators, toll-like receptors and chemokines to be involved in the initiation, and process of phagocytosis. Decreased expression levels of ribosomal RNAs and translation proteins suggests that protein translation in the host is impacted by bacterial infection.

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## 5.4 INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator gene [1, 2]. It is most prevalent among the Caucasian population, affecting 1 in 2,500 new-borns [1]. CF lung disease is the major cause of morbidity and mortality among CF patients and is a result of colonisation and infection of airways with bacteria, fungi and viruses [3]. In early childhood, the CF lung is mainly colonised by

*Staphylococcus aureus* and *Haemophilus influenzae*, although later in a CF patient's life, *P. aeruginosa* typically takes over as the dominant pathogen [4].

*P. aeruginosa* is a versatile Gram-negative microorganism found commonly in both terrestrial and aquatic environments [5, 6]. Its 6.3 Mb sized genome supports its metabolic versatility and, consequently, its adaptability to diverse environments [7]. This opportunistic pathogen can cause acute and chronic infections in immunocompromised people, such as AIDS sufferers and neutropenic patients undergoing chemotherapy, and patients with injuries, catheters, burn wounds and non-CF-associated pulmonary infections [8-12]. *P. aeruginosa* infection often becomes the major cause of morbidity and mortality in CF patients [9].

The respiratory pathogenesis of *P. aeruginosa* can be attributed to an array of key virulence factors, including flagella, type III secretion system, phenazines, the iron scavenging siderophores pyochelin and pyoverdine, lipopolysaccharide, elastase, alkaline proteases, haemolysins (phospholipase and lecithinase), cytotoxins (leukocidin) and exotoxin A [10, 13]. *P. aeruginosa* chronic infections in the CF lung also provoke aggressive inflammatory reactions, such as the host neutrophilic response which releases oxidants and enzymes detrimental to the host tissue [2, 4, 13].

The pathogenesis of *P. aeruginosa* has been studied using diverse model host organisms including *Dictyostelium discoideum, Arabidopsis thaliana, Caenorhabditis elegans, Drosophila melanogaster, Galleria mellonella,* rodents and zebrafish [11]. Zebrafish has been previously used to study aspects of pathogenesis of *P. aeruginosa* [2, 14], as well as other bacterial pathogens including *Salmonella typhimurium*, *S. aureus*, *Burkholderia cenocepacia*, *H. influenzae*, *Leptospira interrogans*, and *Listeria monocytogenes* [15].

Zebrafish is a teleost fish with a total genome size of 1.412 gigabases [16, 17]. Genome comparison has revealed that approximately 70% of human genes have a clear zebrafish orthologue [16]. Remarkable similarities have been observed with human transcriptional regulators, immune

effectors, immune recognition systems, defence signalling pathways and macrophage lineages [15, 18, 19] which make zebrafish a good model for studying host-pathogen interaction.

There are several other advantages in using zebrafish embryos as a model organism for study of human infections. These include ease in handling, low cost, rapid development and the ability of a single pair of adult zebrafish to produce hundreds of offspring every week [15, 20]. It is optically transparent and the availability of transgenic lines with fluorescently marked immune cells allows for real-time visualisation of *in vivo* microbe-phagocyte interactions at high resolution throughout the organism [11, 15, 20].

In zebrafish, the innate and adaptive immune systems develop sequentially. The innate immune response is developed at the embryonic and early larval stages with early lymphocytes making their first appearance in the 4-day-old larvae, and a full adaptive immune system developing at about three weeks of age [21]. The innate immune system is the host's first line of defence against infections and includes physical barriers, cellular, and humoral components such as complement and acute phase proteins [18, 22]. It is responsible for early recognition of pathogens and triggering an appropriate pro-inflammatory response [21, 23, 24]. The main phagocytic cell types of the innate immune system are macrophages and neutrophils [21, 25]. In zebrafish embryos, as early as one day post fertilisation, functional macrophages are capable of sensing and responding to microbial infections [15]. Both neutrophils and macrophages can lead to bacterial clearance by engulfing and killing bacterial pathogens. The bacterial pathogens shown to be engulfed by these phagocytic cells include *P. aeruginosa* and *S. aureus* [11, 25, 26].

Global gene expression studies of wild-type zebrafish embryos using a zebrafish microarray have been conducted following static immersion with *Edwardsiella tarda*, *Escherichia coli, P. aeruginosa* strain PA14 and strain PAO1, whereas systemic infection has been studied with *E. tarda* and *S. typhimurium* [22, 27]. Infection in zebrafish embryos is often established via injection into the blood circulation, and response to infection with various bacterial pathogens has been subjected to microarray analysis [22, 28-31]. Specifically, zebrafish embryos are usually

microinjected with bacterial pathogens directly into the blood circulation at 1-3 day post fertilisation, mostly using the posterior blood island or into the Duct of Cuvier, a wide blood circulation valley on the yolk sac connecting the heart to the trunk vasculature [15].

RNA sequencing (RNA-Seq) has previously been used to study innate immune response of zebrafish embryo to systemic *S. typhimurium* infection [27]. Ordas and colleagues (2011) used a combination of Tag-Seq, RNA-Seq and microarray transcriptome data to compile an annotated reference set of infection-responsive genes in the zebrafish embryos. These included genes encoding transcription factors, signal transduction proteins, cytokines and chemokines, complement factors, proteins involved in apoptosis and proteolysis, proteins with antimicrobial activities, as well as many known or novel proteins not previously linked to the immune response.

Despite the importance of *P. aeruginosa* pathogenesis in CF lung infection, the use of zebrafish as an alternative model to understand host-pathogen interaction until recently had remained unexplored. Diaz-Pascual and colleagues (2017) have recently profiled the global proteome of both zebrafish and *P. aeruginosa* PAO1 following establishment of *P. aeruginosa* PAO1 infection via immersion and injection [14]. However, the interaction of zebrafish and *P. aeruginosa* remains to be elucidated at a global transcriptome scale. Hence, the aim of this study was to investigate the interaction of zebrafish embryos with the virulent *P. aeruginosa* CF isolate PASS1 [32] by visualising macrophage-PASS1 interaction and analysing the simultaneous global gene expression profiles of both organisms via RNA sequencing (RNA-Seq). To our knowledge, this is the first study describing the dual transcriptome of *P. aeruginosa*-zebrafish interaction.

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## 5.5 METHODS

### *P. aeruginosa* strain and growth conditions

The bacterial strains used in this study were *P. aeruginosa* PASS1 [32] obtained from the sputum of a 40-year old female patient and YFP-tagged *P. aeruginosa* PASS1 [33]. The strains were

maintained in a glycerol stock at -80˚C, and prior to each experiment, were grown on Luria Bertani (LB) agar plates and incubated at 37˚C till isolated colonies were obtained. The isolated colonies were then cultured in LB broth overnight at 37 ˚C with constant shaking at 150 rpm. Cells from overnight cultures were washed, pelleted at 6000 *g* for 10 minutes at 4˚C and resuspended into sterile PBS. The cell concentration was estimated by measuring the optical density at 600 nm. Following estimation of cell concentration, the cells were diluted in PBS to an optical density $OD_{600}$ of 2.0. For visualisation of the bacterial suspension during injection of zebrafish embryos an aliquot of phenol red sodium salt stock solution was added to a final concentration of 0.01%.

**Visualisation of PASS-YFP infection in zebrafish embryos by confocal microscopy**

The injection apparatus for the zebrafish embryos was set up as described by Brudal et al. (2014) [34]. Zebrafish embryos derived from adults of the *Tg*(*mpeg1:Gal4, UAS;mCherry-CAAX*) [35] were manually dechorionated and maintained at 29°C prior to injection at 48 hpf. The embryos were anesthetized with 0.005 w/vol % ethyl 3-aminobenzoate methanesulfonate (Tricaine) for 1 to 2 min and placed on 2% agarose plates for injection. PASS1-YFP cells (in a volume of 0.7 to 1 nl) were microinjected into the duct of Cuvier, as visually ascertained under the stereomicroscope. Infected embryos were returned to a petri dish with fresh embryo medium and incubated for 6 hours at 29˚C prior to confocal microscopy. A mock-infection with only sterile PBS was also set-up for comparison with the PASS1-YFP infection. Prior to confocal microscopy zebrafish embryos were anaesthetised with Tricaine as described above, and then transferred to a glass bottom petri dish with glass cover slip containing a mixture of embryo water [36] and Tricaine. The anaesthetised embryos in the petri dish were then covered with 1.3% low-melting-point agarose. Confocal microscopy was performed with an Olympus Fluoview FV1000 IX81 inverted confocal microscope, and images were collected using Olympus Fluoview Ver.3.1a Viewer.

## RNA extraction and RNA-Seq transcriptomics

The zebrafish embryos derived from adults of the AB wild-type line were infected with PASS1 or mocked-infected with PBS with microinjection into the Duct of Cuvier (48 hpf) according to the procedure described above. Infected embryos were returned to a petri dish with fresh embryo medium and incubated for 3 dpi at 29˚C prior to RNA isolation. At 3 dpi, 9 randomly chosen zebrafish embryos from each group were euthanised by a prolonged immersion in an overdose of 50 mg/L Tricaine solution and transferred into 1.5 ml Eppendorf tubes. Three embryos were then pooled to represent one sample to allow for sufficient amount of starting material for RNA isolation. The embryo water was replaced by RNAlater (Ambion) immediately after transfer of embryos to fresh 1.5 ml Eppendorf tubes. The samples were kept at 4˚C until RNA was isolated. For the extraction of total RNA, RNAlater was replaced with 600 µl of Qiazol, and the tissue was homogenised using a pestle motor followed by drawing of sample with a needle 5 times till tissue was completely homogenised. As a control, RNA was isolated from PASS1 cultures grown in LB to an $OD_{600}$ = 1.0. For both the zebrafish and bacterial culture, RNA extractions were performed using the miRNeasy Mini kit (Qiagen) according to the manufacturer's protocols. An additional DNase treatment was performed with a TURBO DNA-free kit (Ambion) according to the manufacturer's protocols. The concentration of the extracted RNA was measured using a NanoDrop Spectrophotometer. The total RNA samples were subjected to ribosomal RNA (rRNA) depletion, zebrafish samples mock-infected with PBS were treated with Ribo-Zero Gold rRNA Removal Kit (Human/Mouse/Rat) (Illumina), zebrafish infected with PASS1 were treated with Ribo-Zero rRNA Removal Kit (Gram-negative Bacteria) (Illumina) followed by Ribo-Zero Gold rRNA Removal Kit (Human/Mouse/Rat) and the PASS1 sample grown in LB was treated with Ribo-Zero rRNA Removal Kit (Gram-negative Bacteria). The depletion steps and subsequent 125 bp paired-end RNA Sequencing on a HiSeq2500 (Illumina) were performed at the Australian Genome Research Facility (Melbourne, Australia).

### Bioinformatic analyses of transcriptomic data

Sequencing data were assessed for quality using FastQC software (Babraham Bioinformatics). The transcriptomes of zebrafish infected with PASS1 and PBS were mapped against the zebrafish genome (Ensembl). Bacterial transcriptomic data were mapped against the PAO1 (NCBI) genome. Transcriptome mapping was undertaken with TopHat2 and normalised based on FPKM differential expression calculation using Cuffdiff [37].

Significantly differentially expressed genes in PASS1 ($p \leq 0.01$ and $\log_2$ fold-changes cut-off -1≥ to ≤1) were mapped to annotated pathways and to cluster of orthologous groups of *P. aeruginosa* strain PAO1, obtained from the *Pseudomonas* Database [38].

Significantly differentially expressed genes in zebrafish ($p \leq 0.01$ and $\log_2$ fold-changes cut-off -1≥ to ≤1) were functionally annotated using Ingenuity Pathway Analysis (Ingenuity Systems Inc., Redwood City, CA). A total of 3238 differentially expressed genes were successfully mapped (Table ST5.6). Functional annotation and gene ontology (GO) classification was conducted separately of the upregulated and downregulated genes in zebrafish-*P. aeruginosa* PASS1 infection using DAVID (The Database for Annotation, Visualisation, and Integration Discovery) version 6.8 [39, 40].

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## 5.6 RESULTS AND DISCUSSION

### Confocal laser scanning microscopy of macrophage-*P. aeruginosa* PASS1 interaction in zebrafish

Macrophages are important effector cells of the innate immune response that can rapidly phagocytose bacteria and alert the immune system to danger [41]. We have previously generated a yellow fluorescent protein (YFP)-labelled derivative of the CF isolate *P. aeruginosa* PASS1, PASS1-YFP [33]. The PASS1-YFP cells were injected into the Duct of Cuvier of transgenic zebrafish embryos *Tg(mpeg1:Gal4, UAS;mCherry-CAAX*) [35] which produce mCherry-labelled

macrophages. This enabled the analysis of macrophage behaviour in zebrafish by confocal laser scanning microscopy (CLSM) visualisation.

Strikingly, within 6 hours post infection (hpi), *P. aeruginosa* PASS1-YFP cells were predominantly found to be associated or engulfed by macrophages (Fig. 5.1). Previous studies have shown that macrophages can kill both Gram-positive and Gram-negative bacteria, including *P. aeruginosa*, via phagocytosis [26]. Brannon and colleagues (2009) have shown the phagocytosis of *P. aeruginosa* strains PAO1 and PAK by macrophages to occur within 2 hpi [26].

A central advantage of the zebrafish embryo model is the ability to monitor infection at a detailed cellular level in real time. Our CLSM results corroborate previous observations that macrophages are capable of phagocytosing and killing *P. aeruginosa* [26, 42]. Earlier studies have used the laboratory strains PAO1 and PAK, this is the first zebrafish study to use a CF isolate. Our previous work showed that PASS1 is non-mucoid and has a mutation in the *lasR* gene, and displays significant phenotypic differences compared with PAO1, including increased biofilm formation and production of virulence factors such as phenazines [32].

**Generation of a dual host-pathogen transcriptome**

The use of a zebrafish embryo model allows the possibility of global gene expression analysis of both host and microbe in parallel. This provides an opportunity to investigate the molecular mechanisms of the interaction between the host innate immune system and the pathogen. The survival of zebrafish embryo infected with PASS1 displayed a gradual decrease in embryo survival rate following 4 days post infection (dpi) (Fig. 5.2). To investigate host-pathogen interaction prior to increase in mortality, we isolated total RNA from PASS1-infected zebrafish (3 dpi), and RNA-Seq was used to examine the zebrafish and PASS1 transcriptomes in parallel. The infected zebrafish transcriptome was compared with phosphate buffered saline (PBS)-injected zebrafish as a negative control. The transcriptome of *P. aeruginosa* PASS1 in zebrafish was compared with PASS1 grown in Luria-Bertani (LB) culture medium.

A total of 214,749,236 sequence reads were generated from the total RNA extracted from three independent biological replicates of PASS1-infected zebrafish. Around 53.2 % of the reads aligned with the *P. aeruginosa* reference genome while 90 % aligned with the zebrafish reference genome (Table 5.1).

**Whole-cell transcriptome analysis of *P. aeruginosa* infected into zebrafish**

Analysis of the *P. aeruginosa* transcriptome data revealed 176 genes to be differentially expressed in *P. aeruginosa* within the infected zebrafish ($p \leq 0.01$ and $\log_2$ fold-changes cut-off $-1 \geq$ to $\leq 1$) with 140 genes upregulated and 36 genes downregulated (Fig. 5.3). Ribosomal RNA genes were the most upregulated transcripts in *P. aeruginosa* PASS1 during zebrafish infection (Fig. 5.3). A number of studies have shown a correlation between growth rate and rRNA concentration [43-49]. Additionally, translation initiation and elongation factor genes were also more highly expressed by PASS1 within the zebrafish (Table ST5.1). This suggests that within the host there is a higher rate of protein synthesis and cell proliferation at 3 dpi compared to the late log ($OD_{600}$ = 1.0) culture of PASS1 in LB medium.

**Expression of virulence genes in PASS1 cells in a zebrafish model**

Vertebrates are known to deplete both inorganic phosphate and iron in response to bacterial infection [50]. Consistent with this the *P. aeruginosa* phosphate transport and iron acquisition genes were highly expressed in zebrafish compared with PASS1 culture in LB (Fig.5. 4). The pyrophosphate porin gene *oprO* and the phosphate-binding protein gene *pstS* were highly upregulated (525-fold and 123-fold, respectively). Phosphate regulation (*phoU*), and DNA degradation (*eddA*) genes were also upregulated. All four genes have been identified to be upregulated under phosphate limitation *in vitro* [51, 52]. *P. aeruginosa* is able to obtain phosphate from the host cell membrane via hydrolysis of the phospholipids using phospholipases [10]. The non-haemolytic phospholipase C gene was highly upregulated 54.6-fold in *P. aeruginosa* PASS1 during zebrafish infection (Fig. 5.4).

Also upregulated were iron scavenging systems of PASS1. pyoverdine biosynthesis genes and the ferri-pyoverdine receptor gene of PASS1 were significantly upregulated in zebrafish. *P. aeruginosa* synthesizes and secretes the siderophore pyoverdine to scavenge ferric iron from host to overcome iron limitation during infection [53-55].

In addition, a range of known *P. aeruginosa* virulence genes were differentially expressed in the zebrafish host compared with the culture in LB medium. These include genes encoding flagella biogenesis and the PasP protease (Fig. 5.4). PasP is an extracellular protease [56] able to cleave collagen, contributing to the loss of epithelial cells [57, 58].

The *P. aeruginosa flaG* flagellin gene was 2-fold upregulated in the zebrafish host. The delivery of bacterial flagellin into the mice macrophage cytosol has been shown to trigger the NLRC4 inflammasome which mediates activation of the protease, caspase-1 (upregulated in our data set by 2-fold) [59, 60]. The activation of caspase-1 promotes the secretion of the proinflammatory cytokines IL-1β and IL-18 as well as pyroptosis, a form of cell death induced by bacterial pathogens [61]. The decrease in the amount of macrophages present in zebrafish embryos infected with *P. aeruginosa* PASS1 compared to zebrafish embryos infected with phosphate buffered saline (Fig. 5.1) could be due to macrophage cell-death via pyroptosis.

Expression of the *cupA1* and *cupC1* genes, encoding fimbrial subunits of chaperone-usher type fimbriae, were both increased in PASS1 in zebrafish. These class of fimbriae are important tissue-specific adhesins in many pathogens, and are presumably playing a role in adhesion to zebrafish cells.

The *pycR* gene encoding a LysR-type transcriptional regulator was upregulated by 5.8-fold. This regulator modulates expression of virulence factors such as lipase/esterase and biofilm formation, as well as genes implicated in lipid metabolism and anaerobic respiration [62] (Fig. 5.5).

The *P. aeruginosa sodM* superoxide dismutase gene was upregulated by 15.6-fold in zebrafish. SodM protects *P. aeruginosa* against toxic effects of superoxides [63], so the increased expression

level of *sodM* may be a defensive response against oxidative killing in the zebrafish macrophage phagolysosome. Iron limitation has been reported to lead to an increase in SodM activity in *P. aeruginosa* [64], which may be an alternate explanation for increased *sodM* expression. All of the *P. aeruginosa* type VI secretion system genes (orthologous to PA1656-PA1671 of PAO1) encoded within the Hcp secretion island-2 (H2-T6SS) showed lower expression levels in zebrafish compared with the culture in LB medium, although not all of them were below the significance threshold ($p \leq 0.01$) (Table ST5.1). This type VI secretion system in PAO1 has been shown to be important in virulence in a worm model and in mammalian cell cultures, and its expression has been shown to be induced by the Fur regulator during iron limitation and by quorum sensing [65]. However, our transcriptomic data indicates that PASS1 is responding to iron limited conditions, thus the downregulation of the H2-T6SS genes in our infected zebrafish model suggests that there are other unknown regulatory pathways governing expression of this secretion system.

**Other differentially expressed PASS1 genes in a zebrafish model**

The PASS1 genes *faoA* and *foaB* located in the *fadBA5* β-oxidation operon were upregulated in the infected zebrafish. β-oxidative enzymes have been shown to be induced *in vivo* during lung infection in CF patients [66]. *In vitro* studies have demonstrated that the *fadBA5* operon is required for phosphatidylcholine (PC) and fatty acid (FA) degradation [66, 67]. The lung surfactant consists of ~10% surfactant proteins and ~90% lipids with phosphatidylcholine (PC) accounting for ~80% of the lipids [68, 69]. The most abundant lipids in the zebrafish embryo are cholesterol, PC, and triglyceride [70]. These lipids are processed within the yolk prior to mobilisation to the embryonic body [70, 71]. The PASS1 *psrA* gene encoding a TetR family transcriptional regulator required for regulation of the *fadBA5* operon also showed increased expression in the zebrafish host (Fig. 5.5). PsrA has been reported to also regulate the electron transfer flavoprotein B-subunit, *etfB* gene during stationary phase of bacterial growth [72], and *etfB* also showed increased expression in PASS1 in zebrafish.

A variety of amino acid utilisation genes, for example, *liuA*, *liuB* and *liuE* encoding enzymes in the branched chain amino acid degradation pathway, showed decreased levels of expression in PASS1 cells in zebrafish. Conversely, a variety of PASS1 amino acid biosynthesis genes showed increased levels of expression in the zebrafish infection model. This most likely reflects the availability of amino acids in LB medium that was used for the *in vitro* control PASS1 culture.

The PA0622 and PA0623 genes in the bacteriocin R2 pyocin gene locus [73, 74] were downregulated in PASS1 cells in the zebrafish model (Fig. 5.3 and Fig. 5.6). This gene cluster has been implicated in the production of a cryptic prophage endolysin that mediates *P. aeruginosa* explosive cell lysis [75]. This cell lysis results in the production of extracellular DNA that facilitates biofilm formation. Other biofilm-related genes, such as GacS/GacA and RetS/LadS two-component systems and quorum-sensing systems including *las*, *rhl* and *pqs* [76] were not significantly differentially expressed (Table ST5.1).

**Whole-cell transcriptome analysis of zebrafish embryos infected with *P. aeruginosa* PASS1**

The transcriptome of zebrafish infected with PASS1 was compared with PBS-injected zebrafish to identify genes upregulated in response to *P. aeruginosa* infection. RNA-Seq analysis revealed 6739 genes to be differentially expressed ($p \leq 0.01$ and $\log_2$ fold-changes cut-off -1≥ to ≤1). This represents a quarter of the protein-encoding genes in the zebrafish genome, suggesting that there is a dramatic transcriptional response to infection. Of the differentially expressed genes, 2510 were found to be upregulated and 4229 were downregulated. The complete list of genes is provided in Table ST5.2, and their $\log_2$ fold change in expression ($p \leq 0.01$, $\log_2$ fold-change cut-off -1≥ to ≤1) are shown graphically in Fig. 5.7.

Two of the most highly upregulated genes in the infected zebrafish are *PSMB8* (20.6-fold) and *TNFRSF18* (9.9-fold) (Fig. 5.7). The *PSMB8* gene has been linked to a number of auto-inflammatory diseases and found to be induced during the innate immune response of zebrafish to bacterial infection [15, 77]. The *TNFRSF18* gene is part of the TNF receptor signalling family and

plays a role in anti-apoptotic signalling via TRAF2 (upregulated 1.3-fold), which is thought to be involved in protection of lymphocytes against activation-induced cell death [78]. Mycobacterial infection of zebrafish has suggested that TNF receptor signalling mediates resistance against mycobacteria [15]. *PSMB8* and *TRAF2* genes may play a similar role in the zebrafish immune response against *P. aeruginosa* PASS1.

The most significantly downregulated genes in the zebrafish infected with *P. aeruginosa* PASS1 were the 5.8S rRNA genes (Fig. 5.7). RNA isolated from both the infected and uninfected control cells underwent an rRNA depletion step, which makes it difficult to conclusively draw inferences about translation, as the differences in the 5S rRNA abundance could be due to artefacts introduced by the depletion process. Nevertheless, 22 mitochondrial ribosomal proteins, 9 ribosomal proteins, as well as several ribosomal proteins modifying enzymes all showed decreased expression in the infected zebrafish cells compared with the uninfected control (Table ST5.2). This suggests that translation in the zebrafish is negatively impacted by the bacterial infection.

The downregulation of transcription of rRNA genes and ribosomal protein genes has been previously reported to occur due to intracellular and extracellular stressors [79, 80]. Stress leads to the induction of processes such as cell cycle arrest, apoptosis or autophagy [79, 81, 82]. Consistent with this the PASS1-infected zebrafish transcriptome was significantly enriched in transcripts related to organismal injury and cell death (Fig. 5.8).

PASS1-infected zebrafish displayed decreased expression of the prohibitin 2 (PHB2) mitochondrial protein, a coordinator/communication protein for cell division, metabolism and cell death [83]. Proteins known to interact with PHB2, including transcription factors ATF2, MEF2A, TEAD3, DNA modifying proteins, SIRT2, HDAC5, RNF2, protease, AFG3L2, RNA binding/ processing proteins, AGO3, DDX20, cell cycle, KIF23, cytoskeleton/structural protein, NUP93, signal transduction, ADRB2, ATP5B, COX4I1, cellular respiration protein, COX6C, mitochondrial transport/translation TIMM50 were also significantly downregulated in PASS1-

infected zebrafish, suggesting that PASS1 infection is impacting a swathe of activities linked to PHB2.

**Cellular and humoral innate immune response in zebrafish infected with PASS1**

Pathway analysis of the zebrafish transcriptome using the Ingenuity package revealed that several canonical pathways within the category of cellular and humoral innate immunity were highly enriched in zebrafish upon infection (Fig. 5.9). These included phagosome maturation, leukocyte extravasation signalling, Fcɣ receptor-mediated phagocytosis in macrophages and monocytes, CXCR4 signalling, clathrin-mediated endocytosis signalling, IL-8 signalling, caveolar-mediated endocytosis signalling, fMLP signalling in neutrophils, production of nitric oxide and reactive oxygen species in macrophages, and macropinocytosis signalling.

In the leukocyte extravasation signalling pathway, the chemokines *CXCR3* and *CXCR4* was upregulated 4.1- and 2.1-fold, respectively (Fig. 5.10). The leukocyte extravasation signalling pathway involves the movement of leukocytes out of the circulatory system and towards the site of tissue damage and infection. Members of the CXC chemokine family have a role in inducing neutrophil recruitment [13]. Previously, the CXC chemokine family has been shown to be involved in the inflammatory response in mice to *Pseudomonas* lung infection [84]. These chemokines are presumably involved in enhancing migration of leukocytes to the site of bacterial infection.

PASS1 cells in zebrafish displayed an upregulation of flagellin, flagellin induces CXCR4 Myeloid-Derived Suppressor Cells which are known to suppress T cell response mechanism for host defense [85]. This suggests that the upregulation of CXCR4 is also beneficial to survival of PASS1 in the in the zebrafish host.

Toll like receptors (TLRs) which are expressed by neutrophils and macrophages play a key role in recognition of bacterial ligands [86, 87]. *TLR3, 4* and *7* were significantly upregulated in the zebrafish transcriptome following PASS1 infection (Table ST5.2). In particular, TLR4, which can recognise bacterial lipolysaccharide, plays a significant role in the response to *P. aeruginosa*

infections in mammalian lungs [88]. TLRs also play important roles in regulating phagocytosis at multiple steps including internalisation and enhancement of phagosome maturation [89, 90]. *RAB7* which is a mediator of late phagosome process [91] was upregulated by 3.3-fold, suggestive of phagosomal activity against PASS1 inside the zebrafish host, which is consistent with interaction between PASS1 and phagosomes observed in our confocal microscopy (Fig. 5.1).

Key transcriptional regulators in the acute phase response to tissue injury, infection and inflammation, including *FOS, JUN* and *STAT3* were upregulated, while the *NFKB2*, *NFKBIB*, *NFKBIE* regulators were downregulated in response to PASS1 infection [92]. Previous studies using zebrafish embryos have shown upregulation of *FOS* and *STAT3* in response to *S. typhimurium* and *M. marinum* [24]. The *JUN* and *FOS* transcriptional regulators together are known as activating protein 1 (AP-1), both are conserved between mammals and zebrafish [15, 27]. AP-1 is involved in cellular expression, cell proliferation and differentiation, with its activation dependent on a variety of stress-related stimuli [93].

The intracellular suppressors of cytokine signalling (SOCS) genes *SOCS1, SOCS2,* and *SOCS3* were upregulated in the acute phase signalling pathway by 3.5-, 2.7-, and 2.0-fold, respectively, in response to PASS1 infection. The SOCS proteins are important regulators of acute phase response and cytokine signalling pathways as they regulate the balance between pro- and anti-inflammatory signals during infection [34, 94].

**Identification of genes previously linked to cell infection**

Based on the Ingenuity Pathway Analysis there were 233 differentially expressed genes related to infection of cells (Fig. 5.11). The 233 differentially expressed genes comprised genes encoding enzymes, G-protein coupled receptors, ion channels, growth factors, kinases, ligand–dependent nuclear receptors, peptidases, transcription regulators, translational regulators, transmembrane receptors, and transporters. The genes with the highest expression changes (> 4-fold) were the transmembrane receptor (tumour necrosis factor receptor superfamily 14, *TNFRS14*), poly (ADP-

ribose) polymerase *PARP9*, transcription regulator (*BTG2*) and the serine protease inhibitor *SERPINA1* genes (Fig. 5.10). This suggests a complex cascade of cellular events in response to *P. aeruginosa* infection.

**Other genes differentially expressed in zebrafish in response to bacteria**

Functional analysis of upregulated genes using DAVID [39, 40] (Table ST5.3 and ST5.4) showed enrichment within the GO category "response to bacterium". The genes upregulated by 4-fold included G protein-coupled receptor 84 (*GPR84*), leukocyte cell-derived chemotaxin 2 like (*LECT2L*), and the tumour necrosis factor receptors *TNFRSF18* and *TNFRSF14*. *GPR84* is expressed in leukocytes, monocytes and macrophages, and is known to play a critical role in immune regulation [95] (Fig. 5.10). The acute phase response LECT2 protein attracts neutrophils [96] and studies of mammalian LECT2 indicate that it plays a role in immune regulation [97]. Infection studies with *Aeromonas salmonicida* and *S. aureus* have shown high induction of *LECT2* in adult zebrafish [98]. *MPX*, which is a zebrafish orthologue of the mammalian *MPO* gene was upregulated suggesting that there is presence of neutrophils at the site of infection which are undergoing apoptosis [99].

**Comparison of zebrafish infection with various pathogens**

Previously Ordas and colleagues [27] have compared Salmonella infection of zebrafish embryos with *M. marinum* infection of adult zebrafish [100]. Transcriptomic datasets from zebrafish were compared to identify the overlap between the up- and downregulated transcripts of the *Salmonella*- and *Mycobacterium*-infected zebrafish. This revealed 288 and 3 commonly up- or downregulated transcripts. Comparison of our dataset to both these infection studies revealed 47 and 1 common up- or downregulated transcripts (Table ST5.5). The one downregulated gene common to these two datasets, as well as our PASS1 zebrafish embryo infection, was *KRT78*, involved in translation.

The common set of 47 upregulated genes included 19 previously implicated in the vertebrate immune response. The *MCL1A* gene was upregulated by 2.0-fold protects against apoptosis during initial steps of differentiation in human macrophages [101]. Complement factor B in macrophages was 2.8-fold upregulated, and its expression was proposed to be facilitated by TLR3, TLR4 and TRIF [102]. The transcriptional regulator *CEBPβ* was upregulated 1.9-fold, and it has been suggested to influence expression of the *IL-1β* gene [103], which, in turn, was 6.5-fold upregulated in our study, and is known to activate neutrophils and macrophages in bacterial phagocytosis [103]. *HIF-1α*, a global regulator of macrophage and neutrophil inflammatory and innate immune functions that is stimulated by TLR4 [104], was upregulated by 1.6-fold.

The gene encoding SRGN which interacts with inflammatory mediators such as IL-1β and TNF [105] was upregulated by 2.7-fold. The protease cathepsin C gene was 2-fold upregulated and is involved in the activation of granule serine peptidases in inflammatory cells [106]. The cathepsin D protease gene was also upregulated (1.8-fold) and has been implicated in macrophage apoptosis [107].

Comparison of transcriptomic data from infection studies with different bacterial pathogens can thus be used to collectively define a common set of innate host genes expressed in response to infection. Comparison of zebrafish embryo infection studies with adult zebrafish infection studies provides an opportunity to dissect the innate immune response separate from the adaptive immune response. The generation of transcriptomics data investigating response to infection to various pathogens is valuable for future host-pathogen interaction studies as well as developing targeted therapeutics.

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## 5.7 CONCLUSIONS

Previously, zebrafish was used as a model organism for *P. aeruginosa* infection by looking at the expression of specific immune related genes and *in vivo* interaction of the pathogen with

phagocytes. In this study we report, for the first time, the simultaneous global gene expression of a zebrafish-*P. aeruginosa* systemic infection. RNA-Seq analysis has yielded a detailed view of both host and pathogen transcriptional responses. During infection, PASS1 displayed increased expression of an array of genes shown previously to be important in pathogenesis. We have also shown that phosphate and iron acquisition genes are significantly upregulated in PASS1, suggesting these are limiting nutrients within the zebrafish host. The response of zebrafish to PASS1 infection involved both humoral and cellular components of the innate immune system. Significant upregulation was observed for genes involved in bacterial recognition and clearance, inflammation and tissue injury. Based on the transcriptomic data, we present a schematic overview of the key response mechanisms in both host and pathogen during PASS1 infection of zebrafish embryos (Fig.5.12).

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## 5.8 AUTHOR STATEMENTS

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*Ethical statement*

All zebrafish experiments were performed with the approval of University of Oslo (Animal ethic approval number: 6981) and Macquarie University (Animal ethic approval number: 5201500513) animal ethics committee and Macquarie University Internal Biosafety Committee (NLRD 5201500584). Zebrafish embryos were utilised 48 hpf for all infection experiments.

*Conflicts of interest*

The authors declared that no competing interests exist.

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## 5.9 ABBREVIATIONS

CF- cystic fibrosis; CLSM- confocal laser scanning microscopy; YFP-yellow fluorescent protein; HPI-hours post infection; DPI-days post infection; PBS- phosphate buffered saline; LB- Luria-Bertani; SOCS- suppressors of cytokine signaling; HPF- hours post fertilisation

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## 5.11 DATA BIBLIOGRAPHY

*1.* Kumar SK. Dryad doi: doi:10.5061/dryad.3vk38 (2018).

## 5.12 FIGURES

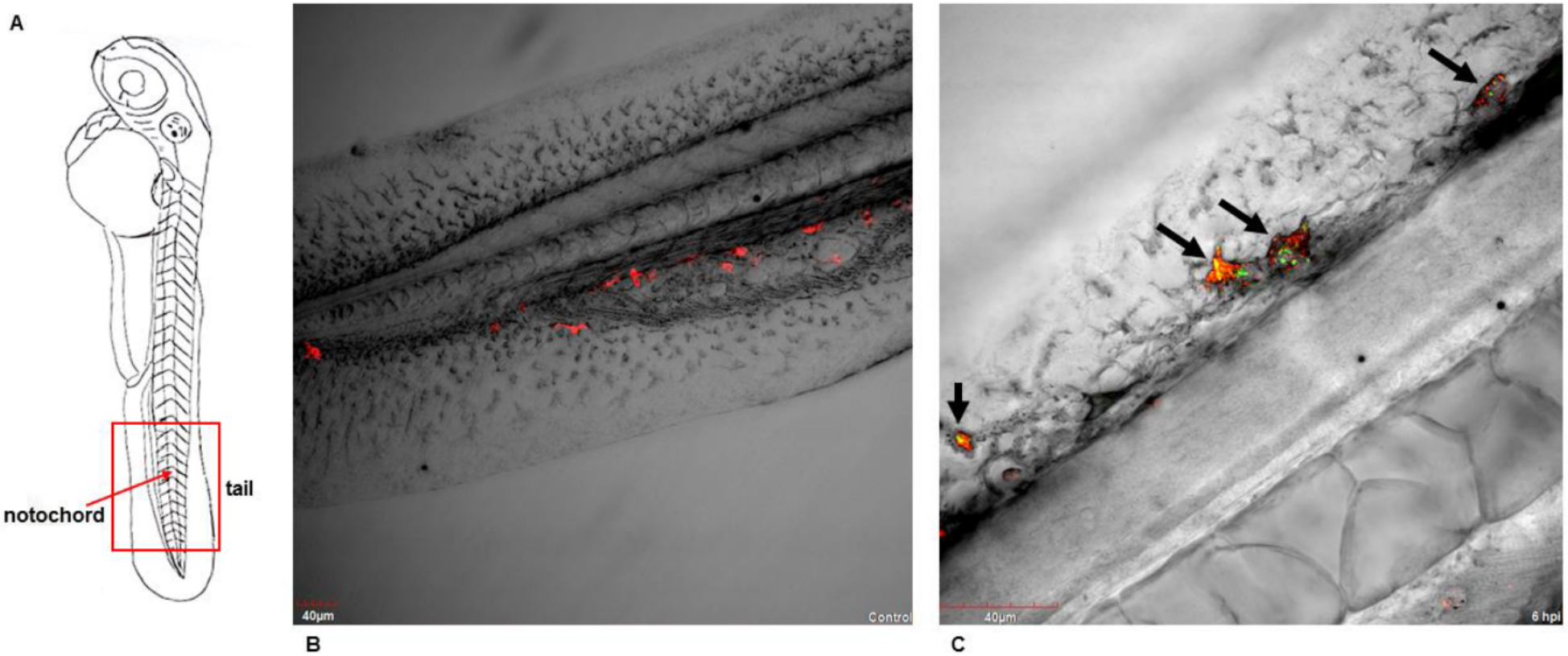


**Fig 5.1. *P. aeruginosa* PASS1-macrophage interaction in zebrafish at 6 hpi.** (A) A schematic view of a zebrafish embryo. The red box represents the tail region, and the red arrow indicates vertebrate notochord. Confocal laser scanning microscopy of transgenic embryos of *Danio rerio Tg*(*mpeg1:Gal4, UAS;mCherry-CAAX*) injected into the Duct of Cuvier with (B) phosphate buffered saline and with (C) YFP-labelled *P. aeruginosa* PASS1 at 48 hpf. (B) Post-infection with phosphate buffered saline, macrophages are localized in the tail region of the vertebrate. (C) The black arrows on the vertebrate notochord in the tail region of zebrafish embryos indicate either an association or engulfment of *P. aeruginosa* PASS1-YFP cells (in green) by macrophages (in red).

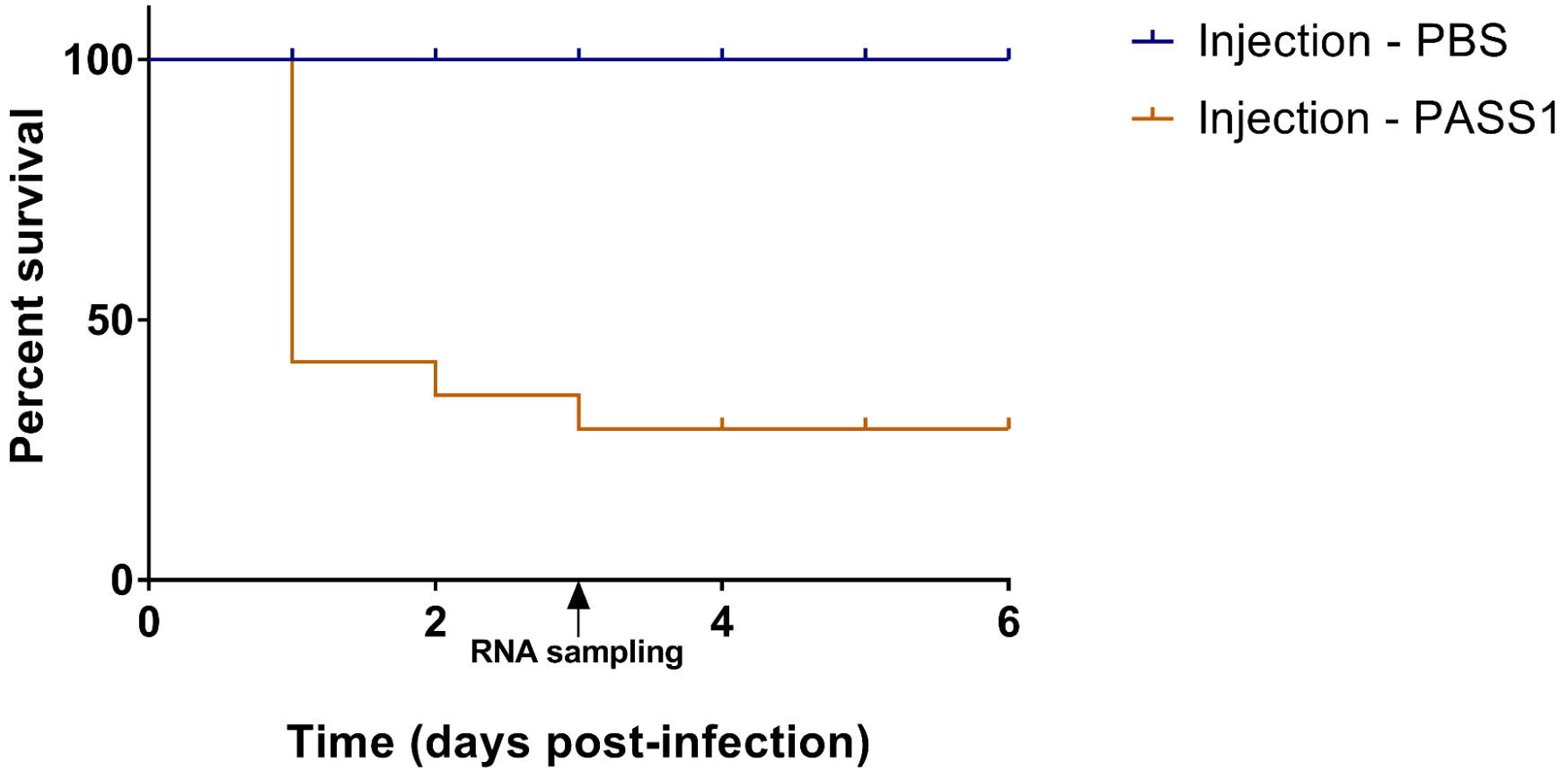


**Fig 5.2. Percentage survival of zebrafish embryos infected with *P. aeruginosa* PASS1.** Kaplan-Meier representation of the survival of zebrafish embryos infected with PASS1 and mock-infected with PBS via injection into the Duct of Cuvier.

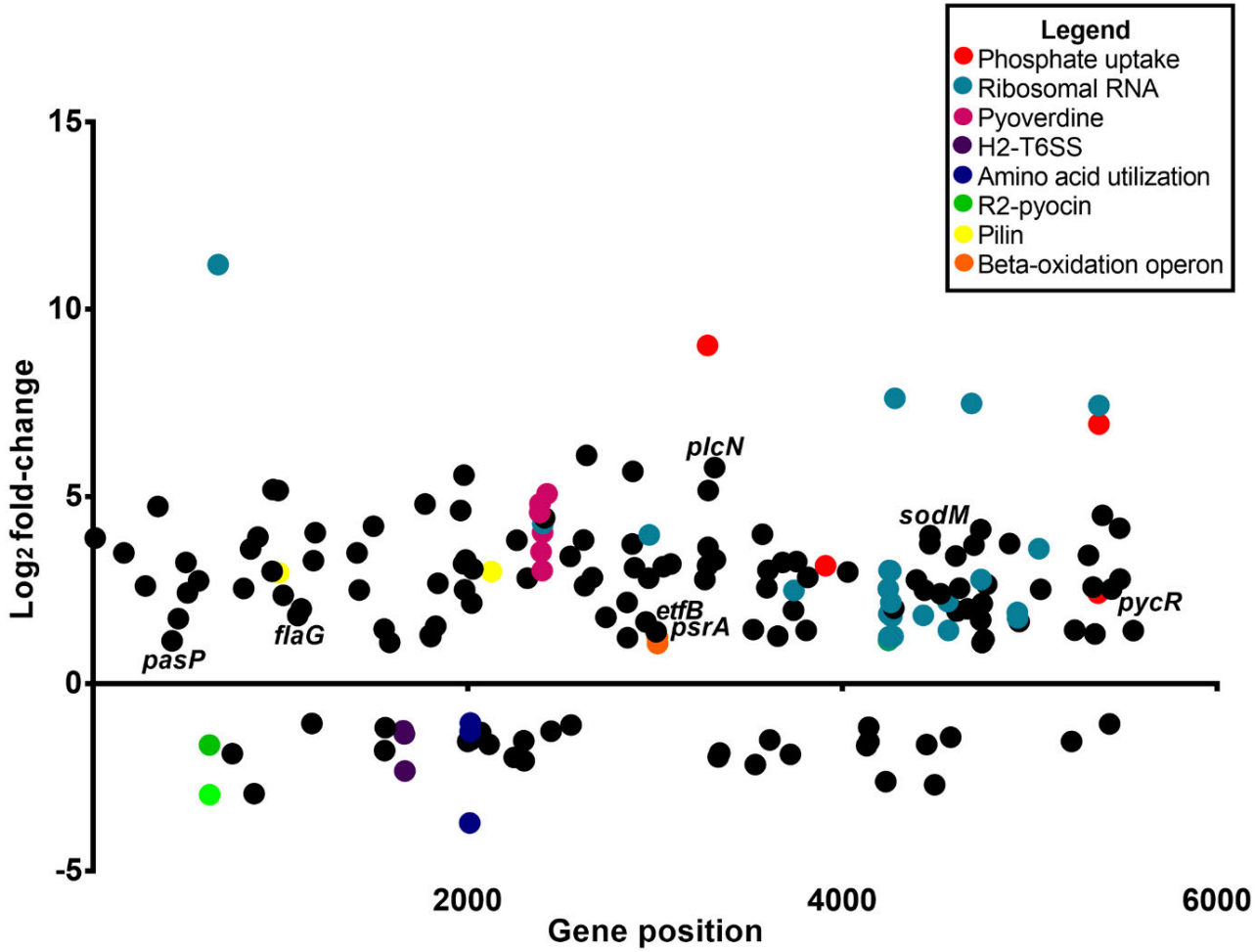


**Fig 5.3. Differential gene expression of P. aeruginosa PASS1 in zebrafish compared to PASS1 cells grown in Luria-Bertani medium.** Each dot represents a gene within the *P. aeruginosa* PASS1 genome (x-axis) and its fold- change ($log_2$) expression *in vivo*, 3 dpi. Only significantly differentially expressed genes are shown ($p \leq 0.01$ and $log_2$ fold-changes cut-off -1≥ to ≤1).

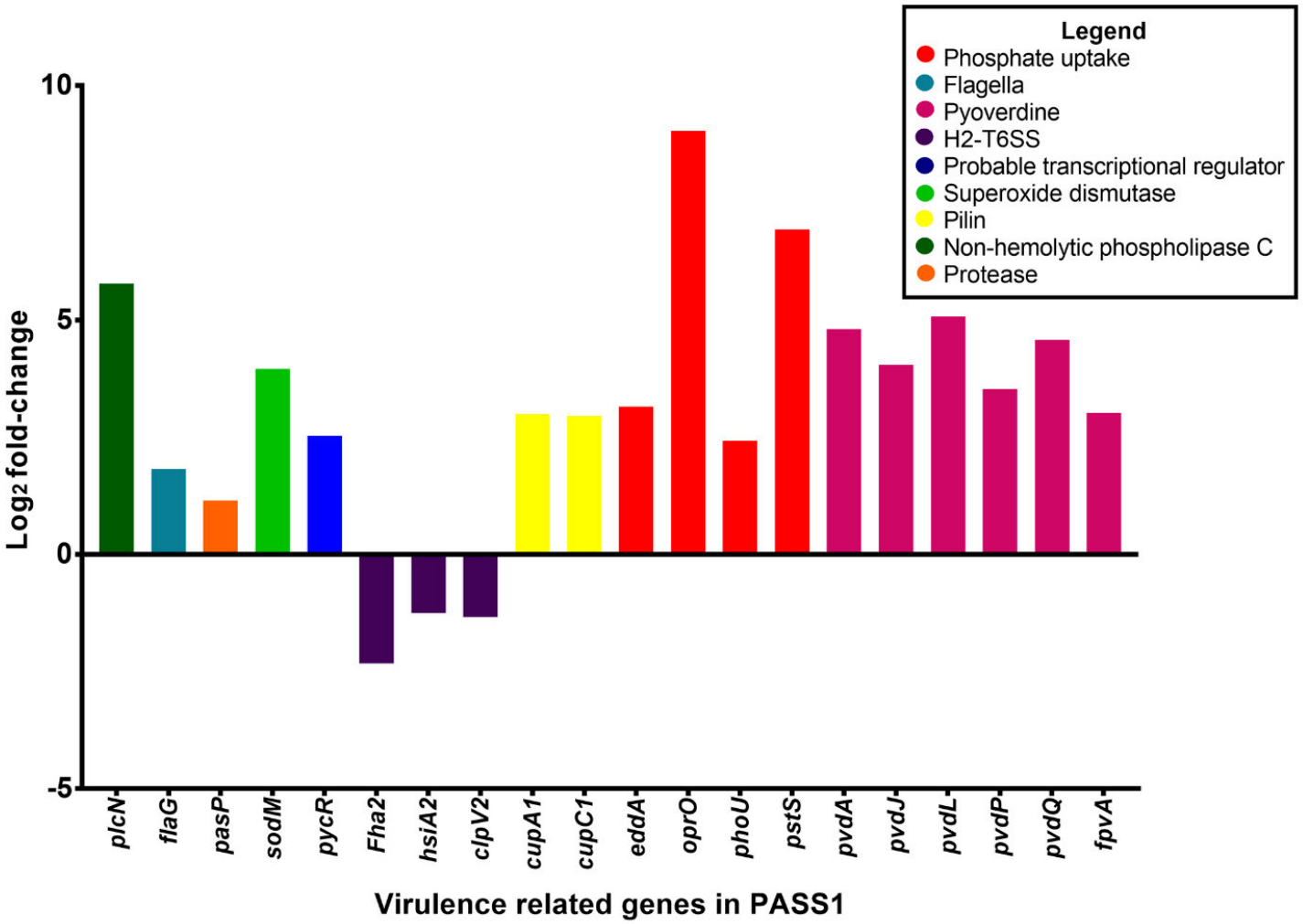


**Fig 5.4. Differential gene expression of virulence-related genes of *P.* aeruginosa PASS1 during infection of zebrafish compared to PASS1 cells grown in Luria-Bertani medium.** $Log_2$ fold change differential expression of known virulence genes ($p \leq 0.01$ and $log_2$ fold-changes cut-off $-1 \geq$ to $\leq 1$).

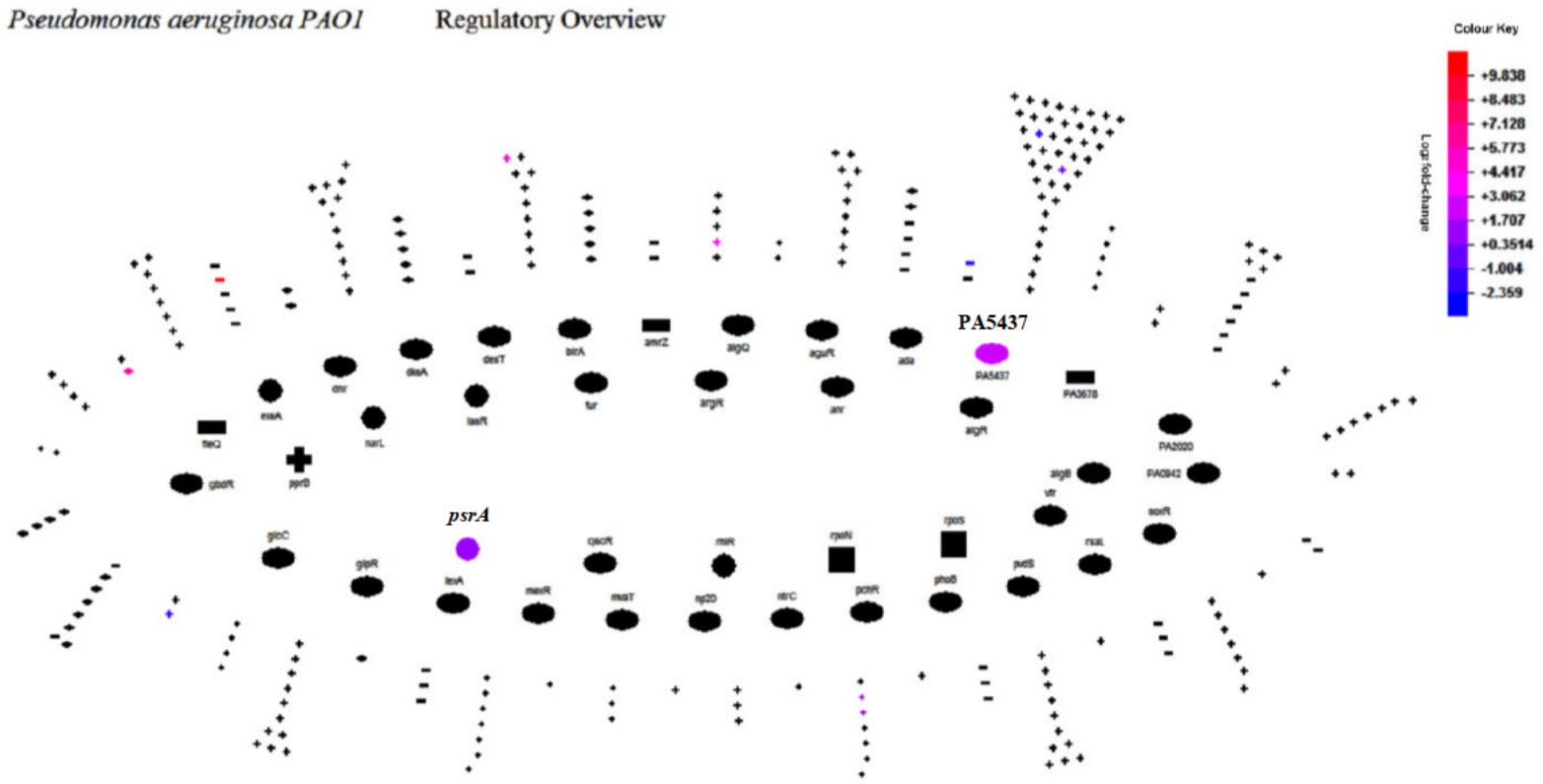


**Fig 5.5. Regulatory overview of *P.* aeruginosa PASS1 grown *in vivo* compared to PASS1 grown in Luria-Bertani medium.** Genes with the $p \leq 0.01$, with $\log_2$ of fold-change of -1≥ to ≤1 cut-off were overlaid on the *P. aeruginosa* PAO1 regulatory overview in biocyc (http://www.biocyc.org). The inner ring consists of master regulators and sigma factors, the outer ring consists of genes that are regulated only, and the middle ring consists of all other genes. The color scale represents the $\log_2$ fold-change expression of the gene. Genes remain black in color if the dataset does not consist of a gene expression value for the particular gene.


**Fig. 5.6 Genome overview of *P.* aeruginosa PASS1 grown in zebrafish compared to growth in Luria-Bertani medium.** Genes with the p-value of 0.01 and $\log_2$ fold-change -1≥ to ≤1 cut-off were overlaid on the *P. aeruginosa* PAO1 genome overview in biocyc (http://www.biocyc.org). The scaffold of the PAO1 genome displays the position of the genes within the genome. The color scale represents the $\log_2$ fold-change expression of the gene. Genes remain navy blue in color if the dataset does not consist of a gene expression value for the particular gene.

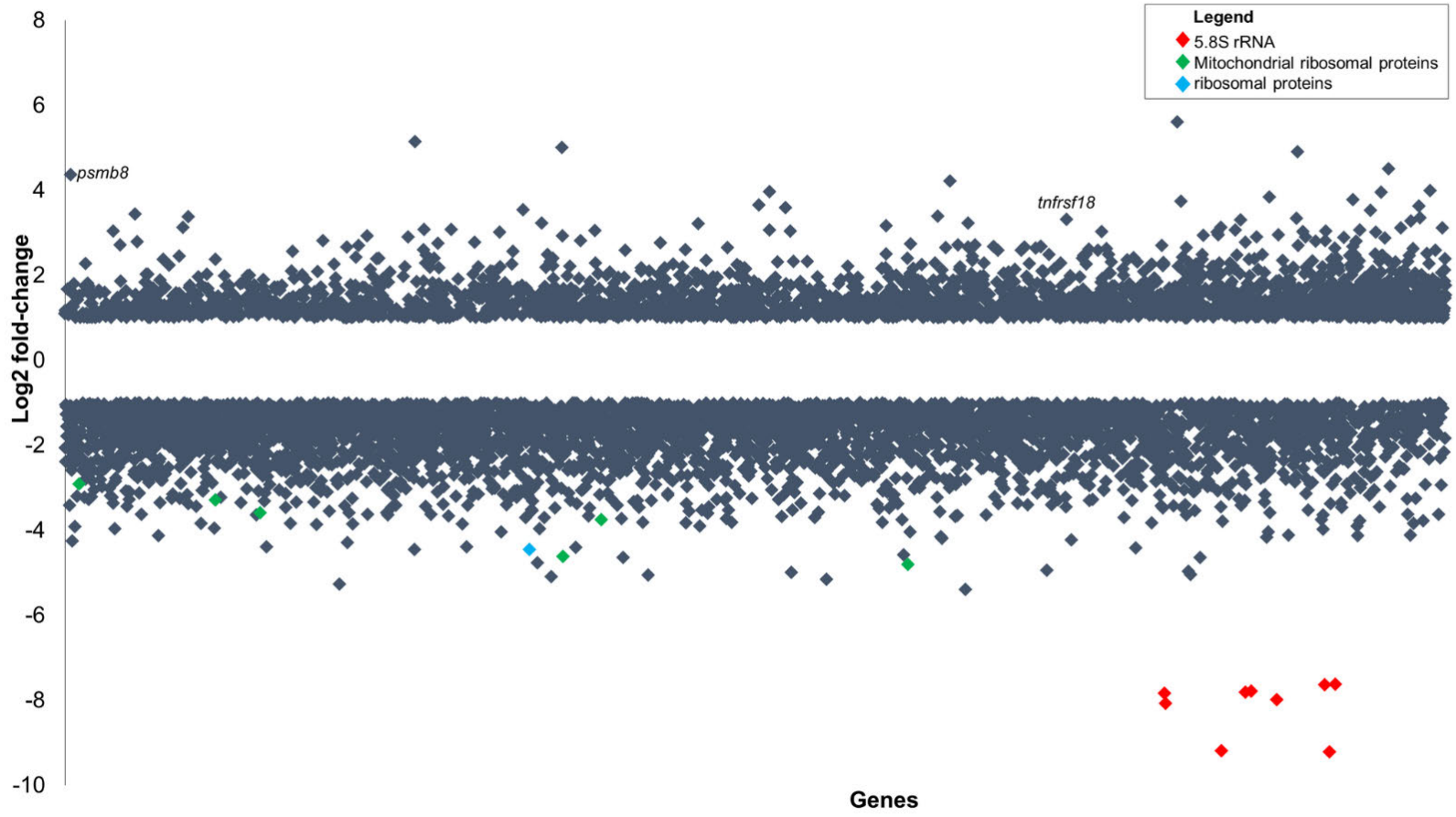


**Fig 5.7 Gene expression changes in zebrafish embryos ($log_2$ fold change) infected with *P. aeruginosa* PASS1 compared to zebrafish embryos injected with phosphate buffered saline.** (A) Each dot represents a gene within the zebrafish genome (x-axis) and its fold- change ($log_2$) expression 3 dpi ($p \leq 0.01$ and $log_2$ fold-changes cut-off $-1 \geq$ to $\leq 1$).

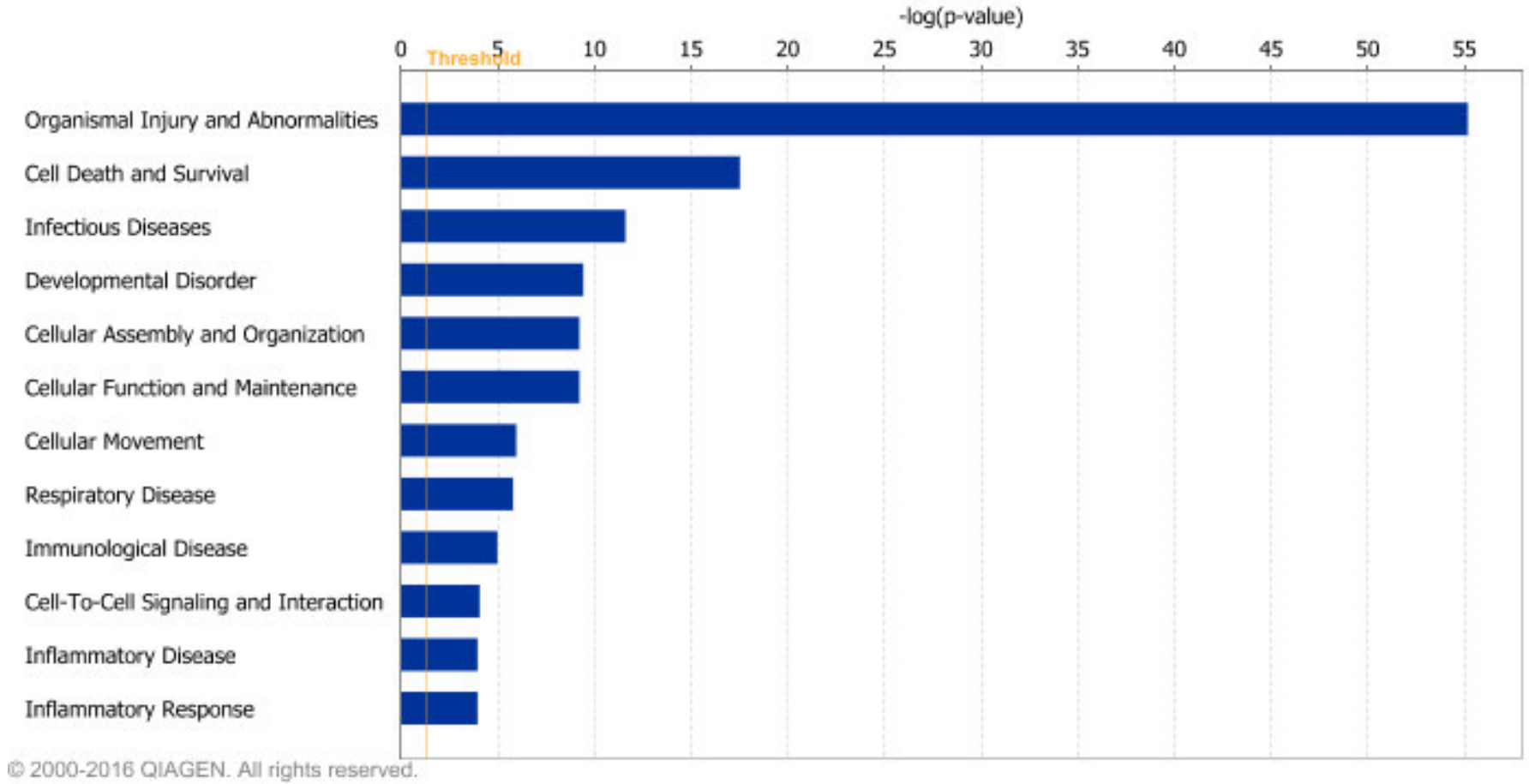


**Fig. 5.8. Molecular and cellular functions in the zebrafish embryo infected with PASS1 versus zebrafish mock-infected with phosphate buffered saline.** The bar graph image obtained via the IPA ingenuity pathway analysis system showing a total of 3238 differentially expressed ($p \leq 0.01$, $\log_2$ fold-change of -1≥ to ≤1 cut-off) annotated genes (S6 Table) assigned to molecular and cellular functions.

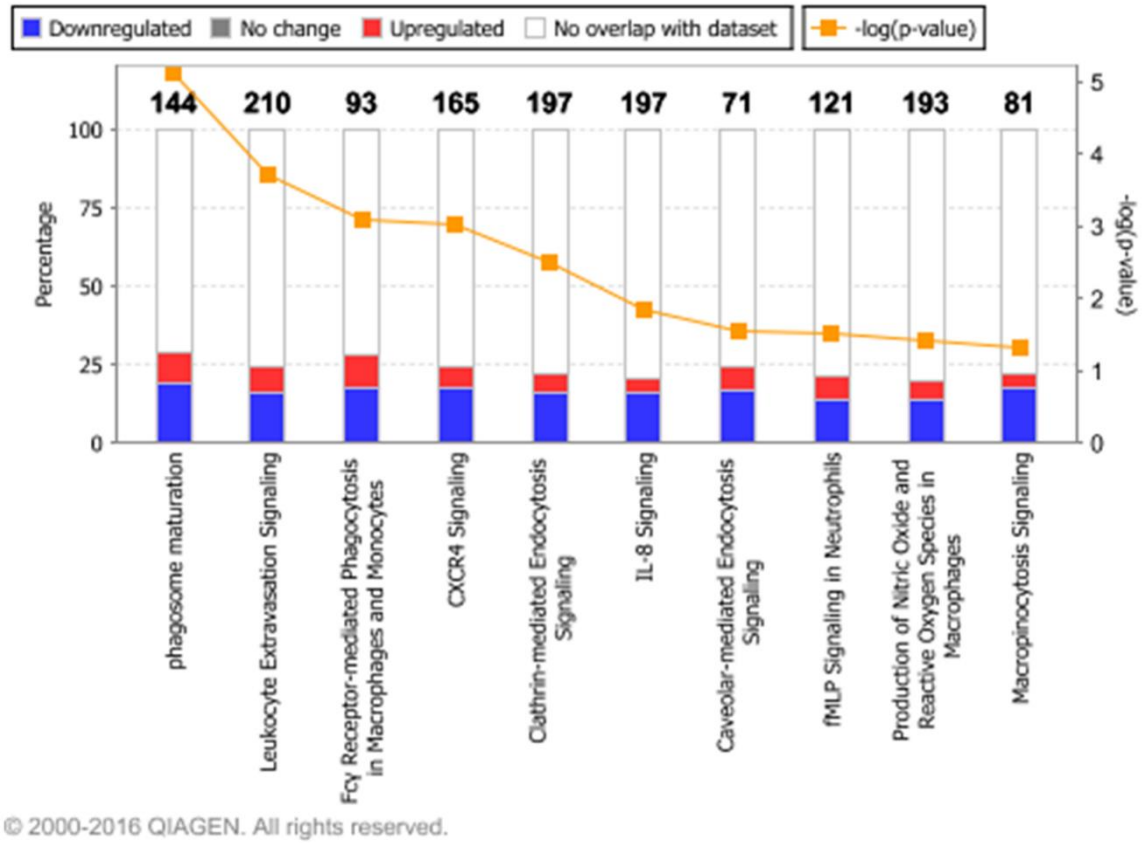


**Fig. 5.9. Cellular and humoral innate immune response signaling pathways enriched in zebrafish infected with PASS1.** The data was obtained via the Ingenuity Pathway Analysis (IPA) of canonical pathways significantly enriched in cellular and humoral innate immune response signaling detected by transcriptomics analysis ($p \leq 0.01$ and $\log_2$ fold-changes cut-off -1≥ to ≤1).

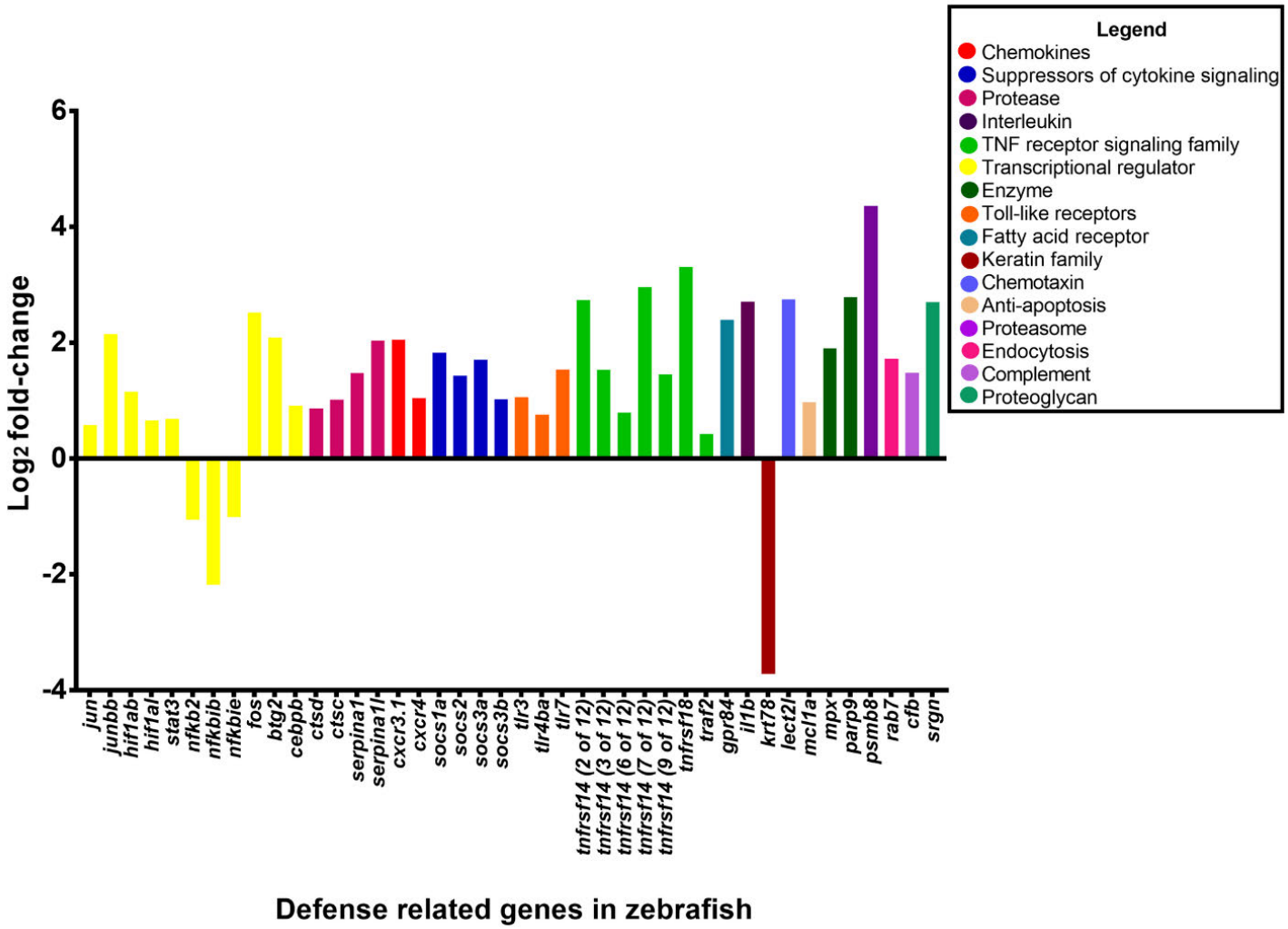


**Fig 5.10. Expression of host defense-related genes in response to infection with *P. aeruginosa* PASS1.** The upregulated genes in zebrafish infected with PASS1 ($p \leq 0.01$ and $\log_2$ fold-changes cut-off $-1 \geq$ to $\leq 1$).

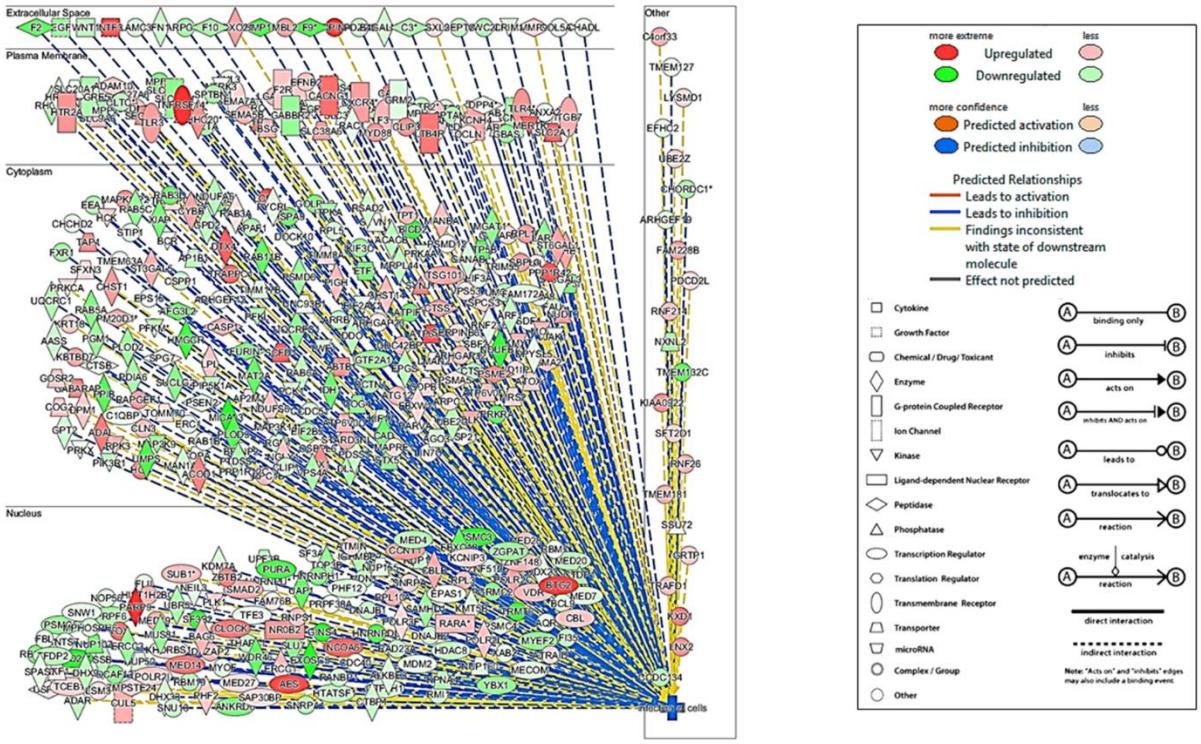


**Fig. 5.11. Network of genes related to infection of cells as revealed via IPA analysis of differentially expressed genes in zebrafish embryo infected with *P. aeruginosa* PASS1.** During infection of zebrafish cells with *P. aeruginosa* PASS1 various genes with varying functions were expressed in the nucleus, cytoplasm, cytoplasm and extracellular space. Significantly upregulated genes are shown in red and downregulated genes shown are in green with the intensity of the color showing the level of upregulated and downregulation of the gene expressed, as log2 of fold-change ($p \leq 0.01$ and $\log_2$ fold-changes cut-off -1≥ to ≤1).

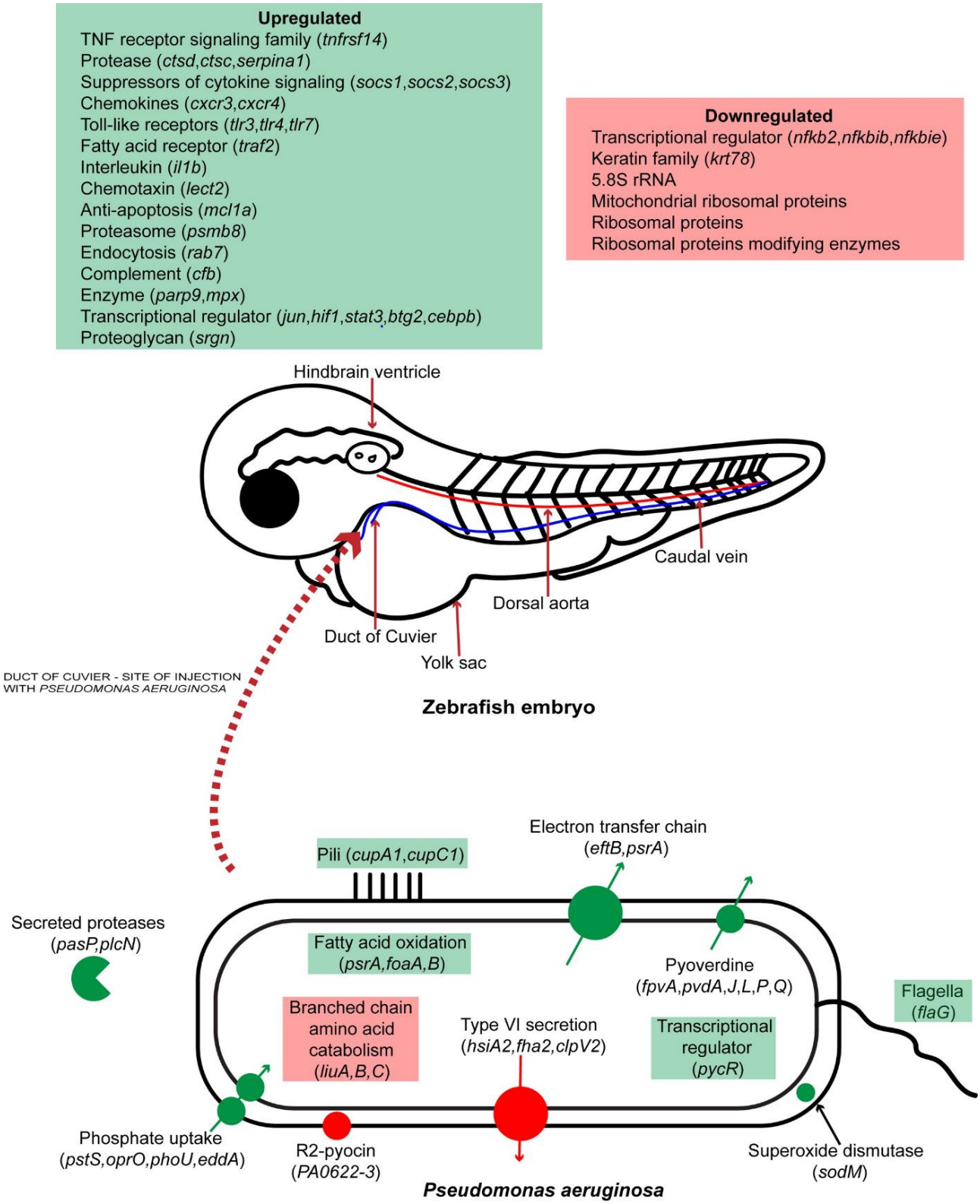


**Fig 5.12. Schematic representation of host-pathogen interactions of zebrafish embryos infected with *P. aeruginosa* PASS1.** Up and down regulated processes and genes are highlighted in green and red, respectively.

## 5.13 TABLES

**Table 5.1. Summary of *P. aeruginosa* PASS1 and zebrafish embryos mapped reads at 3 days post-infection.**

| Condition (3 dpi) | Replicate | Mapped reads | Percentage aligned |
|---|---|---|---|
| **Sequence reads mapped to zebrafish** | | | |
| PASS1-infected zebrafish | 1 | 39709254 | 90.2% |
| | 2 | 43085353 | 89.8% |
| | 3 | 40410278 | 90.0% |
| PBS-infected zebrafish | 1 | 38104344 | 90.2% |
| | 2 | 40702248 | 90.3% |
| | 3 | 39929890 | 90.0% |
| **Sequence reads mapped to *P. aeruginosa* PAO1** | | | |
| PASS1-infected zebrafish | 1 | 27297 | 52.5% |
| | 2 | 15089 | 53.1% |
| | 3 | 21174 | 54.0% |
| PASS1 grown in Luria-Bertani medium | 1 | 12449136 | 94.0% |
| | 2 | 16617581 | 94.3% |
| | 3 | 15580588 | 94.6% |

# 6

# Protoemics of hosts and pathogens in cystic fibrosis

**Pages 199-211 of this thesis have been removed as they contain published material. Removed contents published as:**

Kamath, K.S., Kumar, S.S., Kaur, J., Venkatakrishnan, V., Paulsen, I.T., Nevalainen, H. and Molloy, M.P. (2015), Proteomics of hosts and pathogens in cystic fibrosis. *Proteomics Clinical Applications*, Vol. 9, No .1-2, pp. 134-146. https://doi.org/10.1002/prca.201400122

# 7
# Discussion

## 7.1 Conclusions

Chronic lung infections are the major cause of morbidity and mortality in cystic fibrosis (CF) patients (1). *Pseudomonas aeruginosa* is an opportunistic pathogen that is able to persist in the lungs of CF patients (2) for an extended period of time. In the CF lung environment, *P. aeruginosa* isolates are confronted with a range of selective pressures including nutrient availability, competing microorganisms and host immune responses leading to changes in their genome and phenotype (3).

A variety of genetic and phenotypic adaptations have been reported in *P. aeruginosa* CF isolates. These include development of a mucoid phenotype, changes in biofilm formation, resistance to antibiotics, auxotrophic behaviour, changes in nutrient utilisation and expression of an array of virulence factors (4).

The adapted genotype and phenotype can be beneficial for interaction of *P. aeruginosa* with resident microorganisms and the host innate immune system. The work described here is the first attempt to describe the interactions of *P. aeruginosa* CF isolates with the recently described fungal CF pathogen *Scedosporium aurantiacum* (Chapter 4) (5). Additionally, it is the first study to provide understanding of mechanisms underlying *P. aeruginosa* interactions with the model host organism, zebrafish in the context of the whole cell.

My thesis aims to understand the mechanisms underlying the success of opportunistic pathogen *P. aeruginosa* in CF lung environment by addressing the genetic and phenotypic similarities and differences between CF *P. aeruginosa* isolates (Chapters 2 and 3), the interaction of *P. aeruginosa* with *S. aurantiacum* (Chapter 4), and investigating the molecular basis of the host-pathogen interaction between *P. aeruginosa* and zebrafish (Chapter 5).

## A. Summary of *P. aeruginosa* CF isolates PASS1-4

The *P. aeruginosa* CF isolates PASS1-4 were obtained from adult CF patients. An extensive investigation of their phenotypic differences led to the specific questions addressed in different chapters of this thesis.

*Chapter 2:* The genome and phenotypes of the PASS1-4 CF isolates and the laboratory strain PAO1 was investigated. The published article in Chapter 2 has summary a table (Table 3) describing the genomic and phenotypic characteristics of *P. aeruginosa* CF isolates PASS1-4 and the model strain PAO1.

*Chapter 3*: The investigation of the carbon utilisation profile of the PASS1-4 isolates and PAO1 revealed a distinct difference in the phenome of PASS4 in comparison to PASS1-3. The PASS4 strain respired very well on carbon sources present on the DNA backbone. Therefore, it was hypothesised that the PASS4 strain has preference to utilisation of DNA and further investigation was conducted to characterise the pathways/gene responsible for the selective respiration of PASS4.

*Chapter 4 and 5*: In Chapter 2, *Caenorhabditis elegans* grazing assay, and pyoverdine and phenazine production tests suggested that PASS1 is a highly virulent strain in comparison to CF isolates PASS2-4 with PASS2 being the least virulent. Therefore, we investigated the effect of PASS1 and PASS2 on another resident CF fungal pathogen, *S. aurantiacum* [Chapter 4]. Due to its virulent capabilities, PASS1 strain was chosen as most suitable to identify the virulence mechanisms of *P. aeruginosa* essential for combat with host immunity.

## B. Genetic and phenotypic similarities and differences between *P. aeruginosa* CF isolates

The chronic infection in CF lung represents a highly complex heterogeneous environment with varying selective pressures which gives rise to different genetic and phenotypic traits among *P. aeruginosa* isolates obtained from different patients, as well as those obtained from an individual patient (6-8).

Previous studies have observed genetic diversity among *P. aeruginosa* isolates from CF patients (9-13). In Chapter 2, the genome analysis of *P. aeruginosa* CF isolates PASS1-4 and laboratory strain PAO1 revealed these strains shared a common genomic backbone, but showed a high level of phenotypic diversity in terms of biofilm formation, phenazine and pyoverdine production, metabolic capability, colony morphology, mucin binding, and toxicity to *C. elegans*. This shows the limitations of genomic analysis, while some of these traits could be predicted from the genomic analysis, other phenotypic characteristics are not able to be predicted by *in silico* approaches alone.

*P. aeruginosa* CF isolates are able to thrive in the CF lung environment, which consists of a thick viscous mucus with relatively high availability of amino acids and peptides. Various CF isolates have differences in metabolic phenotypes, with the occurrence of auxotrophy (14) perhaps reflecting an adaptation to a relatively nutrient-rich environment. During host-pathogen interaction studies, the carbon sources available for cell proliferation have been shown to have an influence on biofilm formation and production of extracellular virulence factors (15, 16).

Therefore, the metabolic phenome of *P. aeruginosa* CF strains (PASS1-4) and laboratory strains (PAO1 and PA14) was investigated. PASS2, 3 and 4 all showed reduced carbon catabolic capabilities compared to PASS1 and the non-CF isolates. In particular, PASS4 showed a dramatic narrowing of carbon source utilisation. The phenomic differences among the PASS1-4 CF isolates provides evidence of different mechanisms of adaptation in *P. aeruginosa* in the CF lung environment. This is consistent with a previous phenomic study that suggested that reduction of metabolic capacity is a frequent adaptation to the CF lung, but that the metabolic changes differed between isolates (14). While we mainly focussed on assessing the utilisation of carbon source in different *P. aeruginosa* isolates, the application of Biolog Phenotype MicroArrays nutrient utilisation assays could be extended to include nitrogen, sulphur and phosphorus sources.

Advancing our understanding of the metabolic processes utilised by *P. aeruginosa* isolates can lead to better understanding of their pathogenesis, and may also offer opportunities to design of effective intervention strategies (14, 17). Also, the gain in knowledge of metabolic characteristics

of CF *P. aeruginosa* isolates in context of environment and genetic constraints can allow for enhancements in modelling based studies (17). *In silico* based model predictions complemented with experimental evidence can allow for identification of likely drug targets, as such has been shown for *M. tuberculosis* (17). Since metabolic diversity is a major determinant of virulence in *P. aeruginosa*, a metabolic network reconstruction or perturbations of the metabolic network can serve as an essential component in a multifaceted and effective response to CF lung disease (17).

*P. aeruginosa* physiology and gene expression is dependent on the nutritional environment (18). We undertook a proteomic analysis of the PASS1-4 CF isolates compared with PAO1. As discussed above these isolates share a common genomic backbone, despite this the CF isolates showed a very different protein expression profile compared to the model laboratory strain PAO1. The CF isolates PASS1-4 displayed expression of a broad set of metabolic pathways for the biosynthesis of amino acids, carbohydrates, nucleotides and polyamines. In contrast, PAO1 showed a much more limited expression of biosynthetic pathways, and instead expressed a range of transporters for the uptake of organic nutrients. The difference in groups of proteins expressed suggested that PAO1 is able to transport a diverse set of "ready-made" nutrients from the rich medium, whereas the CF isolates are able to utilise a limited number of nutrients from the medium relying mainly on their own metabolism for synthesis of essential nutrients. Although, the CF isolates collectively varied considerably in protein expression in comparison to PAO1, the protein expression study did not expose the distinct phenomes of the CF isolates.

### C. The *P. aeruginosa* PASS4 isolate may be adapted to utilise eDNA

In Chapter 3, it was hypothesised that PASS4 was specialised to utilise eDNA for growth in the CF lungs since it was able to efficiently utilise components of the DNA backbone, adenosine and inosine. Consequently, studies carried out in Chapter 3 revealed the PASS4 isolate is able to effectively utilise eDNA as a carbon source and furthermore suggested that eDNA may play be an important signal for virulence gene expression. Das et al. (2015) have shown the virulence factor,

pyocyanin to bind to DNA and thus increase its viscosity to facilitate *P. aeruginosa* biofilm formation (19).

Gene and protein expression studies of PASS4 grown in DNA showed the expression of an array of virulence factors and nutrient acquisition systems. Since gene expression and proteomic expression studies did not reveal the reason for metabolic specialisation in wild-type PASS4, its culture was plated on 20 mM N-Acetyl-D-Glucosamine, a carbon source that the wild-type PASS4 strain cannot utilise. Once PASS4 mutant colonies able to grow on N-Acetyl-D-Glucosamine arose, they were cultured in 20 mM N-Acetyl-D-Glucosamine till the mutant was able to grow to an $OD_{600}$=0.7 within ~35 hours. Eight PASS4 mutants obtained were subjected to carbon source testing using Biolog Phenotype MicroArray and genome sequencing of the PASS4 mutants revealed that they all possessed a mutation within the *purK* gene. Upon addition of 2 mM adenosine to carbon sources the parental strain PASS4 was previously unable to utilise had facilitated its growth, confirming that PASS4 is auxotrophic for purine biosynthesis, and thus is only able to grow well in the presence of eDNA or purines. Future work encompassing the complementation of PASS4 *purK* gene into the metabolic versatile PAO1, laboratory strain followed by carbon utilisation screening will provide further evidence for PASS4 purine auxotrophy.

Although, the protein and gene expression studies did not directly provide insights into the mechanisms of metabolic specialisation in strain PASS4, it did show agreement with previous studies (18), that carbon source availability impacts the expression of the virulence factors. Further work focussed on the interactions between *P. aeruginosa* with other microorganisms isolated from CF lungs and with a vertebrate host model.

### D. *P. aeruginosa* inhibits the growth of *Scedosporium aurantiacum*

*S. aurantiacum* is an opportunistic fungal pathogen that is showing an increasing prevalence in the lungs of Australian CF patients. Only a limited number of studies have been undertaken of

interactions between bacterial and fungal pathogens, and virtually nothing was known about interactions between *P. aeruginosa* and *S. aurantiacum.* Chapter 4 of my thesis focuses on interactions between these organisms, and uses a CF *S. aurantiacum* isolate WM 06.482 and a type strain WM 08.202 isolated from wound exudate which displays a lower level of virulence in a *Galleria mellonella* model. Two *P. aeruginosa* CF isolates were used, PASS1 and PASS2, as well as the model strain PAO1. PASS2 has a lower level of virulence than the other strains based on phenotypic and *C. elegans* toxicity studies described in Chapter 2.

Interaction studies conducted between *P. aeruginosa* and *S. aurantiacum* on CF lung mimicking solid plate assays and liquid medium revealed that PASS1 and PAO1 were able to inhibit growth of both *S. aurantiacum* strains in comparison to PASS2, which had little or no growth inhibiting effect (5). However, the highly virulent clinical *S. aurantiacum* isolates WM 06.482 was less susceptible to *P. aeruginosa* strains PAO1 and PASS1 in comparison to less virulent strain WM 08.202.

Interestingly, *P. aeruginosa* strains were able to inhibit growth of *S. aurantiacum* strains as cell lysates and by forming biofilm-like structures on the surface of fungal hyphae in liquid cultures, which was shown by confocal microscopy of fluorescently labelled PASS1 strain (with YFP) and WM 06.482 strain (with mCherry) involved in this interaction. Also, in the absence of cell-cell contact *P. aeruginosa* PAO1 and PASS1 were able to inhibit growth of *S. aurantiacum* strains possibly via bacterial metabolites and/or extracellular signalling molecules. Previous studies have shown that fungal pathogens *C. albicans* and *A. fumigatus* co-exist with *P. aeruginosa.* Specifically, interaction studies with *P. aeruginosa* and *C. albicans* revealed that *P. aeruginosa* can use the bacterial signalling molecule 3-oxo-C12HSL to affect *Candida* morphology, while *C. albicans* can use the fungal metabolite farnesol to affect swarming motility in *P. aeruginosa* and reduce levels of the *Pseudomonas* quinolone signal and pyocyanin in *Pseudomonas* (20). In our study, the ability of *P. aeruginosa* to inhibit growth of *S. aurantiacum* was hampered by commonly

used antimicrobial agent gentamicin. This suggests that therapeutic strategies targeting polymicrobial infections need to take into account the microbial ecology, as changes in abundance of the targeted pathogen may have indirect effects on other pathogenic organisms present.

The resilience of *P. aeruginosa* strain PAO1 and CF isolate PASS1 in bacterial-fungal interaction, as well as the apparently high level of virulence of PASS1 instigated curiosity in the molecular mechanisms involved with interaction of PASS1 with a host system. Specifically, the first line of host defence mechanisms, the innate immune system, in combating highly virulent *P. aeruginosa* isolates.

**E. Interaction of PASS1 with the zebrafish innate immune response**

Since the zebrafish embryos develop the adaptive immune system several months following post-fertilisation and are optically transparent at larval stages allowing for microscopic observations, this model organism was deemed suitable to study the innate immune response of the host in response to virulence mechanisms of *P. aeruginosa* PASS1. Thus, Chapter 5 describes the infection of the zebrafish model host organism with *P. aeruginosa* strain PASS1 enabling the visualisation of direct interactions between zebrafish macrophages and *P. aeruginosa* strain PASS1 and the study of simultaneous gene expression during host-pathogen interaction. This host-pathogen interaction study used dual RNA-Seq and represents the first use of this technology for investigation of the *P. aeruginosa*-zebrafish embryo interaction.

The dual RNA- Seq technique is a valuable approach as it provides knowledge of associated gene expression changes occurring in both host and pathogen during infection, in parallel (20). One of the major benefits of this approach is the potential to monitor gene expression in both organisms to a high level of accuracy and depth (21).

In Chapter 5, microscopic observation revealed *P. aeruginosa* PASS1-YFP cells to be associated or engulfed by macrophages within 6 hours post infection (hpi) (Fig 5.1). Gene expression study

at 3 days post infection (dpi) revealed *P. aeruginosa*-zebrafish embryo interaction to initiate a dynamic cascade of events that culminates in altered gene expression patterns in both interacting organisms. Key genes upregulated in *P. aeruginosa* PASS1 during infection in zebrafish embryos encode the uptake of phosphate, biosynthesis of pyoverdine for iron acquisition and various virulence factors, suggesting that these functions are all important for successful colonisation of the zebrafish host. Simultaneously, the zebrafish embryo was displaying an enrichment of genes involved in humoral and cellular immunity. The host gene expression data also revealed expression of mechanisms involved in infection, inflammation and tissue injury. Therefore, the simultaneous host-pathogen gene expression data indicated that the virulent *P. aeruginosa* PASS1 can mediate vigorous activity in establishment of infection with the host innate immune system responding to the microbial attack.

Our dual transcriptomics approach allows for comparison of genes from both host and pathogen at different time points throughout the infection process — that is, from initial contact through to invasion and, finally, the manipulation of the host (21), however, due to limitations in the financial resources available we could only sequence the initial stages of host-pathogen interaction of zebrafish embryos and PASS1. Additional time points would enable insight into temporal responses during infection as well as, the changes that occur in the cellular networks of both organisms (21).

## 7.2 Future directions

### A. Loss of the psl cluster in PASS2 affects biofilm formation

In *P. aeruginosa* biofilm formation is known to be a major contributor of virulence in the host as it is inherently resistant to antimicrobial treatment (22). In Chapter 2, observation of biofilms of *P. aeruginosa* CF isolates PASS1-4 revealed differences in biofilm architecture, and exposed the lack of biofilm formation ability of PASS2. Therefore, it would be interesting to investigate molecular basis for differences in biofilm formation and architecture between PASS1-4 with the

use of proteomics and transcriptomics techniques as well as gene knockouts and other molecular approaches.

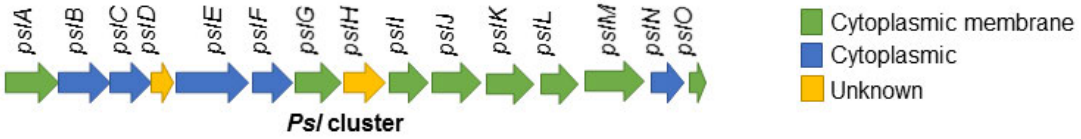


**Figure 7.1:** The *P. aeruginosa* strain PAO1 genome consists of a *psl* cluster consisting of 15 cotranscribed genes which span 18.8 kbp region.

Genome analysis suggests the biofilm formation phenotype of PASS2 is possibly due to lack of the exopolysaccharide biosynthesis cluster, *psl* (Figure 7.1) which previously has been shown to be essential for biofilm formation and its structure (23). The *P. aeruginosa* PAO1 genome contains 15 cotranscribed genes within the *psl* cluster, this cluster of genes could be cloned into *P. aeruginosa* strain PASS2 to see if they can complement the observed biofilm formation defect.

**B. *lasR* deficiency in PASS3 affects phenazine and pyoverdine production**

The QS regulator LasR is known to play a role in production of virulence genes such as pyoverdine and phenazine. Genetic analysis of PASS1-4 strains in Chapter 2 revealed a SNP in PASS1, 6-nucleotide insertion in PASS2, an SNP and deletion in PASS3, with PASS4 being identical to PAO1. Phenotypic observations on the production of phenazine and pyoverdine by the PASS1-4 strains was correlated with the genetic blueprint of each PASS1-4 strain. Although PASS3 did not have PVD biosynthesis deficiency it was unable to produce pyoverdine and phenazine. PASS1 displayed positive production of phenazine and pyoverdine. This suggested that the SNP and deletion in PASS3 has led to complete attenuation of *lasR* which has caused a decrease pyoverdine production in the isolate. To demonstrate the importance of *lasR* in production of pyoverdine, the *lasR* gene from PASS3 can be PCR amplified and then cloned into a suitable expression vector for *P. aeruginosa*. Following cloning the plasmid can be transformed into PAO1. The pyoverdine production can then be measured for the wild-type PAO1 strain in comparison to the PASS3 strain.

## C. Influence of polymicrobial interactions on the host

In addition to investigating the mechanisms involved in biofilm formation of CF isolates it is also important to identify genes involved in communication with other resident microorganisms of the CF lung and interaction of these microorganism with the innate immune system. The complicated interplay between microorganisms during polymicrobial infection of the CF airway is yet to be elucidated, however addressing this knowledge gap has the potential to assist in identification of disease prognosis and development of therapeutics (24).

It is clear from experiments conducted in Chapter 4 and Chapter 5 that bacteria-fungi and bacteria-host interactions are important in order to understand the complexity of CF lung infection. *In vivo* studies of *P. aeruginosa* PASS1-YFP interaction with *S. aurantiacum* strain WM 06.482-mCherry in Chapter 4 revealed that *P. aeruginosa* has a substantial inhibitory effect on the growth of *S. aurantiacum*, however interaction of these microbes in the presence of innate immune response is yet to be explored.

I utilised zebrafish as an infection model to study interactions between *P. aeruginosa* PASS1 and the innate immune system. Transcriptomic studies of the pathogen and host revealed increased expression of an array of virulence genes in PASS1 and a complex array of gene expression changes in the host. It is important to recognise that CF lung infections are a complex polymicrobial disease state, so any single pathogen/host model system cannot fully encompass its complexity.

To investigate the effects of polymicrobial infections on zebrafish embryos as a model host, we conducted a survival study over a 10-day period (Figure 7.2). Zebrafish embryos were injected in the blood vein, or submerged in embryo water with combinations of the three CF pathogen strains: *Burkholderia cenocepacia* S2, *P. aeruginosa* PASS1 and *S. aurantiacum* WM 06.482. Different infections established in zebrafish embryos displayed variation in lethality to the zebrafish embryo host. Systemic infection with PASS1 in zebrafish embryos showed to be most

lethal to zebrafish embryos. The systemic infection with fungal pathogen *S. aurantiacum* WM 06.482, combination of S2 and WM 06.482, and combination of PASS1, S2 and WM 06.482 were also found to be lethal to the embryos.

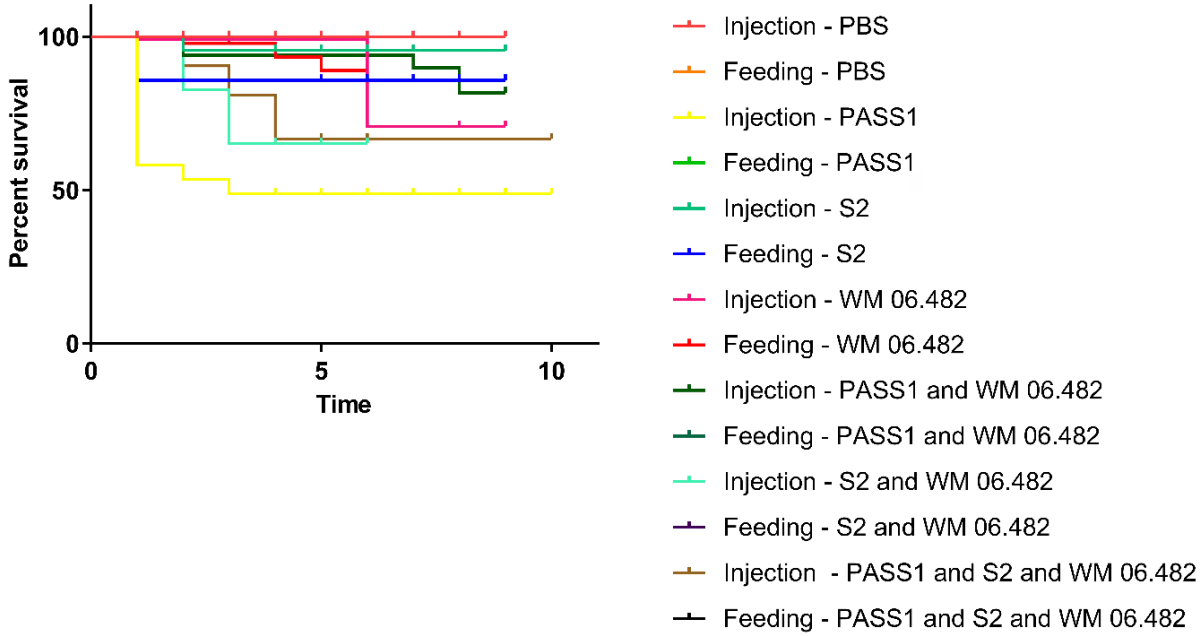


**Figure 7.2:** Kaplan-Meier survival curves are shown for zebrafish embryos with polymicrobial infections. Zebrafish embryos received injections or were submerged in a combination of microorganisms. Different colours on the graph represent different infections and the percentage survival of zebrafish embryos during infection over 10 days post infection.

Previously, *S. aurantiacum* WM 06. 482 has demonstrated high virulence in *G. mellonella*, with 85% of the *G. mellonella* larvae failing to survive after eight days of infection (25). In Chapter 4, we discovered that *P. aeruginosa* can potentially inhibit growth of *S. aurantiacum* via secretion of metabolites. Interestingly, the lethality of PASS1 was lowered in all of the co-infections compared with PASS1 alone, suggesting that the polymicrobial infection decreases the lethality of PASS1 infections due to inhibition/competition between the pathogens or due to a more robust immune response resulting from the polymicrobial infection. In contrast injection with S2 and WM 06. 482 together has a much greater mortality rate for the zebrafish than either of those two strains alone.

Previous studies have outlined poor prognosis and significantly shortened survival rates for patients with *B. cenocepacia* infection in comparison to patients with *P. aeruginosa* infection (26). The lethality of *B. cenocepacia* has been attributed in part to its high production of lipopolysaccharides, which are more toxic and have found to induce an increased neutrophil burst activity and levels of interleukin 8 compared to *P. aeruginosa* (27). In contrast, in the zebrafish model system, *B. cenocepacia* S2 showed a much lower level of lethality than P. aeruginosa. Only a small number of studies have examined polymicrobial infections in host model organisms, the data in Figure 7.2 suggests that polymicrobial infections may differ dramatically from simplistic infection models using a single pathogen.

I was also interested in exploring the transcriptomics of the host and pathogens in polymicrobial infections. RNA was isolated at day 3 and day 10 post infection from co-infection of PASS1 and *S. aurantiacum* WM 06.482; and co-infection of PASS1, S2 and *S. aurantiacum* WM 06.482 into zebrafish embryos. The samples were also fixed in TCA at day 3 and 10 to conduct immunohistochemistry studies on the localisation of the bacterium and fungal pathogen. The samples were not processed further due to financial limitations and were deemed beyond the scope of my PhD, but represent an invaluable source for future investigations.

I also explored using different phenotypically diverse *P. aeruginosa* CF isolates PASS2-4 in the zebrafish host system. Samples for transcriptomics and immunohistochemistry were similarly obtained and archived for future studies. PASS2-4 showed a much lower level of mortality compared with PASS1 (Figure 7.3), which is consistent with their level of toxicity in the *C. elegans* grazing assay (Chapter 2).

RNA-Seq transcriptomics of these samples obtained should provide valuable insights into the complexity of polymicrobial infections such as in the CF lung. It will be possible to investigate the gene expression of the co-infected organisms as well the hosts innate immune response to a polymicrobial infection. Understanding how microbes colonise and persist in the lung environment while destabilising the host immune response is a critical step in developing specifically targeted

anti-inflammatories that can be used in combination with antibiotic and airway clearance therapies (28).

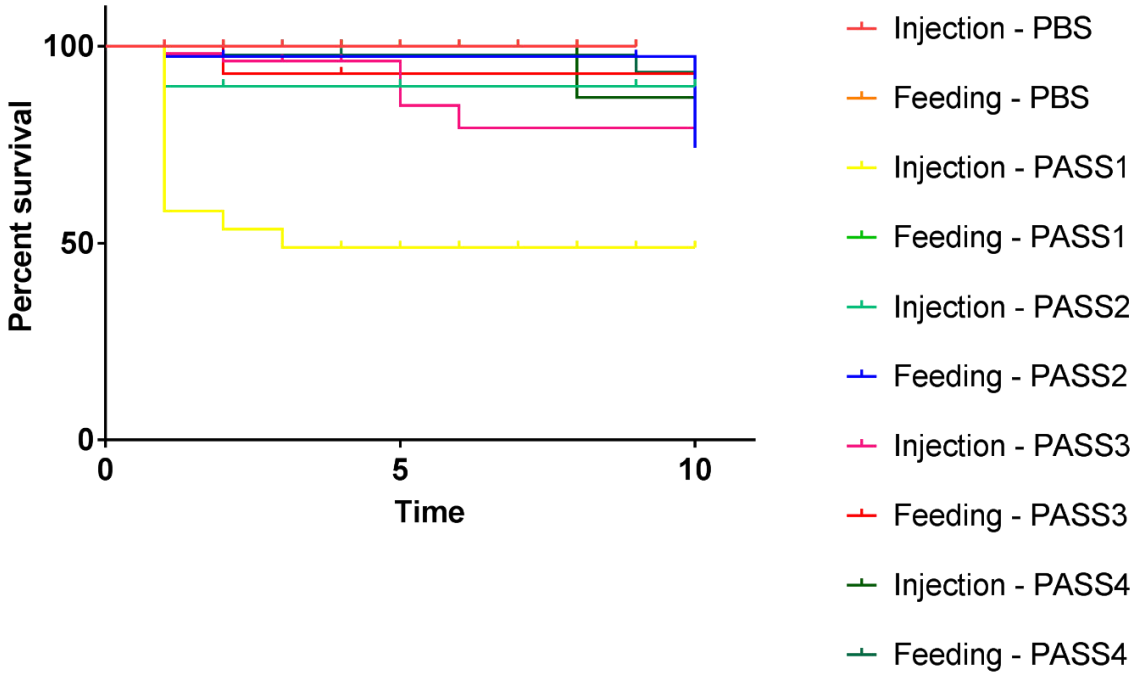


**Figure 7.3:** Kaplan-Meier survival curves are shown for zebrafish embryos infected with *P. aeruginosa* CF isolates (PASS1-4). Zebrafish embryos received injections or were submerged in *Pseudomonas aeruginosa* CF isolates PASS1-4. Different colours on the graph represent different infections and the percentage survival of zebrafish embryos during infection over 10 days post infection.

**D. Analysis of a large cohorts of CF samples is essential**

One of the limitations of this study has been the small group of CF isolates analysed. Microbiome analysis of CF sputum samples with the use of 16S ribosomal RNA sequencing can help identify the composition of the bacterial community over a period of time in patients undergoing different modes of antibiotic therapy. A previous study, utilising 16S rRNA sequencing characterised the lung microbiota of 269 CF patients across a broad range of age, disease stage, clinical status and treatment (29). Their findings revealed that the airways of CF patients only consists of a small number of common taxa which includes *Pseudomonas*, and a significant decrease in CF lung microbiota diversity to occur in both paediatric and adult patients due to treatment with

numerous antibiotics (29). Another study aimed to characterise the bacterial community structure of 126 sputum samples collected over 8–9 years from six age-matched male CF patients. Similarly, they found *Pseudomonas* to be a dominant organism in the CF lung and antibiotic use to be the primary driver of decreasing diversity whilst the bacterial density remained relatively stable (30). A recent deep metagenome sequencing study of children, adolescent, and adult CF patients revealed their lungs to consist of a large repertoire of viruses, fungi, and bacteria (31). Shotgun metagenomic sequencing has the potential to simultaneously unveil the metabolic activity and function of the CF lung microbiota. The metabolic activities of the microbiome can also be characterised with the use of multi-meta-omic techniques including metatranscriptomics, metabolomics and metaproteomics (32, 33). The change in microbiome over time can also be investigated via use of metabolic profiles. Specific phenotypes observed across the microbiome can be investigated further using activity-based protein profiling, whereby synthetic activity-based probes are used to report directly on protein function, regulation, and protein− small-molecule interactions (34).

Despite advances in the field of genomics that are starting to provide a complete snapshot of the CF lung microbiota, it is essential to isolate single organisms for understanding their adaptation to the CF environment. *P. aeruginosa* is a dominant pathogen in the CF lung and its isolates differ genetically and phenotypically making it important to understand its influence on the host and other microorganisms. Each *P. aeruginosa* isolate has a unique mechanism of adaptation to the CF lung, which has been shown in Chapter 2 and 3, thus study of *P. aeruginosa* within a cohort of microorganisms does not necessarily reveal the underlying mechanisms involved in its survival in the host. Detailed genetic and phenotypic analyses of individual strains are also required to paint a full picture of the microbial strategies for flourishing in the CF lung. An example from my thesis is the purine auxotrophy of PASS4 which may be an adaptation allowing to better catabolise DNA as a substrate.

Overall, this thesis has shown that the adaptation and mechanisms of *P. aeruginosa* isolates are vastly diverse. The *P. aeruginosa* CF isolates have variations in their genetic blueprint, phenotype, metabolic and virulence capabilities. Thus, it remains essential to continuously monitor their evolutionary changes and elucidate identified mechanisms essential for *P. aeruginosa* CF isolate adaptation.

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# Appendix I

## Biosafety approval letter

**Office of the Deputy Vice-Chancellor (Research)**

Research Office
C5C Research HUB East, Level 3, Room 324
MACQUARIE UNIVERSITY NSW 2109 AUSTRALIA

**Phone** **+61 (0)2 9850 4194**
Fax +61 (0)2 9850 4465
Email biosafety@mq.edu.au

6 February 2015

Professor Ian Paulsen
Department of Chemistry and Biomolecular Sciences
Faculty of Science and Engineering
Macquarie University

Dear Professor Paulsen,

Re: "Characterizing bacterial transporter, regulator and metabolic genes" (Ref: 5201401141)

**NOTIFICATION OF A NOTIFIABLE LOW RISK DEALING (NLRD)**

The above application has been reviewed by the Institutional Biosafety Committee (IBC) and has been approved as an NLRD, effective 6 February 2015.

This approval is subject to the following standard conditions:

- The NLRD is conducted by persons with appropriate training and experience, within a facility certified to either Physical Containment level 1 (PC1) or PC2.
- Working requiring Quarantine Containment level 2 (QC2) does not commence until the facility has been certified
- Only persons who have been *assessed by the IBC* as having appropriate training and experience may conduct the dealing. This includes persons involved in all parts of the dealing e.g. researchers, couriers and waste contractors. A copy of the IBC's record of assessment must be retained by the project supervisor.
- NLRDs classified under Part 1 of Schedule 3 of the Regulations must be conducted in a facility (or class of facilities) that is certified to at least a PC1 level **and** that is mentioned in the IBC record of assessment as being appropriate for the dealing;
- NLRDs classified under Part 2.1 of Schedule 3 of the Regulations must be conducted in a facility (or class of facilities) that is certified to at least a PC2 level **and** that is mentioned in the IBC record of assessment as being appropriate for the dealing;
- Any transport of the GMO must be conducted in accordance with the Regulator's *Guidelines for the Transport of GMOs* available at: http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/transport-guide-1
- A copy of the IBC's record of assessment has been attached to this approval.
- The record of assessment must be kept by the person or organisation for 8 years after the date of assessment by the IBC (regulation 13 C of the *Gene Technology Regulations 2001*).
- All NLRDs undertaken by Macquarie University will be reported to the OGTR at the end of every financial year.
- If the dealing involves organisms that may produce disease in humans, the NLRDs must be conducted in accordance with the vaccination requirements set out in the Australian Standard AS/NZS 2243.3:2010

- The Chief Investigator must inform the Biosafety Committee if the work with GMOs is completed or abandoned.

Please note the following standard requirements of approval:

1. Approval will be for a period of 5 years subject to the provision of annual reports. If, at the end of this period the project has been completed, abandoned, discontinued or not commenced for any reason, you are required to submit a Final Report. If you complete the work earlier than you had planned you must submit a Final Report as soon as the work is completed. Please contact the Committee Secretary at biosafety@mq.edu.au for a copy of the annual report.

A Progress/Final Report for this study will be due on: 1 February 2016

2. If you will be applying for or have applied for internal or external funding for the above project it is your responsibility to provide the Macquarie University's Research Grants Management Assistant with a copy of this email as soon as possible. Internal and External funding agencies will not be informed that you have final approval for your project and funds will not be released until the Research Grants Management Assistant has received a copy of this email.

If you need to provide a hard copy letter of approval to an external organisation as evidence that you have approval, please do not hesitate to contact the Committee Secretary at the address below.

Please retain a copy of this email as this is your formal notification of final Biosafety approval.

Yours Sincerely

Associate Professor Subramanyam Vemulpad

Chair, Macquarie University Institutional Biosafety Committee

Encl. Copy of record submitted by Macquarie University to the OGTR.

**Biosafety Secretariat**
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Level 3, Research Hub, Building C5C East
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MACQUARIE UNIVERSITY
SYDNEY ~ AUSTRALIA
CRICOS Provider Number 00002J

# Appendix II

## Biosafety approval letter I

MACQUARIE UNIVERSITY

**Office of the Deputy Vice-Chancellor (Research)**
Research Office

C5C Research HUB East, Level 3, Room 324

MACQUARIE UNIVERSITY NSW 2109 AUSTRALIA

**Phone** **+61 (0)2 9850 4063**
Fax +61 (0)2 9850 4465

Email biosafety@mq.edu.au

19th January 2015

Professor Ian Paulsen
Faculty of Science and Engineering
Building E8A
Macquarie University

Dear Ian,

Re: "Host- microbe interaction: Using zebrafish as a model system to investigate virulence mechanisms of cystic fibrosis pathogens" [5201500986]

**NOTIFICATION OF A NOTIFIABLE LOW RISK DEALING (NLRD)**

The above application has been reviewed by the Institutional Biosafety Committee (IBC) and has been approved as an NLRD, effective January 19th, 2016.

This approval is subject to the following standard conditions:

- The NLRD is conducted by persons with appropriate training and experience, within a facility certified to either Physical Containment level 1 (PC1) or PC2.
- Work requiring Quarantine Containment level 2 (QC2) does not commence until the facility has been certified
- Only persons who have been *assessed by the IBC* as having appropriate training and experience may conduct the dealing. This includes persons involved in all parts of the dealing e.g. researchers, couriers and waste contractors. A copy of the IBC's record of assessment must be retained by the project supervisor.
- NLRDs classified under Part 1 of Schedule 3 of the Regulations must be conducted in a facility (or class of facilities) that is certified to at least a PC1 level **and** that is mentioned in the IBC record of assessment as being appropriate for the dealing;
- NLRDs classified under Part 2.1 of Schedule 3 of the Regulations must be conducted in a facility (or class of facilities) that is certified to at least a PC2 level **and** that is mentioned in the IBC record of assessment as being appropriate for the dealing;
- Any transport of the GMO must be conducted in accordance with the Regulator's *Guidelines for the Transport of GMOs* available at: http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/transport-guide-1
- A copy of the IBC's record of assessment has been attached to this approval.
- The record of assessment must be kept by the person or organisation for 8 years after the date of assessment by the IBC (regulation 13 C of the *Gene Technology Regulations 2001*).
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- If the dealing involves organisms that may produce disease in humans, the NLRDs must be conducted in accordance with the vaccination requirements set out in the Australian Standard AS/NZS 2243.3:2010

- The Chief Investigator must inform the Biosafety Committee if the work with GMOs is completed or abandoned.

Please note the following standard requirements of approval:
1. Approval will be for a period of 5 years subject to the provision of annual reports. If, at the end of this period the project has been completed, abandoned, discontinued or not commenced for any reason, you are required to submit a Final Report. If you complete the work earlier than you had planned you must submit a Final Report as soon as the work is completed. Please contact the Committee Secretary at biosafety@mq.edu.au for a copy of the annual report.

A Progress/Final Report for this study will be due on: January 19th 2017

2. If you will be applying for or have applied for internal or external funding for the above project it is your responsibility to provide the Macquarie University's Research Grants Management Assistant with a copy of this email as soon as possible. Internal and External funding agencies will not be informed that you have final approval for your project and funds will not be released until the Research Grants Management Assistant has received a copy of this email.

If you need to provide a hard copy letter of approval to an external organisation as evidence that you have approval, please do not hesitate to contact the Committee Secretary at the address below.

Please retain a copy of this email as this is your formal notification of final Biosafety approval.

Yours Sincerely

Associate Professor Subramanyam Vemulpad
Chair, Macquarie University Institutional Biosafety Committee

Encl. Copy of record submitted by Macquarie University to the OGTR.

**Biosafety Secretariat**
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# Appendix III

## Animal ethics approval letter

# MACQUARIE University ANIMAL RESEARCH AUTHORITY (ARA)

**AEC Reference No.: 2015/022** **Date of Expiry:** 16 July 2016

***Full Approval Duration:** 17 July 2015 to 16 July 2017 (24 months)*

This ARA remains in force until the Date of Expiry (unless suspended, cancelled or surrendered) **and will only be renewed upon receipt of a satisfactory Progress Report before expiry (see Approval email for submission details).**


**Principal Investigator:**
Professor Ian Paulsen
Department of Chemistry and Biomolecular Sciences
Macquarie University, NSW 2109
ian.paulsen@mq.edu.au
0421 013 148

**Associate Investigators:**

| | |
|---|---|
| Sheemal Kumar | 0417 682 598 |
| Ani Penesyan | 0410 320 550 |
| Dasha Syal | 0411 147 916 |
| Emily Don | 0423 387 488 |
| Nicholas Cole | 0431 955 280 |


**In case of emergency, please contact:**
*the Principal Investigator / Associate Investigator named above*
**Animal Welfare Officer** - 9850 7758 / 0439 497 383, *or* **Laboratory Coordinator** 9812 3607 / 0416 243 668

The above-named are authorised by MACQUARIE UNIVERSITY ANIMAL ETHICS COMMITTEE to conduct the following research:

**Title of the project:** **Host- microbe interaction: Using zebrafish as a model system to investigate virulence mechanisms of cystic fibrosis pathogens.**

**Purpose:** **5 - Research: Human or Animal Health and Welfare**

**Aims:** **To infect zebrafish embryos with microbes from the cystic fibrosis patient lung to investigate mechanisms involved in host innate immunity evasion.**

**Surgical Procedures category: 7 - Major Physiological Challenge**

**All procedures must be performed as per the AEC-approved protocol, unless stated otherwise by the AEC and/or AWO.**

**Maximum numbers approved (for the Full Approval Duration):**

| Species | Strain | Age/Weight/Sex | Total | Supplier/Source |
|---|---|---|---|---|
| 23 - Fish | Zebrafish | Larvae/Any | 702 | Cole MND Laboratory |
| | | | 702 | |

**Location of research:**

| *Location* | *Full street address* |
|---|---|
| School of Advanced Medicine | Level 1, F10A, 2 Technology Place, Macquarie University, NSW 2109 |
| Department of Biological Sciences | Level 1, Building E8A, Eastern Road, Macquarie University, NSW 2109 |
| Department of Biological Sciences | Room 240 Level 2, Building E8A, Eastern Road, Macquarie University, NSW 2109 |

**Amendments approved by the AEC since initial approval: N/A**

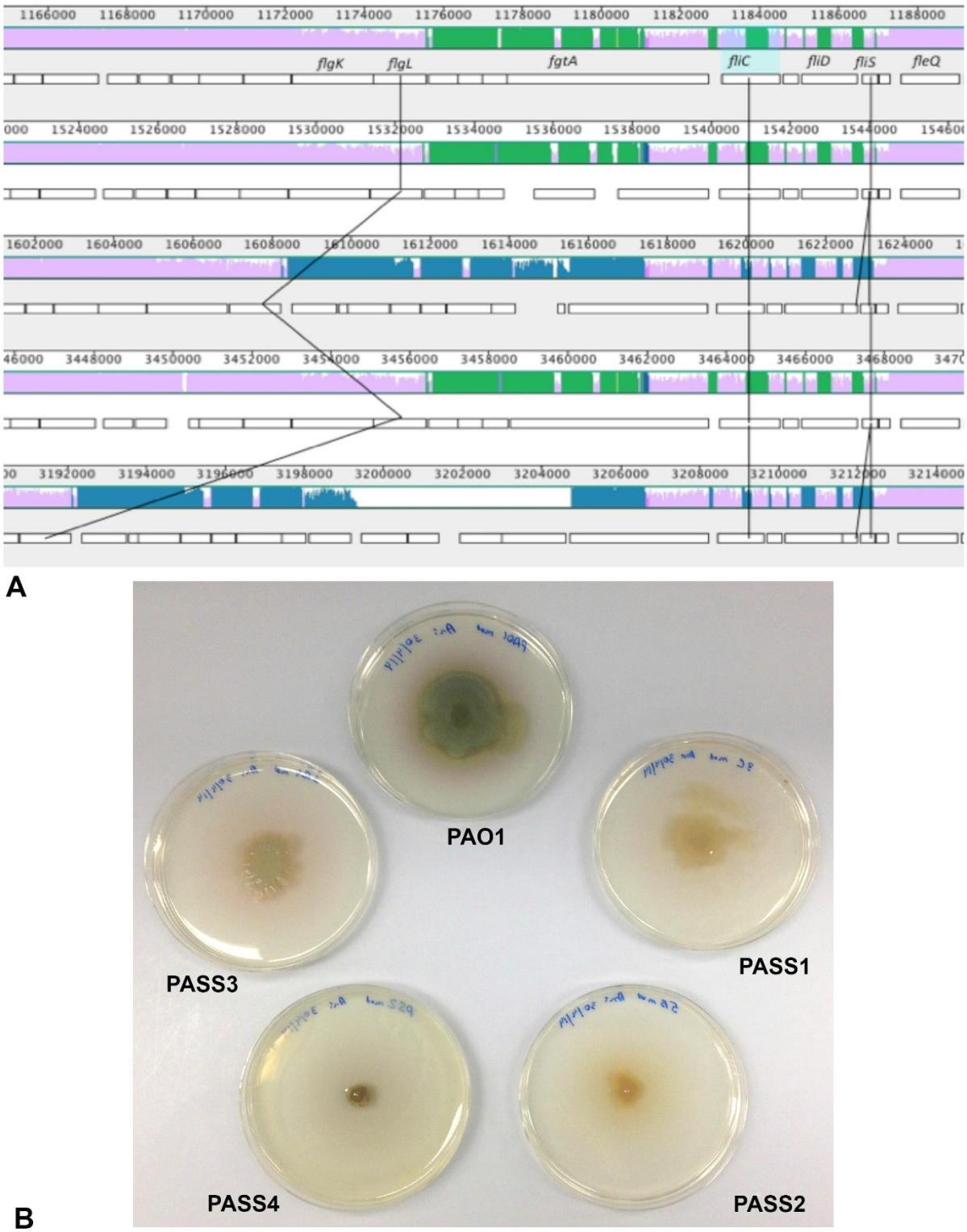
**Conditions of Approval: N/A**

Being animal research carried out in accordance with the Code of Practice for a recognised research purpose and in connection with animals (other than exempt animals) that have been obtained from the holder of an animal suppliers licence.\

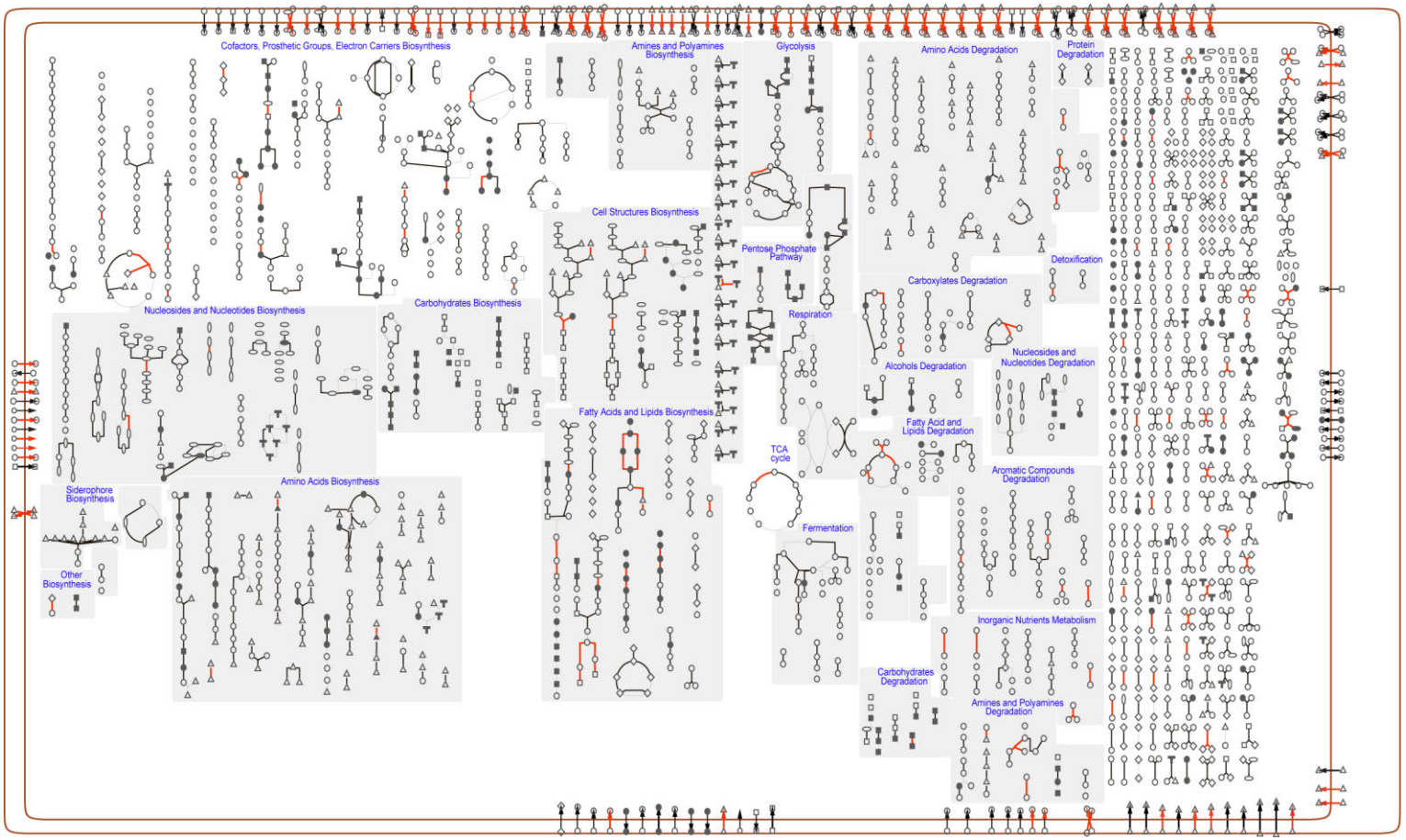
**Professor Mark Connor** (Chair, Animal Ethics Committee) **Approval Date: 16 July 2015**

# Appendix IV

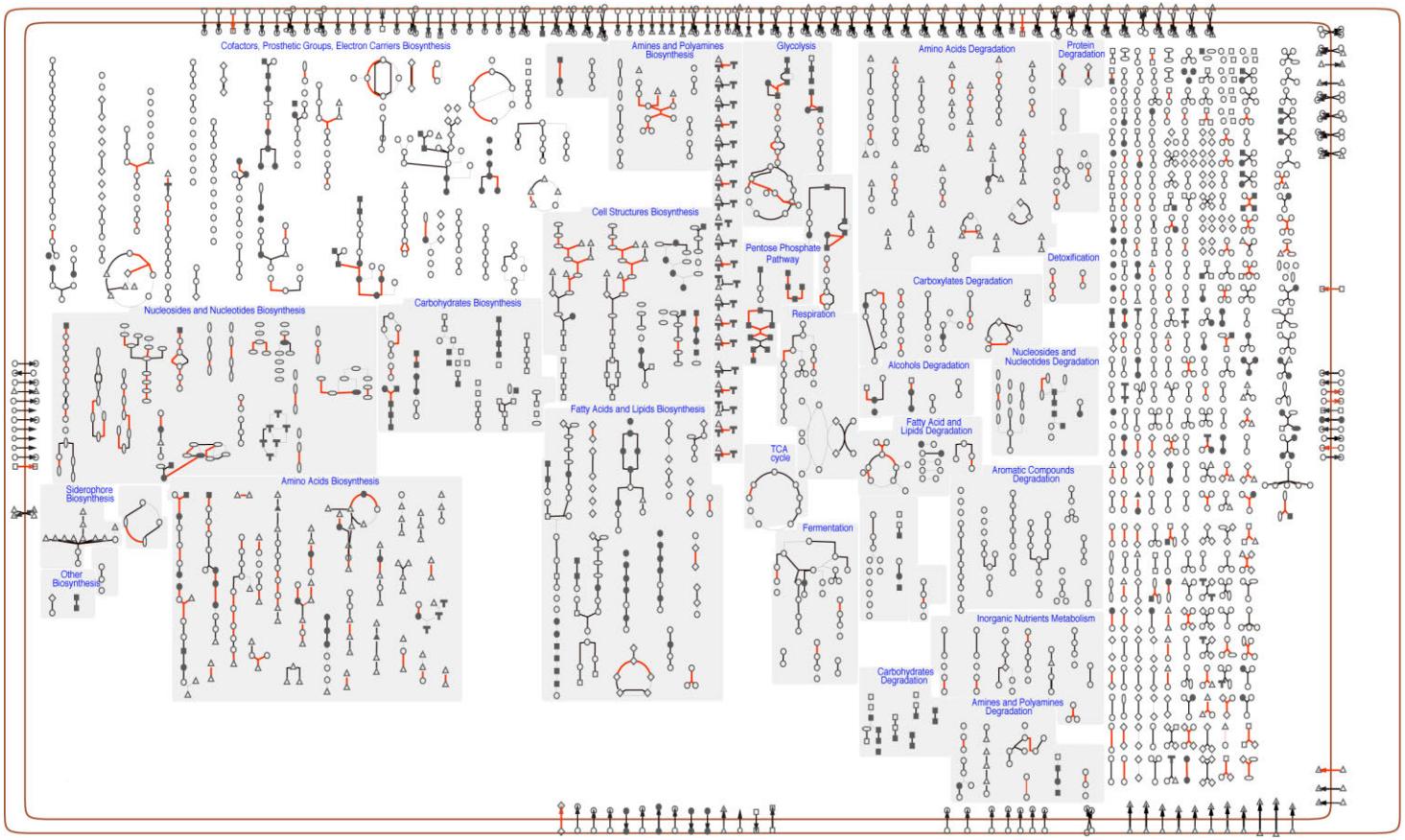
## Chapter 2: Supplementary files




**SF2.1 Fig. Genomic alignment of flagella biogenesis genes in strains PASS1-4 as compared to PAO1 using MAUVE.** Sequences conserved among all 5 isolates are presented in mauve, sequences shared between isolates PAO1, PASS1 and PASS3 are in green, sequences shared between isolates PASS2 and PASS4 are presented in blue (A). Flagella-mediated swimming motility assay for strains PASS1-4 and PAO1 (B).




**SF2.2 Fig. Pathway Tools 17.5 cellular overview diagram showing proteins/pathways expressed uniquely by PAO1, in red.** The shapes on the diagram represent specific types of compounds as follows: square—carbohydrate, triangle—amino acid, upside down triangle—cofactor, ellipse (horizontal)—purine, ellipse (vertical)—pyrimidine, diamond—proteins, T-shape—tRNA, circle—all other compounds. The shading of the icon indicates the phosphorylation state of the compound: shaded compounds are phosphorylated; unshaded compounds are unphosphorylated. Shapes/paths located on outer rectangles represent transporters/membrane proteins. The arrows indicate the direction of the transport: pointing in—uptake, pointing out—export.



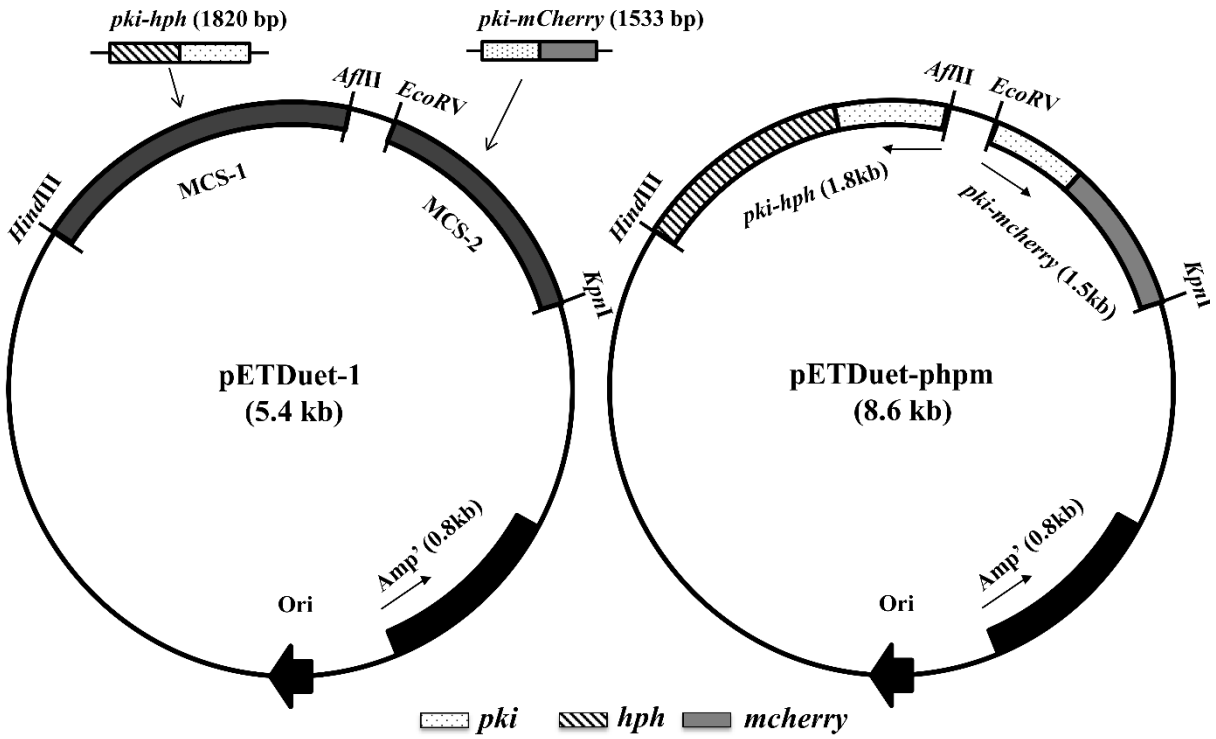

**SF2.3 Fig. Pathway Tools 17.5 cellular overview diagram showing proteins/pathways expressed uniquely by PASS1-4 strains, in red.** The shapes on the diagram represent specific types of compounds as follows: square—carbohydrate, triangle—amino acid, upside down triangle—cofactor, ellipse (horizontal)—purine, ellipse (vertical)—pyrimidine, diamond—proteins, T-shape—tRNA, circle—all other compounds. The shading of the icon indicates the phosphorylation state of the compound: shaded compounds are phosphorylated; unshaded compounds are unphosphorylated. Shapes/paths located on outer rectangles represent transporters/membrane proteins. The arrows indicate the direction of the transport: pointing in—uptake, pointing out—export

**ST2.1 Table. MLST allelic profiles and strain types (ST) of *P. aeruginosa* isolates obtained from the CF sputum in this study.**

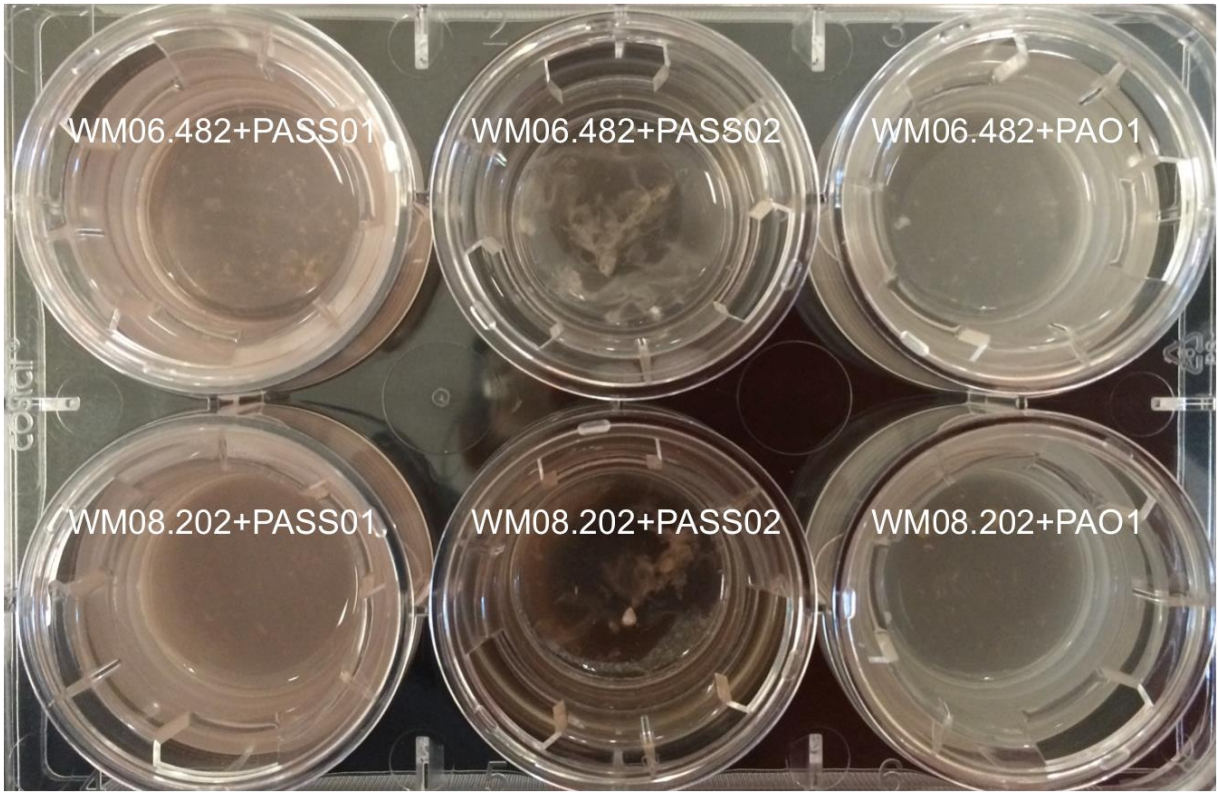
| | acs | aro | gua | mut | nuo | pps | trp | ST |
|---|---|---|---|---|---|---|---|---|
| PASS1 | 17 | 5 | 11 | 72 | 3 | 4 | 3 | new |
| PASS2 | 16 | 5 | 30 | 72 | 4 | 13 | | new |
| PASS3 | 17 | 5 | 11 | 72 | 3 | 4 | 3 | new |
| PASS4 | 11 | 84 | 11 | 3 | 4 | 4 | 7 | 649 |

# Appendix V

## Chapter 4: Supplementary files




**Supplementary Figure SF4.1. Schematic representation of the construction of plasmid pETDuet-phpm containing *pki-hph* (1.8 kb) with *Hind*III and *Afl*II restriction sites and *pki-mcherry* (1.5 kb) with *Eco*RV and *Kpn*I restriction sites.** The plasmid is 8.6 kb long and was made by insertion of *pki-hph* and *pki-mcherry* fragments in two different multiple cloning sites of the pETDuet-1 vector. The patterns used in the image are described in the key below.




**Supplementary Figure SF4.2. Transwell plate showing the formation of red pigment in the co-cultures involving different strains of *S. aurantiacum* (WM06.482 and WM 08.202) and *P. aeruginosa* (PASS01, PASS02 and PAO1).**

**ST4.1 Table. Components for making Synthetic Cystic Fibrosis Medium to a 1-litre volume**

| Compound | Concentration | Grams | Volume (ml) |
|---|---|---|---|
| NaH2PO4 | 0.2 M | | 6.5 |
| $Na_2HPO4$ | 0.2 M | | 6.25 |
| | 1 M | | 0.348 |
| NH4Cl | | 0.122 g | |
| KCl | | 1.114 g | |
| NaCl | | 3.03 g | |
| MOPS | 10 mM | | |
| Deionized water | | | 779.6 |
| l-aspartate | 0.827 | | 8.27 |
| l-threonine | 1.072 mM | | 10.72 |
| l-serine | 1.446 mM | | 14.46 |
| l-glutamate·HCl | 1.549 mM | | 15.49 |
| l-proline | 1.661 mM | | 16.61 |
| l-glycine | 1.203 mM | | 12.03 |
| l-alanine | 1.78 mM | | 17.8 |
| l-cysteine·HCl | 0.16 mM | | 1.6 |
| l-valine | 1.117 mM | | 11.17 |
| l-methionine | 0.633 mM | | 6.33 |
| l-isoleucine | 1.12 mM | | 11.2 |
| l-leucine | 1.609 mM | | 16.09 |
| l-tyrosine | 0.802 mM | | 8.02 |
| l-phenylalanine | 0.53 mM | | 5.3 |
| l-ornithine·HCl | 0.676 mM | | 6.76 |
| l-lysine·HCl | 2.128 mM | | 21.28 |
| l-histidine·HCl | 0.519 mM | | 5.19 |
| l-tryptophan | 0.013 | | 0.13 |
| l-arginine·HCl | 0.306 | | 3.06 |
| Adjust SCFM to pH 6.8 and filter sterilize through a 0.2-µm-pore-size filter. Then add the components listed below. | | | |
| $CaCl_2$ | 1 M | | 1.754 |
| $MgCl_2$ | 1 M | | 0.606 |
| $FeSO4·7H_2O$ | 3.6 mM | | 1 |
| | | **Total** | 1000 |